Antioxidant Activity of Pomegranate Juice and Its Relationship with Phenolic Composition and Processing

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The antioxidant activity of pomegranate juices was evaluated by four different methods (ABTS, DPPH, DMPD, and FRAP) and compared to those of red wine and a green tea infusion. Commercial pomegranate juices showed an antioxidant activity (18−20 TEAC) three times higher than those of red wine and green tea (6−8 TEAC). The activity was higher in commercial juices extracted from whole pomegranates than in experimental juices obtained from the arils only (12−14 TEAC). HPLC-DAD and HPLC-MS analyses of the juices revealed that commercial juices contained the pomegranate tannin punicalagin (1500−1900 mg/L) while only traces of this compound were detected in the experimental juice obtained from arils in the laboratory. This shows that pomegranate industrial processing extracts some of the hydrolyzable tannins present in the fruit rind. This could account for the higher antioxidant activity of commercial juices compared to the experimental ones. In addition, anthocyanins, ellagic acid derivatives, and hydrolyzable tannins were detected and quantified in the pomegranate juices.

Keywords: *Pomegranate; Punica granatum; Punicaceae; juice; phenolics; anthocyanins; ellagic acid; punicalagin; tannins; antioxidant activity; ABTS; DPPH; DMPD; FRAP*

INTRODUCTION

Epidemiological studies show that consumption of fruits and vegetables with high phenolic content correlate with reduced cardio- and cerebrovascular diseases and cancer mortality (Hertog et al., 1997a,b). Phenolic compounds may produce their beneficial effects by scavenging free radicals. In the past few years there has been an increasing interest in determining relevant dietary sources of antioxidant phenolics. Thus, red fruit juices such as grape and different berry juices have received attention due to their antioxidant activity. Pomegranate juice has become more popular because of the attribution of important biological actions (Lansky et al., 1998). Thus, the antioxidant and antitumoral activity of pomegranate bark tannins (punicacortein) (Kashiwada et al., 1992; Su et al., 1988) and the antioxidant activity of the fermented pomegranate juice (Schubert et al., 1999) have been reported. However, detailed investigations of the phenolic compounds and the antioxidant activity of the juice have not yet been carried out.

Pomegranate juice is an important source of anthocyanins, and the 3-glucosides and 3,5-diglucosides of delphinidin, cyanidin, and pelargonidin have been reported (Du et al., 1975). It also contains 1 g/L citric acid and only 7 mg/L ascorbic acid (El-Nemr et al., 1990). In addition, pomegranate bark (Tanaka et al., 1986b), leaf (Tanaka et al., 1985; Nawwar et al., 1994b), and the fruit husk (Mayer et al., 1977) are very rich in ellagitannins and gallotannins. Several apigenin and luteolin glycosides from pomegranate leaves (Nawwar et al., 1994a) and the hydrolyzable tannins punicalagin and punicalin from pomegranate husk have previously been identified (Mayer et al., 1977; Tanaka et al., 1986a).

We report here on the evaluation of the antioxidant activity of pomegranate juice extracted by different procedures and the identification of the compounds responsible for this activity.

MATERIALS AND METHODS

Materials. Four types of pomegranate juices were produced from "Wonderful" pomegranates harvested in California during October 1998. Juice **1** was obtained in the laboratory from pomegranate arils by a hand press reaching a soluble solids (SS) value of 15.5%. Juice **2** was obtained as for juice **1**, but in this case, the arils were frozen and stored for 9 months at −20 °C prior to juice preparation with a SS content of 16.6%. Juice **3** was a single-strength commercial juice produced by Green-Valley Packers (Arvin, CA) with a 16.6% SS, and juice **4** was a commercial concentrate juice produced by the same company. The juice **4** was reconstituted in the laboratory by adding water to decrease SS from 65.0 to 16.3% as in the original juice. Both commercial and experimental juices were stored frozen (−20 °C) until analyzed.

HPLC-DAD Analyses. Three replicates from each juice were centrifuged in an eppendorf tube (2 min at 1400 rpm) and filtered through a 0.45 μm filter. Samples of 20 μL of juice were analyzed using an HPLC system (Hewlett-Packard 1050 pump) coupled with a photodiode array detector (DAD) (series 1040M, series II) and an autosampler (series 1050), operated by HP ChemStation software. A reversed-phase C_{18} Nucleosil column (150 × 4.6 mm; particle size 5 μm) with a guard column

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containing the same stationary phase (Safeguard holder 5001-CS) was used. A quatranary pump was used for mixing the mobile phase to avoid pressure fluctuations due to the mixing of methanol (MeOH) in water. Acetic acid (2.5%) was added to water and methanol to increase peak resolution before preparing the following mobile phases: water (A); 88% water + 12% MeOH (B); 20% water + 80% MeOH (C); MeOH (D). All solvents were HPLC grade. Elution started with 100% A, which remained isocratic until 5 min. A gradient was then installed to reach 100% B at 10 min, holding it isocratic for 3 more minutes. From 13 to 35 min, a linear gradient was installed to reach 50% B and 50% C, and then 100% C at 40 min. The column was then washed with 100% D at 42 min. The flow rate was 1 mL min^{-1} and chromatograms were recorded at 510, 350, and 280 nm. The UV spectra of the different compounds were recorded with a diode array detector.

HPLC-MS Analyses. Electrospray mass spectrometric analyses were performed using a Hewlett-Packard 5989A quadrupole instrument equipped with a electrospray interface (HP 59987A). Nitrogen was used as a nebulizing gas at a pressure of 50 psi and a temperature of 300 °C. The same column and chromatographic conditions as for the HPLC-DAD analyses were used.

