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# Changes in chemical constituents during the maturation and ripening of two commercially important pomegranate accessions

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ABSTRACT

Pomegranate juice is well known for its health beneficial compounds. This study was undertaken to investigate changes in the major chemical composition in arils and peels during fruit maturation in two Israeli commercial accessions, 'Wonderful' and 'Rosh-Hapered.' In both accessions, the levels of total phenolic, antioxidant activity and hydrolysable tannins were reduced in the peels during maturation, while the anthocyanin level increased. The results show that the sugar content in the aril juice increased in both accessions while the levels of acidity and of citric acid decreased. However, these two accessions differed in other parameters in the aril juice, i.e., while the antioxidant and total phenolic contents significantly decreased in 'Rosh-Hapered', these changes were not observed in 'Wonderful'. The anthocyanin level, however, increased in 'Wonderful' but did not change in 'Rosh-Hapered.' This knowledge could help establish the optimum harvest date ensuring the maximum nutritional properties of pomegranates.


## 1. Introduction

The traditional importance of the pomegranate fruit as a medicinal plant (Al-Maiman & Ahnad, 2002) is now supported by data obtained from modern science showing that the fruit contains anti-carcinogenic (e.g., Adhami & Mukhtar, 2006; Bell & Hawthorne, 2008), anti-microbial (Reddy, Gupta, Jacob, Khan, & Ferreira, 2007) and anti-viral compounds (Kotwal, 2007). Recent biological studies have proven that certain compounds contained in pomegranate juice, which has been shown to reduce blood pressure, are anti-atherosclerotic and significantly reduce LDL oxidation (Aviram et al., 2008). These activities are attributed to the pomegranate's high level of antioxidant activity and high total phenolic content (Gil, Tomas-Barberan, Hess-Pierce, Holcroft, & Kader, 2000; Seeram, Zhang, Reed, Krueger, & Vaya, 2006). Due to the extensive knowledge about the pomegranate's health attributes and increasing public awareness about nutritional food, the demand for pomegranate fruit and its by-products has increased tremendously in the Western world. As a result of this trend, the extent of pomegranate growth was increased significantly in many regions throughout the world. Consequently, industries producing pomegranate juice were developed, as well as pharmaceutical companies, which extracted health beneficial compounds from the fruit (Seeram et al., 2006).

Although, knowledge about the importance of pomegranates in human nutrition has increased tremendously in recent years, the chemical composition of the pomegranate fruit during fruit development has not yet been studied in detail. Chemical changes occurring during the fruit maturation stages can affect the nutritional value and health properties of the pomegranate fruit, hence it is important that pomegranates be harvested at the developmental stage having the greatest potential with respect to health beneficial components. An early harvest may impede the development of the characteristic colour, taste and aroma of pomegranates, while late harvested fruit could exhibit a reduced shelf-life and greater sensitivity to diseases.

This study was undertaken to investigate changes in the major chemical composition and antioxidant activity in arils and peels during different stages of fruit development and maturation. In our long-term objectives, we also aimed at establishing the optimum harvest date to ensure that pomegranates reach consumers or the food industry with maximum functional and nutritional properties. We also aimed at studying whether a correlation exists between the colour of the peel during maturation and the chemical composition of the arils. If such a correlation is found, it will enable

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us to select the harvest date according to peel colour. Two commercially important Israeli pomegranate accessions were chosen for this study differing in taste, colour and ripening season.

## 2. Materials and methods

### 2.1. Plant materials and fruit processing

Two different commercial pomegranate accessions were chosen for this study: the 121-2 accession (a landrace of 'Rosh-Hapered'), which represents the early season fruit; and the 101-2 accession (a landrace of 'Wonderful'), which belongs to the 'Wonderful' group of pomegranate varieties representing the late season fruit. These two accessions were chosen from a collection in the Newe Ya'ar research center, ARO [registered in Israel Gene Bank for Agriculture Crops (IBG, website: http://igb.agri.gov.il)] (Holland, Hatib & Bar-Ya'akov, 2008, Chap. 2). The trees were 30 years old and planted at a $3 \times 5$ m distance in 3–5 replicate trees per accession. Sixty young fruitlets of each accession were marked on May 20, 2007. During fruit maturation, the fruits were harvested at one-week intervals as follows: the accession 121-2 fruits were harvested on August 5, 12, 19, 26, September 2, 9, 16, 23, 30 and October 7 (total of 10 tandem weeks); the agricultural harvest of this accession usually takes place on September 9. The accession 101-2 fruits were harvested on September 16, 23, 30, October 7, 14, 21, 28 and November 4 (total of 8 tandem weeks); the agricultural harvest this accession usually takes place on October 7. Five fruits from each accession were harvested every week. The fruits were transported via a ventilated car to the laboratory, where they were characterised by physical (fruit weight and volume) and chemical parameters, as described below.

The peels and arils were separated from every fruit obtained for each pomegranate accession during every harvested week. 10 g of the peels were homogenised together with 40 ml distilled water (Ben Nasr, Ayed, & Metche, 1996) using a Retsch mill (model MM301) to prepare peel homogenate. Aril juice was prepared by squeezing the arils through a nylon sieve. The homogenates and aril juice were then centrifuged (4000 rpm for 15 min) and the sups were frozen at −20 °C for further analysis.

### 2.2. Physical analysis

Every fruit from each accession was weighed on a balance of accuracy of 0.001 g. The volume of the fruit was measured using the liquid displacement method.

### 2.3. Determination of total soluble solids (TSS) and titratable acidity

The measurements were made on fresh aril juice. TSS in the juice was measured using a digital refractometer (ATAGO RR-1 serial no. 602055 Tokyo, Japan, calibrate using distilled water). The instrument was set to measure %TSS with the temperature compensated mode. A Metrohm titration unit (Brinkmann, Metrohm ch-9101 Herisau, Switzerland) equipped with a 719S Titrino titration assembly was used for total titratable acidity determination. 2 ml juice was diluted with 10 ml distilled water and titrated with 0.1 N NaOH to pH 8.2. Titratable acidity was calculated as g citric acid/100 g fresh weight or as a percentage of citric acid. Measurements were replicated twice for each aril juice.

### 2.4. Determination of total phenolic content

For total phenol compounds determination, 1:10 dilutions of the juices were used. Total phenolic were determined using the colorimetric method with a spectrophotometer, which modified the Singleton method for small volumes (Ben Nasr et al., 1996; Singleton & Rossi, 1965). 0.5 ml of the juice samples/standard was mixed with 2.5 ml of diluted Folin–Ciocalteu reagent (1:10 in water). After a short vortex, 20% $Na_2CO_3$ were added and the tubes were mixed for 5 min at 50 °C. Quercetin was used as a standard. The absorbance of the cooled samples was measured at 760 nm.

