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Molecular Profiling - Fruit Carotenoids Components of Six American Heirloom Tomatoes *(Solanum lycopersicum)*

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Abstract

Fruit pigments of six vine-ripening American heirloom tomatoes (Solanum lycopersicum) were analyzed: the green-ripe 'Aunt Ruby's German Green', the red-ripe 'Black from Tula', 'Cherokee Purple' and 'German Johnson Regular Leaf' and the yellow-ripe 'Kellogg's Breakfast' and 'Yellow Brandywine Platfoot Strain' which were grown in Hungary (Godollo). In total, twenty-one type of pigments were determined by Reverse Phase (RP) High-Performance Liquid Chromatography (HPLC): the orange colorations of lutein, β-carotene, β-cryptoxanthin, mutatoxanthin and neoxanthin, the red-orange colorations of lycopene, lycopene-epoxide 1, lycopene-epoxide 2, lycoxanthin, 9-cis-lycopene, 13-cis-lycopene, lycopene-diepoxide 1 and lycopene-diepoxide 2 and the third group of colorations of violaxanthin, neochrome, prolycopene, neurosporene-epoxide, neurosporene, ζ(Zeta)-carotene, ζ-carotene-like, and α(alfa)-cryptoxanthin. Tomato 'Black from Tula' showed the highest content of β-carotene (23.56 g kg^{-1}). The highest lycopene content (19.25 g kg^{-1}) was found in the 'Cherokee Purple' and an extremely high prolycopene (syn.: tetra-cis-lycopene or all-trans-lycopene) content was found in the two yellow fruited tomatoes of 'Kellogg's Breakfast' and 'Yellow Brandywine Platfoot Strain' (100.87 and 70. 99 g kg^{-1}, respectively). Brix indexes did not show significant differences. Based on the results suggestions for growing purposes and further use in metabolomics and molecular and DNA profiling are given.

Introduction

Cultivated tomato (*Solanum lycopersicum*; chromosome number 2n = 24; genome size 0.9 x 109 bp) [1,2] has twelve wild tomato relative species, which is divided into two complexes, the Peruvianum complex with two self-incompatible species *(S. chilense* and *S. peruvianum)* and the Esculentum complex, which includes the intercrossable species of *S. arcanum, S. chmielewskii, S. corneliomuelleri, S. habrochaites, S. huaylasense; S. neorickii, S. pennellii, S. pimpinellifolium,* and the two endemic species of *S. cheesmaniae* and *S. galapagense* [3].

Tomato occupies the top position of the total World production of the ten major fleshy fruits with 28% (about 10^8 MT), followed by banana (20%, 7.6×10^7 MT) apple and grapes (about 15% and 5.6% respectively, a total of 5.9×10^7 MT) pear and pineapple (about 5%, 1.9%, respectively, a total of 1.8×10^7 MT) papaya and strawberry (2% and 1%; 7×10^6MT and 3.4×10^6MT, respectively)[4].

By molecular constitutions, more than 700 carotenoids (including the annotated 281 molecules) [5] have been identified [6,7] from different plant sources [8-12]. By molecular structure, carotenoids are divided into two main groups of the non-oxygenated carotenoids i.e. carotenes and oxygenated carotenoids, i.e. xanthophylls. Carotenes are further divided by acyclic-carotenes (e.g. phytoene, phytofluene, ζ-carotene, neurosporene, and lycopene) and cyclic-carotenes (e.g. δ- and γ-carotene, and α- and β-zeacarotene with one structural ring; and α- and β-carotene with double rings). Xanthophylls (syn. carotenols or hydroxycarotenoids) are also further divided for four types as acyclic (e.g. lycoxanthin and lycophyll) and cyclic groups (e.g. rubixanthin; and α and β-cryptoxanthin, zeinoxanthin, zeaxanthis and lutein). The third group of xanthophylls includes epoxy-carotenoids (e.g. antheraxanthin, auroxanthin, neoxanthin, luteoxanthin, violaxanthin, lutein-5-6-epoxyde and β-carotene-5, 6-epoxide) and the fourth group of xanthophylls comprises some unique carotenoids (e.g. capsanthin, capsorubin, crocetin and bixin) [13]. In the whole carotenoid biosynthesis the phytoene (C40H64) synthase (PSY) is the rate-limiting enzyme [1].

