Changes in color-related compounds in tomato fruit exocarp and mesocarp during ripening using HPLC-APCI⁺-mass Spectrometry

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Abstract Tomato is an important agricultural crop worldwide. Their pigments are very important in many ways. They have been associated with health benefits such as lowering the risk of some chronic diseases. Quantification of chlorophylls by spectrophotometry and Identification of carotenoids using liquid chromatography coupled to mass spectrometry, and quantification by HPLC-DAD was carried out in the exocarp and mesocarp of tomato fruit during 6 different ripeness stages (mature-green, breakers, turning, pink, light-red and red). Four carotenoids have been followed during ripening; β -carotene and lycopene were unequivocally identified, whereas γ -carotene and lycopene-epoxide were tentatively identified. Differences between exocarp and mesocarp were as follows: Most of the ripening period, fruit exocarp had higher quantities of both chlorophyll and carotenoids than mesocarp. In both, exocarp and mesocarp, chlorophylls drastically decreased, lycopene significantly increased, while β -carotene, γ -carotene and lycopene-epoxide only increased slightly during fruit ripening.

Keywords *Lycopersicon esculentum* · Carotenoids · Lycopene · β -Carotene · Chlorophyll

Introduction

During tomato ripening, chlorophyll degradation is concomitantly to carotenoid biosynthesis and both processes are responsible for the change of fruit color from green to red (Hobson and Davies 1971). People prefer the consumption of red tomato, at which point the fruit is believed to possess the highest carotenoids content such as β -carotene and lycopene (Kader et al. 1977; Watada and Aulenbach 1979). High levels of tomato consumption have been consistently correlated with a reduction in the risk of some types of cancer as shown by some epidemiological studies (Clinton et al. 1996; Gerster 1997; Bramley 2000). High levels of tomato consumption imply a high level of carotenoid intake. Carotenoids are lipid-soluble molecules that contribute to color in many fruits and vegetables and some have been associated with several health benefits. Some research reports suggest that lycopene (Bramley 2000; Arab and Steck 2000) and β -carotene (Burri 1997; Erdman et al. 1996) may protect against some forms of cancer and heart disease. Removal and discarding of exocarp and seeds from some fresh fruits and vegetables, is a common practice during processing of some fruits such as tomato (Barret et al. 1998), ignoring that exocarp can be a good source for some nutrients, in some cases even better than the mesocarp as reported for kumquat by Huyskens et al. (1985), and for muskmelon by Flügel and Gross (1982). Some studies have aimed to know the chemical composition of tomato seed oil from tomato processing waste seeking to use it as food ingredient (Sogi et al. 1999; Bhullar and Sogi 2000). It also, attempts have been made to use the removed peels from tomato and incorporate it as lycopene source in dry fermented sausages as reported by Calvo et al. 2008. The objective of this work was to assess the qualitative and quantitative differences of chlorophylls (spectrophotometrically) and carotenoids (by HPLC-APCI-MS) in the exocarp and mesocarp of ‘Caiman’ tomato fruit during six stages of fruit ripening.

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Materials and methods

Chemicals and solvents Standards of lycopene and β -carotene were recently purchased from Sigma-Aldrich (St Louis, MO). Methanol (HPLC-grade), tBME (HPLC-grade) hexane, acetone, ethanol, toluene were obtained from JT baker (Baker Mallinckrodt, México). HPLC grade water for identification analyses was prepared by a Milli-Q plus purification system (Millipore Corp. Bedford, MA). All other chemical reagents used were of analytical grade.

Plant materials Tomato (*Lycopersicon esculentum* Cv Caiman) grown in greenhouses in Acámbaro, Guanajuato, México, was harvested at its mature-green stage of ripeness and transported to the laboratory of phytochemicals and nutrition at the Faculty of Natural Sciences of the Autonomous University in Querétaro.