### 2.5. Antioxidant activity evaluation

Antioxidant activity was measured, as previously recommended, using the FRAP method (Gil et al., 2000; Tzulker et al., 2007). This method was developed to measure the ferric reduction ability of plasma at a low pH (Benzie & Strain, 1996). An intense blue colour is formed when the ferric-tripyridyltriazine ($Fe^{3+}$-TPTZ) complex is reduced to the ferrous ($Fe^{2+}$) form, which was recorded at 593 nm. Standard solution of 1 mM trolox (6-hydroxy-2,3,7,8-tetramethylchroman-2-carboxylic acid) in methanol was prepared. The pomegranate juices were diluted 1:50 in water. Fifty micro-litres of diluted standards of samples were mixed with 950 l FRAP solution prepared by mixing 25 ml acetate buffer (pH 3.6), 2.5 ml TPTZ and 2.5 ml $FeCl_3 \cdot 6H_2O$ solutions. These solutions were left to react for 4 min under continuous stirring. The changes in absorbance were then measured at 25 °C. The results were expressed as trolox equivalent antioxidant capacity (TEAC) (Benzie & Strain, 1996; Gil et al., 2000).

### 2.6. Determination of total anthocyanins content

Anthocyanin pigments undergo reversible structural transformations with a change in pH manifested by strikingly different absorbance spectra (Giusti & Wrolstad, 2001). The coloured oxonium form predominates at pH 1.0 (25 mM potassium chloride buffer) and the colourless form at pH 4.5 (0.4 M sodium acetate buffer). The samples were diluted by a potassium chloride buffer until the absorbance of the sample at a 510 nm wavelength was within the linear range of the spectrophotometer. This dilution factor was later used to dilute the sample with the sodium acetate buffer. The wavelength reading was performed after 15 min of incubation, four times per sample, diluted in the two different buffers and at two wavelengths of 510 and 700 nm. The absorbance was then calculated according to the following equation: $A = (A_{510} - A_{700})_{\mathrm{pH\ 1.0}} - (A_{510} - A_{700})_{\mathrm{pH\ 4.5}}$. Results were expressed as mg of cyanidin-3-glucoside per 1 l of juice, using a molar absorptive coefficient ($\varepsilon$) of 26,900 and a molecular weight of 449.2.

### 2.7. Determination of aril juice colour

The colour of the juice prepared from the arils and the peel surface was determined using a colorimeter (Chroma Meter CR-301, Minolta, Ramsey, N.J. USA) (Solomon et al., 2006), and expressed in dimensions of '$L^*$', '$a^*$', '$b^*$', '$C$' and '$H$' (Aaby, Skrede, & Wrolstad, 2005). These parameters calculated according to the following equation: $(180 - H)/(L + C)$, to obtain what referred to us as the juice 'colour index'. The mean values for five points on the peel surface were calculated for each fruit, and mean values for the different fruits harvested at each developing stage were also calculated.

### 2.8. LC-MS analysis of hydrolysable tannins

#### 2.8.1. Sample preparation

The homogenate samples from these two accessions were diluted with doubled distilled water 1:1000. The samples were further diluted at 1:1 with acetonitrile (Merck cat #30) to achieve final concentrations of 1:2000. The samples were filtered with a 0.45 μm into testing vials and analysed by a LC-MS instrument

using a waters 2790 HPLC system equipped with a Micromass triple quadrupole Quatro-Ultima mass spectrometer in series, consisting of a HPLC quaternary pump, an auto-sampler and a vacuum degasser. The system was controlled by Micromass MassLynx ver. 4.0 software. The chromatographic separations and solvent gradient were the same as previously described for punicalagin, punicalin, gallagic and ellagic acids (Tzulker et al., 2007). The solute was inserted into the mass spectrometer using an Electro Spray Ionisation Probe in the negative mode. The high selectivity identity of the compound was obtained using the Multiple Reaction Monitoring (MRM) method according to their mother and daughter ions. The mother ion (precursor ion) was fragmented by argon using different collision energies, and the daughter ion areas of the standard solutions were compared to those received from the pomegranate samples, as described in Tzulker et al. (2007).

### 2.9. Sugar and organic acid contents

The content of organic acids and sugars was determined in aril juice. The aril juice was diluted 1:10 with distilled water and then filtered through a 0.45 μm Millipore membrane filter. The diluted juices were injected into a Hewlett-Packard HPLC series 1090. The elution system consists of 0.1% phosphoric acid running isocratically with a flow rate of 0.6 ml $min^{-1}$. The organic acids were eluted through an Aminex 87H (Bio-Rad) (30 cm × 7.8 mm) and detected by absorbance 210 nm. Ascorbic acid was detected using UV detector at 254 nm. A standard curve of pure organic acids purchased from Sigma (Poole, Dorest, UK) was used for quantification. Results were expressed as mg of ascorbic acid 100 $g^{-1}$ and g $100^{-1}$ (Aaby et al., 2005) for the remaining acids. For sugar concentrations, the same HPLC elution system, flow rate and column were used. The detection of sugars was obtained by a refractive index detector. A standard curve of pure sugars (glucose, fructose, sucrose and sorbitol) purchased from Sigma was used for quantification. Results were expressed as part per million (ppm) sugars of the juices.

### 2.10. Statistical analysis

The data obtained from this study were analysed statistically using SPSS software adapted to Windows, ver. 16. In this software, Spearman test was used for the correlation studies and their significance. ANOVA test was preformed using the JMP software, ver. 5. Principal component analysis was preformed on the data sets obtained from different measurements with the software TMEV (Saeed et al., 2003) using the default weighted covariance-estimation function. The data were log transformed and normalised to the median of the entire sample set for each parameter before analysis. This transformation reduced the influence of outliers.

## 3. Results and discussion

Two different commercial pomegranate accessions were chosen in order to study the effect of development and ripening stages on phenotype and chemical changes: the 121-2, which represents the early season fruit; and the 101-2 accession, representing the late season fruit (Holland et al., 2008, Chap. 2). These two accessions also differ in peel and aril colour, and taste: accession 121-2 has a white, slightly pink aril/peel colour and a strong sweet taste; accession 101-2 has a deep red aril/peel colour and a sour-sweet taste. In order to determine the changes that occurred during these maturation stages, fruit size and the level of selected chemical compounds were detected to gain greater knowledge about the changes occurring during fruit maturation.

### 3.1. Fruit size gradually increased during fruit maturity and ripening

In both pomegranate accessions, fruits of 121-2 and 101-2 increased in size during the maturation and ripening stages (Fig. 1; Suppl. Table a). This is in agreement with other reports demonstrating that pomegranate fruit size and weight increased during maturation in the 'Molar', 'Taifi' and 'Wonderful' accessions (Al-Maiman & Ahnad, 2002; Ben-Arie, Segal, & Guelfat-Reich, 1984; Gil, Garcia-Vigguera, Artes, & Tomas-Barberan, 1995). This indicates that the pomegranate fruit continues to grow even after the optimum harvesting stage, unlike other fruit types such as tomato, in which two stages can be defined: the first phase where the fruit increases in size; and the second phase where the ripening occurs, characterised by changes in colour, texture and taste. In the latter phase, the fruit does not continue to grow in size (Giovannoni, 2001).