Color of tomato fruit skin and flesh is one of the most important quality components of the tomato in the market. The main acyclic carotenoids of ζ-carotene (its color is light-yellow), neurosporine (yellow-orange), lycopene (red-orange), cyclic-carotenoids of γ-carotenes (pink-orange) and β-carotene (orange) are the main tomato fruit colorant, and slightly depended on the elution solvents used for the analyses [13,14]. The amount of β-carotene, the main orange colorant and lycopene which causes red-to-orange coloration, are the predominant tomato pigments [15]. Chlorophylls, the green pigments of unripe fruits, breaks down during ripening, except in cultivars with 'black' colored fruits, where persistent chlorophyll content gives purplish-brown color together with the red-to-orange colorations. Persistent chlorophyll content is regulated by gf (green flesh) gene, that encodes a Stay-Green senescence-related regulator, and it finally results in the mix of red and green color, which seems black/brown/chocolate color [16].

Functionally, carotenoids, especially β-carotene, primarily act as accessory and photoprotective pigments for chlorophyll a and b of LHCs (Light Hirvesting Comlex) of photosystem I and II (PSI and PSII) during photosynthesis [17] in leaves, fruit skins and flowers. They absorb the sunlight in a broader range of the blue spectrum (400-500 nm) than chlorophylls, and they transfer this absorbed extra energy to chlorophyll a of the photosynthetic reaction center. Carotenoids also supply substrates for the biosynthesis of the plant growth regulator abscisic acid (ABA) [18]. All carotenoids can became crystallized in the chromoplasts during the transition of chloroplast to chromoplast, or transported and accumulated in lipid bodies.

In animals, ceto-carotenoid type astaxanthin is responsible for the orange color of salmon meat and lobster shell. Feather colors of the birds also came from carotenoids [19]. Chicken egg yolks are rich in lutein and zeaxanthin [20]. In human nutrition and health, carotenoids act as anti-aging and anti-cancer substances and provide provitamin-A (e.g. β-carotene, β-cryptoxanthin and α-carotene) [10]. Wide ranges of carotenoids of algae, fungi and bacteria have also been identified and characterized [21].

Genes of enzymes involved in carotenoids synthesis are encoded in plant nuclear genomes and gene products are transported either to the cytoplasm (including mitochondria) (i.e. mevalonate pathway) or to the plastids (i.e. non-mevalonate pathway) where they are post-translationally modified and activated [17].

Tomato fruits are also rich in phenols and polyphenols (like gallic acid, catechin, rutin, ferulic acid etc.) and vitamin E (α and γ-tocopherol), which are also responsible for the antioxidant capacity of the soluble phase of fruit sap [22].

The aim of the study presented was to determine the carotenoids content of six American heirlooms for pigment compositions and to describe the differences with the aim of utilizing the information for future breeding purposes.

Materials and Methods

Plant materials

A greenhouse study was conducted in the summer (June 1 to Sept 31) of 2014 at the spring at Experiment Station, Szent István University, Hungary, Europe. Tomatoes were seeded into Canadian Growing Mix 2 (Conrad Fafard Inc., Agawan, MA) in 72-cell flats and transplanted into trade gallon pots after five weeks in accordance with standard transplant production (Peat Brown OPM Multipack 025W, Kekkila). Transplants were fertilized once a week with a 20N-4.4P-16.6K water soluble fertilizer (Peter's Water Soluble Plant Food 20-10-20) (Scotts Co, Marysville, OH) at a rate of 265 mg/L of N. Plants were allowed to grow for three weeks and then potted into three-gallon (24.13 cm tall, 27.94 cm diameter 11.36 L) containers.

Plants were watered twice daily and fertigated weekly with 20N-4.4P-16.6K water soluble fertilizer (Peter's Water Soluble Plant Food 20-10-20) (Scotts Co, Marysville, OH) at a rate of 265 mg/L of N. Tomato plants were staked with three foot bamboo stakes attached with twist ties. All suckers below the first flower cluster were removed in accordance with Kemble et al. [23]. Treatments were randomly assigned to six individual plants in a completely randomized design with three replications.

