PAPER

PHENOLIC COMPOUNDS , CAROTENOIDS AND ANTIOXIDANT ACTIVITY IN FIVE TOMATO (*LYCOPERSICON ESCULENTUM* MILL.) CULTIVARS

A. ZANFINI[1], G.G. FRANCHI[2], P. MASSARELLI[2], G. CORBINI[1] AND E. DREASSI*[1]

[1]Dipartimento di Biotecnologie, Chimica e Farmacia, Università degli Studi di Siena, Via A. Moro 2, 53100 Siena, Italy

[2]Dipartimento di Scienze Mediche, Chirurgiche e Neuroscienze, Università degli Studi di Siena, Strada delle Scotte 6, 53100 Siena, Italy

*Corresponding author. elena.dreassi@unisi.it

ABSTRACT

In this study we examined the antioxidants content (polyphenols and carotenoids) and the total hydrophilic (HAA) and lipophilic (LAA) antioxidant activity of red (cv. Shiren and Red Pear), yellow (cv. Yellow Pear-shaped), pale yellow (cv. Snowball) and black (cv. Black Trifele) ripe tomato fruits. For each studied cultivars, the HAA was higher than the LAA. The correlation between antioxidants (polyphenols and carotenoids) and TEAC values (HAA and LAA) was also estimated and the only significative correlation was obtained between rutin and TEAC-HAA. Statistical analysis shows significative differences in antioxidants content (especially for lycopene and β-carotene) between the analyzed tomato cultivars.

Keywords: ABTS, antioxidants, β-carotene, HAA, LLA, lycopene, rutin, TEAC, tomato

1. INTRODUCTION

The nutraceutical quality of tomato fruits is related to their antioxidant and antitumoral properties. The habitual consumption of tomatoes has been associated with decreased risk of chronic degenerative diseases including certain types of cancer and heart diseases (LAVELLI *et al*., 2000; GIOVANNUCCI, 1999, 2002; RAO and AGARWAL, 1998). Epidemiological findings confirmed that the beneficial health effects are due to the presence of bioactive molecules such as carotenoids, particularly lycopene, and polyphenolic compounds, particularly flavonoids. Lycopene has been considered the most efficient for quenching singlet oxygen (DI MASCIO *et al*., 1989). Its protective role in human health has been well described in numerous papers which showed that the dietary intake of lycopene is associated with a decreased risk of prostate cancer and degenerative diseases (GIOVANNUCCI, 1999, 2002; RAO and AGARWAL, 1998, 1999). In human diet, tomatoes and tomato products are the predominant sources of lycopene. The lycopene contents and the qualitative and quantitative composition of tomato fruits depend on cultivar, ripening stage, climatic conditions, etc (ABUSHITA *et al*., 2000; ZANFINI *et al*., 2007). Many authors have focalized their research on the variations in the carotenoid profile in relation to different cultivars (ABUSHITA et al., 1997, 2000; LEONARDI *et al*., 2000; ZANFINI *et al*., 2007). Similar studies focused their attention on the polyphenolic compounds such as flavonoids and hydroxycinnamic acids. The phenolic fraction of tomato fruits contains quercetin, naringenin, rutin and chlorogenic acid as the main compounds (CLIFFORD, 1999; HERTOG *et al*., 1992; JUSTESEN *et al*., 1998). Their quantitative changes in relation to cultivar, season and country of origin were also investigated and the ability of these compounds as scavengers of peroxyl radicals has been well described (HALLIWELL, 1999; STEWART *et al*., 2000).
The antioxidant capacity of tomato fruits has been widely investigated and a clear influence of the genetic factors (type of cultivar) has been found. The tomato cultivars characterized by a high carotenoid contents showed the highest antioxidant activity and a strong correlation between the antioxidant power and lycopene content was also found (RAFFO *et al*., 2002, 2006; ZANFINI *et al*., 2010).
Today, especially in the Mediterranean area, black, white or yellow tomato fruits are commonly present in local markets and used as fresh products for salads or for culinary preparations. The aim of the present study was to examine the polyphenolic fraction, the lycopene and β-carotene contents and the hydrophylic and lipophilic antioxidant activities of red, yellow, pale yellow and black tomato fruits. This work is justified by the fact that the color of fruits and vegetables can be an index of the antioxidant potential and may be assist in the selection of food consumption (CHÁVEZ-MENDOZA *et al*., 2015). We analyzed five different cultivars: two red cultivars, the cv. Read Pear which is characterized by a typical pear shaped fruit and the more commercial cv. Shiren which is a conventional cherry type tomato; the cv. Yellow Pear-shaped with yellow pear shaped fruits; the cv. Snowball with pale yellow fruits and the black cultivar cv. Black Trifele.

