Effects of maturity status on biochemical content, polyphenol composition and antioxidant capacity of pomegranate fruit arils (cv. ‘Bhagwa’)

Olaniyi Amos Fawole, Umezuruike Linus Opara *

Postharvest Technology Research Laboratory, South African Research Chair in Postharvest Technology, Faculty of AgriSciences, Stellenbosch University, Private Bag X1, Stellenbosch 7602, South Africa

1. Introduction

Pomegranate (Punica granatum L.) belongs to the family Punicaceae, and has gained popularity in recent years due to its multi-functionality and great nutritional benefit in the human diet. It is now grown globally in many geographical regions, satisfying the nutritional and medicinal needs of populations of various countries (Holland et al., 2009). Commercial orchards of diverse pomegranate cultivars are grown in countries such as Iran, India, Egypt, China, Israel, Tunisia, Syria, Lebanon, Turkey, Greece, Cyprus, Italy, France, Spain, Chile, Portugal, USA, Oman and most recently in South Africa (Al-Said et al., 2009; Holland et al., 2009; Fawole et al., 2011).

For most fruits, advancing maturity corresponds to a number of coordinated physiological, biochemical, and structural processes that result in changes in size, color and flavor, making the fruit desirable for consumption (Moing et al., 2001; Nunes et al., 2009; Wilson and Downs, 2012; Baloch and Bibi, 2012). In pomegranate fruit handling and marketing, important quality attributes include size, color, juiciness, taste, flavor, and seed hardness (Jalikop, 2007; Holland et al., 2009; Opara et al., 2009). The edible part of the fruit (aril) contains considerable amounts of acids, sugars, polyphenol and important minerals (Al-Maiman and Ahmad, 2002). The fruit is usually consumed as fresh aril, or as processed products, mostly juice. Because pomegranate fruit external skin color does not indicate the extent of ripening degree or its readiness for consumption (Holland et al., 2009), parameters such as color of aril, total soluble solids and organic acids contents are commonly considered for fruit quality assessment and classification (Ben-Arie et al., 1984; Cristosto et al., 2000; Martinez et al., 2006). Due to an increasing consumer interest and awareness on the health benefits of the fruit juice, numerous studies have reported the content and composition of major health and medicinal components in various pomegranate fruit cultivars (Gil et al., 2000; Shwartz et al., 2009; Tezcan et al., 2009; Elfalleh et al., 2011). The beneficial health qualities of pomegranate are attributed to the exceptionally high antioxidant capacity that strongly correlates with high content and unique composition of phenolic compounds (Gil et al., 2000; Borochov-Neori et al., 2011; Fischer et al., 2011; He et al., 2011). Consequently, the importance of health beneficiing phenolic content in fruit juice as part of the target traits by breeders in the context of accurate evaluation of fruit quality is being considered in others to satisfy export requirements (Holland...
et al., 2009). Significant variations in organic acids, sugars and phenolic composition in pomegranate juice have been reported by various authors (Ben-Arie et al., 1984; Gil et al., 1995; Melgarejo et al., 2000; Poyrazoglu et al., 2002; Al-Maiman and Ahmad, 2002; Mirdehghan and Rahemi, 2007; Holland et al., 2009). According to the authors, changes in chemical, phenolic and antioxidant properties in fruit juice considerably differ among cultivar types, growing regions and degree of fruit maturity at harvest.

Commercial production of pomegranates is fairly new in South Africa and the cultivar ‘Bhagwa’ is one of the most widely grown in South Africa and globally. As far as we know, apart from the recent article by Fawole et al. (2011) on the phytochemical and antioxidant properties of selected fruit cultivars at commercial harvest, there is no information on the changes that occur in chemicals and phenolic composition of pomegranate fruit during maturation. In order to fully understand the nutritional value offered by pomegranate during maturity stages of the fruit, it is imperative to study the changes in the nutritional contents of commercially grown pomegranate cultivars. The objective of this study was to quantify the chemical and phenolic composition and antioxidant capacity of pomegranate fruit (cv. ‘Bhagwa’) at different fruit maturity stages and to investigate the relationships among the investigated nutritional parameters.