Data gathered included germination vigor and fruit quality characteristics. The first six ripened fruit grown on the same vine nodes were collected and processed for HPLC and Brix analyses according to Pek et al. [24] and Daood et al. [25].

Extraction of carotenoids

Lipids and fat soluble pigments collected from the raw tomatoes were extracted according to Abushita et al. [22] with slight modifications. Five-gram samples of tomato fruits were taken from each variety in four replicates (6 varieties x 4 replicates = 24 samples) and grind in a crucible mortar with quartz sand followed by adding 20 mL cc. methanol. The mixture was then transferred quantitatively to a 100 mL conical flask and 70 mL of a 6:1 dichloroethane:methanol solution was added. The mixture was shaken for 15 min by a mechanical shaker till the dichloroethane phase was clearly separated from the polar phase (water + methanol). The two phases were separated and the lower layer containing lipids dissolved in dichloroethane was dried over anhydrous sodium sulphate. Finally, the organic solvent was evaporated under vacuum by rotary evaporator at 40˚C. The residues were re-dissolved in HPLC-grade acetone before injection onto HPLC column [25].

HPLC analysis

For Reverse Phase (RP) High-Performance Liquid Chromatography (HPLC) a Chromaster Hitachi HPLC instrument was used coupled with diode-array detector (Model 5430) and an auto sampler (Model 5210) and a gradient pump (Model 5110). The instrument and analyses was operated by EZchrom Elite software (version 3.3.2.SP2). The separation of carotenoids was performed on Accucore (Thermo Scientific) C-30, 2.7 µ 150 x 4.0 mm column with gradient elution of (a) tetra-butyl-methyl-ether (TBME) and (b) methanol (MetOH). The gradient elution started with 100% TBME, and changed to 30% TBME in MetOH for 25 min, stayed isocratic for 5 min and finally turned to 100% TBME for 5 min according to Daood et al. [25].

HPLC peak identification was based on the comparison of spectral properties and retention time of carotenoids separated with those of available molecular standards of lycopene, β-carotene and zeaxanthin (Sigma-Aldrich, Budapest, Hungary). In case of carotenoids with no available standards, the peaks were identified according to their spectral characteristics and chromatographic retention according to Ritter and Purcell [26] Liaaen-Jensend and Lutences [27] and Borsarelli and Mercadante [28]. The cis isomers of lycopene were identified on the basis of appearance of an extra absorption wavelength at 340 nm and 361 nm. The 9-Z- and 13-Z cis lycopene isomers were identified according to the II-value, which equals to absorbance at 361 nm over absorbance at the maximum wavelength according to Liaaen-Jensend and Lutences [27]. The column effluents were detected and integrated at their maximum absorption wavelength for quantitative determinations and were quantified as either lycopene- or β-carotene equivalents (µg g-1 equal to g kg-1) according to their spectral characteristics according to Rodriguez-Amaya [13].

Statistical analysis

Mean values of four (n=4) HPLC measurements and 95 % Confidence Interval for Mean (CI95% = x ± d) confidence intervals were calculated at P 95% confidence level by SPSS program package. For dendrogram (using Average Linkage Within Group) and Canonical Discriminant Functions Analyses the SPSS program package was also used.

Discussion

Morphology and genetics: The germinations vigor of the heirloom's seeds was found the strongest in 'Kellogg's Breakfast' [5] followed by 'Yellow Brandywine Platfoot Strain' [6], 'Cherokee Purple' [3], 'German Johnson Regular Leaf' [4], 'Aunt Ruby's German Green' [1] and 'Black from Tula' [2]. The 'Cherokee Purple' [3] was found to be a 'potato leaved' type (Figure 1).

Figure 1: Germination vigor of the six American heirloom tomatoes (Solanum lycopersicum) studied (three pots each) (1) 'Aunt Ruby's German Green' (2) 'Black from Tula' (3) 'Cherokee Purple' (4) 'German Johnson Regular Leaf' (5) 'Kellogg's Breakfast' and (6) 'Yellow Brandywine Platfoot Strain'.