2. MATERIALS AND METHODS

2.1. Reagents and standards

All solvents used were of HPLC grade from BHD (Poole, England). The β-carotene, lycopene, vanillin, kaempferol-3-O-glucoside, naringin, naringenin, and the acids gallic, protocatechuic, vanillic, chlorogenic (4-CQA), caffeic, benzoic and cinnamic were purchased from Sigma-Aldrich Chemie Gmbh (Steinheim, Germany); lutein, quercetin

and rutin standard from ICN Biochemical Inc. (Ohio, USA); Folin-Ciocalteau reagent, magnesium oxide and silica gel 60 (0.063-0.200 mm) from Merck (Darmstadt, Germany); β-apo-8'-carotenal and ABTS from Fluka Chemie (Buchs, Switzwrland); Trolox from Hoffman La Roche Aldrich Chem. Co. (Saint Louis, MO, USA).

2.2. Plant material and tomato sampling

Different tomato cultivars were investigated: *Lycopersicon esculentum* Mill. cv. Red Pear, cv. Snowball, cv. Black Trifele and cv. Yellow pear-shaped. Seeds were from Graines Baumaux - Mirecourt - France and from Baker Creek Heirloom Seeds Co., Mansfield (MO), USA. The commercial cherry type cv. Shiren was also analyzed.
Seeds were allowed to germinate under glass in February-March 2014, then plants were transplanted and cultivated in open air nearby Siena (Southern Tuscany, Italy, 43°15'11.8"N 11°36'11.5"E) under the climatic conditions typical of the Mediterranean area.
Figure 1 shows for illustration purpose the colors of various tomato cultivar and below are listed their morphological characteristics.

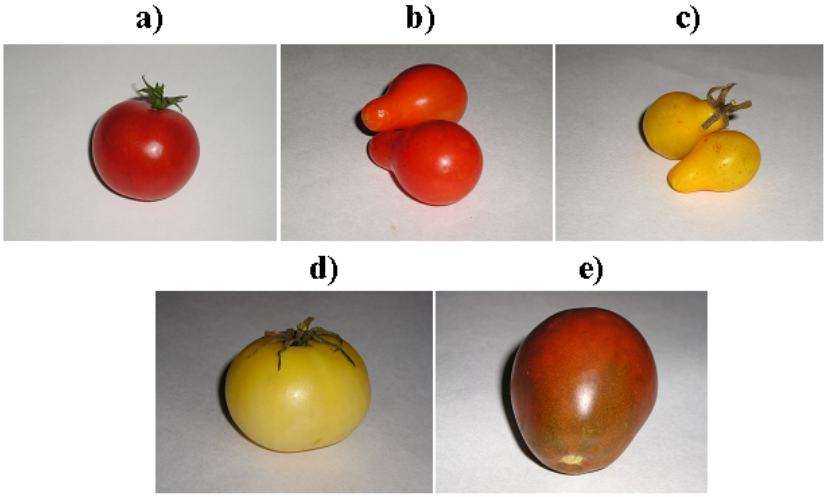

Figure 1. Sample of various tomatoes fruits used in the analysis: (a) cv. Shiren; (b) cv. Red Pear; (c) cv. Yellow Pear-shaped; (d) cv. Snowball and (e) cv. Black Trifele.