2. Materials and methods

2.1. Plant material and processing

Pomegranate fruit (Bhagwa cultivar) grown in a commercial orchard located in Porterville, South Africa (33°01′00″S, 18°58′59″E) was used for this study. Plants were 4 years old with spacing of 3 m × 5 m. The orchard has a double drip irrigation system delivering about 32 l per day per tree. Sampling started in January when it was possible to squeeze juice from the arils and ended in May at commercially full-ripe stage. A sample of twenty fruits of the same size and without physical defect was randomly collected from 10 trees at 54, 82, 110, 140 and 165 days after full bloom (DAFB). Fig. 1 shows the appearance of whole fruit and arils at different stages of maturity. Arils were removed manually from each fruit and juice content was extracted from arils using cheesecloth after blending with a blender (Mellerware, South Africa). After measuring the pomegranate juice (PJ) content, a portion (5 ml) was centrifuged at 5000 rpm for 10 min at 4 °C to determine juice color based on absorbance at 530 nm using a Helios Omega UV–vis spectrophotometer (Thermo Scientific technologies, Madison, USA). Crude PJ sample (1 ml) was extracted with 29 ml of cold 50% aqueous methanol. The resulting mixture was vortexed and then sonicated in dim light, aqueous methanolic extract of PJ (15 µl) was diluted with methanol (735 µl) in test tubes followed by the addition of methanolic DPPH solution (750 µl, 0.1 mM). The mixtures were incubated at room temperature for 30 min in the dark, and the absorbance was measured at 517 using a UV–vis spectrophotometer. Absorbance was compared with the standard curve (ascorbic acid, 0.2–2.0 mM). The free-radical activity of PJ was expressed as ascorbic acid (mM) equivalent per ml PJ (mM AAE/ml).

2.2. Chemical properties

2.2.1. Titratable acidity, total soluble solids and pH

Titratable acidity (TA) was determined replicate by titration using a Metrohm 862 compact titratosampler (Herisau, Switzerland), and the results were expressed as percentage citric acid. Total soluble solids (TSS) were measured using a digital refractometer (Atago, Tokyo, Japan) and the results were expressed as °Brix. The pH of PJ was determined at room temperature by using a pH meter (Crison, Barcelona, Spain). All measurements were made in triplicate.

2.2.2. HPLC analysis of individual sugars and organic acids

Sugars and organic acids in PJ at each maturity stage were analyzed using high performance liquid chromatography (HPLC) (Agilent 1100 Series, Waldron, Germany) equipped with diode array detector (DAD). A sample of 10 µl of extracted juice sample was injected onto the HPLC and optimal separation was performed in an isocratic mobile phase of 5 mM H2SO4 (560 µl of H2SO4 in 2 l) using an HPX 87H column (Aminex, 300 mm × 7.78 mm). A refraction index detector was utilized at 55 °C at a flow rate of 0.5 ml/min with UV detection set at 210 nm. Sample preparation and chromatographic procedure were based on the method of Castellari et al. (2000). Identification and quantification of sugars and organic acids composition were made by comparison of peak retention times, peak areas and spectra with those of external standards. Total sugar was calculated by summation of individual sugar (Melgarejo et al., 2000).

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2.2.3. Ascorbic acid content

Ascorbic acid was determined according to Klein and Perry (1982) with slight modifications (Barros et al., 2007). Briefly, PJ sample (0.5 ml) was mixed with metaphosphoric acid (1%, 14.5 ml), the resulting mixture was vortexed and then sonicated in ice for 5 min followed by centrifuging at 10000 rpm for 5 min at 4 °C. The supernatant (1 ml) was mixed with 2, 6-dichloroindophenol (9 ml) and the absorbance was measured within 30 min at 515 nm against a blank using a UV–vis spectrophotometer (Helios Omega, Thermo Scientific technologies, Madison, USA). Ascorbic acid content was calculated using the calibration curve of authentic L-ascorbic acid (0.01–0.1 mg/ml), and the results were expressed as ascorbic acid equivalent (AAE) per milliliter crude juice (mg AAE/ml juice).

2.2.3. DPPH radical-scavenging activity

The DPPH assay was carried out in triplicate, according to the method used by Karioti et al. (2004) with some modifications. Briefly, under dim light, aqueous methanolic extract of PJ (15 µl) was diluted with methanol (735 µl) in test tubes followed by the addition of methanolic DPPH solution (750 µl, 0.1 mM). The mixtures were incubated at room temperature for 30 min in the dark, and the absorbance was measured at 517 using a UV–vis spectrophotometer. Absorbance was compared with the standard curve (ascorbic acid, 0–2.0 mM). The free-radical activity of PJ was expressed as ascorbic acid (mM) equivalent per ml PJ (mM AAE/ml).

2.3. Changes in antioxidant capacity and polyphenols concentration

2.3.1. Sample preparation

Crude PJ sample (1 ml) was extracted with 29 ml of cold 50% aqueous methanol. The resulting mixture was vortexed, and then sonicated in ice for 20 min in a cold water bath followed by centrifuging at 10000 rpm for 5 min at 4 °C. The supernatant was subsequently collected and assayed for antioxidant capacity and phenolic components.

