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Analysis of phenolic compounds in different parts of pomegranate (*Punica granatum*) fruit by HPLC-PDA-ESI/MS and evaluation of their antioxidant activity: application to different Italian varieties.

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Abstract

In this study, the analysis of pomegranate phenolic compounds belonging to different classes in different fruit parts was obtained by of high performance liquid chromatography (HPLC) coupled to photodiode array (PDA) and mass spectrometry (MS) detections. Two different separation methods were optimized for the analysis of anthocyanins and hydrolysable tannins together to phenolic acids and flavonoids. Two C18 columns, core-shell and fully porous particles stationary phase, were employed. Phenolic compounds separation parameters were optimized considering chromatographic resolution and analysis time. A total of 35 phenolic compounds were found and 28 of them were tentatively identified belonging to five different phenolic compounds classes namely anthocyanins, phenolic acids, hydrolysable tannins and flavonoids. Quantitative analysis was performed using a mixture of nine phenolic compounds belonging to phenolic compounds classes representative of pomegranate. The method was then fully validated in terms of retention time precision, expressed as RSD%, limit of detection (LOD), limit of quantitation (LOQ) and linearity range. Phenolic compounds were analysed directly in pomegranate juice, and after solvent extraction employing a mixture of water and methanol with a small percentage of acid in peel and pulp samples. The accuracy of the extraction method was also assessed obtaining satisfactory values. Finally, the method was used for to the study of identified analytes in pomegranate juice, peel and pulp of six different Italian and one international variety. On the same samples total phenols and antioxidant activity were evaluated trough colorimetric assays and results were correlated among them.

Keywords: pomegranate, by-products, phenolic compounds, HPLC-PDA-ESI/MS, preparative liquid chromatography.

Introduction

Among other components phenolic compounds have great significance in fruits nutritional, organoleptic and commercial features. Great attention has been recently paid not only to the edible fruit parts but also to the phenolic compounds content of fruit processing by-products like peels, seeds, hulls, etc. In fact, the food and agricultural products processing industries produce significant amount of phenolics-rich by-products, which could be important natural origin of antioxidants.

Among fruits pomegranate (*Punica granatum* L.) represents an interesting source of phenolic compounds because of their presence in different parts of the fruit. Recently pomegranate has increased popularity and interest owing to potential health promoting benefits due to consumption of the fruit and its derivate products like juice, jam, jelly, vinegar, wine, oil and dietary supplements [1]. Pomegranate constituents have exhibited a broad range of bioactivities such as anti-carcinogenic, anti-microbial, anti-oxidant and anti-inflammatory activities [2-4]. Vitamin C and phenolic compounds contents are considered responsible for pomegranate antioxidant properties [1]. The most represented phenolic compounds in pomegranate fruit and in their derived products are mainly anthocyanins, phenolic acids, flavonoids and ellagitannins.

Juice is obtained from the fruit endocarp, consisting of the red-colored arils which also contain seeds, other parts of the fruit are the non-edible exocarp (peel) and the mesocarp, a membrane-like structure that divide the arils. Several studies report how all the pomegranate fruit parts contain bioactive molecules [5-7]; considering that about 25% of the pomegranate fruit, harvested annually, is employed for making juice and other food products, a considerable amount of waste material is produced after juice extraction [8]. Given its bioactive molecules content, this material is a very interesting potential source of active principle for the dietary supplements and nutraceuticals industry representing a valuable commercial resource [9-11]. Specifically, polyphenolic composition of the pomegranate juice, mainly consisting in anthocyanins and hydrolysable tannins, have been extensively studied, as well as their antioxidant properties, showing variations among

cultivars depending on region of production and growing conditions [12]. Beside the juice, peel represents an agricultural biomass waste having capacity for higher antioxidant activity than the edible portion [9]. Pomegranate peel is a rich resource of phenols, specifically ellagitannins and in particular punicalagin isomers that have been isolated [13-16]. Seeds are also a source of bioactive molecules, although less studied due to their small size and difficult retrieval and isolation procedure [17, 18]. As an additional waste product containing bioactive molecules, pomegranate pulp was also object of investigation regarding its functional properties; pomegranate peel extract resulted more effective as an antioxidant compared to the pulp or seed extracts [19, 20].