Yellow Pear-shaped (or Yellow Pear): plants are indeterminate and hardy. They produce bright yellow, pear-shaped cherry tomatoes with a sweet, mild flavor. They ripe in 75-80 days and are 25-50 mm long. Red Pear (or Red Pear-shaped) is almost analogous, with bright red fruits. Snowball (or White Beauty) is a cultivar introduced in North America in the mid-1800s. Plants are indeterminated and yield very heavy crops of 140 up to 220-340 g, 65-75 mm, yellow-white, somewhat flattened round tomatoes with very mild sweet flavors. They ripe in 80-85 days. Skin and flesh are pale yellow to parchment or creamy white, with a pink blush on the blossom end. Black Trifele (or Japanese Black Trifele) is, despite the name, a cultivar of Russian origin. Pear-shaped fruit has green-streaked shoulders, deepening to a burnished mahogany and finally to a darkened, nearly black base. They ripe in 80-85 days, reach 65-75 mm long and wide, 170 g weight. Shiren is a more recently obtained cultivar. Plants are indeterminate, compact and particularly suitable for full-sun or greenhouse cultivation. The plant is very resistant to many viruses and produces a very elegant "Fishbone" cluster with long shelf life fruits. Fruits are

cherry-like globose in shape, with a diameter of 35 mm and a weight of 10-20 g; they ripe in 55-68 days.
Full ripe (4.5-5.5°Brix) and healthy fruits harvested during July and August 2014 were analyzed for antioxidant content as well as for hydrophilic (HAA) and lipophilic antioxidant activity (LAA). For the analysis ten tomatoes were sampled from four different representative plants and combined into one sample which is analysed as reported below. The ripeness was determined both visually and by determination of ° Brix after chopped and centrifuged an aliquot of the various tomato samples with Exacta Optech GmbH (Munchen, Germany) refractometer.

2.3. LC–UV-MS analysis of hydrophilic extracts

LC-MS system consisted of an Agilent 1100 series system (Agilent Technologies, Palo Alto, CA) including a vacuum solvent degassing unit, a binary high-pressure gradient pump, an UV detector and a 1100 MSD model VL benchtop mass spectrometer with API-ES interface. Nitrogen was used as nebulizer gas and drying gas (350°C). The nebulizer gas, the drying gas, the capillary voltage, and the vaporizer temperature were set at 40 psi, 9 L/min, 3000 V and 350°C, respectively and fragmentor 70 eV. The LC-ESI-MS determination was performed by operating the MSD in both positive and negative ion mode. Mass spectra were acquired over the scan range m/z 50-1500 using a step size of 0.1 μ. The chromatographic separation was performed using a Pursuit C18 (3 μm, 5x2.0 mm) (Varian) column. The sample was injected (20 μl) after filtration. The separation was performed by using linear gradient elution for 60 min with a mobile phase of 0.2% (v/v) formic acid in water and acetonitrile (from 90:10 to 30:70 v/v in 60 min) at the flow rate of 1.5 ml/min. After the chromatographic separation an aliquot of the eluent (400 μl/min) was directed to MSD for spectra analysis. The MS analysis was used for the identification of the main compounds present in the polyphenolic fraction. Their identification was obtained for comparison with the retention time and with the fragmentation pattern of the relative standards. The UV-Vis analysis was used for quantitative purposes. The quantitative analysis was performed for the main identified compounds using calibration curves obtained by injecting the standard solutions at various concentrations. All analyses were run in triplicates and results were expressed with the standard deviation of the means.

2.4. Lycopene, β-carotene and lutein analysis

Carotenoids and xanthophylls were extracted using a procedure previously published with small variations (SETIAWAN et al., 2001). A sample of 5 g of homogenized fresh tomatoes was extracted using 10 ml THF in presence of 0.01% butylated hydroxytoluene (BHT) and internal standard (β-apo-8'-carotenal). This extraction was performed twice. The organic fractions were collected and evaporated to dryness under nitrogen. The residue was dissolved in chloroform, appropriately diluted with the mobile phase mixture (methanol: acetonitrile: dichloromethane 50:48:2), then filtered (0.45 mm Minisart SRP 4 filter, Sartorius, Germany) and analyzed by using HPLC. LC 410 Series Perkin-Elmer apparatus (Norwalk, Connecticut, USA) equipped with a UV/VIS LC295 Perkin-Elmer detector set at 290 nm and TotalChrom ver.6.3.1 software were used (Perkin Elmer). Chromatographic separation was done using a reversed-phase LiChrospher 100 RP 18 column (5 μm, 125 x 4.6 mm) (Merck). Elution was carried out using a mixture of methanol: acetonitrile: dichloromethane (50:48:2) at a flow rate of 1.0 ml/min. Quantitative analysis of lutein, lycopene and β-carotene was based on the internal standard method. Three replications were carried out to examine each sample.