2.3.2. DPPH radical-scavenging activity

The DPPH assay was carried out in triplicate, according to the method used by Benzie and Strain (1996) with a few modifications (Fawole et al., 2011). The FRAP working solution containing mixtures of 300 mM acetate buffer (50 ml), 10 mM 2,4,6-tripryidylyl-s-triazine (TPTZ) (5 ml) and 20 mM ferric chloride (5 ml) was freshly prepared and incubated in a water bath at 37 °C before being used. In triplicates, diluted aqueous methanolic PJ extracts (150 µl) were added to 2850 µl of the FRAP working solution before incubation in the dark for 30 min. The reduction of the Fe3+-TPTZ complex to a colored Fe2+-TPTZ complex at low pH by PJ extracts was monitored by measuring the absorbance at 593 nm.Trolox (100–1000 μM) was used for the calibration curve, and the results were expressed as trolox (mM) equivalents per ml PJ (mM TE/ml PJ).

2.3.3. Ferric ion reducing antioxidant power (FRAP)

The antioxidant power of PJ was measured colorimetrically according to the method of Benzie and Strain (1996) with a few modifications (Fawole et al., 2011). The FRAP working solution containing mixtures of 300 mM acetate buffer (50 ml), 10 mM 2,4,6-tripryidylyl-s-triazine (TPTZ) (5 ml) and 20 mM ferric chloride (5 ml) was freshly prepared and incubated in a water bath at 37 °C before being used. In triplicates, diluted aqueous methanolic PJ extracts (150 µl) were added to 2850 µl of the FRAP working solution before incubation in the dark for 30 min. The reduction of the Fe3+-TPTZ complex to a colored Fe2+-TPTZ complex at low pH by PJ extracts was monitored by measuring the absorbance at 593 nm. Trolox (100–1000 μM) was used for the calibration curve, and the results were expressed as trolox (mM) equivalents per ml PJ (mM TE/ml PJ).
2.3.5. Total flavonoids content

The total flavonoids content (TFC) was determined using the method described by Yang et al. (2009) and results were expressed as catechin equivalents (CAE) per 100 ml PJ.

2.3.6. Total monomeric anthocyanin content

Total monomeric anthocyanin content (TMA) was quantified using the pH differential method (Wrolstad, 1993). In triplicates, PJ extracts (1 ml) were mixed with 9 ml of pH 1.0 and pH 4.5 buffers,
separately. Absorbance was measured at 520 and 700 nm in pH 1.0 and 4.5 buffers and result was expressed as cyanidin 3-glucoside using the following equations:

\[ A = (A_{510} - A_{700})/\varepsilon x 10^{-l} - (A_{510} - A_{700})/\varepsilon x L \]  

(1)

Total monomeric anthocyanin (mg/ml) = \( \frac{A \times MW \times DF}{x L} \)  

(2)

where \( A = \) Absorbance, \( \varepsilon = \) Cyd-3-glucoside molar absorbance (26,900), \( MW = \) anthocyanin molecular weight (449.2), \( DF = \) dilution factor, and \( L = \) cell pathlength (1 cm).

Final results are expressed as equivalent per 100 ml PJ (mg C3gE/100 ml PJ).

2.3.7. Rhodanine assay for gallotannins

Determination of gallotannins content in juice methanolic extracts was carried out as described by Makkar (2000). In triplicates, diluted extracts (50 μl) were mixed with 150 μl of 0.4 N sulphuric acid followed by 600 μl rhodamine. After 10 min, 200 μl of 0.5 N KOH was added and subsequently distilled water (4 ml) after 2.5 min. The absorbance was read at 520 nm against a blank that contained aqueous methanol instead of sample after 15 min incubation at room temperature. Gallic acid was used for the standard curve. Gallotannins content was calculated from the standard curve and expressed as gallic acid equivalent (GAE) per 100 ml PJ.