Sample preparation Fruit were ripened in air at 25 °C and sampled for evaluation of whole fruit and skin weights, color, chlorophylls and carotenoids at six different ripeness stages: mature-green, breakers, turning, pink, light-red and red. These ripeness stages were established using the color classification requirements in tomatoes from the United States Standards for Grades of Fresh Tomatoes. At sampling, exocarp (thickness ~1 mm) was separated from the mesocarp using a potato peeler. Both exocarp and mesocarp were collected for analysis, and seeds were discarded. Exocarp samples were frozen and freeze-dried, and mesocarp samples were frozen at –80 °C. Both were light-protected and stored at –80 °C, until analysis. Moisture content was determined in exocarp and mesocarp by freeze-drying until constant weight.

Fruit physiological stage assessment Color (values of L^* , a^* , b^* , C^* and hue) was measured on fresh tomatoes using a Minolta spectrophotometer (Minolta Co. Ltd, Japan). External (exocarp) color was measured on four points around the blossom-end area, while the internal color (mesocarp) was measured on the central flat point of each half of every fruit.

Chlorophyll estimation Chlorophyll extraction was done according to Yahia et al. (2007) with some modifications. Frozen tomato mesocarp (5 g) or freeze-dried exocarp (0.4 g) samples were homogenized in the dark with 10 mL ethanol, sonicated for 20 min at room temperature and centrifuged at 10,000× g during 15 min. The supernatant was recovered and the extraction process repeated on the pellet. Both supernatants were mixed and the absorbance was measured at 642.5 and 660 nm, using a DU-65 Spectrophotometer (Beckman Coulter, Inc., Fullerton, CA). Since chlorophyll in plants is

present in two different chemical forms, chlorophyll content was calculated as chlorophyll a, chlorophyll b and total chlorophyll in mg/L according to 940.03 AOAC method (1997) following the equations: Chlorophyll a = $(9.93 \times A_{660}) - (0.777 \times A_{642.5})$, chlorophyll b = $(17.6 \times A_{642.5}) - (2.81 \times A_{660})$, and total chlorophyll = $(7.12 \times A_{660}) + (16.8 \times A_{642.5})$.

Total carotenoid estimation Extraction of carotenoids was done according to 970.64-AOAC method (2000) with some modifications by Soto-Zamora et al. (2005). Frozen tomato mesocarp (5 g) or freeze-dried exocarp (0.4 g) samples were homogenized for 1 min with 10 mL of extractant solution (hexane:acetone:ethanol:toluene at 10:7:6:7), after which 1 mL of 40 % methanolic KOH was added and heated at 56 °C for 20 min for saponification. It was then immediately cooled in water at room temperature. Ten mL of hexane were added and stirred for 1 min, and 10 mL of 10 % sodium sulfate were added and stirred for 1 min. After phase separation the upper phase was used for carotenoid analysis of this saponified extracts. A non-saponified extract was obtained following the same procedure but neither the 40 % methanolic KOH solution was added, nor the extract was heated at 56 °C. The measurement of total carotenoids was done on top-phase aliquots from the saponified and non-saponified carotenoids extracts in a Beckman DU-65 spectrophotometer at 450 nm. A calibration curve was done using β -carotene in hexane as standard and hexane as the blank.

HPLC-APCI-MS analysis For identification of specific carotenoids, aliquots from the saponified and non-saponified carotenoids extracts (50 μ L) were filtered through 0.45 μ m nylon membrane and injected into the HPLC (HP 1100, Agilent Technologies Co, Palo Alto, USA) with DAD detector coupled to a Time-of-Flight (TOF) mass spectrometer (TOF 6210, Agilent Technologies Co, Palo Alto, USA), using a YMC C30 3- μ m column, 4.6×150 mm in reverse phase at 15 °C. The mobile phase was composed of methanol (A) and tBME (B) following a linear gradient of 100 % (A), and 0 % (B) at minute zero and 0 % (A) and 100 % (B) after 40 min, with a flow rate of 1 mL/min. The mass spectrometer system was equipped with an APCI⁺ interface operating in the positive ionization mode. Nitrogen was the drying gas at a flow rate of 5 L/min, and the nebulizer pressure and temperature were 50 psi and 400 °C, respectively. Fragmentor and capillary conditions were 200 and 4000 V, respectively. A Mass Hunter manager software (version A.02.01) was used. Some carotenoids were identified by comparing the peak chromatographic data (visible spectral characteristics, retention times) and peak-mass-spectrum data with standards. Some carotenoids were tentatively identified by the mass spectrum data (molecular

