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Evaluation of Biochemical Composition and Enzyme Activities in Browned Arils of Pomegranate Fruits

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Abstract
Aril browning threatens production, consumption, and exports of pomegranates, because affected fruit cannot be externally distinguished from healthy fruit. This study compared the mineral, biochemical composition, and related enzyme activities in affected brown arils with healthy ones in 'Malase Saveh' pomegranates. The results indicated that concentrations of Cu in the aril and K, Mg, and Mn in the peel were higher in the healthy fruit than in the affected fruit. The total soluble solids, titratable acidity, total phenolics, total flavonoids, total anthocyanins, antioxidant activity, and color parameters (L*, a*, b*, hue, and chroma) decreased in the browned arils of pomegranates, whereas fruit respiration rate and acidity, peroxidase (POD), and polyphenol oxidase (PPO) enzyme activity were higher in the browned arils. No difference was found for phenylalanine ammonia lyase (PAL) activity. There were positive correlations between total anthocyanins and both color values and total phenols, and a negative correlation between PPO and POD activities was observed. Overall, the nutritional and functional value of the affected fruit is anticipated to be far less than that of the healthy fruit.

Keywords: Anthocyanins, aril browning, biochemical composition, disorders.

Abbreviations: A3GlcT, anthocyanidin 3-O-glucosyltransferase; **POD,** peroxidase; **PAL,** phenylalanine ammonia lyase; **PPO,** polyphenol oxidase; **ROS,** reactive oxygen species; **SOD**, superoxide dismutase; **TA,** titratable acidity; **TSS,** total soluble solids**.**

Introduction

The pomegranate (*Punica granatum* L.) is one of the oldest known edible fruits (Cam *et al.*, 2009). It is native to the area extending from present day Iran to the Himalayas in northern India and has been cultivated since ancient times throughout the Mediterranean region of Asia, Africa, and parts of Europe (Fawole and Opara, 2013). The edible part of the pomegranate is its arils, which are usually consumed fresh and in processed forms such as fresh juice, canned beverages, jelly, jam, and paste. It is also used for flavoring and coloring drinks (Zaouay *et al.*, 2012). Pomegranate juice is rich in organic acids, sugars, vitamins, polysaccharides, polyphenols, and minerals (Al-Maiman and Ahmad, 2002). It has high antioxidant activity (Gil *et al.*, 2000), which is attributed to its large amounts of phenolic compounds and sugar-containing polyphenolic tannins and anthocyanins (Çam *et al.*, 2009; Gil *et al.*, 2000). Phenolic compounds are among the most important groups of secondary metabolites in fruits, as they are partially responsible for color, astringency, bitterness, flavor, and nutritional qualities in fruits and vegetables (Gould *et al.*, 2009).

Anthocyanins are part of the plant-derived flavonoid compounds that produce colors ranging from orange and red to blue and purple in various fruits and vegetables.

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The individual anthocyanins are not particularly stable and are susceptible to degradation. In general, this is affected by numerous factors, such as temperature, light, oxygen, pH, enzymes, and metallic ions (He *et al.*, 2010). Previous studies have shown that low temperatures (25°C) during the maturation of grape berries favor anthocyanin biosynthesis, whereas high temperatures (35°C) are associated with anthocyanin degradation and inhibition of anthocyanin accumulation (He *et al.*, 2010). Borochov-Neori *et al.* (2011) reported that pomegranates matured and ripened under extremely hot temperatures had lower external and internal color and accumulated less anthocyanins compared to those under moderate climate conditions. Additionally, reducing soil nitrate and phosphate to a critical level can also enhance anthocyanin production in grape berries (Bao-Do and Cormier, 1991). Anthocyanins are one of the most important quality characteristics of pomegranates. Six anthocyanin pigments, such as 3-mono- and 3, 5-diglucosides of cyanidin, delphinidin, and pelargonidin, are responsible for the red color of pomegranate peels and arils (Miguel *et al.*, 2004).