### 3.2. The levels of antioxidant capacity, total phenolic and total anthocyanin contents changed in arils juice during fruit maturation

The beneficial health effects attributed to pomegranate fruit consumption are related, at least in part, to their antioxidant activity (Vaya & Aviram, 2001). Since pomegranate arils are largely consumed in our region, we first examined the level of antioxidant activity during the fruit maturation. The FRAP method was used (Tzulker et al., 2007) and the values calculated as equivalent to Trolox. The level of antioxidant activity was significantly reduced during the 10 weeks of maturation in accession 121-2 from 10.3 to 7.8 mM. However, only a slight reduction was observed in accession 101-2 representing the late season ripening pomegranate fruit (Fig. 2; Suppl. Table b).

In pomegranates as well as in many other fruits and vegetables, the level of antioxidant activity can be attributed to the level of total phenolic content (e.g., Gil et al., 2000; Solomon et al., 2006; Tzulker et al., 2007). Therefore, total phenolic levels were measured in these juices. Consistent with the reduction found in antioxidant activity, the total phenolic level was also significantly reduced in accession 121-2 during the 10 weeks of maturation from 3.9 to 1.9 mM. However, such a reduction in phenols was not observed for accession 101-2 (Fig. 2; Suppl. Table b). Yet, total

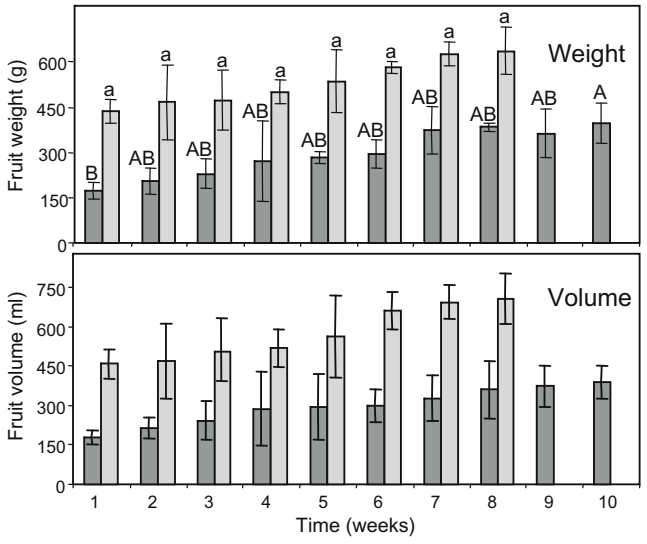


**Fig. 1.** Changes in fruit size, weight and volume in two pomegranate accessions, 121-2 (■) and 101-2 (□), during fruit maturation. The data presented represent the mean ± SD of five replicates from each accession. ANOVA was used to determined statistically significant difference at ($p < 0.05$) as identified by different letters.

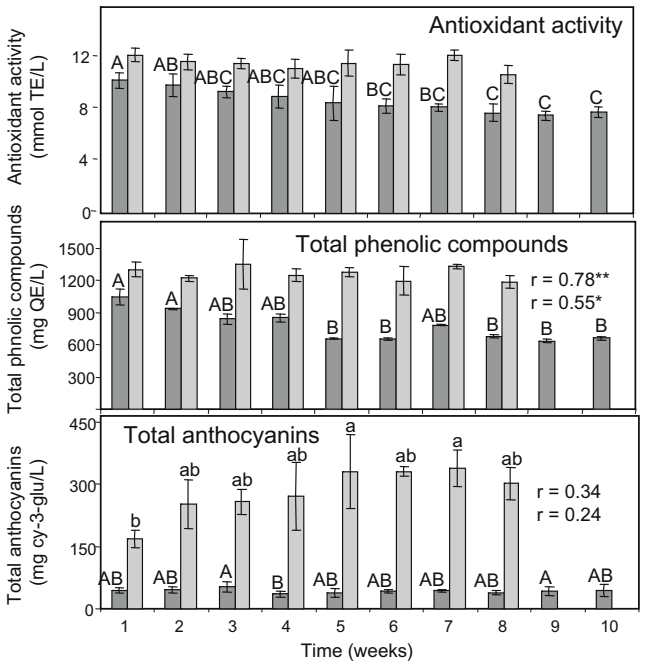


**Fig. 2.** Changes in antioxidant activity, represented as mmol of trolox equivalents (TE) per L juice, total phenolic compounds content, represented as mg quercetin equivalents (QE) per L juice, and total anthocyanin level, represented as mg cyanidin 3-glucoside equivalents per L juice, in aril juices prepared from two pomegranate accessions, 121-2 (■) and 101-2 (□) during fruit maturation. The data presented represent the mean ± SD of five replicates from each accession. The *r* value was calculated against the antioxidant activity. ANOVA was used to determined statistically significant difference at ($p < 0.05$) as identified by different letters.

phenolic content and antioxidant activity correlated significantly in the aril juice of both accessions ($r = 0.78$ for 121-2 and $r = 0.55$ for 101-2, $p < 0.01$).

A reduction in total phenolics compounds in the aril juice during ripening and maturation was previously reported for the 'Ganesh' pomegranate accession (Kulkarni & Aradhya, 2005). A decrease in phenolic compounds during ripening was also reported for other fruits, such as pear (Amiot, Tacchini, Aubert, & Oleszek, 1995) and guava (Bashir, Abu-Bakr, & Abu-Goukh, 2003). A reduction in total phenolic that correlated to antioxidant capacity was found in strawberry (Wang & Lin, 2000). The decline in total phenolic may be due to the oxidation of phenolic content by polyphenol oxidase that characterises these stages of maturity (Amiot et al., 1995). Since total phenolic, especially the hydrolysable tannins, are major contributors to the astringent taste of fruit, their reductions are important in adjusting the fruit to feed animals, who, in the later stages of maturation, eat the fruit and spread the seeds. The level of hydrolysable tannins, which belong to the total phenolic compounds such as punicalgin, punicalin, gallagic and ellagic acids that characterise the pomegranate, were not determined in the current study in the aril juice since it was previously shown that their levels in aril juice of the these two accessions were very low, almost below detection level (Tzulker et al., 2007).

While the level of total phenolic was reduced in accession 121-2 and in several other fruits, the level of total phenolic, and accordingly their antioxidant activity in some fruits, increased during maturation. This was found in muscadine grapes (Lee & Talcott, 2004) and in sweet cherry (Serrano, Guille'n, Martínez-Romero, Castillo, & Valero, 2005). In these latter fruit types, the increase in total phenolic was associated with the greatest accumulation of anthocyanins, a group of phenolic compounds that accumulated in these fruits (Serrano et al., 2005). The accumulation of anthocyanins during ripening was also found in blackberry and raspberry (Siriwoharn, Wrolstad, Finn, & Pereira, 2004; Wang & Lin, 2000).