fragmentation patterns) from the chromatographic/spectrometric peaks. For quantification of β -carotene and lycopene, the filtered carotenoids extracts (50 μ L) were injected in triplicate into the HPLC-DAD equipment. The carotenoid detection was monitored at 450, 470 nm. The additional analytical conditions were established as mentioned above. Calibration curves were prepared from commercial standards of β -carotene (0.02 to 0.10 mg/mL) and lycopene (0.01 to 0.10 mg/mL).

Statistical analysis All values in figures are means of three determinations $\pm$ standard error. Analysis of variance (ANOVA) was conducted and the significance of difference between means was determined by Tukey method at a 5 % significance level. A correlation analysis was performed among color parameters and skin pigment content.

Results and discussion

Fruit characteristics Whole fruit weight ranged from 220 to 260 g. Exocarp weighed about 10 % of the whole fruit. Exocarp and mesocarp from ‘Caiman’ tomato fruit had a moisture content of 91–93 %. External color for different ripeness stages of the tomato fruit exhibited significant changes for L^* , a^* , C^* and hue (h°) values, but not for b^* value (Table 1). Exocarp from “mature-green” tomatoes was completely green, having an a^* value of -7.1 to -6.7 . “Breaker” tomatoes showed a pinkish-reddish pigmentation on the blossom-end area with a^* values from -2.9 to 0.1 . “Turning” tomatoes showed an extended pigmented exocarp area from blossom-end (no more than 30 % of the whole fruit) and an increased a^* value ranging from 6.5 to 12.1 . “Pink” tomatoes showed a more extended reddish area (no more than 60 % of the whole fruit) with a^* values ranging

from 13.8 to 16.4 . “Light-Red” tomatoes exhibited a pinkish-red surface (no more than 90 % of the whole fruit) with a^* values between 20 and 24 , and “red” tomatoes showed a completely red-colored exocarp with a^* values ranging from 24 to 26.6 . L^* value showed a slight decreasing tendency after the “turning” ripeness stage in both, exocarp and mesocarp, whereas the b^* value showed no differences during ripening, neither in exocarp nor in mesocarp. However, the exocarp b^* value was higher than the mesocarp b^* value at every ripeness stage. Exocarp color parameters correlated with tomato pigments. High correlations (r) were observed between total carotenoids and a^* , L^* , C^* and h° values; being 0.924 ($p=0.008$), -0.916 ($p=0.01$), 0.929 ($p=0.007$) and -0.919 ($p=0.009$), respectively. Similarly, lycopene content was highly correlated with a^* (0.94 , $p=0.005$), L^* (-0.946 , $p=0.004$), C^* (0.951 , $p=0.004$), h (-0.933 , $p=0.001$) and with total carotenoids (0.996 , $p=0.001$).

Chlorophyll and carotenoid compounds in exocarp and mesocarp Mature-green tomato exocarp had higher total chlorophyll content than mesocarp (more than twice), but total chlorophyll drastically decreased until disappearance in both exocarp and mesocarp during ripening (Fig. 1). In both exocarp and mesocarp, mature-green tomato had higher chlorophyll a than chlorophyll b content; more than three times in exocarp and more than twice in mesocarp. Furthermore, the exocarp showed higher contents of chlorophyll a (more than twice) and chlorophyll b (about twice) than mesocarp. A similar behavior of chlorophyll degradation and rates of chlorophyll a/chlorophyll b were reported by Kozukue and Friedman (2003). Four carotenoids have been followed in this report during ripening; β -carotene and lycopene were unequivocally identified, whereas γ -carotene and lycopene-epoxide were tentatively identified by means