In 2001, an incidence of a physiological disorder called 'aril browning' or 'aril paleness' was observed in the Ferdows region of the South Khorasan Province, Iran, and it thereafter spread to other areas (Fig. 1). Aril browning is usually reported in over-ripe fruit during harvest (Kulkarni, 2005) or during postharvest storage (Elyatem and Kader, 1984), but this disorder is initiated during fruit development. There are no visible external symptoms on the fruit; in fact, they often have a good external appearance. The disorder is detected only after cutting the fruit. Affected arils are soft, light creamy-brown to dark blackish-brown, deformed, and undesirable for consumption (Shivashankara *et al.*, 2004). Therefore, consumption of the affected fruit has threatened the popularity of the pomegranate. Furthermore, it is a serious challenge to quality control for export. Previous studies have shown that aril browning is affected by various factors, including genetic background, variety, pruning, season growth, harvest time, fruit size, and pathogens (Jalikop *et al.*, 2010; Shivashankar *et al.*, 2012), but the causative factor has not been determined.

Phenylalanine ammonia lyase (PAL) is the first enzyme in the phenylpropanoid pathway and plays an important role in the synthesis of phenolic compounds such as flavonoids, anthocyanins, isoflavonoids, and other secondary metabolites (Tomas-Barberan and Espin, 2001). Usually anthocyanin production is associated with an increase in PAL activity (He *et al.*, 2010). The direct oxidation of phenolic compounds by polyphenol oxidase (PPO) and peroxidase (POD) enzymes is a major cause of fruit tissue browning (Tomas-Barberan and Espin, 2001). Stress conditions during fruit development might promote aril browning, because enzymatic browning is a direct consequence of membrane disintegration. Therefore, the causes of browning must be sought in processes which affect membrane integrity (Franck *et al.*, 2007).

Under optimal conditions, the produced reactive oxygen species (ROS) are efficiently removed by the antioxidant system. However, stress conditions, including drought stress and desiccation, salt stress, chilling, heat shock, heavy metals, mechanical stress, nutrient deprivation, pathogen attack, and high light stress, enhance the production of ROS (Mittler, 2002), which results in membrane degradation and possible browning reactions (Franck *et al.*, 2007). Shivashankara *et al.* (2004) reported that the browning of pomegranate arils is caused by oxidative damage to membranes leading to higher enzymatic browning by PPO and POD. In litchi (*Litchi chinensis* Sonn.) fruit, pericarp browning is also caused by the degradation of anthocyanins by PPO and POD. It has been suggested that anthocyanase could first remove the sugar moiety from litchi

anthocyanins, which produces anthocyanidin, and finally POD would cause the degradation of the anthocyanidin in the presence of hydrogen peroxide (Zhang *et al.*, 2005). Ghasemnezhad *et al.* (2013) found that chitosan coating may inhibit PPO activity and reduce anthocyanin degradation in pomegranate arils during storage. Furthermore, a significant increase in POD activity is associated with the degradation of anthocyanins (Zhang *et al.,* 2005). Overall, the role of these enzymes in aril browning of pomegranates is not completely clear. This study was undertaken to compare the status of the biochemical and mineral compositions and some enzymes related to anthocyanin synthesis and degradation.

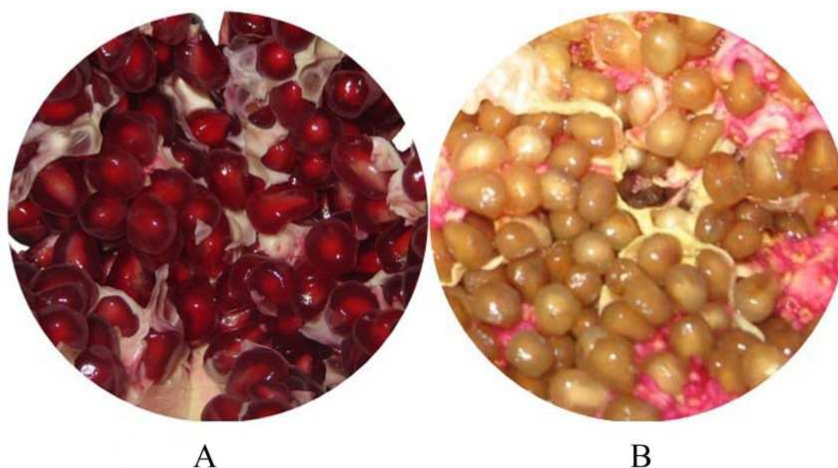

Fig. 1. Healthy (A) and browned (B) arils of 'Malase Saveh' pomegranate, Markazi Province, Iran