A number of published paper reported the analysis of phenolic compounds in pomegranate juice and other fruit parts of different cultivars worldwide through colorimetric assay like Folin-Ciocalteau together to the evaluation of samples antioxidant activity through *in vitro* chemical tests. Most of the obtained results are reported in a recently published review [12]. While the separation and quantification of pomegranate phenolic compounds in fruit juice and extracts from different fruit parts is carried out by high performance liquid chromatography (HPLC), which is the method choice using spectrophotometric and mass spectrometry detection systems through electrospray ionization interface (ESI) [11, 15, 21-24]. Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry was also applied to determine in particular the tannin oligomer structures [25]. In the last few years, some applications, dealing with the determination of pomegranate phenolic compounds by ultra-high performance liquid chromatography (UHPLC), have also been presented [9, 26, 27].

Some of these works aimed to develop and validate the analytical procedure for pomegranate phenolic compounds analysis employing liquid chromatography-based methods [11, 17, 24, 28, 29]. Particular interest has been paid to the optimization of phenolic compounds extraction procedure, which is a critical step for the analysis of phenolic compounds in pomegranate by-products. Among employed techniques, solvent extraction is the classical and most employed procedure. Different solvents have been tested for phenolic compounds extraction from peel, pulp and seed pomegranate

samples. Among them methanol, ethanol, acidified water, acetone, diethyl ether and their combination have been widely used [6, 17, 19, 28, 30, 31, 32]. Green procedures like microwave extraction and pulsed ultrasound extraction techniques have been optimized for the extraction of phenolic compounds from pomegranate peel, pulp and marcs [31, 33-35]. Moreover a green extraction procedure employing non-toxic and eco-friendly solvents, e.g., deionized water or aqueous solutions of cyclodextrins, has been recently proposed as a sustainable method for the extraction of phenolic compounds from whole pomegranate fruit [36].

In a previous work, we studied phenolic compounds profile and antioxidant activity of juices obtained from six Italian pomegranate cultivars. In total, 13 phenolic compounds were identified, 6 belonging to anthocyanins family and 7 belonging to other families. Anthocyanins concentration was determined in all juices and the results were correlated with antioxidant activity. Differences among analyzed cultivars were observed [37].

Based on the previously published paper, in this study a HPLC-PDA/ESI-MS for the analysis of different classes of phenolic compounds in pomegranate was optimized and validated. Different parts of fruits were analyzed namely juice, peel and pulp. Juice samples were directly analyzed after simple centrifugation and sample dilution. Phenolic compounds from pomegranate by-products like peel and pulp were extracted using non-toxic solvents like water and ethanol. The method was then applied to the analysis of different pomegranate Italian varieties, being autochthonous pomegranate germplasm from semi-abandoned orchards or as sparse plants collected and cultivated by the Department of Agricultural and Forestry Sciences (DAFNE), at Tuscia University (Viterbo, Italy). On the same samples, total phenols were determined by Folin-Ciocalteau assay and antioxidant activity was evaluated through two widely used free radical scavenging assays, namely DPPH scavenging activity and Trolox equivalent antioxidant capacity (TEAC) method.

Materials and methods

Materials

Solvents employed for the extraction procedure and for HPLC-MS analyses were ethanol (EtOH), formic acid, methanol (MeOH), water and acetonitrile (ACN) and were purchased from Merck KGaA (Darmstadt, Germany). The standard compounds and reagents namely gallic acid, ellagic acid, caffeic acid, p-coumaric acid, quercetin, catechin, potassium persulfate, sodium carbonate, Folin reagent, ABTS (2,2-Azinobis-(3-ethylbenzothiazoline-6-sulfonic)-diammoniumsalt), Trolox(6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and DPPH (2,2-diphenyl-2-picryl-hydrazyl) were purchased from Merck KGaA (Darmstadt, Germany). Cyanidin-3-glucoside was obtained from Extrasynthese (Genay Cedex, France). Punicalagins α and β were obtained by preparative RP-HPLC/UV separation from pomegranate peels. Stock standard solutions of analytes (1000 mg/L) were prepared dissolving each compound in ACN solvent and stored at -18°C.