Table 1 Color parameters for exocarp and/or mesocarp of tomato Cv Caiman at different ripeness stages

Exocarp					
Ripeness stage	L^*	a^*	b^*	C^*	h°
Mature-Green	58.6 ± 2.13^a	-6.9 ± 0.24^a	25.6 ± 1.85^a	26.5 ± 1.83^a	105.2 ± 0.79^a
Breakers	57.4 ± 2.65^{ab}	-1.4 ± 1.53^b	27.7 ± 2.31^a	27.8 ± 2.34^a	92.9 ± 3.09^b
Turning	55.3 ± 1.65^b	9.3 ± 2.80^c	28.4 ± 3.27^a	29.9 ± 3.93^a	72.1 ± 3.36^c
Pink	50.2 ± 2.22^c	15.0 ± 1.31^d	28.0 ± 3.29^a	31.9 ± 3.24^{ab}	61.6 ± 2.66^d
Light red	45.2 ± 1.28^d	22.0 ± 2.03^e	26.3 ± 2.43^a	34.3 ± 3.08^b	50.0 ± 1.28^e
Red	44.5 ± 2.53^d	25.3 ± 1.30^f	24.0 ± 3.20^a	34.9 ± 2.81^b	43.4 ± 3.32^f
Mesocarp					
Mature-green	62.4 ± 7.58^a	-3.0 ± 1.09^a	14.9 ± 4.87^a	15.3 ± 4.99^a	101.3 ± 4.44^a
Breakers	56.9 ± 10.6^a	4.2 ± 2.70^{ab}	15.5 ± 0.63^a	16.2 ± 1.31^a	75.0 ± 8.67^{ab}
Turning	59.1 ± 13.7^a	9.5 ± 5.29^{bc}	12.6 ± 3.99^a	16.5 ± 0.02^a	53.1 ± 20.4^b
Pink	58.0 ± 5.98^a	12.9 ± 1.07^{bcd}	13.3 ± 2.42^a	18.6 ± 0.98^{ab}	45.7 ± 7.68^c
Light red	58.8 ± 8.56^a	14.3 ± 5.52^{cd}	12.8 ± 2.73^a	19.9 ± 2.99^{ab}	43.3 ± 16.0^c
Red	49.7 ± 5.11^a	18.9 ± 2.26^d	11.5 ± 2.48^a	22.2 ± 2.81^b	31.3 ± 4.56^c

Each observation is a mean $\pm$ SD of three replicate experiments. Values in the same column with different superscripts differ significantly ($p < 0.05$)

Fig. 1 Changes in chlorophyll (total, a and b), lycopene and β -carotene content in exocarp and mesocarp of ‘Caiman’ tomato fruit during ripening. MG mature-green; B breakers; T turning; P pink; LR light- Red; R red. Each observation is a mean of three replicate experiments

of their protonated molecular masses (m/z); 537 and 553 for γ -carotene and lycopene-epoxide, respectively (Fig 2). The upper section of Fig. 2 shows the carotenoids chromatographic profile for mesocarp of “red” (fully ripe) tomato. Qualitatively, mesocarp chromatographic profile was similar as that of the exocarp (data not shown). Figure 2 also shows the total ion chromatogram (TIC) for mesocarp in section B, the spectrometric ion extraction for the m/z ion 537 in section C and the spectrometric ion extraction for the m/z ion 553 in section D. The chromatographic peaks in section A were named peak β -carotene, peak 1, peak 2 and peak lycopene and they were also found in the mass-spectrometric profile (TIC: Total Ion Chromatogram) in