Anthocyanins are water-soluble pigments primarily responsible for the attractive red–purple colour of many fruits, including pomegranate juice, and they are well known for their antioxidant activity (e.g., Seeram & Nair, 2002). In order to determine if anthocyanins content is altered in aril juice during maturation, a factor that influences the juice pigmentation and one of the important quality factors of pomegranate marketing, we next measured anthocyanins levels. The anthocyanins levels in the aril juice were significantly increased during maturation in accession 101-2 (from 165 to 328 mg/l), which has a strong red aril colour, but not in accession 121-2, which has a white–pink aril colour (Fig. 2; Suppl. Table b). Accession 101-2 belongs to the 'Wonderful' group of pomegranate fruit (Holland et al., 2008, Chap. 2), and our results agreed with those previously reported for this accession by Ben-Arie et al. (1984), showing that the red colour in the aril juice significantly increased during ripening. A rapid increase in anthocyanin pigment concentration during ripening was also reported for the 'Mollar' and 'Ganesh' pomegranate accessions (Gil et al., 1995; Kulkarni & Aradhya, 2005). The results suggest that in some pomegranate accessions, the level of anthocyanins increases, while in others, such as accession 121-2, which has basically a low content of these pigments, the level does not significantly change.

Anthocyanins contribute to fruit colour, however some of these compounds do not have a strong red–purple colour. To determine the relationships between anthocyanins levels and aril juice colour, the juice colours were measured using a colorimeter, and the '$L^*$', '$a^*$', '$b^*$', '$C$' and '$H$' values were recorded (Table 1). These values did not change significantly during maturation in both 121-2 and 101-2 accessions. We previously used the 'colour index' to determine the juice colour of pomegranates, since the values obtained correlated well to the colour examined by the naked eye and to the anthocyanins level (Tzulker et al., 2007). The 'colour index' correlated significantly to the anthocyanins level of accession 101-2 ($r = 0.59$, $p < 0.01$) but not to that of accession 121-2, whose colour does not change significantly during maturation. The low correlation value obtained in accession 121-2 suggests that other compounds in addition to anthocyanins, such as hydroxycinnamic

**Table 1**
Colour determination in pomegranate aril juices at different stages of maturity in accessions 121-2 and 101-2.

| Arils / Harvested weeks | $L^*$ | $a^*$ | $b^*$ | $C$ | $H$ | Colour index |
|---|---|---|---|---|---|---|
| *121-2* | | | | | | |
| 1 | 21.9 ± 0.5 | 4.7 ± 0.3 | 0.8 ± 0.3 | 4.8 ± 0.3 | 10.3 ± 4.3 | 6.3 ± 0.0 |
| 2 | 20.6 ± 0.8 | 5.5 ± 0.3 | 1.4 ± 0.1 | 5.7 ± 0.3 | 14.1 ± 1.2 | 6.3 ± 0.1 |
| 3 | 20.2 ± 0.5 | 6.6 ± 0.1 | 2.0 ± 0.2 | 6.9 ± 0.1 | 16.9 ± 1.9 | 6.0 ± 0.1 |
| 4 | 20.0 ± 0.5 | 5.3 ± 0.4 | 1.0 ± 0.1 | 5.4 ± 0.4 | 11.3 ± 1.1 | 6.6 ± 0.1 |
| 5 | 23.2 ± 0.7 | 3.6 ± 0.9 | 0.5 ± 0.3 | 3.7 ± 1 | 7.9 ± 3.3 | 6.3 ± 0.1 |
| 6 | 22.0 ± 0.9 | 4.6 ± 0.4 | 1.5 ± 0.2 | 4.9 ± 0.5 | 18.7 ± 1.5 | 5.9 ± 0.2 |
| 7 | 20.0 ± 0.7 | 5.9 ± 0.4 | 1.9 ± 0.2 | 6.3 ± 0.5 | 18.2 ± 0.7 | 6.1 ± 0.2 |
| 8 | 20.5 ± 0.4 | 5.4 ± 0.2 | 1.8 ± 0.2 | 5.7 ± 0.2 | 18.4 ± 2 | 6.1 ± 0.1 |
| 9 | 22.4 ± 0.4 | 4.8 ± 0.8 | 1.3 ± 0.3 | 4.9 ± 0.9 | 15.2 ± 1.3 | 6.0 ± 0.1 |
| 10 | 17.1 ± 0.5 | 6.5 ± 0.9 | 2.7 ± 0.3 | 7.1 ± 0.9 | 22.8 ± 1.1 | 6.5 ± 0.2 |
| *101-2* | | | | | | |
| 1 | 17.1 ± 1.1 | 5.8 ± 0.3 | 2.8 ± 0.3 | 6.6 ± 0.4 | 25.7 ± 1.6 | 6.4 ± 0.0 |
| 2 | 18.7 ± 0.3 | 4.8 ± 0.4 | 2.1 ± 0.5 | 5.3 ± 0.5 | 24 ± 4.3 | 6.5 ± 0.2 |
| 3 | 18.6 ± 0.6 | 4.2 ± 0.3 | 2.0 ± 0.1 | 4.5 ± 0.3 | 25.4 ± 0.4 | 6.7 ± 0.1 |
| 4 | 18.3 ± 1.1 | 4.9 ± 1.3 | 1.9 ± 0.6 | 4.7 ± 1.4 | 25.3 ± 4.1 | 6.7 ± 0.2 |
| 5 | 19.6 ± 0.1 | 4.2 ± 0.8 | 1.8 ± 0.5 | 4.6 ± 1 | 22.6 ± 2.5 | 6.6 ± 0.1 |
| 6 | 18.6 ± 0.4 | 5.1 ± 0.4 | 2.1 ± 0.4 | 5.7 ± 0.5 | 23.8 ± 3.8 | 6.5 ± 0.2 |
| 7 | 20.0 ± 0.4 | 3.5 ± 0.3 | 1.3 ± 0.3 | 3.7 ± 0.3 | 21 ± 3.4 | 6.8 ± 0.1 |
| 8 | 20.5 ± 0.6 | 3.4 ± 0.5 | 1.7 ± 0.2 | 3.6 ± 0.6 | 28.5 ± 1.1 | 6.3 ± 0.3 |

acids, influence aril colour. To determine the nature of these compounds, further studies are required.

### 3.3. The levels of sugars and some organic acids increase during maturation, while the levels of other organic acids decrease

One of the processes occurring in fruit during ripening is the hydrolysis of starch that accumulates into simple sugars in the early stages of fruit development. As a result, the fruit get its sweetness, which attracts birds and mammalians to eat the fruit and spread the seeds. It was found that accession 121-2, which has a strong sweet taste, has similar levels of glucose and fructose to those found in accession 101-2, which has a sweet–sour taste (Fig. 3; Suppl. Table c). Pomegranate aril juices showed a significantly increased content of these two sugars during fruit maturation in both accessions (Fig. 3). The increase in total soluble sugars also affects total soluble solids (TSS) content, which is one of the most widely used parameters measured during fruit ripening and characterises its quality. The TSS value indeed increases significantly during these maturation stages (Fig. 3; Suppl. Table c), and a strong correlation was found between TSS and glucose and fructose levels during fruit development ($r = 0.99$, $p < 0.01$). A similar increase in TSS level was also reported for other pomegranate accessions during fruit development (Al-Maiman & Ahnad, 2002; Gil et al., 1995; Kulkarni & Aradhya, 2005). However, in previous experiments with the 'Wonderful' accession, it was found that the TSS increased significantly during the first 4.5 months from the flowering period through fruit development, and then generally remained at a fairly constant level (Ben-Arie et al., 1984). This was not observed in accession 101-2 considered to be a 'Wonderful' landrace (Fig. 3; Suppl. Table c), in which the TSS content increased gradually throughout all of the fruit maturation stages. The differences found between the above study and this study may be attributed to the fact that these are similar accessions, and not the same accession, and the fruits were grown in different years and in different regions in Israel, which may affect the processes occurring during the maturation stages. The TSS level also increased in fruits such as mango and banana (Bashir et al., 2003), sweet cherry (Serrano et al., 2005) and blackberry (Siriwoharn et al., 2004).