By fruit shape, all heirlooms showed extremely puffy fruits with hollow locules (i.e. fruit cavity with seeds) which suggested the presence of spf2 genes (superpuff) with bell pepper shaped fruits [29]. Fruits of 'Aunt Ruby's German Green' [1] showed serious radial crakes, which phenomenon is regulated by two dominant genes of CE and RA. All the other tomatoes showed some radial crack resistance, which is controlled by recessive alleles of cr and ra however 'Black from Tula' [2] was found susceptible to fruit bursting, which is regulated by dominant gene BT (burst types) [29].

The first three American heirlooms [1-3] were found to carry Abg (Aubergine) gene, which cause purple fruit epidermis particularly on shoulder, and the 'Yellow Brandywine Platfoot Strain' [6] was found to probably carry fs (fruit stripe) gene, which causes dark green radial stripes at the opposite locules (Figure 2) (Table 1).

Figure 2: Fruit samples of the six American heirloom tomatoes (Solanum lycopersicum) studied. (1) 'Aunt Ruby's German Green' (2) 'Black from Tula' (3) 'Cherokee Purple' (4) 'German Johnson Regular Leaf' (5) 'Kellogg's Breakfast' and (6) 'Yellow Brandywine Platfoot Strain'.

Gene Symbol	Gene Name	Description
Abg	Aubergine	Purple fruit epidermis purple particularly on shoulder
Af	Anthocyanin fruit	Anthocyanin in green and ripe fruit (absent when shaded)
ant	Aurantia (ant1)	Short thick stems, light green pinnae; light orange fruit with colourless pericarp
at	Apricot (yellow)	Yellow-pink colour of fruit flesh
aur	Aurantiaca (aur1)	Small, pointed, yellowish light-green pinnae, and orange fruit
B	β-carotene	High β-carotene, low lycopene in ripe fruit
Bc	crimson (ogc)	Increased fruit lycopene content, phenotype similar to Bog
Bm	minutum	High β-carotene, low lycopene in ripe fruit
Bog	old gold (og)	Corolla tawny orange; increased fruit lycopene
del	Delta	Reddish-orange fruit, due to inhibition of lycopene, and increase of δ-carotene
dg	dark green	Dark green colour appears as fruit develops, then persists until onset of ripening
dps	diospyros	Fruit tissue is dusky orange
gdf	Gold Fleck	Small dark green spots on immature fruit, which turn yellow on ripe fruit
gf	green flesh	Persistent chlorophyll giving ripe fruit purplish-brown colour
glu	glutinosa (glu1)	Dark green, shiny fruit with sticky epidermis; poor germination
gr	green ripe (gr)	Resembles gf, except that center of fruit turns red

gs	green stripe	Radical green stripes in epidermis of unripe fruit; golden in ripe fruit
hp-1	high pigment (hp2)	Intensified chlorophyll, carotenoids and ascorbic acid contents
r-	yellow flesh	Yellow colour of ripe fruit flesh
r(1s)	yellow flesh (1s)	Yellow colour of ripe fruit flesh
r(2s)	yellow flesh (2s)	Yellow fruit flesh; lighter yellow flowers
rprov4	yellow flesh	Yellow colour of ripe fruit flesh
rprov5	yellow flesh	Yellow colour of ripe fruit flesh
ry	yellow flesh	Reddish yellow: Likely allele of r with reddish flesh tones in ripe fruit
sh	sherry	Fruit flesh yellow with reddish tinge
t	tangerine	Fruit flesh and stamens orange coloured
vo	virescent orange	Fruit flesh orange, redder in outer walls
y	Colourless fruit epidermis	Fruit epidermis lacks pigmentation (clear skin)

Table 1: Tomato genes encoding for fruit colours (Tfm 2014; Tgc 2012)

Genetically, the synthesis of plant carotenoids links to the processes of fruit ripening (i.e. cell wall softening), which is regulated about 50 genes in tomato [1]. Regulation/modulation of fruit ripening of all fleshy fruit plant species has profound agronomic importance. Recently, fresh market tomatoes include only 'long shelf-life' varieties, which are natural mutants, and carry ripening inhibitor (RIN) gene(s). The main RIN genes are the rin (ripening-inhibitor MADS-box gene) nor (non-ripening transcription factor gene) nr (never-ripe ethylene signaling) nr-2 (never-ripe 2) / gr (green-ripe) and cnr (colorless non-ripening) [30,31]. Of them, one of the earliest tomato fruit ripening mutants was the dominant NR (Never-ripe) mutation [32]. This mutation was shown to be the consequence of a single amino acid change in one of the seven ethylene receptors (LeTR1-7) [23]. The first registered RIN tomato, the 'Daniela'(FA144) an indeterminate long-self life hybrid, was released in about 1992 by the BonTom Tomato Breeding Group (Faculty of Agriculture, Hebrew University of Jerusalem, Israel) [3]. Green-Ripe (GR) and its allele, NR-2, were also found as a dominant nonripening mutation [31-35]. One of the other unique natural tomato mutants is a dwarf type 'Micro-Tom' [36] with obviously small fruits.