Materials and Methods

Plant material

Acidulous-tasting 'Malase Saveh' pomegranate fruit were harvested at the commercial mature stage from a commercial orchard in Markazi Province, Iran and immediately transported to the horticulture laboratory at the University of Guilan, Rasht, Iran. Initially, the respiration rate of some fruits was measured randomly; thereafter, the fruit peels were carefully cut at the equatorial zone with a sharp knife, and then healthy and affected (browned aril) fruit were separated into two groups. The arils were manually extracted and washed in clean water for 2 min. About 25g of pomegranate arils from each of the two groups was frozen in liquid nitrogen and stored at -80˚C for enzyme assays. Arils were juiced using a hand press, and the aril juice was centrifuged (1252×g for 15 min) and used for color measurement or kept frozen at -20°C until further analysis. The peels and arils from each group were dried at 65˚C in a hot air oven and used for nutrient element analyses.

Fruit respiration rate and juice color values

Respiration rate was measured by placing one fruit in 1-L flask which was then capped with a rubber stopper for 3 h. Thereafter, 1-mL gas samples were withdrawn from the headspace by syringe to determine CO_2 levels using a gas chromatograph (Model: Agilent 7890A) equipped with a Poropak column and a thermal conductivity detector. The column, injector, and detector temperatures were 90°C, 120°C, and 100°C, respectively. Helium was used as the carrier gas at a flow rate of 60 mL min^{-1}. Respiration rate was expressed as mg CO_2 kg^{-1} h^{-1} on five replicates.

Pomegranate juice color was measured using a colorimeter (Chroma Meter, Minolta, Japan). Juice color was assessed

according to the Commission International del'Eclairage (CIE) and expressed as L*, a*, b*, C, and H° color values. In this coordinate system, the L* value is a measure of lightness, ranging from 0 (black) to +100 (white); the a* value ranges from −100 (greenness) to +100 (redness), and the b* value ranges from −100 (blueness) to +100 (yellowness). The hue angle (h*) and chrom or intensity (C*) were calculated according to the following equations: $h^\circ = \arctan(b^*/a^*)$ and $C = (a^{*2} + b^{*2})^{1/2}$. For the hue color index, 0° or 360° represent red-purple and 90°, 180°, and 270° represent yellow, green, and blue, respectively.

Nutrient element analyses in fruit peels and arils

Nutrient element compositions in the peels and arils of healthy and affected fruit were analyzed for the macro- and micronutrient content. Nitrogen was analyzed using the Kjeldahl method (Buresh *et al.*, 1982) and P spectrophotometrically (Chapman and Pratt, 1961), and K, Ca, Mg, Mn, Cu, Zn, Fe, and B were analyzed by Perkin-Elmer (400) atomic absorption spectrometer.

Total soluble solids (TSS), titratable acidity (TA), and pH

TSS is an index of soluble sugar content in fruit. TSS (°Brix) in juice samples was determined with a digital refractometer (Euromex RD 635, Holland) at room temperature. TA was determined by titrating aliquots (40 mL) of juice samples with 0.1N NaOH to an endpoint of pH 8.2 and expressing the result as % of citric acid. The pH was measured at room temperature using a Metron model pH meter (WTW 526, Germany).

Total anthocyanin content

Total anthocyanin content in juice was evaluated spectrophotometrically using the pH differential method (Giusti and Wrolstad, 2001). Absorbance was measured at 510 and 700 nm in buffers at pH 1.0 and 4.5 using a UV–visible spectrophotometer (T80+, PG Instruments, Leicester, UK) and then calculated according to the following equation: A= [(A510/A700) pH1.0 - (A510 / A700)pH4.5]. Results were expressed as mg of cyanidin-3-glucoside per 100 ml of juice, using a molar absorptive coefficient (ε) of 26900 and a molecular weight of 449.2.

Total phenolic, flavonoid, and anthocyanin content

Total phenolic content in juice was determined using the Folin–Ciocalteu method (Ghasemnezhad *et al.*, 2012) with some modification. Briefly, 300 µL of diluted juice was mixed with 1.5 mL of 10-fold diluted Folin–Ciocalteu reagent and 1.2 mL of 7.5% sodium carbonate. The mixture was allowed to stand for 90 min at room temperature in the dark before the absorbance was measured using a UV–visible spectrophotometer at 760 nm. The results were expressed as mg gallic acid equivalent in 100 mL of juice (mg GAE/100 mL of juice).