Fig. 2 HPLC and mass spectroscopic chromatograms from ‘Caiman’ tomato fruit mesocarp. A HPLC-DAD chromatogram at 470 nm; B total ion chromatogram (TIC) from HPLC-APCI⁺-MS; C ion extraction for m/z 537; D ion extraction for m/z 553. Peak 1 is tentatively γ -carotene and peak 2 is tentatively lycopene-epoxide

section B with a high similarity in the signal level. The ion extraction procedure applied to the m/z ion of 537 resulted in the profile exhibited in section C in correspondence with the peak of β -carotene, peak 1, and peak of lycopene, confirming the m/z of β -carotene and lycopene ions being 537. Peak 1 with the same m/z ion value (537) should be a hydrocarbon-carotene, whereas peak 2 derived from the extraction ion for m/z 553 should correspond to an epoxy-carotene ($537+16$). Mass spectra data for these four peaks are presented in Table 2 where the fragmentation pattern for β -carotene, lycopene and peak 1 corresponded with the characteristic m/z ion value for some hydrocarbon-carotenes being this 537. Whereas peak 2 exhibited an ion fragmentation where the m/z ion 553 was the main ion (base peak) and showed a fragment of 537, as a possible product of de-epoxidation of the ion 553. From this, peak 2 can be tentatively identified as lycopene-epoxide. The four chromatographic peaks were monitored during ripening (Fig. 3). Tomato exocarp and mesocarp had the lowest total carotenoids content at their mature-green stage and both tissues exhibited a significant increase during ripening, being 8- and 4-fold at “red” ripeness stage for exocarp and mesocarp, respectively. In the exocarp, lycopene increased about 25

Table 2 Spectrometric data of carotenoids from saponified extracts of ‘Caiman’ tomato fruit. Peaks are from Figs. 2 and 3

Peak	Tr (min)	Molecular mass [M]	APCI ⁺ -MS Ion fragmentation (m/z) [M+H-ion; base peak %]
β-Carotene	19.5	536	537 [M+H; 100]; 457 [M+H-80, 8.6]; 391 [M+H-146, 8.5]
1 (tentatively γ-Carotene)	25.8	536	537 [M+H; 100]; 457 [M+H-80, 2.7]; 391 [M+H-146, 1.9]; 409 [M+H-128, 5.7]
2 (tentatively Lycopene-epoxide)	28.6	552	553 [M+H; 100]; 537 [M+H-16, 15.6]; 461 [M+H-92; 4.5]
Lycopene	31.6	536	537 [M+H; 100]; 431 [M+H-106, 3.0]; 391 [M+H-146, 1.6]

times, whereas in mesocarp it increased about 9 times. This increase was observed mainly at the very last period of ripening (Fig. 3) in agreement with the results of Giovanelli et al. (1999) in tomato pericarp. β-carotene slightly increased in exocarp and increased about 4 times in mesocarp during fruit ripening. The two carotenoids that were tentatively identified as γ-carotene and lycopene-epoxide slightly increased during ripening. Tomato fruit changes its color from green to red during ripening, as a consequence of chlorophyll degradation simultaneously to carotenoid biosynthesis as it has been observed in our results similarly to the reported by Hobson and Davies (1971) and by Yahia et al. (2007) who observed a significant decrease of chlorophylls a and b and high increases in β-carotene and lycopene content for the ‘Rhapsody’ tomato variety.

The changes in *a** value were expected to be a good indicator for color changes, because of *a** value represent the green-to-red axis at the CIELAB color system. Hue angle value was also a good indicator for color changes in tomato exocarp showing no overlapping among the different