During the transgenic GM programs, the first FDA-approved transgenic food of Flavr-Savr tomato was released in 1994 [37] followed by further delayed ripening tomatoes (DNAP, Zeneca/Peto and Monsanto) [38].

In our work presented, none of the studied heirlooms showed the presence of any RIN genes by visual observation (Figure 2), as the fruits were ripened and softened very quickly (in some days immediately after the total fruit size development). These fruit characters obviously suggest the marketing of these heirlooms for fresh consumption and tinned juice production.

All heirlooms also showed 'indeterminate' growing habit. Nearly a century ago, a spontaneous mutation in SP (self-pruning) gene family spawned the 'determinate' tomato development which now dominate the tomato market being beneficial for mechanical harvesting [1].

Pigments characteristics: Fruit pigments compositions of the heirlooms showed three main groups (Figures 3) and (Figure 4) (Table 2). In the irst group, on the contrary of the extremely low carotenoids compositions (Table 2) 'Aunt Ruby's German Green' [1] was found delicious taste due probably to its tasty compositions of other organic fruit components of carbohydrates and organic acids [39].

In the second group, 'Black from Tula' [2] showed the highest content of β-carotene (23.56 ± 9.17 ug g^{-1} FW). his β-carotene content showed very high level compared tomato cv. 'Strombolino' (3.27 ± 0.17 ug g^{-1} FW) measured in our lab by using the same technology (Helyes et al.,) [12]. he highest lycopene content (19.25 ± 14.25 ug g^{-1} FW) was found in the 'Cherokee Purple' [3] (Table 1) which level was similar to that of 'German Johnson' [4] (17.93 ± 6.29 ug g^{-1} FW). hese lycopene contents showed lower levels then in tomato cv. 'Strombolino' (62.6 ± 2.77 ug g^{-1} FW) [12] however it was higher than in a vine-ripened tomato 'Lemance F1', which yielded 4.5± 1.40 ug g^{-1} FW [24].

he third group comprised the two yellow fruited tomatoes of 'Kellogg's Breakfast' [5] and 'Yellow Brandywine Platfoot Strain' [6]. Both heirlooms showed extremely high level of prolycopene content (100.87 ± 51.4 and 70.99 ± 15.27 ug g^{-1} FW, respectively) (Table 1) which were found as extreme levels compared to the studies of Kachanovsky et al. [40], which was 4.5 ± 0.8 ug g^{-1} FW.