2.5. Lipophilic and hydrophilic extracts and their antioxidant activities determination

Total antioxidant activity was measured both, for hydrophilic and lipophilic extracts of the various tomato cultivars. In brief, 5 g of fresh tomato sample was extracted with 10 ml of CH_2Cl_2 and then centrifuged at 1620 g for 10 min. The extraction was performed twice and the supernatant fractions were collected and evaporated to dryness under nitrogen. The dried residue was dissolved in 3 ml of CH_2Cl_2 and analyzed for the determination of lipophilic antioxidant activity (LAA). The pellet was extracted with 10 ml of 60% methanol in Milli-Q water. The samples were then sonicated with Sonorex RK103H apparatus (Bandelin electronic, Berlin, Germany) for 10 min and centrifuged at 1620 g for 10 min. The supernatant was then transferred to a new tube and used for determination of hydrophilic antioxidant activity (HAA) and polyphenol content.
The antioxidant activity was measured using ABTS radical cation ($ABTS^{\cdot}$) decolorization assay (RE et al., 1999). In brief, 1 ml of the ABTS solution was added to different volumes of the lipophilic or hydrophilic extract (20, 40 or 60 μl) and diluted at a final volume of 2 ml using ethanol. The solution was vortexed for 10 s and the decolourization produced by the presence of antioxidants was measured at 751 nm (UV/Visible Lambda 2 spectrophotometer, Perkin-Elmer, Norwalk, Connecticut, USA), 10 min after initial mixing. The TEAC assay is a standard method used for antioxidant activity assessment of vegetables with numerous advantages such as reproducibility, simplicity and a good estimate of the antioxidant activity of pure compounds and complex matrices (THAIPONG et al., 2006). Trolox was used to prepare the standard curve and the activity was reported as equivalent millimolar Trolox related to fresh weight (mM TEAC/100 g FW). The LAA and HAA were measured in triplicates for each extract.

2.6. Statistical evaluation of data

All analyses were run in triplicates and results were expressed with the standard deviation of the means. A one-way analysis of variance (ANOVA) was performed to test the significance of the observed differences (using Stata 9.0, StataCorp LP). Data were analyzed considering the tomato cultivar as experimental factor and when the observed differences were significant ($P \leq 0.05$) the mean values were then compared by Bonferroni's multiple comparison test.

3. RESULTS AND DISCUSSION

3.1. Polyphenolic fraction analysis

3.1.1 Identification of the main polyphenolic compounds by LC-UV-MS

In the present study 13 different polyphenolic compounds were detected and identified in the extracted samples. The identified compounds belonging to five groups of phenolic compounds (benzoic acids, hydroxycinnamic acids, phenolic aldehydes, flavonols and flavanones) are listed in Table 1. Identification of the chromatographic peaks was made by comparison of mass spectra with those provided by commercial standards and by comparing their retention times in correlation to MS fragmentation patterns.

Table 1. List of polyphenolic compounds identified in tomato samples.

Compound	Retention time (min)	$[M+H]^+$ (*m/z*)	$[M+H]^-$ (*m/z*)	Identification
1	5.1	---	169	Gallic acid
2	9.5	155	153	Protocatechuic acid
3	19.7	169	167	Vanillic acid
4	20.4	355	353	Chlorogenic acid
5	21.3	181	179	Caffeic acid
6	23.4	153	151	Vanillin
7	28.4	123	121	Benzoic acid
8	30.1	611	609	Rutin
9	32.6	287	285	Kaempferol-3-O-glucoside
10	33.1	581	579	Naringin
11	41.1	149	147	Cinnamic acid
12	44.7	303	301	Quercetin
13	47.1	273	271	Naringenin