Pomegranate samples

Fruits of six old pomegranate Italian varieties called: Gaeta 1 (A), Gaeta 3 (B), Gaeta 4 (C), Tordimonte A (D), Itri A (E) and Formia (G), and from the international cultivar Wonderful (F), used as standards in this trial, were collected during the first decade of November 2016 in the experimental farm of Tuscia University. Each variety was replicated four times; plants were trained to bush with 3-5 stems spaced at 2 m within row and 4 m between rows. Agricultural practices were conducted as previously reported [30]. Fruits were harvested when commercially ripe, after 15 weeks from flower set, and considering external changing colour. The fruits were stored at room temperature for a few days until used. The measure of pomegranate fruit and longitudinal length and equatorial diameter per fruit was determined and fruit shape was calculated as the ratio length/diameter. Arils were manually selected from five fruits per tree of each variety to detect fresh

weight of total aril content per fruit, and the percentage of arils per fruit was calculated as the ratio between total arils fresh weight and fruit fresh weight.

Pomegranate phenolic compounds extraction

Fruits were washed and hand-peeled. Arils (50 g) were squeezed using a commercial an electric juice extractor (Robodiet–DeLonghi, Italy) to obtain juice. Twelve replicates per variety were executed. Before the analysis, the juices were centrifuged at 800 x g for 5 min at room temperature to remove pulps and then stored at -80°C until analysis. Total soluble solids content (TSS) of each fruit, detected as °Brix, was determined on juice samples, using a temperature compensating hand-held refractometer (ATAGO PR-101). The pH of fruit juice was evaluated on each replicate by a digital pH meter. Titratable acidity (TA), reported as per cent citric acid, was found out using standard methodology. Maturity index (MI) expressed as TSS/TA ratio was also estimated.

Pomegranate peel was finely ground with a laboratory grinder, and then stored at -80°C until phenolic compounds extraction. By-product samples (peel and pulp) were subjected to solvent extraction before HPLC-PDA-ESI/MS analysis for determination of phenolic compounds. The extraction method was performed on 1 g of each dried sample, extracted with three aliquots of 10 mL of water, water/MeOH (1:1 *v/v*) and MeOH. The extracts were combined, and brought to dryness in a rotary evaporator. The obtained extracts were solubilized in 2 mL of water/MeOH (1:1 *v/v*), filtered on Acrodisc filter 0.45 μm Sigma-Aldrich (Milan, Italy), diluted ten and one hundred times for pulps and peels, respectively, and analysed. Juice samples were analysed without any pre-treatment. Each sample was analyzed in triplicate.

Isolation of punicalagin anomers by preparative HPLC/UV

Punicalagin anomers were isolated from pomegranate peel extracts by preparative HPLC analysis. A Shimadzu system (Shimadzu, Milan, Italy), including a SCL-10A VP controller, two LC-10 AD VP pumps, a SPD-20A UV/Vis detector and a CTO-10ASVP column oven was used. Two grams of grinded pomegranate peels were extracted with 50 mL of water/MeOH mixture (1:1 *v/v*) and the extract was brought to dryness in a rotary evaporator. The obtained extract was dissolved in 1 mL of water/MeOH mixture (1:1 *v/v*) and subjected to preparative-HPLC separation using a Discovery® HS C18 (250 × 10 mm I.D. × 5 μm) (Supelco) preparative column. After five consecutive injections seven fractions were collected, but only fraction 5 contains punicalagin anomers. Fraction 5 was concentrated to 0,4 mL and subjected to preparative-HPLC separation for a further purification.