Phenolic Compounds Identification and Quantification. The phenolic compounds in pomegranate juice were identified by their UV spectra, recorded with a diode-array-detector, and HPLC-MS (electrospray), and, wherever possible, by chromatographic comparisons with authentic markers. Individual anthocyanins were quantified by comparisons with an external standard of cyanidin 3-glucoside (Apin Chemicals Ltd., U.K.). Ellagic acid derivatives as an external standard of ellagic acid**,** hydrolyzable tannins as gallic acid, and gallagic-derived tannins as punicalagin (isolated in the present work). Concentrations were expressed as micrograms per milliliter of juice. Reproducibility of the analyses was ±5%.

Antioxidant Activity Evaluation. Four methods were used to test the antioxidant activity of pomegranate juices including three based on the evaluation of the free-radical scavenging capacity of the juices, and one based on measuring their iron-reducing capacity. The antioxidant activity of the different pomegranate juices was compared to those of red wine and green tea, two well-known food antioxidants (Ghiselli et al., 1998; Yokozawa et al., 1998; Cao et al., 1996). A commercial 1997 Cabernet Sauvignon wine from California was used as red wine. Infusions of 1 g of green tea brewed for 5 min with 100 mL of boiling water were prepared. In addition, the antioxidant activity of a water extract of pomegranate husk (1 g of fruit rind homogenized with 10 mL of water) was also tested. The first method generated the $ABTS^{\bullet+}$ by addition of H_2O_2 and horseradish peroxidase (Cano et al., 1998), which is a colored free radical, whose neutralization was easily followed by reading the decrease in absorbance at 414 nm after the addition of the antioxidant. This assay is similar to that described by Rice-Evans and Miller (1994) and to the commercial RANDOX method. The second method assayed used a commercially available free radical ($DPPH^{\bullet+}$, 2,2 diphenyl-1-picrylhydrazyl) which is soluble in methanol (Brand-Williams et al., 1995), and the antioxidant activity measured by decrease in absorbance at 515 nm. The third radical-scavenging method generates a colored free radical ($DMPD^{\bullet+}$) by addition of Fe^{3+} to *p*-phenylene diamine (Fogliano et al., 1999), and the absorbance at 505 nm was measured. The FRAP method was developed to measure the ferric reducing ability of plasma at low pH (Benzie and Strain, 1996). An intense blue color is formed when the ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex is reduced to the ferrous (Fe^{2+}) form and the absorption at 593 nm was recorded. Standard solutions of 5.7 mM L-ascorbic acid (Aldrich, Germany) in deionized water and 10 mM TROLOX (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Aldrich, Germany) in methanol were prepared. For all the antioxidant activity methods, diluted samples in water of 1:20 (v:v) for red wine and green tea, 1:5 and 1:10 for husk and 1:50 for pomegranate juices were used, except when using the DMPD method in which dilutions of 1:100 to 1:200 of pomegranate juices were needed. Diluted standards or diluted juice samples were used on the day of preparation except the ascorbic acid solutions that were used within 1 h of preparation. Fifty microliters of diluted standards (or juice samples) was mixed in an Eppendorf tube with 950 μL of the free-radical (or Fe^{3+}) solutions. These solutions were left to react for a period of time (15 min for the DPPH method, 4 min for the FRAP assay, and 10 min for the DMPD and the ABTS methods) under continuous stirring. The changes in absorbance were then measured at 25 °C. The results were expressed as Trolox equivalent antioxidant capacity (TEAC) and ascorbic acid equivalent antioxidant capacity (AEAC) (Cano et al., 1998; Cao et al., 1998; Cao and Prior, 1998; Wang et al., 1996).

Cation-Exchange Resin. A Bio-Rad cation-exchange resin AG50W-X8 was used to remove the anthocyanins from the pomegranate juices. Activation of the resin was carried out for 15 min with 1N HCl followed by another 15 min with 1 N NaOH and repeated three times. Then, the resin was washed with water to end the activation with acetate buffer (pH 4.0). Pomegranate juice was loaded onto the resin, and the anthocyanins were retained. The supernatant was passed through an activated C-18 solid-phase extraction cartridge. The eluted water and methanol fractions were analyzed with DPPH, FRAP, and DMPD methods for antioxidant activity.

Total Phenolics. For total phenolic determinations, dilutions of 1:10 and 1:20 for red wine, green tea, and 1:5 and 1:10 for husk and pomegranate juices were used. Total phenolics were determined by the Folin−Ciocalteu reagent (Singleton and Rossi, 1965). Dilutions were carried out per duplicate and calculated by a calibration curve obtained with *p*-coumaric acid. The absorbance was measured at 660 nm.

RESULTS AND DISCUSSION

Antioxidant Activity of Pomegranate Juices. The antioxidant activity shown in Figures 1 and 2 are equivalent to those of Trolox and ascorbic acid solutions with the indicated concentrations in millimolar. Red wine and green tea provided similar results using all four antioxidant activity methods and TEAC and AEAC values were within the ranges previously published: 6−12 TEAC for red wine (Fogliano et al., 1999; Ghiselli et al., 1998) and 8.3 TEAC for green tea infusions (Prior and Cao, 1999). The analyzed Cabernet Sauvignon red wine showed a free-radical scavenging activity equivalent to that of a Trolox solution 6 mM with the ABTS method, 7.5 mM with the DPPH method, 8.7 mM with the DMPD method, and 9.4 mM with the FRAP method. The values calculated as ascorbic acid equivalents were very similar to those found for Trolox equivalents. Green tea showed antioxidant activity values similar to those of red wine, with the exception of the values obtained with the DMPD method in which the green tea values were significantly lower (4.4 TEAC) than those found for red wine (8.7 TEAC). The differences observed could probably be explained by the interference of organic acids present in wine (tartaric) with the DMPD method (see below).