Under the experimental conditions used, the most intensive signals detected for the main compounds was the pseudomolecular ion ($[M+H]^+$ or $[M-H]^-$).
Some benzoic acid derivates or phenolic acids were identified. Gallic acid comes out at Rt 5.1 min, giving the characteristic molecular ion at 169 *m/z*. The acids p-hydroxybenzoic acid, vanillic acid, protocatechuic acid were also identified by their retention times, their pseudomolecular ion ($[M+H]^+$ or $[M-H]^-$) and mass fragmentations in comparison with standard solutions. A comparison with the MS spectra described in literature was also carried out (GUASH-JANÉ *et al*., 2004; TIAN *et al*., 2005).
Hydroxycinnamic acids were also identified. The presence of chlorogenic acid and caffeic acid was detected in all the analyzed samples. The phenolic aldehyde vanillin was also detected. Flavonols were mainly characterized by quercetin (303 *m/z*) and rutin (611 *m/z*). The presence of kaempferol-3-O-glucoside (449 *m/z*) was also detected. Flavanones were mainly represented by naringenin (273 *m/z*).
Comparison of total ion content (TIC) and UV (289 nm) chromatograms obtained from the LC-UV-MS analysis of the various cultivars revealed similar profiles (same compounds were observed even if in different quantity). Additionally, we found that the profiles obtained from the analysis of the various cultivars were essentially identical with some qualitative differences detected for minor components (unknown compounds).

3.1.2 Quantitative analysis of the main identified compounds

The main polyphenolic components identified were quantified as previously reported and the most abundant polyphenols detected in tomato cultivars are reported in Table 2. The most abundant were chlorogenic acid, rutin and quercetin. The determination of phenolic compounds showed that Snowball and Black Trifele cultivars contained the lowest concentration of antioxidant compounds, while the cultivar that possessed the highest concentration, especially of rutin (40.18 μg/g of FW), was Shiren. These observations are perfectly in agreement with HAA values showed below.
Quantitative determination of phenolic compounds showed that contents were in agreement with the few analytical data reported on red, purple or yellow cultivars

(MARTÍNEZ-VALVERDE *et al*., 2002; VALLVERDÚ-QUERALT *et al*., 2011; GARCÍA-VALVERDE *et al*., 2013; CHOI *et al*., 2014; RAIOLA *et al*. 2016).

Table 2. Mean content (μg/g of FW)±SD of the main polyphenolic compounds in the analyzed tomato cultivars.

	Tomato cultivar				
	Red Pear	**Snowball**	**Black Trifele**	**Yellow Pear**	**Shiren**
Gallic acid	1.03±0.06^{c}	1.09±0.08^{c}	0.04±0.01^{b}	nd ^{a}	0.62±0.02^{d}
Chlorogenic acid	12.53±1.09^{b}	15.08±1.03^{b}	9.26±0.83^{a}	17.54±1.26^{b}	8.86±0.06^{a}
Caffeic acid	0.69±0.02^{c}	0.47±0.05^{b}	0.01±0.01^{a}	0.03±0.01^{a}	1.32±0.09^{d}
Rutin	14.46±1.11^{c}	9.96±0.95^{a}	12.40±0.92^{b}	26.32±1.86^{c}	40.18±2.45^{d}
Kaempferol-3-O-glucoside	0.13±0.01^{a}	0.10±0.02^{a}	0.13±0.01^{a}	0.54±0.02^{c}	0.20±0.01^{b}
Naringin	0.12±0.01^{b}	0.06±0.01^{a}	0.23±0.01^{c}	0.23±0.01^{c}	0.50±0.02^{d}
Quercetin	23.91±1.16^{d}	16.92±1.23^{c}	8.01±0.75^{b}	15.34±1.14^{c}	0.15±0.01^{a}
Naringenin	3.63±0.27^{b}	0.54±0.04^{a}	10.14±1.01^{c}	5.36±0.43^{b}	10.21±0.92^{c}

Different letters in the rows represent statistically significant differences (P>0.05).
nd = not detected.