Mobile phases: water (solvent A) and acetonitrile (solvent B); flow rate: 3 mL/min. gradient: 0 min, 0% B, 15 min, 20% B, 30 min, 30% B, 50 min, 100% B. Purification of fraction five was performed in isocratic mode employing water/ACN (95:5, *v/v*). 200 μL was the injected sample volume. Data were acquired with an UV detector at 280 nm utilizing a Shimadzu LCsolution software ver 1.24 SP1. The recovered fractions were evaporated by a rotary evaporator to attain pure punicalagin α (10 mg) punicalagin β (12 mg), whose structures were confirmed by ESI-MS analysis.

HPLC-PDA-ESI/MS analysis

HPLC experiments were carried out with a Shimadzu Prominence LC-20A instrument (Shimadzu, Milan, Italy), including a CBM-20A controller, two LC-20 AD XR dual-plunger parallel-flow pumps, a DGU-20A3 on-line degasser, an autosampler SIL-10ADvp and a CTO-20AC column oven. An SPD-M10Avp PDA detector and an HPLCMS-2010, with an ESI interface (Shimadzu), were employed for quantification and characterization of phenolic compounds, respectively. MS data were acquired with the LCMSsolution Ver. 3.7 software (Shimadzu).

Phenolic compounds present in pomegranate samples were analysed with two different methods. Method A: Anthocyanins analyses were performed on a LiChrosorb RP-18, 200 x 4.6 mm I.D., 5 μm particle size (Hewlett Packard, PA, USA). 2 μL the injected sample volume, while mobile phase contained water/formic acid (90:10, *v/v*) (solvent A) and water/ACN/formic acid (40:50:10, *v/v/v*) (solvent B). The following step-wise gradient profile was applied: 0 min, 12% B, 35 min, 30% B, 36 min, 100% B, 40 min, 100% B. Flow-rate was 1.0 mL/min, data acquisition was done with a PDA detector in the range 400-600 nm and analytes were detected at 518 nm. Time constant was 0.64 s and sample frequency 1.5625 Hz. Data acquisitions were done with a Shimadzu LCMS solution software ver 3.70.

Method B: ellagitannins, phenolic acids and flavonoids analyses were achieved on a Poroshell120 SB-C18, 150 x 2.1 mm I.D. with particle size of 2.7 μm (Agilent, CA, USA). The injection volume was 2 μL, mobile phase contained water/formic acid (99.9:0.1, v/v) (solvent A) and water/acetonitrile/formic acid (39.9:60:0.1, *v/v/v*) (solvent B). The step-wise gradient program was as follows: 0 min, 0% B, 15 min, 20% B, 30 min, 30% B, 50 min, 100% B, 60 min, 100% B, 65 min 0% B. Flow-rate was 0.2 mL/min. Data were acquired using a PDA detector in the range 210-400 nm and the chromatograms were extracted at 283 and 325 nm. Time constant was 0.64 s and sample frequency 1.5625 Hz. Data acquisition was done with Shimadzu LCMS solution software ver 3.70.

MS acquisition was done with ESI, in both negative and positive mode. ESI conditions: mass spectral range, m/z 150-1400; interval, 1.0 sec; scan speed, 2000 amu/s; nebulizing gas (N_2) flow, 1.5 L/min; ESI temperature, 250°C; heat block, 300°C; DL (desolvation line) temperature, 250°C; CDL, voltage -34V; interface voltage, +4.5 kV; Qarray voltage, 1.0 V and detector voltage, 1.5 kV.

HPLC-PDA method validation

A mixture of nine phenolic standard compounds (catechin, quercetin, ellagic acid, cyanidin-3-glucoside, punicalagin α, punicalagin β, gallic acid, caffeic acid and p-coumaric acid), belonging to phenolic classes representative of pomegranate, was employed for quantitative analysis and method validation. Stock standard solutions of each compound were prepared at concentration 1000 mg/L in acetonitrile for linearity study. External standard calibration curve was obtained by using five data points (concentration range 0.1-100 mg/L) analysing solutions prepared by diluting the stock solution, using acetonitrile as a solvent. Five analyses were performed for each concentration level. The content of the different analytes present in pomegranate extracts was assessed by using the calibration curves of the compounds with the same chromophore. By means of the calibration curves described above, it was possible to quantify anthocyanins, ellagic acid and derivatives, gallic acid and derivatives and flavonoids. For reference compounds, the limit of detection (LOD) and the limit of quantification (LOQ) were determined following the EURACHEM guidelines [38]. The accuracy of the chromatographic method was estimated by means of the recovery test. This involved the addition of a known quantity of standard compounds on a sample of lemon pulp and peel that do not contain the studied standard molecules [39]. Every extract thus obtained was analysed in triplicate. Recovery was obtained applying equation 1.