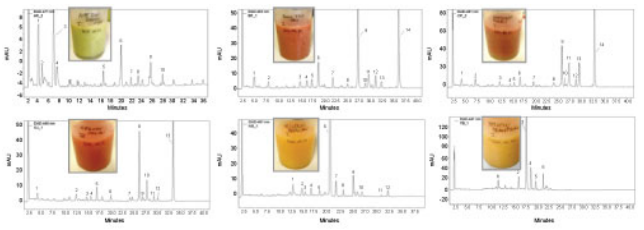

Figure 3: HPLC profiles of carotenoids of the six American heirlooms tomatoes (Solanum lycopersicum) studied AR - 'Aunt Ruby's German Green'.: 1 - Lutein, 2 - Chlorophyllid B, 3 - Chlorophyll B, 4 - Chlorophyll B, 5 - Neoxanthin, 6 - β-carotene, 7-13-cis-lycopene, 8 - Rubixanthin, 9 - Lycopene, 10 - γ-carotene. BF - 'Black from Tula': 1 - Lutein, 2 - Chlorophyll B, 3 - Lycopene diepoxide, 4 - Mutatoxanthin, 5 - Neoxanthin, 6 - cis-neoxanthin, 7 - Lycopene-epoxide 1, 8 - cis-β-carotene, 9 - β-carotene, 10 - Lycoxanthin, 11 - 13-cis-lycopene, 12 - Rubixanthin, 13 - 9-cis-lycopene, 14 - Lycopene. CP - 'Cherokee Purple': 1 - Lutein, 2 - Chlorophyll B, 3 - Lycopene diepoxide, 4 - Mutatoxanthin, 5 - Neoxanthin, 6 - Cis-neoxanthin, 7 - Lycopene-epoxide 1, 8 - Lycoxanthin, 9 - β-carotene, 10 - 15-Cis-lycopene, 11 - 13-Cis-lycopene, 12 - Rubixanthin, 13 - 9-Cis-lycopene, 14 - Lycopene. GJ - 'German Johnson Regular Leaf': 1 - Lutein, 2 - Lycopene diepoxide, 3 - Mutatoxanthin, 4 - Neoxanthin, 5 - Cis-neoxanthin, 6 - Lycopene-epoxide 1, 7 - Lycoxanthin, 8 - β-carotene, 9 - 15-Cis-lycopene, 10 - 13-Cis-lycopene, 11 - Rubixanthin, 12 - 9-Cis-lycopene, 13 - Lycopene. KB - 'Kellogg's Breakfast': 1 - Prolycopene epoxide, 2 - Mutatoxanthin, 3 - Neoxanthin, 4 - Cis-neoxanthin, 5 - Neochrome, 6 - Prolycopene, 7 - Proneurosporene, 8 - Violaxanthin, 9 - β-carotene, 10 - 13-Cis-lycopene, 11 - 9-Cis-lycopene, 12 - Lycopene; YB - 'Yellow Brandywine Platfoot Strain': 1 - Prolycopene epoxide, 2 - Neochrome, 3 - Prolycopene, 4- Proneurosporene, 5 - ζ -carotene, 6 - α-cryptoxanthin; Photos of the meshed fruit saps prepared for HPLC analysis are indicated.

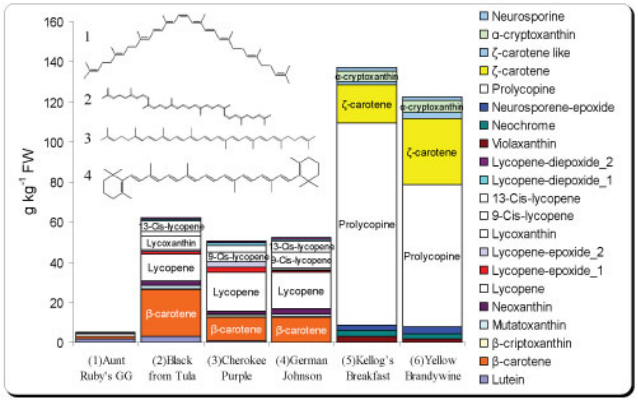

Figure 4: Cumulative carotenoids components (Table 1) of the six American heirloom tomatoes (Solanum lycopersicum) studied. Molecular formulas of the main non-oxygenated carotenoids of neurosporene, lycopine, β-carotene and ζ-carotene are indicated. The main metabolic steps (numbers 1 to 4) and molecular formulas from non-cyclic carotenoids of (1) ζ-carotene → (2) prolycopene → (3) lycopine to, → cyclic carotenoid (4) β-carotene are indicated.

Carotenoids	Mol. Formulas	MW	I.'Aunt Ruby's German Green' (1)	II.a. 'Black from Tula' (2)	II.b. 'Cherokee Purple' (3)	III.a. 'German Johnson' (4)	III.b. 'Kellog's Breakfast' (5)	III.c. 'Yellow Brandywine' (6)
ζ-carotene	C40H60	540.904	0	0	0	0	19.14 ± 15.39	32.65 ± 22.21
ζ-carotene like	C40H60	540.904	0	0	0	0	1.68 ± 1.58	3.33 ± 3.21
Neurosporene	C40H58	538.890	0	0	0	0	1.55 ± 0.59	1.42 ± 0.38
Neurosporene-epoxide	C40H58O	549.449	0	0	0	0	2.49 ± 1.76	3.68 ± 2.33
Prolycopene (tetra-cis-lycopene; all-trans-L.)	C40H56	536.889	0	0	0	0	100.87 ± 51.4	70.99 ± 15.27
Neochrome	C41H58O3	598.8974	0	0	0	0	3.1 ± 1.54	2.26 ± 0.41
α-cryptoxanthin	C40H56O	552.872	0	0	0	0	5.33 ± 3.12	5.96 ± 1.29
Violaxanthin	C40H56O4	600.870	0	0	0	0	2.92 ± 1.17	1.89 ± 0.65
Lycopene	C40H56	536.873	0.23 ± 0.26	13.33 ± 7.48	19.25 ± 14.25	17.93 ± 6.29	0	0