Using the ABTS and the DPPH methods, the antioxidant activity of the experimental pomegranate juice obtained from fresh arils (Juice **1**) was twice those of red wine and green tea (Figure 1). The activity was lower in the experimental juice prepared from frozen arils (Juice **2**), showing that during the freezing process some antioxidant compounds are degraded or transformed, but this juice still showed a higher antioxidant activity than red wine and green tea. The antioxidant activity of both commercial pomegranate juices (Juices **3** and **4**) was even higher (nearly three times that of wine and tea) (Figure 1) and suggested that the industrial process to obtain the juices either increased the

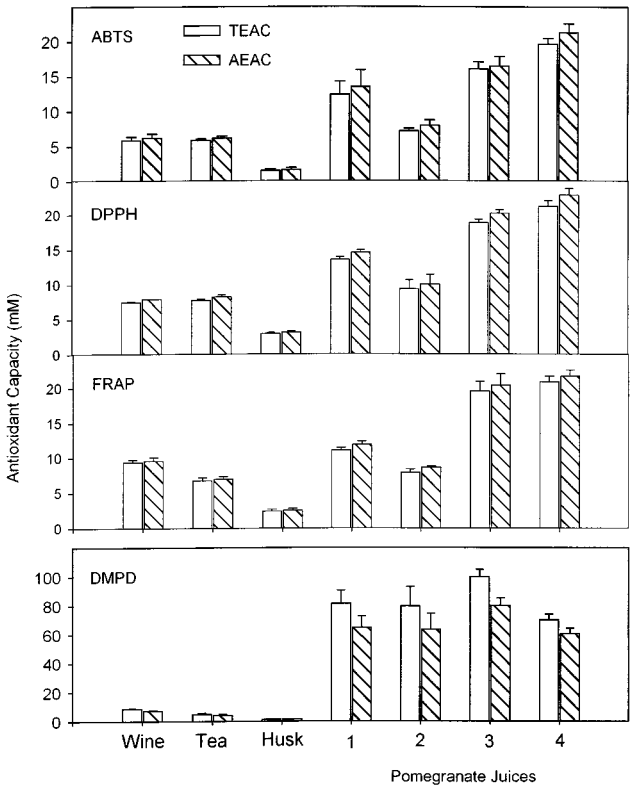

Figure 1. Antioxidant capacity of pomegranate juices compared to those of red wine, green tea and a water extract of pomegranate fruit husk (rind) evaluated by the ABTS, DPPH, FRAP, and DMPD methods. The values represent the Trolox equivalent antioxidant capacity (TEAC) and ascorbic acid equivalent antioxidant capacity (AEAC). The antioxidant capacity of the different food products is equivalent to those of Trolox and ascorbic acid solutions with the indicated concentrations (mM).

content of pomegranate antioxidants or enhanced their activity. The FRAP method also showed a higher antioxidant capacity for the experimental juice produced from fresh arils with respect to red wine or green tea, and a smaller activity for the juice produced from frozen arils (Juice **2**). Again, the activity of the commercial juices (**3** and **4**) was 2-fold that of the experimental ones (**1** and **2**), supporting that the method of juice extraction has an important role in this activity.

When measuring the antioxidant activity of pomegranate juices by the DMPD method (Figure 1), an extraordinary high activity was observed compared to the other free radical scavenging activity methods. However, the antioxidant activities of red wine, green tea, and pomegranate husk extract measured by the DMPD method were in the same ranges as those measured with the ABTS, DPPH, and FRAP methods. This clearly shows that there is something in pomegranate juice that neutralizes the DMPD free radical, and that this juice constituent is not a main constituent in wine, tea, or the water extract of pomegranate husk. The antioxidant activity for the four analyzed juices evaluated by the DMPD method was in the same range. This antioxidant test was repeated at least four times to confirm the observed high activity. To determine the reason for the high activity observed in pomegranate juices with the DMPD method, the commercial single strength juice (**3**) was fractionated. A cation-exchange resin was used to remove anthocyanins from the juice and the remaining supernatant was fractionated by filtration trough a C-18 solid-phase extraction cartridge.