Recovery % = [(Conc. Sample Fortified – Conc. Sample Unfortified)/Fortification]*100 (1)

In order to study the method's precision, intra- and inter-day multiple injections of standard compounds mixture were done and then the %RSD of retention times were calculated.

Trolox equivalent antioxidant capacity assay

The antioxidant capacity was assessed by TEAC test method described by Re and co-workers [40] with some modifications. $ABTS^{\bullet+}$ radical cation was generated by reacting 7 mM ABTS solution

and 2.45 mM potassium persulfate solution in the dark at room temperature for 16 h. Subsequently, $ABTS^{\bullet+}$ solution was diluted in ethanol to an absorbance of 0.70 ± 0.05 at λ = 734 nm. Before the analyses, pomegranate pulp, juice and peel extracts were diluted ten, fifty and two hundred times, respectively. The reacting mixtures were prepared mixing 10 μL of each diluted sample with 190 μL of $ABTS^{\bullet+}$ solution in a 96-multiwell insert system (Greiner Bio-one, Germany). After 10 min of incubation in darkness, absorbance was recorded at 734 nm by a multifunctional microplate reader (InfiniteM, 200 PRO, Tecan, Italy) in a sample dispensed in triplicate. Trolox was used as reference standard and TEAC data were calculated from the Trolox standard curve (50-600 μmol/L). The antioxidant capacities of the pomegranate pulp, juice and peel were accounted as Trolox equivalent antioxidant capacity (TEAC) and reported as µmol of Trolox equivalents per g of fresh samples.

Total phenolic content

Total phenolic content of pomegranate pulp, juice and peel extracts was determined by a modified Folin-Ciocalteau method [41] using gallic acid as standard. The pomegranate juice and peel extracts were opportunely diluted, while pulp extracts were analysed without any sample dilution. The reacting mixtures were prepared by mixing 10 μL of each sample with 100 μL of Folin reagent. After 8 min, 300 μL of a 20 % (w/v) sodium carbonate (water solution) was added. After 2 h of incubation at room temperature in darkness, 200 μL of the mixture was transferred into a 96-multiwell insert system (Greiner Bio-one, Germany). The absorbance of solutions was measured at 760 nm by a multifunctional microplate reader (InfiniteM, 200 PRO, Tecan, Italy) in the samples dispensed in triplicate. Total phenolic content was determined from the gallic acid calibration curve (10-110 μmol/L). The results were reported as mg of gallic acid equivalents per g of fresh samples.

DPPH radical scavenging assay

The free radical scavenging activity of samples was evaluated by DPPH according to the previously reported method by Padmanabhan and Jangle [42]. The method is based on electron-transfer that produces a violet solution in ethanol. This free radical in the presence of an antioxidant molecule is reduced, giving a change in colour from deep violet to light yellow. A 0.1 mM solution of DPPH in ethanol was daily made-up. A volume of 180 μL of this solution was mixed with 20 μL of ethanol (control reaction) and 20 μL of the extracted sample of pomegranate pulp, juice and peel, opportunely diluted. 200 μL of each mixture solution was added to the 96-wells microplate (Greiner Bio-one, Germany) and incubated in dark, at room temperature, for 30 min. The decrease in absorbance was measured at 518 nm by a multifunctional microplate reader (InfiniteM, 200 PRO, Tecan, Italy). The experiment was performed in triplicate. The percentage of DPPH consumption was changed to Trolox equivalents (TE) using a calibration curve (R^2 = 0.992) with trolox standard solutions (20-800 μmol/L).