2.3.8. HPLC-DAD/MSn of polyphenol and anthocyanin composition

Liquid chromatography–mass spectrometry (LC–MS) analysis of phenolics and anthocyanin components in PJ at different maturity stages was performed according to Fischer et al. (2011) with slight modification, using a Synapt G2 mass spectrometer UPLC™ system (Waters Corp., Milford, USA) connected to a photo diode array detector and a BEH C18 column (1.7 μm particle size, 2.1 x 100 mm, Waters Corp.). The mobile phases were 5% formic acid in water (v/v) as eluent B. The ow rate was 0.2 ml/min and the column temperature was set at 40 °C. The electrospray ionization (ESI) probe was operated in the positive mode with capillary voltage, 3 kV; and cone voltage, set at 40 °C. The electrospray ionization (ESI) probe was operated in the positive mode with capillary voltage, 3 kV; and cone voltage,

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2.4. Statistical analysis

All data are presented as mean values (± S.E.). Graphical presentations were made using GraphPad Prism software version 4.03 (GraphPad Software, Inc. San Diego, USA). Analysis of variance (ANOVA) was also performed using SPSS for windows (SPSS Inc., Chicago, II, USA). Where there was statistical significance (P<0.05), the mean values were further separated using Duncan’s multiple range test. Correlation coefficients (r) were determined by Pearson correlation matrix method also using SPSS for windows. Relationships among the investigated fruit parameters were explored with Pearson correlation coefficient and principal component analysis (PCA) using the statistical XLSTAT software Version 2012.4.01 (Addinsoft, France).

3. Results and discussion

3.1. Changes in chemical composition

The evolution of chemical composition of PJ during fruit developmental stages is shown in Table 1. Fruit maturity had a significant (P<0.05) effect on all parameters studied along the days after full bloom (DAFB). Juice content was 29.31% at 54 DAFB and reached 54.93% at full-ripe stage. Previous studies have reported juice content ranging between 25 and 45% during pomegranate fruit developmental stages of the ‘Wonderful’ pomegranate cultivar (Shulman et al., 1984). There was a steady increase in absorbance as juice color changed from white to pink between 54 and 110 DAFB, and then increased sharply as the color changed from pink to deep red (Fig 1). The overall increase in juice absorbance during fruit developmental stages is largely as a result of the accumulation of anthocyanins (Shulman et al., 1984).

Total soluble solids (TSS) content increased significantly during fruit development with approximately 1.5-fold increase from 54 DAFB to 165 DAFB (Table 1). The TSS found in fully ripened fruits averaging 16.2 Brix was higher than 15.3% reported by Kulkami and Aradhya (2005) for the ‘Ganesh’ cultivar grown in India. According to Ben-Arie et al. (1984), pomegranate fruit of ‘Wonderful’ cultivar grown in Israel attained optimum quality when the TSS of the fruit reached 15%. The increase in TSS could be attributed to the hydrolysis of starch to sugars as pomegranate fruit advancing maturity (Kulkami and Aradhya, 2005).

On the contrary, fruit titratable acidity initially decreased sharply in late immature stage (82 DAFB) then followed by a continuous but slow decrease with advancing fruit maturity (Table 1). Titratable acidity (TA) ranged from 0.62 to 0.38% (tartaric acid), representing more than 38% decrease between 54 and 165 DAFB. The TA values obtained in this study are within the range reported by Kulkami and Aradhya (2005) for ‘Ganesh’ cultivar. High acidity during immature stage of the fruit could be attributed to the organic acids content and composition at this stage of fruit development. The decrease in acidity coincided with increase in pH. The pH of the juice decreased from early immature (3.57) to early half-ripe stage (3.18), but did not differ significantly (P>0.05) until the full-ripe stage. As a result of variations in TSS and TA contents, the ratio of TSS/TA varied considerably from 54 DAFB (16.68) up to 140 DAFB (39.19), and thereafter showed no significant increase (P>0.05) till 165 DAFB (41.83). This suggests that the fruit cultivar may have attained its characteristic taste and flavor before commercial full-ripe. The ratio of TSS/TA has been reported as one of the most reliable indicators of fruit maturity in some pomegranate cultivars although it is largely dependent on cultivar types (whether the fruit is sweet, sweet-sour or sour), as well as the agro-climatic conditions (Ben-Arie et al., 1984; Al-Maiman and...
through fruit maturation (Fig. 2) in the cultivar studied. Literature addition, the concentration of fructose was higher than glucose sugars with the principal sugar fractions being fructose and glucose. In Al-Said et al., 1984). The ratio of TSS/TA was found useful for the classi' study could provide useful cues for objective maturity indexing of changes in TSS and TA during fruit development found in the present 1999; Martinez et al., 2006). This literature evidence suggests that the of many Spanish and Italian pomegranate cultivars (Hernandez et al., 2004; Zarei et al., 2011). Adequate knowledge of the pattern of sugar accumulation in the studied cultivar could be useful for the characterization of the fruit sensory quality (Al-Maiman and Ahmad, 2002).