ripeness stages. In this respect, a specific tomato ripeness stage can be determined by *a** or *h*^o values of exocarp. Although color parameters such as *L** and *C** values showed significant changes during ripening, and high correlations with total carotenoid and lycopene contents, these parameters showed a high overlapping between subsequent tomato ripeness stages. Yahia et al. (2007) results exhibited a significant decrease in values *h* and *L** in ‘Rhapsody’ tomato. In the case of chlorophyll, there were significant differences in chlorophyll content between exocarp and mesocarp. The significantly higher chlorophyll content in exocarp could be hypothetically explained assuming that exocarp cells being directly exposed to sunlight should be more photosynthetically active than the mesocarp cells. On the other hand, there were no qualitative differences between the types of carotenoids found in exocarp and mesocarp, and there were no qualitative differences between the carotenoid chromatographic profile of saponified extracts and the profile for the non-saponified extracts, suggesting that the carotenoids naturally found in tomato Cv ‘Caiman’ are not esterified. Peak 1 showed a *m/z* of 537 and a retention time of 25.8 min, which is a time-equidistant value in relation to β-carotene (observed at 19.5 min) and lycopene (observed at 31.6 min). Peak 1 seems to be a hydrocarbon-carotene that according to the column elution method based on a reverse phase linear gradient, suggest to have an equidistant molecular polarity in relation to β-carotene and lycopene. According to this reasoning, peak 1 should correspond to γ-carotene that is structurally formed by half a molecule of β-carotene and half a molecule of lycopene (Fig. 4). Furthermore, it seems that the biosynthetic pathway of carotenoid in ‘Caiman’ tomato is as follows: γ-carotene is derived from the cyclization of one end of the lycopene molecule, and β-carotene is derived from the cyclization of the remaining acyclic end of the γ-carotene molecule (Rodriguez-Amaya 2000). In our results, the dominant pathway of carotenoid biosynthesis seems to be the conversion of lycopene to γ-carotene and then the conversion of γ-carotene to β-carotene, accumulating mainly lycopene. On the other hand, the route of conversion of lycopene to δ-carotene, then to α-carotene, then to α-cryptoxanthin and then to lutein, seems to be absent in this tomato variety (Fig. 4). Furthermore, in our results, neither

Fig. 3 HPLC-DAD carotenoids chromatograms at 470 nm of ‘Caiman’ tomato fruit mesocarp. MG mature-green; B breakers; T turning; P pink; LR light-red; R red. Peak 1 is tentatively γ-carotene and peak 2 is tentatively lycopene-epoxide

Fig. 4 Partial pathway of carotenoid biosynthesis (*top*) and chemical structures for some carotenes (*bottom*)

lutein nor other hydroxy-carotenoid was detected in our saponified or non-saponified extracts. Khachick et al. (2002) reported the presence of lycopene-epoxides in tomatoes and they suggested that the oxidation of lycopene may be part of the natural metabolism in tomatoes or this oxidation can also take place because of the exposure of tomato-based food products to severe heat processing at relatively high temperatures. Our results suggest that lycopene-epoxide was derived from the natural metabolism because no heat treatments were

applied in our experimental approach. Furthermore, because of lycopene-epoxide was detected in both, saponified and non-saponified extracts, the epoxidation of lycopene seems not to be a product of the saponification step during the extraction process. Abushita et al. (2000) reported similar total-carotenoids content in mesocarp to our results, and also reported the presence of lutein, lycopene-epoxide, lycopene and β-carotene in different tomato cultivars. Khachick et al. (1992) using HPLC, reported the presence of low levels of

chloroplast xanthophylls such as neoxanthin, violaxanthin, lutein-epoxide and lutein, but only when highly concentrated extracts of tomato were obtained from a large batch of raw tomato (2 Kg). Tomato exocarp is sometimes removed by some consumers, and also in some processing operations (Barret et al. 1998). However, from the results obtained in our work, it is recommended that exocarp should not be discarded, and should be consumed along with mesocarp, or it can be utilized as source of nutrients due to their high content of nutritional components such as some carotenoids, as lycopene with their antioxidant properties (Stahl and Sies 1996), and their potential reduction of the risk of some diseases (Clinton et al. 1996; Gerster 1997).

Conclusions

Unripe (mature-green) exocarp of 'Caiman' tomato fruit possesses significantly higher contents of total chlorophyll, chlorophyll a and chlorophyll b than mesocarp, whereas, when ripe, exocarp is a much better source of total carotenoids and lycopene than mesocarp. Carotenoids increase in number and quantity during tomato fruit ripening in both exocarp and mesocarp. β -Carotene made its appearance (very slightly) at the mature-green stage, whereas lycopene and γ -carotene at the turning ripeness stage, and finally lycopene-epoxide appeared at the red ripeness stage. Lycopene accumulated at outstanding levels in the fully ripe tomatoes.

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