Statistical analysis

Each data was repeated three times and expressed as mean ± standard deviation (SD). In the TPC and antioxidant activity tests, comparison of the groups was made by one-way ANOVA using Graphpad Prism 4 statistical software package (Graphpad, San Diego, CA, USA). Differences among means were considered significant at $P<0.05$ using Tukey's honest significant difference test. Correlations among mean values of TPC and antioxidant activity was determined by the Pearson coefficient. Probability values of $P\leq0.05$ were selected as the criteria for statistically significant difference. Multivariate statistical analysis by means of principal component analysis (PCA) was executed using the PLS-Toolbox SW (Eigenvector, Wenatchee, WA, USA, in the Matlab environment). Data were previously auto scaled.

Results and Discussion

Fruit quality

Fruit weight of cultivar Wonderful (***F***) and Italian varieties Gaeta-1 (***A***) and Tordimonte A (***D***) resulted the highest (Table 1), while the fresh weight of the other varieties ranged from the lowest value of Formia (***G***, 228.5 g) to 291.6 g of Gaeta-3 (***B***). Considerable variations between the varieties were observed for the equatorial diameter and longitudinal length of the fruit (Table 1). The fruits of ***F*** showed the largest size, with the shape slightly rounded than the other varieties. The maximum percentage of arils was found in fruits of ***A*** and ***F*** (63.2 and 62.3%, respectively), while the variety ***D*** showed the lowest value (46.7%).

Total soluble solid, pH and titratable acidity

TSS ranged from 16.12 °Brix for ***F*** to 13,92 for ***B*** (Table 2). The TSS value of fruit of the variety ***G*** was closer to that of ***F***, and among the other varieties, no important differences were observed, with the values closer to ***B*** (Table 2). The fruit juice of the varieties ***D*** and ***F*** showed the highest value of titratable acidity (TA), contrariwise to those obtained in all the other varieties (Table 2). The pH value of pomegranate fruit juice was significant lower for the variety ***D*** and ***F*** (Table 2), in comparison with the values of the other varieties. The maturity index (MI) has significantly separated the variety in two groups (Table 2), and the lowest values were observed for ***F*** (7.53) and ***D*** (5.88).

HPLC method development and validation

In this study, a fused core C18 stationary phase column (150 x 2.1 mm I.D. with particle size of 2.7 μm) was employed for the analysis of hydrolysable tannins, phenolic acids and flavonoids. Optimization of separation conditions was assessed on pomegranate samples in order to have the best separation of all detected phenolic compounds. To optimize the separation, in terms of resolution of analytes and analysis time, the mobile phase composition, including the formic acid concentration and the step gradient program were studied. The effect of ACN and MeOH as organic modifiers was evaluated. The use of an ACN/water mixture allowed the best separation of sample's compounds in the shortest time. Best conditions, in terms of resolution of analytes and analysis time, were obtained applying a step gradient elution mode (see materials and methods section). The use of a column with partially porous particle stationary phase allowed obtaining good chromatographic performance due to the increased column efficiency respect to a classical fully porous particles stationary phase.

A different method was employed for anthocyanins separation, which requires strong acidic media due to the necessity to displace the equilibrium of these analytes to the flavylium cations structure. A fully porous C18 column (200 x 4.6 mm I.D. with particle size of 5 μm) was employed using an acidified mobile phase with 10% HCOOH resulting in a better resolution and increased absorbance between 515 and 540 nm.

A mixture of nine standard compounds belonging to phenolic compounds classes' representative of pomegranate was used for quantitative analysis. In order to validate the HPLC method, retention time precision, expressed as RSD% of retention time (t_R), limit of detection (LOD), limit of quantitation (LOQ), linearity range and recovery, were considered (Table S1).

Retention time precision of the method was studied by analyzing the standard mixture of phenolic compounds six times on the same day and in two different days (n=9). The calculated RSDs% values of retention times were in the range 0.28% and 4.89%.

Calibration curves of catechin, quercetin, ellagic acid, cyanidin-3-glucoside, punicalagin α, punicalagin β, gallic acid, caffeic acid and *p*-coumaric acid were constructed under the same chromatographic conditions optimized for samples analysis. Peak areas were plotted as a function of concentration expressed as mg/L, obtaining good values of correlation coefficients, R^2, between 0.989 to 0.999, without the employment of an internal standard. The linearity of the optimized method was calculated in the concentration range between LOQ value and 100 mg/L.