3.2. Changes in individual sugars

As shown in Fig. 2, ‘Bhagwa’ pomegranate contains mainly reducing sugars with the principal sugar fractions being fructose and glucose. In addition, the concentration of fructose was higher than glucose throughout fruit maturation (Fig. 2) in the cultivar studied. Literature studies indicate that there are variations, and little consistency with respect to the relative concentrations of glucose and fructose in pomegranate cultivars. Our result is in agreement with Melgarejo et al. (2000) and Carbonell-Barrachina et al. (2012), who reported higher level of fructose than glucose in pomegranate cultivars grown in Spain. However, other studies have shown that glucose was higher than fructo in other pomegranate cultivars (Al-Maiman and Ahmad, 2002; Miguel et al., 2004; Ozgen et al., 2008). The content of glucose and fructose increased considerably during fruit maturation, with ratios of glucose to fructose (G/F) ranging from 0.67 to 0.85, and relatively higher fructose content than glucose. Fructose content ranged from 375.02 to 819.01 mg/100 ml while glucose contents ranged from 251.03 to 695.10 mg/100 ml juice. Rapid increase in the total sugar was observed from the immature to the early half-ripe stage (54–110 DAFB), probably due to intense fruit expansion during the maturity stages. This was followed by a relatively slower but significant accumulation of sugars until the full-ripe stage. The accumulation of simple sugars is one of the processes occurring during the final developmental stages of fruit, resulting in increases in sweetness as fruit approach ripeness (Shwartz et al., 2009; Zarei et al., 2011).

3.3. Changes in individual organic acids

The amount and trend of organic acids measured during matura period are shown in Fig. 3. Tartaric, citric, malic and ascorbic acids were detected as main organic acids in Pj during the fruit developmental stages. Acid content is an important maturity parameter in pomegranate fruit because it plays a major role in the development of juice flavor. Among the acids quantified by HPLC analysis in this study, tartaric acid had the highest value of 39.5 and 79.6% of total acids at 54 DAFB and 165 DAFB, respectively. Tartaric, citric and malic acid contents decreased insignificantly (P>0.05) during fruit maturation until 110 DAFB, followed by significant decrease as the fruit reached the final stages of maturation. This pattern of acidity evolution corroborates with the general phenomenon that organic acids accumulate during fruit growth and are used as respiratory substrates in mature fruit (Diakou et al., 2000; Moing et al., 2001). On the contrary, the result showed that the ascorbic acid content increased with advancing maturity (Fig. 3). This trend is similar to that reported by Shwartz et al. (2009) for ‘Wonderful’ cultivar, but in contrary to Al-Maiman and Ahmad (2002), Kulkami and Aradhya (2005) and Zarei et al., 2011 where ascorbic acid content decreased significantly.

Fig. 2. Concentration of individual and total sugars in pomegranate fruit juice at different maturity stages. Average values (±S.E.) are presented. Different letters on bars of the same color mean statistically significant differences (P<0.05) between concentrations of individual sugars at different maturity stages.

Fig. 3. Concentration of individual organic acids in pomegranate fruit juice at different maturity stages. Average values (±S.E.) are presented. Bars with different letter(s) are statistically significant differences (P<0.05).
with advancing fruit maturity in ‘Ganesh’, ‘Taif’ and ‘Rabbab-e-Fars’ cultivars, respectively. Further studies are required to explain these differences in ascorbic acid accumulation among pomegranate fruit cultivars.

3.4. Changes in total phenolics content and antioxidant capacity

The changes in total phenolics and antioxidant capacity (as measured by DPPH and FRAP assays) of PJ during maturation stages are shown in Fig. 4. Highest content of total phenolic content (TPC) was observed in fruit at the early immature stage (54 DAFB, 2027.46 mg), after which the TPC decreased significantly (P<0.05) at the half-ripe stage, particularly at 110 DAFB (550.25 mg), and then remained significantly stable (P>0.05) until the full-ripe stage (583.72 mg). Overall, there was a decrease of 73% in total phenolics during fruit development and maturation. The decrease in total phenolic content has been attributed to the oxidation of polyphenols by polyphenoloxidase during fruit maturity (Amiot et al., 1995; Kulkami and Aradhya, 2005; Shwartz et al., 2009). Our results showed that total phenolic content was dependent on the maturity stage, and the decrease in TPC with advancing fruit maturity has been reported for different pomegranate cultivars (Kulkami and Aradhya, 2005; Al-Maiman and Ahmad, 2002; Shwartz et al., 2009).