Total flavonoid content in juice was determined by a colorimetric method described by Park *et al.* (2008) with slight modification. Briefly, 300 µL juice, 30% ethanol, 0.5 M sodium nitrite, and 0.3 M aluminum chlorides were mixed. After 5 min, 1 mL of 1.0 N NaOH was added, and the mixture was measured at 506 nm. Total flavonoid content was expressed as mg catechin equivalents (RE) per 100 mL of juice.

The free radical scavenging activity of pomegranate juice was measured according to the DPPH method reported by Brand-Williams *et al.* (1995) with modifications. Briefly, 100 µL of juice diluted with methanol in the ratio of 1:10 was mixed with 1.9 mL of 0.1 mM DPPH in methanol. The mixture was vortexed and allowed to stand at room temperature in darkness. After 15 min the absorbance was measured at 515 nm using a UV–visible spectrophotometer (T80+, PG Instruments). For each sample, three separate

determinations were recorded. Antioxidant activity was expressed as the percentage decline in absorbance relative to the control, corresponding to the percentage of DPPH scavenged (%DPPHsc), which was calculated as follows: %DPPHsc = [(Acontrol−Asample)/Acontrol] × 100.

Enzyme Assays

Pomegranate arils (5 g) were frozen in liquid nitrogen and ground with 10 mL of extraction buffer (100 mM L^{-1} potassium phosphate buffer, pH 7.0, 0.5 mM L^{-1} ethylenediaminetetraacetic acid, 60 g L^{-1} polyvinyl polypyrrolidone). The homogenate was centrifuged at 15339×g for 20 min, and the supernatant was used for enzyme activity determination. The supernatant was used as crude extract for assays of POD and PPO activities according to Ghasemnezhad *et al.* (2012). For PAL extraction, 10 g of pomegranate aril was homogenized in 50 mL of a 50mM sodium borate buffer (pH 8.8) solution containing 4% of insoluble PVPP, 5 mM mercaptoethanol. The homogenate was centrifuged for 20 min at 15339×g for 20 min and the supernatant was used for the enzyme assays (Qin *et al.*, 2003).

The POD (EC 1.11.1.7) activity was assayed by measuring the increase in absorption at 470 nm according to the method of Zhang *et al.* (2005). The reaction mixture contained 225 mM H_2O_2, 100 mM potassium phosphate buffer (pH 7.0), 45 mM guaiacol, and 0.1 mL enzyme extract. Enzyme activity is expressed as unit g^{-1} fresh weight per min.

PPO (EC 1.14.18.1) activity was determined by measuring the initial rate of increase in absorbance at 420 nm as described by Gonzalez *et al.* (1999). The activity was assayed in 3 mL of reaction mixture consisting of 2.5 mL potassium phosphate buffer (pH 6.0), 0.3 mL 0.5 M pyrocatechol, and 0.2 mL crude enzyme. The blank consisted of 3 mL potassium phosphate buffer (pH 6.0).

PAL (EC 4.3.1.25) activity was assayed according to the method of Assis *et al.* (2001) slightly modified with 500 μL enzyme extract, 2 mL of 50 mM borate buffer (pH 8.8), and 500 μL 20 mM L-phenylalanine for 60 min at 37°C. The reaction was stopped with 100 μL HCl (6 N). PAL activity was determined by the production of cinnamate, which was measured by absorbance at 290 nm. Specific enzyme activity was defined as nmol cinnamic acid $h^{-1}g^{-1}$ fresh weight.

Statistical analysis

Data was analyzed using the ANOVA procedure of SAS software Version 9.1 and the difference between means was determined by Duncan's multiple range tests. Differences at $P< 0.05$ were considered statistically significant. The results were presented as mean values ± SE. The Pearson correlation coefficients were determined among the results of measured traits and aril browning.

Results and Discussion

Fruit respiration rate and juice color values

Different respiration rates were observed between healthy and affected pomegranates (Table 1). These results are consistent with the higher respiration rates in the brown arils of pomegranate fruit cultivar 'Ganesh' (Shivashankara *et al.*, 2004). Respiration is a basic physiological process that provides the energy for plant biochemical processes. Carbohydrates, lipids, and organic acids are substrates that are broken down in this process (Fonseca *et al.*, 2002). The higher respiration rate in affected fruits indicates a faster overall metabolism and deterioration (Chung and Moon, 2009).