The recovery of extraction procedure was estimated by spiking a sample of lemon pulp and peel, which do not contain the studied standard molecules [39] (Russo et al., 2014) with standard solutions of catechin, quercetin, ellagic acid, cyanidin-3-glucoside, punicalagin α, punicalagin β, gallic acid, caffeic acid and *p*-coumaric acid at concentration levels in the range of calibration curve (10 mg/Kg, final added concentration. Satisfactory recovery values for all the analytes, ranging from 56 to 96%, were obtained considering also the complexity of studied food matrix (Table S1).

Identification and quantification of phenolic compounds in pomegranate extracts

Analytes were tentatively identified in pomegranate samples combining the information obtained from PDA and MS detectors and by comparison with literature data. When possible, the identification of compounds was confirmed by comparison with standards commercially available. The UV/Vis chromatograms of a juice sample and peel extract at wavelengths of 280 and 540 nm are shown in figure 1.

In general, a total of 35 phenolic compounds belonging to different classes were found and 28 of them were tentatively identified (Table 3). Among them, four classes of compounds were identified namely phenolic acids, anthocyanins, hydrolysable tannins and other flavonoids. UV/Vis spectra allowed to distinguishing among phenolic compounds belonging to the different classes. MS spectra were acquired in negative ionization mode for phenolic compounds different from anthocyanins

while in positive ionization mode for anthocyanins, which consist of species that contain flavylium cation moiety [43].

The optimized and validated method was then applied to the analysis of phenolic compounds in different parts of pomegranate fruit, namely juice, peel and pulp of all varieties (six Italian and one international), all fruit samples with the same state of maturity. Pomegranate juice was directly analysed after sample centrifugation while peel and pulp phenolic compounds were extracted by a water and methanol mixture containing a small percentage of acid.

The phenolic extracts of the seven pomegranate varieties yielded similar qualitative HPLC profile for each kind of sample. Table 3 reports quantitative results as mean value together to concentration range (minimum-maximum) of the seven analysed samples. Phenolic compounds concentration is expressed as mg/g weight for peel and pulp samples while as mg/L for juice samples. Table 4 reports the percentage distribution of the different classes of phenolic compounds in the three pomegranate fruit parts, juice, peel and pulp.

Pomegranate peel samples are the quantitative richest in studied phenolic compounds (medium total concentration 121.1 mg/g), while pomegranate juices samples are the poorest in molecules of our interest (medium total concentration 0.5 mg/mL) even if with a major number of detected compounds. Pomegranate juice usually contains anthocyanins, between 29.9 and 73.2% of total concentration of detected phenolic compounds. Six different anthocyanins were detected and identified in juice samples namely cyanidin-3,5-diglucoside, pelargonidin-3,5-diglucoside, delfinidin-3,5-diglucoside, cyanidin-3-glucoside, pelargonidin-3-glucoside and delphinidin-3-glucoside. The most representative one is cyanidin-3-glucoside, followed by pelargonidin-3,5-diglucoside. In addition, pulp samples contained anthocyanins in a lower percentage than juice (range 4.9-23.1%). Instead, anthocyanins were below the limit of detection in peel extracts samples.

The main compounds in peel samples were ellagitannins (range 39.7-84.2%), which were present in lower percentages also in pulp and juice samples. More than 70% of the total amount of ellagitannins (94.4 mg/g) in pomegranate peel samples is given by the two punicalagin anomers (α

and β) and the two ellagic acid glucoside isomers, 17.1 mg/g, 18.3 mg/g, 17.8 mg/g and 15.2 mg/g respectively. While total ellagitannins amount in pulps is given only by the presence of ellagic acid (0.7 mg/g).

Total flavonoids content in pomegranate pulps is characterized by the presence of catechin and syringetin hexoside, while juice and peels are rich in catechin. Phenolic acids are contained below the 8% of the total phenolic compounds amount for all analysed the samples.