The antioxidant capacity of PJ at different stages of fruit maturation in relation to TPC is shown in Fig. 4. Antioxidants may act in various ways in different antioxidant assays (Cam et al., 2009). Antioxidant capacity has been determined by several methods based on both the free radical scavenging and the oxidation-reduction mechanisms, although the action mechanism set in motion by the antioxidant activity of these compounds is still not clearly understood (Viuda-Martos et al., 2010). The trend of TPC at different maturity stages reflected the antioxidant capacity of the fruit juice in the antioxidant assays employed in this study. This is in agreement with previous studies where different antioxidant assays were used to assess pomegranate arils during fruit maturity (Gil et al., 2000; Kulkami and Aradhya, 2005; Shwartz et al., 2009; Zarei et al., 2011). Immature fruit at the 54 DAFB showed high values of free radical scavenging activity (1.57 mM AAE/ml) and ferric reducing power (1.67 mM TE/ml). Moreover, these values decreased significantly (P<0.05) by up to 42% for DPPH and 10% for FRAP. The radical scavenging activity further decreased significantly (P<0.05) to the lowest value (0.56 mM TE/ml) at early half-ripe stage (110 DAFB) and then remained stabilized with further significant (P>0.05) changes (0.39 mM TE/ml), reflecting insignificant (P>0.05) decrease in total phenolic content, while the ferric reducing power only stabilized at 140 DAFB. The reduction in antioxidant activities during pomegranate fruit maturation may be associated with the relative decrease in content of various polyphenol compounds that constitute the total phenolic content in the fruit juice (Gil et al., 2000; Fischer et al., 2011). The decrease in total phenolics corresponded with significant (P<0.05) decrease in total flavonoids and gallotannins during fruit development and maturation (Fig. 5). This result was expected as flavonoids and gallotannins are phenolic compounds and should contribute to the total phenolic content of the fruit juice (Gil et al., 2000; Fischer et al., 2011). The decrease in total flavonoids and gallotannins is desirable as it reduces the astringency of pomegranate juice, especially in sour cultivars (Al-Said et al., 2009; Zarei et al., 2011). However, the content of total monomeric anthocyanin in pomegranate fruit increased with advancing fruit maturity. Anthocyanins were undetectable at early immature fruit stage (54 DAFB), and then increased significantly (P<0.05) thereafter. This increase could be as a result of the contribution of phenolic compounds to the biosynthesis of flavlyium ring of anthocyanins (Kulkami and Aradhya, 2005). It is worth noting that although anthocyanins are known as antioxidant compounds, their increase during fruit development constituted only small proportion of total phenolic contents in fruit juice, hence the change in flavonoids showed a much more significant influence than anthocyanins on juice antioxidant capacity.

Pomegranate juice is rich in phenolic compounds which have synergistic and/or additive effects on its pharmacological activities (Seeram et al., 2005). Some of the phenolic compounds which have been reported to have potent antioxidant activity in pomegranate juice include punicalagin, ellagic acid, gallic acid, caffeic acid, protocatechuic acid and p-coumaric acid (Gil et al., 2000; Seeram et al., 2005; Elfalleh et al., 2011; Fischer et al., 2011; He et al., 2011).

3.5. Changes in polyphenol and anthocyanin composition

Five individual phenolic compounds were identified and quantified by HPLC-DAD in ‘Bhagwa’ fruit juice. These compounds include flavonols;
composition during the late immature stage studied (82 DAFB) showed that cyanidin 3,5-diglucoside was the predominant pigment, especially during late ripening stage (Fig. 7B). Delphinidin 3,5-diglucoside was the second most abundant anthocyanin in the fruit arils, while pelargonidin 3-glucoside had the lowest concentration. In Spanish cultivars, Hernandez et al. (1999) reported delphinidin 3,5-diglucoside as main pigment in the juice of pomegranate cultivars studied, while Borochov-Neori et al. (2011) found that cyanidin 3-glucoside is the predominant pigment, with values of individual anthocyanins being ten times more than those found in our study. This disparity is reflected in the changes in the mono- and di-glucose derivatives of cyanidin (cy), delphinidin (dp) and pelargonidins (pg), with cy:dp:pg ratio of 50:49:1 and 61:36:3 at 82 and 165 DAFB, respectively (Fig. 7C). This suggests that there was distinct composition and accumulation of pigment in fruit arils at the investigated maturity stages. Overall, the content of both mono- and diglucosylated anthocyanins increased steadily from the late immature stage till full ripe stage (Fig. 7D), with a total anthocyanin accumulation of approximately 91% and mono- and diglucosylated anthocyanins ratio of approximately 1:1 and 1:2 at 82 and 165 DAFB, respectively.