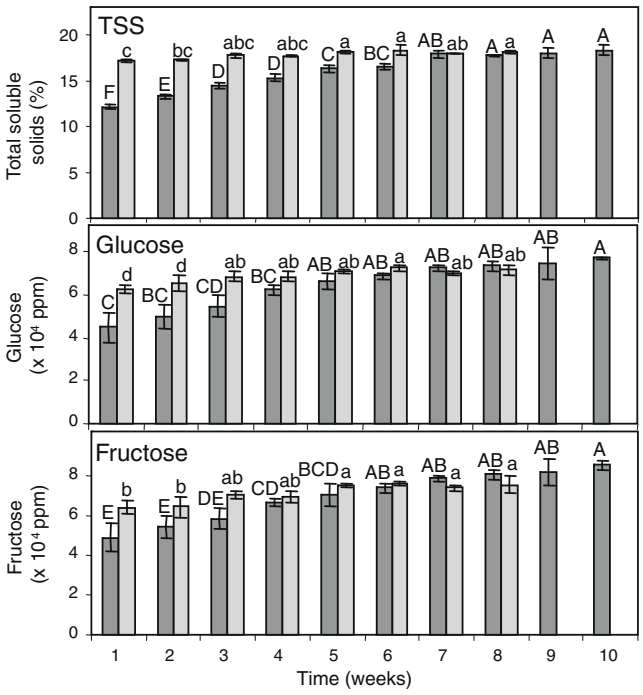


**Fig. 3.** Changes in TSS, glucose and fructose in aril juices prepared from two pomegranate accessions, 121-2 (■) and 101-2 (□), during fruit maturation. The data presented represent the mean ± SD of five replicates from each accession. ANOVA was used to determined statistically significant difference at ($p < 0.05$) as identified by different letters.

In addition to TSS, the titratable acidity content was also used to identify the fruit and juice qualities. Actually, the ratio between TSS and titratable acidity defines the "taste" of the fruit, and for the 'Wonderful' accession the values varied from 11 to 16 (Ben-Arie et al., 1984). As expected from the general taste of the arils in both accessions (121-2 and 101-2), the titratable acidity content of accession 101-2 having a sour–sweet taste was significantly higher (about 3-fold in the first measured stage) than accession 121-2 having a sweet taste. The titratable acidity is significantly reduced in the aril juice of accession 101-2 but not in accession 121-22, while the pH values were slightly increased in accession 121-22 but were not significantly altered in accession 101-2 during fruit development and maturation (Fig. 4; Suppl. Table d). Elevation in the pH of the aril juice was observed during pomegranate fruit development in the 'Taifi' and 'Mollar' pomegranate accessions (Al-Maiman & Ahnad, 2002; Gil et al., 1995). A reduction in titratable acidity during fruit development was also found in the 'Ganesh', 'Taifi' and 'Wonderful' pomegranate accessions (Ben-Arie et al., 1984; Gil et al., 1995; Kulkarni & Aradhya, 2005). A reduction in titratable acidity was also reported for other fruits, such as blackberry (Siriwoharn et al., 2004), which reflect an adjustment of fruit taste for animals that eat the fruit and spread the seeds.

It was previously suggested that although several organic acids were found in pomegranate aril juices, the major acid accounting for titratable acidity is citric acid (Melgarejo, Salazar, & Artes, 2000). The result obtained by HPLC analysis show that this might also be the case for accession 101-2, since citric acid is found to be the major organic acid in this juice (Fig. 4; Suppl. Table d) and is significantly correlated to titratable acidity ($r = 0.99$, $p < 0.01$). However, the percentage of citric acid in aril juice prepared from accession 121-2 was the lowest compared to other organic acids such as malic, oxalic and succinic acid, and only slightly higher than ascorbic acid (Fig. 4; Suppl. Table d). In addition, citric acid was not significantly correlated to the titratable acidity level in this accession. Although, the titratable acidity content in accession 121-2 was significantly lower than that found in accession 101-2, the juice of the former has two additional organic acids that were not detected in the aril juice of accession 101-2, namely, oxalic and succinic acids. The levels of these two organic acids were significantly reduced in the aril juice of accession 121-2 during maturation (Fig. 4; Suppl. Table d). However, the levels of malic and ascorbic acids increased in both accessions during fruit development, while that of citric acid was significantly reduced. Changes in the levels of organic acids during fruit maturation were also reported for sweet cherry, where the level of malic acid that is predominant in the fruit increases through developmental, while those of citric and succinic acids were not changed significantly (Serrano et al., 2005).

Ascorbic acid (Vitamin C) is abundant and has many biological functions in fruits, which include roles in many aspects of redox control and antioxidant activity that prevent, for example, the browning of tissues (Kulkarni & Aradhya, 2005). While measurements of ascorbic acid content show that its levels decreased significantly with ongoing maturity in the 'Ganesh' and 'Taifi' pomegranate accessions (Al-Maiman & Ahnad, 2002; Kulkarni & Aradhya, 2005), we found that its levels increased in both accessions tested in the current study (Fig. 4). A significant elevation in ascorbic acid content during maturation was also found in sweet cherry (Serrano et al., 2005), while reductions during fruit development have been often recorded for other fruits such as guava, mango and banana (Bashir et al., 2003).