The juice color values, including L*, a*, b*, chroma, and hue angle, were measured using a colorimeter and are summarized in Table 1. L* was increased in the juice of affected fruit. This indicates that the aril color becomes brighter in affected fruit. Zaouay *et al.* (2012), in agreement with our results, reported that darker juice contains higher levels of antioxidants and total phenolics. In contrast, values a* and b* were higher in the healthy pomegranate

juice. These results indicate that these pomegranates have more of the red and yellow color components, respectively. The chroma value (C), which represents the purity or intensity of a color, and hue angle (h˚) of the juice were lower in the affected fruit because of the sharp decrease in a* and b*values. These results are in agreement with the pattern of changes in anthocyanins. A decrease in a*, b*, and hue angle can be an indication of the appearance of aril browning.The red color is one of the factors that affected pomegranate consumer's behavior (Zaouay *et al.*, 2012). Venkatachalam and Meenune (2012) reported that maturation of longkong (*Aglaia dookkoo* Griff.) fruit on-tree is associated with an increase in browning that is followed by an increase in a* and decreased L* and b* values.

Table 1. Comparison of fruit respiration rates and color values of juice in healthy and browning-affected 'Malase Saveh' pomegranates, Markazi Province, Iran

Parameters	Healthy fruit	Affected fruit
Respiration (mg CO_2 kg^{-1} h^{-1})	21.3 ± 1.1b	32.5 ± 1.8 a
L*	27.7 ± 0.6 b	35.5 ± 0.9 a
a*	16.6 ± 0.5 a	7.2 ± 0.6 b
b*	10.8 ± 0.2 a	5.9 ± 0.6 b
Chroma	19.8 ± 0.6 a	9.4 ± 0.8 b
Hue	39.4 ± 0.39a	33.2 ± 1.0b

Values in the same row with different letters are significantly different (P<0.05). Data are mean ± SE (n=5).

Peel and arils nutrient element concentrations

The relative amounts of macronutrient in the arils of both affected and healthy fruit were N > k > P > Ca > Mg, whereas in the peels they were K > N > Ca > Mg > P (Table 2). No significant differences for macronutrients were observed between healthy and affected brown arils, however the peel showed significant differences for K and Mg (Table 2). The peel of the healthy fruit showed higher K concentration than that of the affectedfruit, but the opposite was true for Mg. In general, B, Zn, and Cu concentrations were higher in pomegranate arils than in fruit peel. Differences were observed in the Cu and Mn concentrations in arils and peel, respectively, between healthy and affected fruit. Both Cu and Mn concentrations were higher in healthy fruit peels and arils than in affected ones. The results of this experiment differ from the findings of Shete and Waskar (2005), who reported that concentrations of Ca and P decreased, but N, K, Mg, and B increased in affected arils.

Table 2. The comparison of macro- and micronutrient element concentrations in peels and arils of healthy and browning-affected 'Malase Saveh' pomegranates, Markazi Province, Iran

Fruit		Macronutrient (g Kg^{-1})					Micronutrient (mg kg^{-1})				
		N	P	K	Ca	Mg	B	Fe	Zn	Cu	Mn
Aril	Healthy	14.1	1.5	10.5	1.4	0.9	56.3	37.4	26.4	25.2 a	6.5
	Affected	15.9	1.8	10.8	1.3	1.2	52.2	34.8	30.6	16.5 b	7.3
Peel	Healthy	10.1	12.7	15.6 a	6.5	2.3 a	35.5	58.1	16.9	22.6	13.6 a
	Affected	10.8	10.6	9.1 b	6.8	1.5 b	37.0	42.5	14.7	20.1	6.2 b

Values within the same column and section with different superscripts are significantly different (P<0.05). Data are mean ± SE (n=3).