3.6. Multivariate analysis of investigated fruit attributes

Pearson correlation was used to investigate the relationship between selected attribute associated fruit maturity (Table 2) of the pomegranate cultivar. Interestingly significantly ($p<0.05$) strong correlations were revealed. These include significantly ($p<0.05$) strong and positive correlation that TSS showed with individual sugars (fructose: $r^2=0.993$; glucose: $r^2=0.995$), TSS/TA ($r^2=0.991$) and anthocyanin ($r^2=0.943$). Also, TSS correlated negatively strongly with TA ($r^2=-0.985$), this relationship reflected the correlations between individual sugars and organic acids (except ascorbic acid). It is worth noting also, that the relationships between total phenolic content and antioxidant capacities (DPPH and FRAP) were significant ($P<0.05$) and positive (DPPH = 0.993; FRAP = 0.961). Anthocyanin correlated negatively with antioxidant capacity in FRAP assay ($r^2 = -0.881$; $P<0.05$), while the relationship in the DPPH assay was negative but insignificant ($r^2 = -0.819$; $P>0.05$). This further suggests that the free radical scavenging and ferric reducing capacities found at the different fruit maturity stages may not be attributed to the anthocyanin content in fruit juice. Furthermore, the relationships between ascorbic acid and the antioxidant capacities

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(+\text{ catechin}, (-\text{ epicatechin and phenolic acid; protocatechuic acid, gallic acid and ellagic acid (Fig. 6). Among the investigated phenolics, gallic acid had the highest concentration ranging from 42.5 mg/L (54 DAFB) to 10.5 mg/L (165 DAFB). The content of gallic acid at commercial harvest (165 DAFB) was lower than the values reported (13.71–20.55 mg/100 ml) by El-Alaieh et al. (2011) in Tunisian pomegranate ecotypes at commercial harvest. The concentration of (+) catechin and ellagic acid decreased significantly ($P<0.05$) at the half-ripe stage, and then remained unchanged till the last stage of maturity. In contrast, the content of protocatechuic acid increased during the later maturity stages.

The anthocyanin profile of the PJ at different maturity stages studied is shown in Fig. 7. Six major anthocyanins: cyanidin 3,5-diglucoside, cyanidin 3-glucoside, delphinidin 3,5-diglucoside, delphinidin 3-glucoside, pelargonidin 3,5-diglucoside and pelargonidin 3-glucoside were identified. Total anthocyanin level (Fig. 7A) was calculated as the summation of the individual anthocyanins. Anthocyanins were undetectable at the early immature fruit stage (54 DAFB). Total anthocyanin obtained at fruit harvest (56.5 mg/L) is within the range of those previously published for some Spanish cultivars (Gil et al., 1995; Hernandez et al., 1999; Melgarejo et al., 2000). The anthocyanin

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\text{Fig. 6. Individual phenolic compounds in pomegranate juice at different maturity stages. Different letters on bars mean statistically significant differences ($P<0.05$).}
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\text{Fig. 7. Anthocyanin composition in pomegranate juice at different maturity stages. Cy 3,5-cyanidin 3,5-diglucoside; Cy 3-cyanidin 3-glucoside; Dp 3,5-delphinidin 3,5-diglucoside; Dp 3-delphinidin 3-glucoside; Pg 3,5-pelargonidin 3,5-diglucoside; Pg 3-pelargonidin 3-glucoside.}
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were insignificant \((P > 0.05)\). Studies by Wang et al. (1997) and Prior et al. (1998) underlined the significance of the role of phenolic acids and flavonoid compounds to antioxidant capacity of fruit, and concluded that the antioxidant capacity of many types of fruit is due primarily to non-ascorbic acid phytochemicals.

Furthermore, to obtain a broad view on the metabolic changes that occurred during fruit maturation, the whole data set was subjected to principal component analysis (PCA). The total variability is explained by four factors (F1–F4), with the first two factors of the PCA showing correlation of 96.12% of the total variance explained (Fig. 8). Acceptable explanations for variation in fruit maturity stages can be drawn from the factor (F1) which accounted for over 85% of the total variance (Fig. 8). A general view of the PCA showed that young and immature fruits had higher acidity (associated with organic acid concentrations) and phenolic contents, while half-ripe to fully ripened fruits have high TSS which correlated with sugars (fructose and glucose) as well as in attributes (such as juice absorbance and anthocyanins) reflecting the color intensity of fruit juice. Negative scores along F1 corresponded to early and late immature fruits at 54 DAFB and 82 DAFB, respectively. Ripened and full-ripened fruits at 140 DAFB and 165 DAFB had high positive scores along F1, while fruits with low negative scores were half-ripe at 110 DAFB (Fig. 9B). The beginning of ripening process in the cultivar could be well ascribed to fruit at 110 DAFB, where the concentrations of fruit biochemical indices seemed to be at equilibrium (Fig. 9A and B). Short distances between phenolic compounds and antioxidant capacities further suggest their significant contributions to the antioxidant capacity in DPPH and FRAP assays.