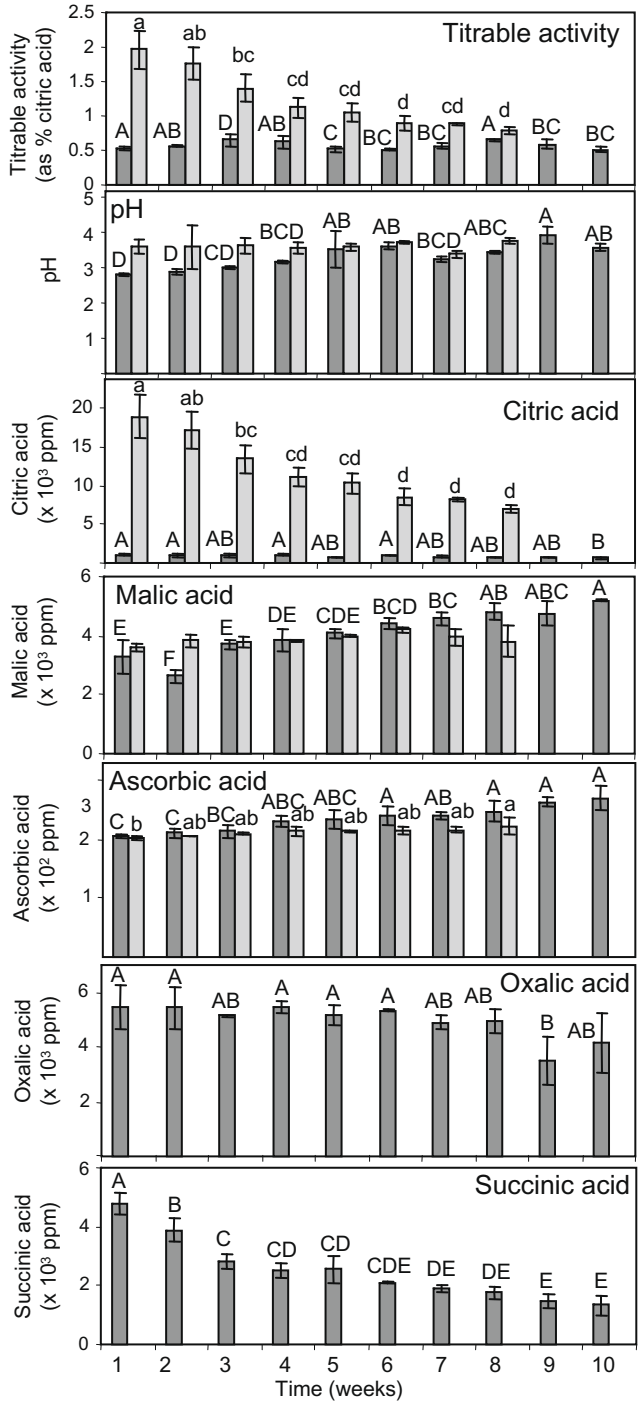


**Fig. 4.** Changes in titratable acidity, pH and organic acids found in aril juices prepared from two pomegranate accessions, 121-2 (■) and 101-2 (□), during fruit maturation. The data presented represent the mean ± SD of five replicates from each accession. ANOVA was used to determined statistically significant difference at ($p < 0.05$) as identified by different letters.

### 3.4. Changes occur during the ripening period in homogenates prepared from the peels

It was previously found that pomegranate peels contain a high concentration of antioxidant compounds (Aviram et al., 2008; Tzulker et al., 2007). The high correlation between antioxidant activity and the level of hydrolysable tannins, such as punicalagin, punicalin and gallagic acid, which are found mainly in peels, suggest that these latter compounds contribute significantly to the antioxidant activity of peels (Tzulker et al., 2007). Thus, we next examined whether antioxidant activity and total phenolic content in peels change during ripening and fruit maturation. To this end, the peels of these accessions were separated from the other fruit fractions and homogenates with water. The results (Fig. 5; Suppl. Table e) show that in both accessions, antioxidant activity and total phenolic content were significantly reduced during maturation. This reduction is in accordance with a reduction in the levels of punicalagin, gallagic and punicalin, the major compounds of hydrolysable tannins of the peels (Tzulker et al., 2007) (Fig. 5). This

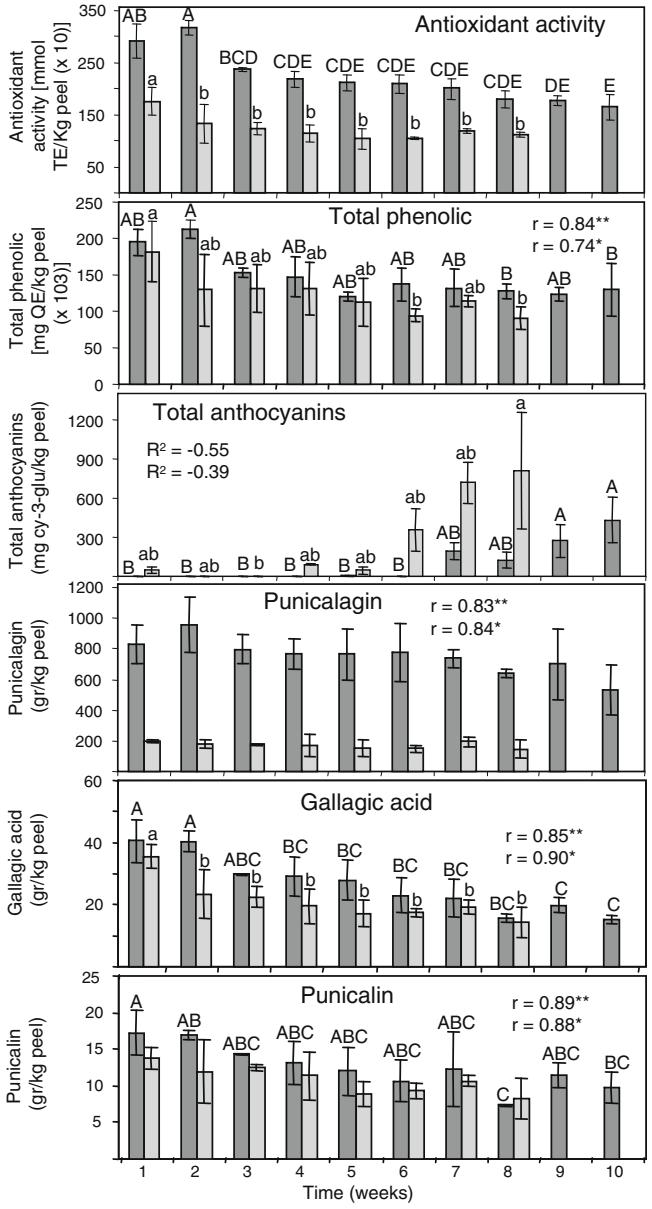


**Fig. 5.** Changes in antioxidant activity, represented as mmol of trolox equivalents (TE) per kg peel, total phenolic compounds content, represented as mg quercetin equivalents (QE) per kg of peel, and total anthocyanin level, represented as mg cyanidin 3-glucoside equivalents per kg peel, and the three major hydrolysable tannins, punicalagin, gallagic acid and punicalin in homogenates prepared from peels of two pomegranate accessions, 121-2 (■) and 101-2 (□), during fruit maturation. The data presented represent the mean ± SD of five replicates from each accession. The r value was calculated against the antioxidant activity. ANOVA was used to determined statistically significant difference at ($p < 0.05$) as identified by different letters.

suggests that total phenolic, which play a role in protecting the fruit from plant diseases and pests that are needed in the first stages of fruit development, are required less during the final stages of maturation. Moreover, since the hydrolysable tannins might be responsible for astringency, a reduction in these compounds in the fruit suggests that the fruit adjusts itself to the taste of animals that consume the fruit and spread its seeds. The reductions in hydrolysable tannins were in accordance with the reduction in the level of ellagic acid, which belongs to this group of compounds in strawberry (Williner, Pirovani, & Guemes, 2003). However, the level of ellagic acid increased during ripening stages in the skin and juice of muscadine grapes (Lee & Talcott, 2004), demonstrating that different fruits vary in their ability to reduce or accumulate hydrolysable tannins during the ripening processes.