β-carotene	C40H56	536.873	1.15 ± 1.98	23.56 ± 9.17	11.51 ± 3.32	12.2 ± 3.34	0	0
9-Cis-lycopene	C40H56	536.873	1.35 ± 2.31	2.2 ± 3.82	4.5 ± 5.39	7.84 ± 2.24	0	0
13-Cis-lycopene	C40H56	536.873	0.08 ± 0.02	5.04 ± 13.06	3.68 ± 3.15	5.28 ± 2.77	0	0
Lycopene-epoxide 1	C40H56O	552.433	0	1.45 ± 0.74	2.55 ± 2.63	0.81 ± 0.36	0	0
Lycopene-epoxide 2	C40H56O	552.433	0	0.81 ± 0.74	2.83 ± 5.61	0.76 ± 0.18	0	0
β-cryptoxanthin	C40H56O	552.872	0	0.35 ± 0.22	0.96 ± 0.1	0.67 ± 0.19	0	0
Lycoxanthin	C40H56O	552.872	0	7.13 ± 13.3	0	0.77 ± 0.29	0	0
Lycopene-diepoxide_1	C40H56O2	568.428	0.2 ± 0.28	0.77 ± 0.7	1.1 ± 0.07	1.04 ± 0.35	0	0
Lycopene-diepoxide_2	C40H56O2	568.428	0.2 ± 0.28	1.06 ±1.27	1.03 ± 0.14	1.09 ± 0.13	0	0
Lutein	C40H56O2	568.871	1.77 ± 1.99	2.93 ± 2.87	0.99 ± 1.2	0.19 ± 0.04	0	0
Mutatoxanthin	C40H56O3	584.871	0	1.62 ± 0.74	0.75 ± 0.28	1.25 ± 0.27	0	0
Neoxanthin	C40H56O4	600.870	0	2.11 ± 2.41	1.52 ± 1.05	2.69 ± 0.5	0	0
Total carotenoid content (µg/g)			5,19 ± 0	64,04 ± 0	51,73 ± 0	53,32 ± 0	140,16 ± 0	122,18 ± 0
Brix			4,48 ± 0,17	4,7 ± 0,17	4,32 ± 0,3	4,25 ± 0,16	4,63 ± 0,24	4,84 ± 0,39

Table 2: Fruit carotenoids components (µg g-1 FW) of the six American heirloom tomatoes (*Solanum lycopersicum*) studied. The three pigment groups are indicated I to III.

As the later stages of main metabolic desaturation steps of the synthesis of plant acyclic carotenoids (C40H60), which goes through ζ-carotene → neurosporene → prolycopene (and → lycopene), we may suggest that heirlooms 'Kellogg's Breakfast' [5] and 'Yellow Brandywine Platfoot Strain' [6] may be the most economic tomatoes by blocking (and saving metabolic energy) the further enzymatic reactions at the stage of prolycopene, which would include the cyclizations of lycopene either to α-zeacarotene → δ-carotene → ε-carotene, or to β-zeacarotene → γ-carotene → α- and β-carotene [41].