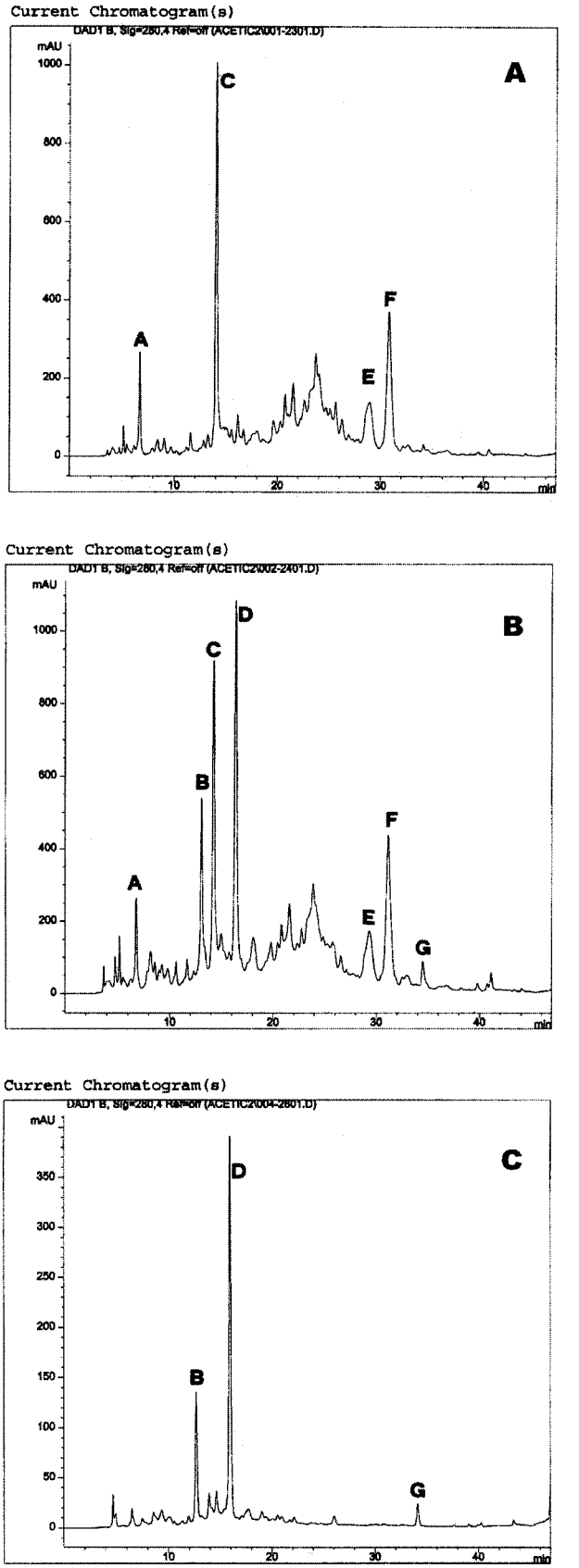

Figure 2. HPLC chromatograms of pomegranate phenolics recorded at 280 nm. (Panel A) Juice **1** directly obtained from arils; (Panel B) Commercial single-strength juice **3** (extracted from whole pomegranates); (Panel C) Husk water extract (1 g homogenized in 100 mL water). **A** = galloylglucose; **B** = punicalagin isomer; **C** = hydrolyzable tannin; **D** = punicalagin isomer; **E** = delphinidin 3-glucoside; **F** = cyanidin 3-glucoside; **G** = ellagic acid.

Table 1. Antioxidant Activity of Fractions of Commercial Single-Strength Pomegranate Juice Evaluated by Three Methods

fractions	percentage of antioxidant activity of the original juice		
	DPPH	FRAP	DMPD
ion-exchange resin, nonretained	28.9	27.7	63.0
ion-exchange resin, retained	71.1	72.3	36.8
nonretained fraction separated by C-18 solid-phase extraction cartridge,			
water extract	0.0	0.0	74.6
methanol extract	26.4	27.7	15.2

The water-soluble compounds of the supernatant were eluted from the cartridge with water and the phenolic compounds retained were then eluted with methanol. The percentage of the antioxidant inhibition of the fractions evaluated with DPPH, FRAP, and DMPD methods are shown in Table 1. After removing the anthocyanins, the compounds remaining in the supernatant fraction conferred a 28% of the total antioxidant activity of the commercial pomegranate juice for the DPPH and FRAP methods. However, when the DMPD method was assayed, the supernatant fraction was the responsible of the 63% of the total antioxidant activity of the juice. When the supernatant was fractionated through the sep-pack, only the methanol fraction showed activity when the DPPH and FRAP methods were used. The water fraction did not contain any compound with antioxidant activity for DPPH and FRAP methods. However, a high activity of 74.6% was shown when the water fraction was evaluated with the DMPD method, and only a 15.2% of the activity was due to the methanol soluble compounds. This fact suggested that some water soluble constituents of pomegranate juice reacted with the DMPD free radical and showed an enhanced antioxidant activity. These water soluble compounds did not show free-radical scavenging activity with the other two methods. This prompted us to study the free radical scavenging activity of the main organic acid in pomegranate (citric acid). In addition, the activity of malic acid, a common organic acid of many other fruits, and tartaric acid, the main acid in grapes and red wine, were also tested. None of the organic acids showed antioxidant activity when were evaluated with the DPPH and FRAP methods. However, citric, malic and tartaric acids showed antioxidant activity when the DMPD method was tested. Citric acid had an important activity neutralizing the DMPD radical, while the other assayed organic acids showed considerably less activity (data not shown). These results show that the DMPD method should be used with caution for evaluation of total antioxidant capacity, especially in those food products which are rich in organic acids (especially citric acid).

We conclude that both experimental and commercial pomegranate juices showed an antioxidant activity that was always higher than those of red wine and green tea. In addition, the commercial single-strength juice (juice **3**) and the juice from concentrate (juice **4**) had a higher antioxidant activity than those experimental juices (**1** and **2**) obtained by pressing the arils without including the rind or husk.

HPLC Qualitative Analysis of Pomegranate Juices and Phenolic Compounds Identification. Due to the differences observed in the antioxidant activity of experimental and commercial juices, their phenolic compounds were studied by HPLC on reversed-phase column coupled with diode array detector (HPLC-DAD) and mass spectrometry detector (HPLC-MS). In addition, the phenolic compounds present in water extracts of pomegranate rinds were also analyzed. Both commercial and experimental pomegranate juices were characterized for their typical red color produced by a combination of delphinidin, cyanidin and pelargonidin 3-glucosides and 3,5-diglucosides, which were easily detected in the HPLC chromatograms recorded at 510 nm (Gil et al., 1995). Pelargonidin 3,5-diglucoside was only present as traces in the different juices and this prevented its quantification. In addition, another anthocyanin with larger retention time and UV−vis spectra as a delphinidin derivative was detected in minor amounts, but its small concentration prevented its identification.