3.2. Lutein, Lycopene and β-carotene analysis

We carried out a quantitative analysis of lutein, lycopene and β-carotene in the different investigated tomato cultivars. All samples exhibited a similar chromatographic profile, showing that the two carotenoids lycopene and β-carotene were the main component of the lipophilic extracts for the cultivars with red fruits. The compounds were identified by comparing the retention time with those obtained from the reference standard injections.
The quantitative data were obtained using the internal standard method and are presented in Table 3. Lycopene content ranged from 1.02 μg/g (recorded for Yellow pear tomatoes) to 184.42 μg/g of fresh weight (recorded for Shiren tomatoes), while β-carotene ranged from 2.60 μg/g (recorded for Yellow pear tomatoes) to 64.81 μg/g (recorded for Shiren tomatoes). Additionally, the quantitative data showed that the Yellow pear cultivar had a β-carotene content more abundant than the lycopene content. The two investigated carotenoids were not detected in the Snow ball cultivar in which only lutein was detected. Lutein was also very abundant in Black Trifele cultivar.

Table 3. Principal carotenoids (lycopene and β-carotene) and xantophylls (lutein) content of various tomatoes cultivars.

Tomato cultivar	mg/g of fresh fruit		
	lutein	**lycopene**	**β-carotene**
Red Pear	0.18±0.02^{b}	42.18±2.31^{e}	12.4±0.12^{d}
Snowball	0.08±0.01^{a}	nd^{a}	nd^{a}
Black Trifele	1.12±0.04^{d}	60.9±1.92^{c}	8.42±1.66^{c}
Yellow Pear	0.20±0.02^{b}	1.02±0.28^{b}	2.60±0.84^{b}
Shiren	0.21±0.02^{bc}	184.42±2.61^{d}	64.81±0.37^{e}

Different letters in the columns represent statistically significant differences (P>0.05).
nd = not detected.

From the results of table 3 emerged, that there is a significant difference in the ability of tomatoes to synthesize carotenoids in relation to cultivar. The cultivar with the highest content of carotenoids was Shiren, on the contrary, the Yellow pear cultivar had low ability in carotenoids production.
Characterization of carotenoid content of yellow tomatoes and the differences with the red ones dates back to the 50s (JENKINS and MACKINNEY, 1955) and many authors have determined significant influence of cultivars on the content of tomato carotenoids (ABUSHITA *et al*., 2000; BINOY *et al*., 2004; CHOI *et al*. 2014; ILAHY *et al*., 2011; RAIOLA *et al*. 2016; ZANFINI *et al*. 2007). Our results are in accord with data reported by these authors and demonstrate that lutein, lycopene and β-carotene content is significantly related with the cultivar and the color of the tomatoes as earlier reported by other authors (LI *et al*., 2013).

3.3. Antioxidant activity

The antioxidant activity of tomato lipophilic (LAA) and hydrophilic (HAA) extracts was also performed using the Trolox equivalent antioxidant capacity (TEAC) assay. For each studied cultivar, the HAA was higher than the LAA and the Shiren type tomatoes, characterized by a high carotenoid and total phenolic contents, showed the highest antioxidant activity (Table 4).

Table 4. TEAC values (μM Trolox/100 g FW) measured for each investingated cultivar.

Tomato Cultivar	HAA - TEAC	LAA- TEAC
Red pear	502.8±52.3^{a}	75.4±2.0^{c}
Snow ball	472.8±48.7^{a}	47.7±1.3^{a}
Black Trifele	499.2±60.3^{a}	121.7±0.9^{d}
Yellow pear	643.5±54.1^{ab}	58.3±1.0^{b}
Shiren	706.0±19.3^{b}	131.0±2.1^{e}

Different letters in the columns represent statistically significant differences (P>0.05).

Statistical analysis shows significative differences in antioxidants activity only for LAA between the analyzed tomato cultivars.
The correlation between the content of the various antioxidant (independent variable) and the TEAC value (dependent variable) was also estimated. The only significative correlation was obtained between rutin and TEAC HAA ($r^2 = 0.982$, $p<0.05$).

4. CONCLUSIONS

This study has confirmed the important role played by cultivar in determining the antioxidant potential of fresh raw tomatoes. Results show that phenolic compounds but especially carotenoids are responsible for the differences among tomatoes in accord with the cultivar. In effect the values of HAA and LAA reflect the contribution of different antioxidants to the total antioxidant activity of the various tomato cultivars.

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