Next, the levels of anthocyanins, which also belong to the total phenolic compounds, were measured in these homogenates. It was found that while the total phenolic reduced in their levels, the level of total anthocyanins significantly increased during the maturation stages (Fig. 5; Suppl. Table e). The negative correlations between these two parameters (Fig. 5) can be explained, as previously suggested, since the flavylium ring required for anthocyanins formation is made from phenolics compounds and this may lead to a reduction in their content and an elevation in anthocyanins levels (Kulkarni & Aradhya, 2005).

Anthocyanins can contribute significantly to peel colour, therefore we also measured the peel's skin colour directly by determining '$L^*$', '$a^*$' '$b^*$' $C$ and $H$ values using a colorimeter. The '$L^*$' value is reduced in both accessions during fruit maturation. This trend was particularly noted in accession 121-2 (Table 2), indicating that the peel colour becomes darker as the fruit matures. The most notable changes were detected in the '$a^*$' value, which increased significantly in both accessions with ripening. This is in accordance with the replacement of the green colour with the red colour, which increased during this period. A similar trend was also reported for the 'Mollar' pomegranate accession (Gil et al., 1995). The '$b^*$' value was reduced significantly in the peels of both accessions, indicating that the yellow colour is replaced by blue pigments during maturation. The 'colour index' increased significantly in both accessions during maturation (Table 2). To further study whether the anthocyanins contributed significantly to peel colour, correlations were performed between the levels of anthocyanins in the peels and the colour parameters. It was found that total anthocyanins and the colour index are significantly correlated in both accessions, 101-2 and 121-2 ($r = 0.82$, $p < 0.05$ and $r = 0.81$, $p < 0.01$, respectively), indicating that the total anthocyanins content contributed significantly to the peel's skin colour.

Since peel colour significantly changed during maturation in both accessions, we next studied if these changes are correlated to chemical changes occurring in the arils. To this end, the colour index of the peels was correlated to the parameters measured in the aril juice, as well to aril colour index. It was found that the two accessions differ in their correlations. In accession 121-2, the colour index of the peels was significantly positively correlated to the following aril juice parameters: TSS, glucose and fructose content, pH, and to the levels of malic and succinic acids (Suppl. Table g). This colour index is significantly negatively correlated to antioxidant activity, total phenolic content and the contents of citric, oxalic and ascorbic acids (Suppl. Table g). On the other hand, the colour index of accession 101-2 was positively correlated only with ascorbic acid, and negatively correlated with acidity and citric acid (Suppl. Table f). This suggests that the fruit colour of accession 121-2 can provide a good indication of aril quality and the content of its major compounds, while such an indication can only slightly be obtained for accession 101-2.

### 3.5. Principal component analysis suggests that different stages of fruit maturation are associated with distinct metabolites changes

To obtain a broad view on the metabolic changes that occurs during fruit maturation, we also analysed the data set by principal component analysis (Fig. 6). In this analysis, the samples of given time point were relatively indiscriminant from one another and the distance between samples types increased across the first component with the weeks of fruit maturation. The results of this analysis have shown that samples of arils and the peels derived from various stages of developments were clearly separated on the basis of metabolites differences and other measured parameters (Fig. 6A and B). The two accessions were differs, in their metabolites changes, both in the arils and peels, during their maturation as expected from their different primary phenotypes, colour and taste (Fig. 6A and B).

Next, this analysis was further used to study the distances between the different parameters that were measured in the arils and peels. Such analysis can lead to more knowledge on the rela-

**Table 2**
Colour determination in pomegranate peel surface at different stages of maturity in accessions 121-2 and 101-2.

| Peels<br>Harvest date | $L^*$ | $a^*$ | $b^*$ | $C$ | $H$ | Colour index |
|---|---|---|---|---|---|---|
| *121-2* | | | | | | |
| 1 | 73.9 ± 5.0 | 12.4 ± 5.4 | 36.5 ± 2.5 | 38.9 ± 3.1 | 71.5 ± 7.6 | 0.9 ± 0.1 |
| 2 | 68.4 ± 7.4 | 19.5 ± 8.1 | 35.4 ± 2.7 | 41.5 ± 4.7 | 62.2 ± 13.4 | 1.1 ± 0.2 |
| 3 | 63.7 ± 4.9 | 26.3 ± 6.1 | 29.2 ± 4.1 | 41.4 ± 3.8 | 45.8 ± 8.7 | 1.3 ± 0.1 |
| 4 | 63.2 ± 3.6 | 26.7 ± 7.8 | 28.4 ± 3.3 | 37.9 ± 4.9 | 49.9 ± 10.4 | 1.3 ± 0.0 |
| 5 | 59.3 ± 4.9 | 35.2 ± 6.3 | 25.1 ± 3.9 | 43.6 ± 5.2 | 35.9 ± 10.8 | 1.4 ± 0.1 |
| 6 | 58.1 ± 5.0 | 36.5 ± 4.6 | 24.7 ± 2.8 | 44.2 ± 3.9 | 34.3 ± 4.9 | 1.4 ± 0.1 |
| 7 | 34.2 ± 4.0 | 37.3 ± 5.8 | 14.9 ± 2.0 | 39.9 ± 6.3 | 20.3 ± 3.6 | 1.9 ± 0.3 |
| 8 | 37.5 ± 11.5 | 42.6 ± 7.1 | 18.2 ± 2.6 | 46.9 ± 6.4 | 24.8 ± 7.5 | 2.1 ± 0.2 |
| 9 | 33.7 ± 9.6 | 38.4 ± 9.2 | 15.9 ± 4.2 | 38.1 ± 9.3 | 25.2 ± 6.1 | 2.2 ± 0.6 |
| 10 | 37.7 ± 0.5 | 39.6 ± 0.8 | 14.2 ± 2.8 | 42.1 ± 6.9 | 23.1 ± 2.8 | 2.2 ± 0.2 |
| *101-2* | | | | | | |
| 1 | 59.4 ± 16.4 | 13.1 ± 11.4 | 35.4 ± 4.7 | 37.1 ± 6.7 | 54.7 ± 13.3 | 1.2 ± 0.2 |
| 2 | 47.1 ± 11.5 | 38.9 ± 7.8 | 19.1 ± 3.2 | 46.9 ± 6.4 | 24.8 ± 7.6 | 1.3 ± 0.1 |
| 3 | 58.6 ± 8.4 | 34.4 ± 7.7 | 31.7 ± 6.1 | 45.1 ± 4.2 | 46.1 ± 11.6 | 1.3 ± 0.2 |
| 4 | 52.8 ± 7.5 | 35.4 ± 7.0 | 27.7 ± 2.0 | 45.1 ± 6.5 | 38.5 ± 4.2 | 1.4 ± 0.1 |
| 5 | 52.5 ± 7.6 | 39.4 ± 6.6 | 23.9 ± 1.9 | 46.5 ± 5.7 | 31.5 ± 4.8 | 1.5 ± 0.1 |
| 6 | 41.4 ± 6.6 | 43.2 ± 5.2 | 23.7 ± 3.8 | 52.1 ± 5.6 | 27.1 ± 3.5 | 1.6 ± 0.1 |
| 7 | 39.7 ± 14.4 | 43.6 ± 10.0 | 24.2 ± 6.5 | 47.1 ± 5.8 | 32.7 ± 14.3 | 1.8 ± 0.4 |
| 8 | 32.4 ± 5.7 | 43.9 ± 9.3 | 14.8 ± 5.8 | 40.1 ± 11.7 | 20.5 ± 3.4 | 2.3 ± 0.6 |