Both colored and noncolored phenolics are clearly shown in the chromatograms of the different pomegranate juices (Figure 2). The experimental juices obtained directly from arils are characterized by two main compounds **A** and **C**, in addition of the anthocyanin peaks (**E**, delphinidin 3-glucoside; **F**, cyanidin 3-glucoside), and many other minor peaks that were observed in the chromatograms (Figure 2A). The 3,5-diglucosides of delphinidin and cyanidin which were clearly resolved in the chromatogram at 510 nm, appeared as broad peaks that overlapped with other phenolic peaks in the chromatograms at 280 nm (t_R 20−25 min). The chromatograms of commercial juices showed in addition two other main peaks (**B** and **D**) and a minor, but quite distinctive, peak at higher retention time (**G**) (Figure 2B). The water extract of pomegranate rind (Figure 2C) was characterized by the presence of **B**, **D**, and **G** and the absence of **A** and **C** and the typical pomegranate juice anthocyanins. This clearly shows that the phenolic pattern of the commercial pomegranate juice includes additional phenolics to those present in the arils juice and that the industrial process to produce pomegranate juices also extracts some phenolic compounds from the fruit rind.

The main phenolic compounds in pomegranate juice were identified by their MS fragments and UV spectra (Table 2). Compound **A** had a UV spectrum with a maximum at 278 nm, with a shape similar to that of gallic acid but with a slight shift in its maximum (Figure 3A). This compound was quite water soluble and was not adsorbed on the solid-phase extraction cartridges (RP-18), where it eluted together with all the water soluble compounds (sugars, organic acids, etc.). Its HPLC-MS (electrospray) analysis showed a quasi-molecular ion at 333 m/z $(M-H)^-$, in accordance with galloyl-glucose, a common constituent of plants containing hydrolyzable tannins.

Compound **C** was the main UV absorbing compound in the aril juices. This compound showed a UV spectrum with a maximum at 266 nm (Figure 3C). This compound was partially purified from the aril juice by removing the anthocyanins using ion-exchange chromatography, solid-phase extraction on a reversed-phase cartridge, and LH-20 chromatography with methanol. This produced an enriched fraction that was HPLC-MS analyzed. This compound gave a quasi-molecular ion at 1397 and characteristic fragments at 935, 785, 765, 613, 451, and 301 m/z. A fragment at 173, corresponding to gallic acid, was also observed. These analyses showed that this was a hydrolyzable tannin containing at least a molecule of ellagic acid (301 m/z) a molecule of gallic

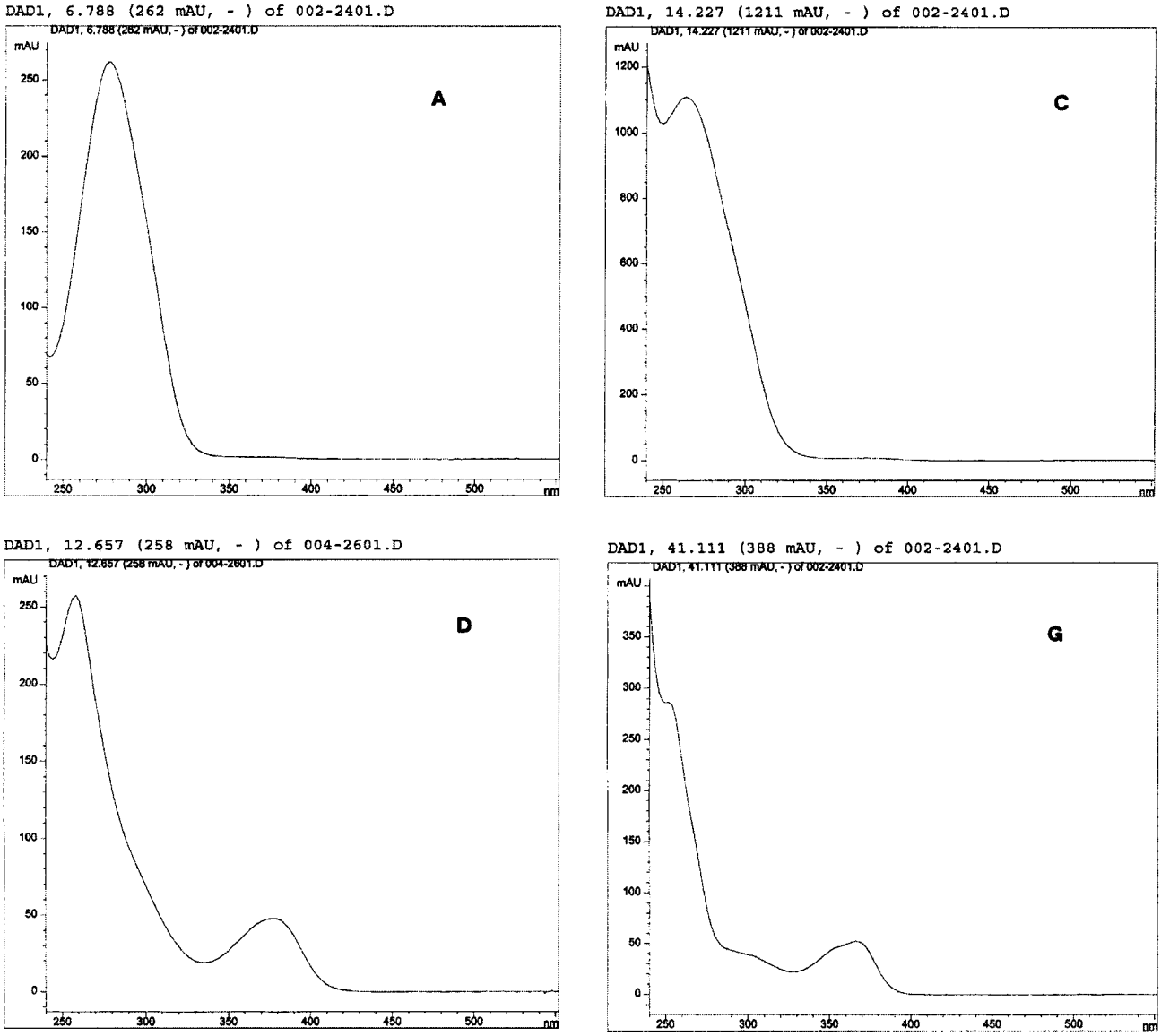

Figure 3. UV spectra of pomegranate juice characteristic compounds. **A** = galloylglucose; **C** = hydrolyzable tannin; **D** = punicalagin; **G** = ellagic acid.