Gould *et al*. (2009) showed that the activity of some enzymes involved in the biosynthesis of anthocyanin, including A3GlcT (anthocyanidin 3-O-glucosyltransferase), is completely inhibited by 1 mM Cu^{2+}, Mn^{2+}, and Zn^{2+}. The inhibitory effect of these free metals under *in vitro* conditions may be due to the destruction of substrate anthocyanins. In this study, we found that brown arils have higher Mn^{2+}, and Zn^{2+} concentrations than healthy ones. However, no significant difference was found between healthy and affected arils for these elements (Table 2). In contrast to the findings of Gould et al. (2009), Cu^{2+} concentrations were higher in healthy arils than in brown ones. Metal ions are common cofactors in the PPO (Mayer, 2006) and antioxidant enzymes such as superoxide dismutase (Gill and Tuteja, 2010). Enzyme activity is affected by the concentration of metal ions. Latha *et al.* (2013) reported that in Indian gooseberries (*Phyllanthus emblica* Linn.), copper sulphate and zinc sulphate serve as activators for PPO enzyme activity. On the contrary, in sugarcane (*Saccharum officinarum* L.) PPO activity was markedly inhibited by metal ions (Cu^{2+}, Al^{3+}, and Mg^{2+}) at 1 and 10 mmol L^{-1} (Zhao *et al.*, 2011). In algae (*Anabaena variabilis* Kutz.), with increasing concentrations of metal salts ($CuSO_4$ and $ZnSO_4$), superoxide dismutase (SOD) activity increased in direct proportion with the metal concentration (Padmapriya and Anand, 2010). Overall, little information has been finding regarding the roles of metal ions in the anthocyanin degradation of pomegranate arils.

TSS, TA and pH

In terms of quality of a fruit, acids and sugars are important components which provide characteristic taste and flavor to fruits and their products. The major soluble sugars found in pomegranates are glucose and fructose (Al-Maiman and Ahmad, 2002).

The levels of TSS and TA was lower in affected fruits (Table 3). These results are in agreement with those reported by Shete and Waskar (2005). Jalikop *et al.* (2010) found that aril browning is affected by the levels of TSS in pomegranates; for every unit increase in TSS, there was an increase in the severity of aril browning. There was a negative correlation ($p < 0.05$) between TA and respiration rate ($r=-0.86$) (Table 5). This is probably due to the higher respiration rate in affected fruit; thus, it could be inferred that organic acids are the main respiratory substrates (Nanda *et al.*, 2001). Affected fruit had a higher pH than healthy fruit (Table 3). These results are in accordance with previous studies on other pomegranate cultivars (Shivashankar *et al.*, 2004; Shivashankar *et al.*, 2012). The increased pH in affected fruit is probably due to reduced TA. As mentioned in a previous study, organic acids are the main respiratory substrates in pomegranates (Sayyari *et al.*, 2011). The higher respiration rate found in affected fruits (Table 1) is associated with the further reduction of organic acids, followed by increasing pH values. Shivashankar et al. (2012) reported that increasing pH and decreasing moisture content in affected arils led to an imbalance between oxidative and reductive processes that was followed by a loss of membrane integrity facilitating enzymatic oxidation of phenolic compounds to brown-colored polymers and consequent browning reactions in arils.

Total phenolic, flavonoid and anthocyanin contents

In this study, the total phenolic and flavonoids decreased dramatically in affected brown arils (Table 3), suggesting that phenolic compounds were oxidized in the browning process (Tomas-Barberan and Espin, 2001). In agreement with our results, Shivashankar *et al.* (2012) reported that phenol contents in pomegranate juice decreased from 1.38 in healthy fruit to 1.075 (mg 100 g^{-1} fw) in affected fruit.

Phenolic compounds have many biological and functional activities for fruit quality and human health. They are highly varied in species, cultivars, and fruit tissue (Tomas-Barberan and Espin, 2001). Oxidation of phenolic compounds is the main cause of browning in fruit and vegetables, which are finally polymerized to brown or black pigments (Holderbaum *et al.*, 2010).

Fruit browning is usually associated with changes in pigment concentrations. Total anthocyanin content was lower in affected pomegranates (Table 3).

Table 3. Comparison of TSS, TA, pH, total phenolic and flavonoid, total anthocyanins and antioxidant activity in the juice of healthy and browning-affected 'Malase Saveh' pomegranates, Markazi Province, Iran

Parameter	Healthy fruit	Affected fruit
TSS (˚Brix)	17.7 ± 0.3 a	16.2 ± 0.2 b
TA (%)	1.5 ± 0.1 a	0.8 ± 0.1 b
pH	3.5 ± 0.2 b	4.2 ± 0.2 a
Total phenolic (mg GAE per 100 mL)	135.6 ± 3.8 a	78.9 ± 5.0 b
Total flavonoid (mg RE pre 100 mL)	73.8 ± 2.9 a	60.7 ± 2.6 b
Total anthocyanins (mg per 100 mL)	14.8 ± 0.8 b	7.0 ± 0.5 a
Antioxidant activity (% DPPHsc)	65.7 ± 3.6 b	45.0 ± 2.1 b

Values in the same row with different letters is significantly different (P<0.05). Data are mean ± SE (n=5).