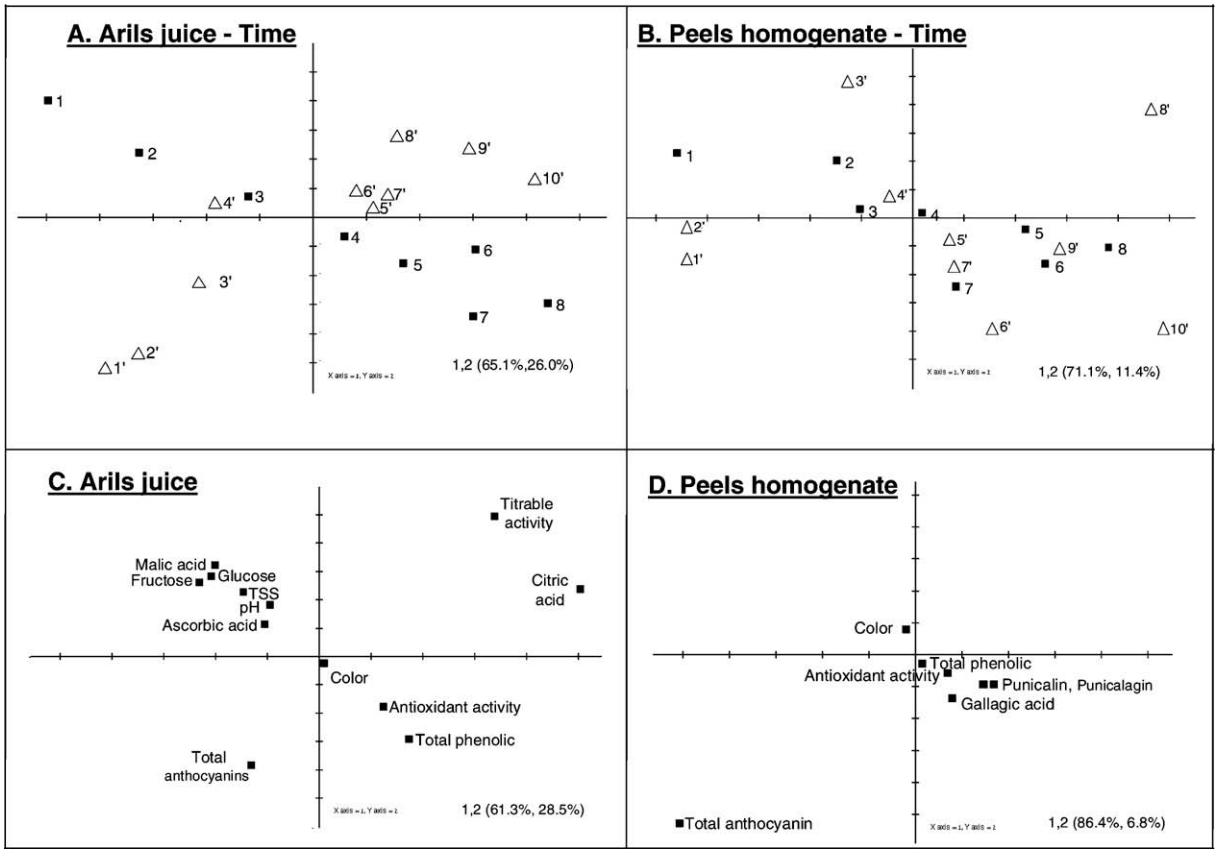


**Fig. 6.** Principle component analysis of different parameters and metabolite analyses measured during pomegranate fruit development. Principal component analysis results of distinct developmental stages during fruit maturation of accession 101-2 (■) and 121-2 (△) as measured in arils juice (A) and in peels homogenate (B). Principal component analysis results of different parameters measured in arils juice (C) and peels homogenate (D) of the two accessions in all of the developmental stages. The variance explained by each component is given within parentheses.

tion between these different parameters. To this end, the data sets obtained from both accessions were combined for each parameter that has been tested in the aril juice. Principal component analysis has shown that antioxidant activity and total phenolic have short distanced of separation (Fig. 6C). TSS, glucose and fructose are grouped together, as well as citric acid and titratable acidity. These suggest that the sugars are major part of the TSS and that in general citiric acid can contribute significantly to titratable acidity. To further test these relations, correlation matrix (Spearman test) was conducted on data obtained from aril's juices of the two accessions (Suppl. Tables f and g). The results shown that in both accessions the antioxidant and the total phenolic content are significantly correlated as well as the levels of the sugars and TSS, while the level of citric acid and titratable acidity were correlated in accession 101-2 but not in 121-2 (Suppl. Tables f and g).

In peels homogenates, the results obtained from the principal component analysis shown that antioxidant activity and total phenolic have short distance to the three hydrolysable tannins (Fig. 6D), suggesting that these compounds significantly contribute to the antioxidant activity. Indeed, high correlations were found in both accessions between these parameters, which also accordance with previous results (Gil et al., 2000; Tzulker et al., 2007).

### 3.6. Final remarks

During fruit development and maturation, significant changes were found in the physical parameters (fruit weight and volume) and chemical profile of pomegranate arils and peels. Basically, the two accessions that were tested showed a similar trend in most of the aril parameters examined, although, the significance of the trend differed between them. For example, the levels of antioxidant activity, total phenolics compounds and citric acid were reduced during maturation in the aril juice, while the levels of TSS, glucose and fructose, as well as of malic and ascorbic acids, increased. Some trends can be found in one accession but not in the other. For example, the total anthocyanin content was not significantly altered during maturation in the accession 121-2 aril juice, which has very a low anthocyanin level, but significantly increased in accession 101-2. In addition, the titratable activity decreased significantly in accession 101-2 but not in accession 121-2. The changes observed in the peels, however, were very similar in both accessions (Fig. 5).

Changes between the two accessions were also found in the relationship between colour development of the fruit's skin and aril juice quality. Since significant changes occur during pomegranate fruit maturation, it is important to establish correlations between the external pigmentation of the fruit and that of the aril juice during maturation in order to achieve fruit quality determinations by colorimetric online non-destructive methods. It was found that while the peel colour of accession 121-2 was significantly correlated to antioxidant activity, total phenolic content, sugars and organic acids levels of arils, that of accession 101-2 was correlated only to acidity level and citric acid content. Hence, in future, it is expected that such a study will be performed for each commercial pomegranate accession in order to determine if the peel colour can give indications of the quality of the aril juices. This is required for marketing the fruit as a functional food with the best nutritional value and health properties. In addition, this

information could help pomegranate juice producers and industrialists to gain an indication of aril juice quality when harvesting the fruit. However, in future, new methods should be developed in order to assess fruit quality in accessions, such as accession 101-2 in which its peel colour did not give a strong indication of arils quality.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2009.01.036.

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