Table 2. HPLC-DAD-MS Analyses of Pomegranate Phenolics

compds	MS fragments	UV spectra
(**A**) galloylglucose	331 (M−H)	278
(**B**) punicalagin	1083 (M−H); 781 (M−ellagic), 601 (gallagic acid), 301 (ellagic acid).	378, 258
(**C**)	1397 (M−H), 935, 785, 765, 613, 451, 301, 275.	294sh, 266
(**D**) punicalagin	1083 (M−H); 781 (M−ellagic), 601 (gallagic acid), 301 (ellagic acid).	378, 258
ellagic acid glucoside	463 (M−H), 301 (ellagic acid)	360, 342, 300, 254
(**G**) ellagic acid	301 (M−H)	362, 346, 300, 256
(**F**) Cy 3-GLC	447 (M−H), 284 (cyanidin aglycone).	518, 278
(**E**) Dp 3-GLC	463 (M−H), 300 (dephinidin aglycone)	526, 276
Pg 3-GLC		506, 276
Cy 3,5-dglc	609 (M−H), 447 (M−glucose), 285 (cyanidin)	518, 276
Dp 3,5-dglc	633 (M−H), 463 (M−glucose), 301 (delphinidin)	526, 276

acid (173 *m*/*z*) and a molecule of tertgallic acid (451 *m*/*z*), and the absence of gallagyl residues (lack of 601 *m*/*z* fragment). The MS spectrum of this compound was consistent in all the HPLC-analyses carried out with the different juices and fractions, in which the same molecular ion and fragments were observed. Its UV spectrum supported that this was an ellagitannin containing at least one galloyl residue and lacking gallagyl residues. The presence of a gallagyl residue in **C** would render the compound yellow, and with a UV spectrum showing maxima around 375 and 265 nm (as compounds **B** and **D**) (Figure 4D). The ion at 935 was consistent with a molecule containing two hexahydroxydiphenyl molecules (precursors of ellagic acid) and one gallic acid residue on glucose. The peak at 783 was consistent with a galloyl loss, and the peak at 765 entails an additional loss of a water molecule. An additional loss of a galloyl residue leads to fragment 613, and the loss of glucose yields the peak at 451 that can further fragment to render ellagic acid (301 *m*/*z*). So this

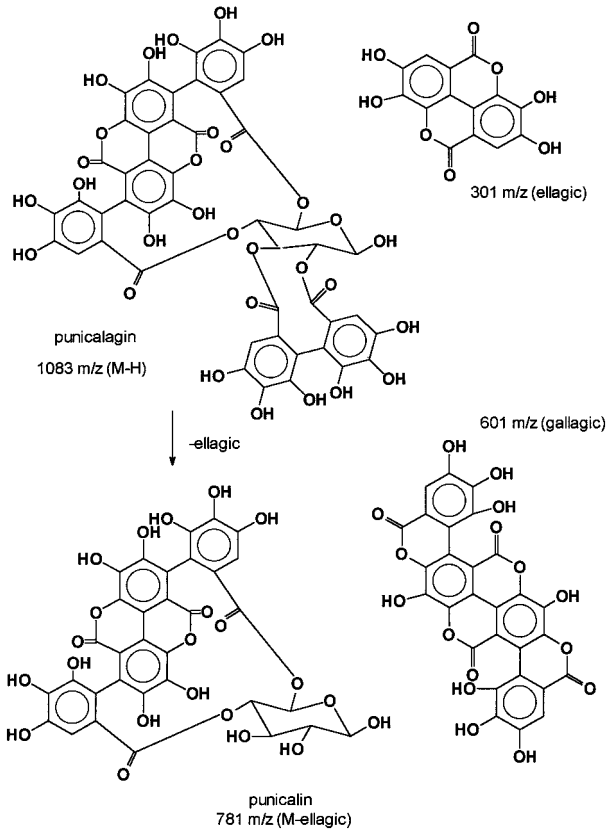

Figure 4. HPLC-MS (electrospray ionization) fragmentation of compounds **B** and **D** (punicalagin isomers). The fragments punicalin (781 *m*/*z*), gallagic (601 *m*/*z*) and ellagic acid (301 *m*/*z*) are observed.

compound was tentatively identified as a digalloyl, tertgalloyl ester of glucose. Its full identification will only be possible after NMR studies of the isolated compound.

Compounds **B** and **D** had the same UV spectra with maxima at 378 and 258 nm (characteristic of gallagic acid derivatives) (Figure 3D). These compounds were the main constituents of a water extract of pomegranate husk, and had a characteristic yellow color. They were purified from the water husk extracts by LH-20 chromatography with methanol. Both compounds interconverted rapidly when in solution to render approximately 30% **B** and 70% **D**, this behavior is similar to that already described for α and β isomers of punicalagin (position isomers at the anomeric carbon of the glucose) by Doig et al. (1990). The HPLC-MS analyses of compounds **B** and **D** showed identical spectra for both compounds with a quasimolecular ion at 1083 *m*/*z* (M−H) in accordance to punicalagin (glucose + gallagyl + hexahydroxydiphenoyl) (Figure 4). This is a complex ellagitannin characteristic of pomegranate peel, which contains glucose, ellagic acid, and gallagic acid. Several isomers have been previously described in pomegranate fruit peel and also in leaves and bark. Fragments for the loss of ellagic acid (781 *m*/*z*) and for the gallagic (601 *m*/*z*) and ellagic acid (301 *m*/*z*) residues were the main fragments observed in the HPLC-MS spectrum, supporting the nature of these compounds.