As the worldwide monoculture of tomatoes (i.e. less then ten cultivars are cultivated in the World), these unique heirlooms may provide basal material for feeding experiments and medical studies. e.g., eggs of carotenoid-fed female birds *(Larus fuscus)* were found with high carotenoid contents but low Ig immune globulins (i.e. passive immunity). Whereas, control females produced eggs containing low carotenoid but high Ig content, which results indicated a carotenoid-mediated effects of phenotypes for ecological fitness of mother birds and their offspring [8]. Carotenoids of lutein and zeaxanthin supplemented in male zebra finch birds *(Taeniopygia guttata)* showed elevated blood carotenoid levels with increased cell mediated and humoral immune responses than control birds, which were coupled with brighter beak coloration, which suggested that carotenoids-based colour signals in birds may directly signal male health via the immunostimulatory action of ingested carotenoid pigments [28]. In depletion of carotenoids of nineteen healthy adult people who were fed at controlled low carotenoids diets for 10 weeks, of the six major human serum carotenoids of lycopene, β-carotene, α-carotene, lutein, zeaxanthin and β-cryptoxanthin, the lycopene concentration showed sharp decrease compared to other carotenoids [12]. This reference indicated that lycopene appears to be the physiologically most important antioxidant of human body [13].

When a comparative dendrogram (Figure 5a) analysis were carried out for carotenoids contents (Table 1) it revealed based on the four replicated measurements [1-24] that 'Aunt Ruby's German Green' [1] produced the most homogenous fruit set by giving single separate Clade 1 (samples of AR 1-4), which was close to 'Kellogg's Breakfast' [5] (samples of KB 17-20) and 'Yellow Brandywine Platfoot Strain' [6] (samples YB 21-24) (Clade 2 of Figure 5a). Heirlooms of 'Black from Tula' [2] (samples BF 5-8), 'Cherokee Purple' [3] (samples CP 9-12) and 'German Johnson Regular Leaf' [4] (samples 13-16) showed divers/not stable carotenoids compositions (Figure 5a).

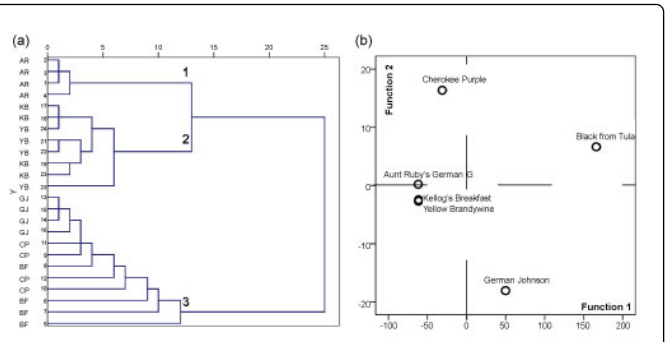

Figure 5: Dendrogram (a) and Canonical Discriminant Functions (b) analysis of the carotenoids contents of six American heirloom tomatoes (Solanum lycopersicum) studied. AR (1 to 4) – 'Aunt Ruby's German Green' (1) BF (5 to 8) – 'Black from Tula' (2) CP (9 to 12) 'Cherokee Purple' (3). GJ (13 to 16) – 'German Johnson Regular Leaf' (4) KB (17-20) – 'Kellogg's Breakfast' (5) YB (21-24) – 'Yellow Brandywine Platfoot Strain' (6) The level of dissimilarity (i.e. Squared Euclidean Distances) (0 to 25) the three main clades and the numbers of the four measurements of each heirloom are indicated.

Based on the whole carotenoids compositions, which comprised 21 pigments, we tried to present an ultimate biochemical genotype identificication [11] by using discriminant analysis (Figure 5b). The result revealed a close group of three heirlooms of 'Aunt 'Ruby's German Green' [1], 'Kellogg's Breakfast' [5] and 'Yellow Brandywine Platfoot Strain' [6] with the most similar carotenoids compositions [42].

Conclusion

In conclusion, as a result of high prolycopene and ζ-carotene contents, the two yellow fruited heirloom tomatoes 'Kellogg's Breakfast' [5] and 'Yellow Brandywine Platfoot Strain' [6] are suggested to be involved in breeding programs to identify further gene markers for yellow fruit coloration. Heirlooms 'Aunt Ruby's German Green' [1] - due to its fruit taste and three red-fruited heirlooms of 'Black from Tula' [2] 'Cherokee Purple' [3] and 'German Johnson' [4] due to their high lycopene and β-carotene contents, seems to have high potential for more intensive recultivation purposes. The molecular profiling applied in this study also provides further use for metabolomics.

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