### 4. Conclusions

This study provides information on the chemical and phenolic composition, as well as antioxidant capacities of ‘Bhagwa’ pomegranate fruit which is an important commercial pomegranate cultivar. Results obtained showed that major compositional changes in the fruit are developmentally regulated. In general, there were significant differences in the pattern of accumulation of sugars, ascorbic acid and anthocyanins, while there are significant declines in the levels of organic acids, total phenolics and antioxidant capacity during fruit development and maturation. The results also showed that fructose and glucose were the major sugars, while tartaric acid was the major organic acid in the PJ. According

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**Table 2**

Pearson’s correlation coefficients (\(r\)) among selected chemical indices associated fruit maturity.

| Variables | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|-----------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|
| 1. Juice abs | 1 | | | | | | | | | | | | | | | |
| 2. pH | 0.953 | 1 | | | | | | | | | | | | | | |
| 3. TSS | 0.926 | 0.867 | 1 | | | | | | | | | | | | | |
| 4. TA | 0.889 | -0.791 | -0.985 | 1 | | | | | | | | | | | | | |
| 5. TSS/TA | 0.941 | 0.856 | 0.991 | -0.991 | 1 | | | | | | | | | | | | |
| 6. T. phenolics | -0.762 | -0.563 | -0.945 | 0.959 | -0.925 | 1 | | | | | | | | | | |
| 7. T. flavonoids | -0.813 | -0.780 | -0.948 | 0.908 | -0.902 | 0.950 | 1 | | | | | | | | | |
| 8. Galloanthins | -0.537 | -0.480 | -0.771 | 0.745 | -0.703 | 0.893 | 0.920 | 1 | | | | | | | | |
| 9. Anthocyanins | 0.996 | 0.923 | 0.943 | -0.917 | 0.961 | -0.802 | -0.830 | -0.573 | 1 | | | | | | | |
| 10. DPPH | -0.846 | -0.736 | -0.965 | 0.995 | -0.976 | 0.961 | 0.881 | 0.736 | -0.881 | 1 | | | | | | |
| 11. FRAP | -0.782 | -0.684 | -0.946 | 0.945 | -0.921 | 0.993 | 0.971 | -0.919 | -0.919 | 0.938 | 1 | | | | | |
| 12. Glucose | 0.919 | 0.834 | 0.995 | -0.985 | 0.989 | -0.955 | -0.950 | -0.793 | 0.942 | -0.966 | -0.962 | 1 | | | | |
| 13. Fructose | 0.932 | 0.849 | 0.993 | -0.979 | 0.988 | -0.943 | -0.949 | -0.784 | 0.952 | -0.956 | -0.954 | 0.999 | 1 | | | |
| 14. Ascorbic | 0.989 | 0.916 | 0.877 | 0.846 | 0.906 | -0.700 | -0.742 | -0.457 | 0.987 | -0.802 | -0.725 | 0.878 | 0.894 | 1 | |
| 15. Tartaric | -0.839 | -0.756 | -0.978 | 0.892 | -0.972 | 0.982 | 0.931 | 0.803 | -0.670 | 0.991 | 0.965 | -0.976 | -0.966 | -0.781 | 1 |
| 16. Citric | -0.938 | -0.944 | -0.969 | 0.913 | -0.942 | 0.993 | 0.941 | 0.734 | -0.952 | 0.872 | 0.880 | -0.950 | -0.956 | -0.880 | 0.906 | 1 |
| 17. Malic | -0.721 | -0.634 | -0.922 | 0.928 | -0.890 | 0.995 | 0.961 | 0.934 | -0.760 | 0.928 | 0.993 | -0.934 | -0.922 | -0.651 | 0.961 | 0.850 | 1 |

Values in bold are significantly different from 0 with a significance level alpha = 0.05.

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**Fig. 8.** Scree plot of variance explained by each factor of the principal component.

**Fig. 9.** Principal component analysis of the first two factors (F1 and F2) showing dispersion of ‘Bhagwa’ pomegranate fruit cultivar based on the measured parameters at fruit maturity stages; Variable plot (A) and observation plot (B).
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