In addition, two other ellagic acid derivatives (with characteristic UV spectra showing maxima at 362, 346, 300, and 256 nm) (Figure 3G) were detected. One of them was identified as ellagic acid (MS 301 *m*/*z*) (**G**), and another as ellagic acid hexoside (MS 463 *m*/*z* and a fragment at 301 for the loss of a hexose), and was tentatively identified as ellagic acid glucoside. In addition, an ellagic acid pentoside (M−H, 433 *m*/*z*), and an ellagic acid rhamnoside (M−H, 447 *m*/*z*) were also detected in some samples.

Quantitation of Phenolic Compounds in the Juices and Antioxidant Activity of the Isolated Phenolics. The main phenolic compounds present in pomegranate juice can be arranged into four groups. A first group includes the anthocyanin pigments, which are easily quantified by HPLC with detection in the visible region at 510 nm, using cyanidin 3-glucoside as an external standard. The second group includes the hydrolyzable tannins of the gallagyl type, which are characterized by a typical UV spectrum with two maxima at 378 and 258 nm. This group includes the punicalagin isomers (**B** and **D**), punicalin (gallagylglucose), and other related compounds, and they were quantified as punicalagin by HPLC with UV detection at 350 nm. All these compounds showed HPLC-MS spectra with the characteristic fragment al 601 *m*/*z* corresponding to gallagic acid. The third group of pomegranate juice phenolics includes ellagic acid (**G**) and its glycosides. These compounds are characterized by the typical UV spectrum of ellagic acid (UV max, 362, 346, 300, 256) and by HPLC-MS spectra with the fragment at 301 *m*/*z* corresponding to ellagic acid. These compounds were quantified at 350 nm as ellagic acid. The fourth group of pomegranate phenolics includes a very wide group of hydrolyzable tannins with undefined UV spectra showing only maxima below 280 nm. These compounds are different combinations of glucose, gallic acid, hexahydroxydiphenic acid (which gives rise to ellagic acid after hydrolysis), and tertgallic acid. Their HPLC-MS spectra are characterized by the presence of fragments at 173 (gallic), 301 (ellagic), and 451 (tertgallic) mass units. All these compounds, which include **A** and **C**, were quantified at 280 nm as gallic acid.

Both experimental and commercial juices had the same anthocyanin pigments, but significant quantitative differences were found (Table 3). It seems that when arils are frozen and stored prior to juice extraction (juice **2**), the anthocyanins are partly degraded and/or transformed into other products. Something similar was observed in the anthocyanins of the commercial juice obtained from concentrate (juice **4**). However, the experimental juice obtained from arils (juice **1**) and the commercial single-strength juice (juice **3**) contained similar amounts of anthocyanins (Table 3).

The main difference observed between the commercial and the experimental juices was the high content of punicalagins and ellagic acid derivatives in the commercial juices. The other hydrolyzable tannins remained quite constant in the different juices. One possible explanation for the high content of the rind constituents (punicalagin isomers and ellagic acid derivatives) in the commercial juices is that the hydrostatic pressure to crush the whole fruit to release the juice from the arils, also extracts the water-soluble ellagitannins from the rind that pass to the juice in proportion to the force used. Other factors include the juice processing conditions such as added enzymes, thermal treatments and concentration process.

The phenolic content calculated by the Folin−Ciocalteu method of pomegranate juice, both experimental and commercial, was in the same range as red wine (generally above 2000 mg/L) and in this case it was twice that

Table 3. Phenolic Compound Composition (mg/L) of Pomegranate Juices

phenolic compounds	pomegranate juices[a] 1	2	3	4
1st group: anthocyanins				
delphinidin 3,5-diglucoside	42.9	38.8	61.1	21.1
cyanidin 3,5-diglucoside	53.0	46.4	71.4	31.4
delphinidin 3-glucoside	76.0	23.6	95.2	37.8
cyanidin 3-glucoside	128.3	59.5	151.1	67.0
pelargonidin 3-glucoside	5.9	3.9	8.5	4.6
total anthocyanins	306.0	172.2	387.4	161.9
2nd group: gallagyl-type tannins				
punicalagin **B**	12.7	14.4	421.3	434.9
punicalagin **D**	10.1	11.1	838.5	918.2
other	45.1	102.5	302.0	525.6
total gallagyl-type tannins	67.9	128.1	1561.7	1878.8
3rd group: ellagic acid derivatives				
ellagic acid glucoside	17.9	17.9	83.2	91.3
ellagic acid	15.3	8.7	37.9	172.8
total ellagic derivatives	33.2	26.5	121.1	264.0
4th group: other hydrolyzable tannins				
galloyl glucose	51.1	43.9	49.3	65.5
compound **C**	224.5	203.6	116.5	229.0
other compounds	264.1	277.7	251.5	262.1
total hydrolyzable tannins	539.2	525.2	417.3	556.6

[a] (**1**) Juice from fresh arils; (**2**) juice from frozen arils; (**3**) single-strength commercial juice; (**4**) commercial juice from concentrate. Commercial juices are extracted by crushing whole pomegranates.