Reduction of anthocyanin content was probably due to anthocyanin degradation in the affected fruit. Anthocyanin is one of the most important quality factors of the pomegranate, and the color of pomegranate juice is attributed to these compounds (Zaouay *et al.*, 2012). The deterioration of color in fruit juices containing anthocyanins is mainly the result of the degradation of monoglucosidic anthocyanins (Turfan *et al.*, 2011), because diglucosidic anthocyanins are more stable than monoglucosides, whereas monoglucosidic anthocyanins possess a deeper color than diglucoside forms (He *et al.*, 2010). Shivashankar *et al.* (2004) have reported that anthocyanin content is lower in brown arils than in healthy arils, and the absorption of pomegranate juice at 540 nm for healthy and affected fruit were 0.531 and 0.321 (ΔA540 g^{-1} fw), respectively. He *et al.* (2010) found that high, stressful temperatures (>35˚C) during fruit maturation and ripening are associated with anthocyanin degradation. Hence, one of possible factors for the decreased anthocyanin content in pomegranates in recent years could be the results of global warming (Borochov-Neori *et al.*, 2011). High, stressful temperatures might result in cell membrane degradation and start browning reactions (Franck *et al.*, 2007).

There were strong correlations ($p < 0.01$) between total anthocyanins and total phenolic content ($r = 0.97$), color parameters, and pH (Table 5). This suggests that the decrease in anthocyanin content is associated with a decrease in a* and increases in L* values. In agreement with our results, Zaouay *et al.* (2012) reported a positive correlation between a*, chroma, and total anthocyanin content. It indicates that the red-colored juices are generally rich in anthocyanin pigments (Zaouay *et al.*, 2012). This is due to the fact that, at the physiological pH of 3.0 in the plant vacuole, anthocyanins exist in a stable red flavylium ion form giving the arils their bright red color. As the pH increases to about 3.5, however, the arils undergo a reversible structural transformation to the anhydro base forming colorless chromenols and giving rise to arils with reduced color intensity (Shivashankar *et al.*, 2012; Zhang *et al.*, 2001).

Antioxidant activity

In this study, antioxidant activity was lower in affected fruit than in healthy fruit (Table 3); this decrease is related to the reduced total phenolic and anthocyanin contents in affected fruit. A positive correlation was found between antioxidant activity and total phenolic, flavonoid, and anthocyanin contents (r = 0.94, 0.98 and 0.93, respectively). These results are in agreement with previous reports that the antioxidant activity of pomegranates is attributed to phenolic compounds (Gil *et al.*, 2000; Cam *et al.*, 2009; Zaouay *et al.*, 2012). These results also corroborate the findings of Gil *et al.,* (2000) who found that total anthocyanins were well correlated to antioxidant activity; but, it is in disagreement with results reported by Zaouay *et al.* (2012).

The beneficial health effects of the pomegranate are related to its antioxidant activity, which is associated with the high level of phenolic compounds and anthocyanins (Gil *et al.*, 2000). The antioxidant activity of anthocyanins arises from their high reactivity as hydrogen or electron donors (Duan *et al.,* 2007). Gracia-Alonso *et al.* (2004) studied the antioxidant activity of 28 different fruits and reported that fruits with high antioxidant activity were all rich in anthocyanins.

Enzyme activity

In this study, there were no differences in PAL activity between healthy and affected fruit (Table 4). These results are in agreement with those of Yingsanga *et al.* (2008), who reported that changes in PAL activity in rambutan (*Nephelium lappaceum* Linn.) were not closely related to the development of browning. In other words, it suggests that the synthesis of phenolic compounds may not be affected.

Table 4. Comparison of enzyme activity in arils of healthy and browning-affected 'Malase Saveh' pomegranates, Markazi Province, Iran

Enzyme Activity	Healthy Fruit	Affected Fruit
PAL (nmol cinnamic acid $h^{-1}g^{-1}$ fw)	1824.0 ± 173.3 a	1729.4 ±175.4 a
PPO (u g^{-1} fw)	18.6 ± 3.1 b	74.3 ± 5.6 a
POD (u g^{-1} fw)	11.5 ± 0.5 b	33.1 ± 3.1 a

Values in the same row with different letters are significantly different ($p<0.01$). Data are mean ± SE (n=5).