Table 4. Total Phenolics by Folin−Ciocalteu Method of Pomegranate Juices compared with Red Wine and Green Tea

item	total phenolics (mg/L ± sd)
red wine[a]	2036 ± 59
green tea[b]	1029 ± 36
pomegranate husk[c]	316 ± 23
juice from fresh arils (**1**)	2117 ± 95
juice from frozen arils (**2**)	1808 ± 26
single-strength commercial juice (**3**)	2566 ± 131

[a] Cabernet Sauvignon, 1997 (California). [b] One gram of green tea in 100 mL of boiling water for 5 min. [c] One gram of pomegranate husk homogenized in 10 mL of water.

found in green tea (Table 4). The total phenolics calculated as an addition of the individual phenolics in the HPLC chromatogram of single-strength pomegranate juice, reached 2487 mg/L, which was in good agreement with the value found with the Folin−Ciocalteu method (2566 mg/L). This analysis also confirmed that the commercial juices had a higher phenolic content (above 2500 mg/L) than the juices produced in the laboratory from arils (1800−2100 mg/L) (Table 4).

To calculate the contribution of the different groups of phenolics to the total antioxidant activity of pomegranate juice, the antioxidant capacity values of 1 mM solutions of gallic acid, cyanidin 3-glucoside, ellagic acid, and punicalagin were calculated and quoted as TEAC and AEAC. The concentration of Trolox or ascorbic acid required giving the same radical scavenging capacity as 1 mM test substance is shown in Table 5. The higher antioxidant activity was observed for punicalagin, as could be expected for a large molecule with 16 phenolic hydroxyls per molecule. Cyanidin 3-glucoside was the less active showing antioxidant activity in the same range as ellagic acid (both having four free phenolic hydroxyls). Gallic acid showed a relatively high antioxidant activity (2.5 TEAC) although it had only three free phenolic hydroxyls per molecule. This value is in agreement with previously reported data that determined a TEAC of 2.6 for methyl gallate (Hagerman et al., 1998).

Table 5. Antioxidant Activity of Isolated Phenolics Measured by DPPH Method[a]

compd	antioxidant activity TEAC[a]	AEAC[b]	no. of free phenolic OH
gallic acid	2.5	2.7	3
cyanidin 3 glucoside	0.8	0.8	4
ellagic acid	1.1	1.2	4
punicalagin	6.3	6.7	16

[a] TEAC is the concentration of Trolox required to give the same antioxidant capacity as 1mM test substance (Rice-Evans and Miller, 1994). [b] AEAC is the same with ascorbic acid.

Table 6. Antioxidant Activity of Individual Phenolic Groups in Commercial Single-Strength Pomegranate Juice Measured by the DPPH Method

phenolic groups	phenolics (mg/L)	antioxidant activity TEAC	AEAC
anthocyanins	387.4	1.4	1.4
punicalagins	1561.7	9.8	10.5
ellagic acids	121.1	0.5	0.6
hydrolyzable tannins	417.3	6.2	6.6
total phenolics	2487.5		
calculated activity		17.9	19.0
measured activity		20.5	22.1

The antioxidant activity of the individual compounds was then used to calculate the contribution of the different phenolic compounds to the total antioxidant capacity of the single-strength commercial pomegranate juice (Table 6). The total antioxidant activity of this pomegranate juice was equivalent to that of a solution 20.5 mM of Trolox calculated experimentally by the DPPH method. When the contribution of the different phenolics groups to the juice antioxidant activity was calculated, the anthocyanins accounted for only 1.4 mM of Trolox, and the ellagic acids only 0.5 mM of Trolox. The punicalagins, however, accounted for 9.9 mM Trolox and the other hydrolyzable tannins (calculated as gallic acid) reached 6.2 mM of Trolox. When all the calculated activities were added this reached 17.9 mM of Trolox, which explains 87% of the antioxidant activity experimentally determined for this juice. This clearly shows that the antioxidant capacity of pomegranate juices is

mainly due to the hydrolyzable tannins including punicalagins. The increase observed in punicalagin derivatives in commercial juices is responsible for their higher antioxidant activity when compared with the juices obtained experimentally from arils, which have only the antioxidant activity due to the other hydrolyzable tannins. These results support previously reported work in which the antioxidant capacity of high molecular weight polyphenolics (tannins) was reported to be 15-30 times more effective at quenching peroxyl radicals than simple phenolics or Trolox (Hagerman et al., 1998).

These results are especially interesting as indirect evidence shows that pomegranate tannins can be absorbed in the intestine (Filippich et al., 1991). In fact it has been reported that the ellagitannins of pomegranate are hydrolyzed extensively in mice, leading to the excretion of ellagic acid in the feces and urine (Castonguay et al., 1994).

Commercial pomegranate juices show an antioxidant activity three times higher than red wine and a green tea infusion. The activity was higher in commercial juices than in the experimental ones obtained in the laboratory by hand pressing the arils. This difference in activity seems to be due to the presence of pomegranate rind tannins in commercial juice. The main antioxidant compounds in pomegranate juice are hydrolyzable tannins, but anthocyanins and ellagic acid derivatives also contribute to the total antioxidant capacity of the juice. From the methodological point of view the DPPH and FRAP methods are recommended as easy and accurate methods for measuring the antioxidant activity of fruit and vegetable juices or extracts. The DPPH method is less sensitive than the other methods for hydrophilic antioxidants, while FRAP is a simple test with a wide dilution juice range. The results are highly reproducible and comparable to other free radical scavenging methods such as ABTS. The DMPD method should be used with caution in those extracts rich in organic acids.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid; DPPH, α,α-diphenyl-β-pycrylhydrazyl; DMPD, *N*,*N*-dimethyl-*p*-phenylenediamine; FRAP, ferric reducing ability of plasma; TROLOX; TEAC, Trolox equivalent antioxidant capacity; AEAC, ascorbic acid equivalent antioxidant capacity.

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