Differences for PPO and POD activity were observed between healthy and affected fruit (Table 4). Increased PPO and POD activity in affected arils is associated with a reduction in phenol levels. This result indicates that browning in affected arils was apparently due to the enzymatic oxidation of phenolic compounds (Shivashankar *et al*., 2012). PPO catalyzes the hydroxylation of monophenols to diphenols and the oxidation of diphenols to diquinones followed by the non-enzymatic formation of melanines. Additionally, POD can oxidize phenols to quinones in the presence of hydrogen peroxide (Degl'Innocenti *et al*., 2005). Yingsanga *et al*. (2012) also reported that higher activities of PPO and POD in spinterns as compared to the peel of the rambutan fruit is the main reason for the higher browning in spinterns. Positive correlations were found in the present study between total phenolic compounds and both PPO and POD enzyme activity (Table 4). In other fruits such as the apple (*Malus sylvestris* var. Domestica) (Holderbaum *et al*., 2010), litchi (Zhang *et al*., 2005), rambutan (Yingsanga *et al*., 2008), and longkong (Venkatachalam and Meenune, 2012). It has been reported that tissue browning is a result of the oxidation of phenolic compounds by PPO and/or POD.

Table 5. Correlation (Pearson test) among various traits of fruit juice of 'Malase Saveh' pomegranates, Markazi Province, Iran

Traits	Titratable acidity	Total phenolics	Total flavonoids	Total anthocyanins
Titratable acidity	1.00			
Total phenolics	0.98**	1.00		
Total flavonoids	0.88**	0.86*	1.00	
Total anthocyanins	0.97**	0.97**	0.88*	1.00
pH	-0.89 ns	-0.88*	-0.81 ns	-0.89*
Antioxidant activity	0.99**	0.94**	0.98**	0.93**
L*	0.93**	-0.87*	-0.90*	-0.92**
a*	0.95**	0.96**	0.84*	0.96**
Chroma	0.94**	0.95**	0.83*	0.95**
PAL	0.67 ns	0.66 ns	0.63 ns	0.60 ns
PPO	-0.96**	-0.93*	-0.87*	-0.98**
POD	-0.89**	-0.90**	-0.75 ns	-0.92**
Respiration rate	-0.86*	-0.87*	-0.71 ns	-0.92**

The r value of the correlation is given and its significance: * significant at P< 0.05, **significant at P< 0.01and ns= not significant.

The anthocyanin content of pomegranate juice and PPO and POD activity were correlated (r= -0.98 and -0.92, respectively). Increased PPO and POD activity is associated with reduced anthocyanin (Table 5). Anthocyanin degradation by enzymatic browning reaction has been reported in other fruits including: litchi (Zhang *et al.*, 2001), longkong (Venkatachalam and Meenune, 2012), and strawberry (*Fragaria ananassa* Duch.) (Lopez-Serrano and Barselo, 1999). In litchi (*Litchi chinensis* Sonn.), it was suggested that the anthocyanase could first remove the sugar moiety from the litchi anthocyanins, producing anthocyanidin, and finally POD would caused the degradation of anthocyanidin in the presence of hydrogen peroxide (Zhang *et al.*, 2005). Pang *et al.* (2008) suggested that, during the pericarp browning of lychee fruits, PPO first catalyses the oxidation of phenols, anthocyanidins, and/or their degraded products to form quinones, which then oxidize anthocyanins to brown by-products, leading to decoloration, browning, and reduced anthocyanin content.

Although several factors have been reported for the aril browning of pomegranate fruits, the main reason for it is still not clear and to date remains a conundrum. Therefore, further research should be conducted to clarify this issue. Affected fruits have poor quality and are not suitable for fresh consumption. The occurrence of aril browning phenomena in pomegranates has caused many consumers to doubt its purchase. Overall, the role of PPO and POD in aril browning of pomegranates is not completely clear. However, Ghasemnezhad *et al.* (2013) found that chitosan coating may inhibit PPO activity and reduce anthocyanin degradation during storage of pomegranate arils.

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