For all juices, the anthocyanins are contained around 40%, while the sample F contains more than 65%.

Looking to pulps samples, they can be divided in two groups: one (A, D, and F) with a total amount around 2.5 mg/g, and the other one (B, C, E, and G) around 1.5 mg/g. The percentage of phenolic compounds is very similar for all the pulp samples, except for sample D where ellagitannins are higher (70%) than the others; meanwhile flavonoids percentage is lower (4%). For all the peel samples the total phenolic compounds content is higher than 100 mg/g, except for sample D that the total content is around 50 mg/g. The same behaviour was found also in the percentage of phenolic compounds were ellagitaninns are higher than 70% for all the samples except for sample D where the amount is lower than 40%.

From results described here, pomegranate peel represents a rich source of ellagitannins in particular of two isomers of punicalagin. This result is in agreement with literature data [19, 24, 29, 33]. A way to recover the ellagitannins present in pomegranate peels could be to isolate these molecules by means of preparative HPLC system. The optimized preparative RP-HPLC/PDA method allowed the purification and isolation of 15 mg of punicalagin α and 16 mg of punicalagin β, with a yield of 50% from 2 g of dried pomegranate peel.

Ellagitannins, isolated and purified using preparative LC analyses, were crystallized and subjected to HPLC/PDA analysis applying the same optimized chromatographic conditions for the pomegranate samples, to verify the purity degree. The two isolated isomers had purity higher than 95%. The identification of the two punicalagin isomers was confirmed by ESI-MS data.

The quantitative data obtained from HPLC analyses were applied to carry out a principal component analysis (PCA) to compare the phenolic compounds composition of three pomegranate parts (juice, pulp and peel) of the seven studied varieties. A multidimensional input was built: an array whose rows are all the analyzed samples and columns are the concentration of the 32 detected compounds. Figure 3 reports the results achieved applying the PCA data model. Here the scores and loading plot of the first two Principal Components (PCs) is reported explaining up to 65% of the total variance. The position of each variable in the loading plot describes its relationship with the other variables. Variables that are close to each other have high correlations. As shown in figure 3, the first component, PC1 accounted for 49.34% of the total variation while the second component (PC2) represented 15.90% of variability. The biplot showed three distinct groups. The first group showed high positive correlation between peel extract of the seven varieties and ellagitannins. In particular, high levels of punicalagin α and β and ellagic acid derivatives are reported. This finding confirmed that pomegranate peel extracts are characterized by the high percentages of ellagitannins [19, 24, 29, 33]. The second group included pulp extract samples and a positive correlation can be observed with compounds like syringetin hexoside and ellagic acid. The third group included juice samples from the seven varieties. This group was characterized by high concentration of anthocyanins in particular of cyanidin-3-glucoside for almost all the analyzed samples.

Antioxidant activity of pomegranate extracts

The total phenolic content (TPC) was obtained by Folin-Ciocalteau assay. Antioxidant activity was tested with two well-known chemical assays, TEAC and DPPH scavenging. Trolox was used as reference standard and the results were reported as μmol Trolox equivalents per mL or g of sample for juice and peel/pulp, respectively. Table 4 reports values of TPC and antioxidant activity. The antioxidant properties of samples were studied by using the two free radical scavenging methods, each directed towards a specific free radical ABTS and DPPH.

TPC values varied between 0.87 and 1.93 mg GAE/mL for juice sample. Comparison of the absolute values with the one's reported in other papers are difficult because they are related to different analytical methods, cultivar, maturity stage, and environmental conditions. However results obtained for juice samples are comparable to that one's obtained in a previous work published by our research group on pomegranate Italian cultivars [37]. Among analysed samples, juice extracted from Gaeta 1 (A) fruits exhibited the highest TPC whether Gaeta 4 (C) showed the lowest TPC. The highest value for TEAC was obtained for juice extracted from Gaeta 1 (A) fruits while for DPPH for juice extracted from Wonderful (F) fruits.

TPC values varied between 3.19 and 8.89 and between 90.0 and 137.3 mg GAE/g of weight for pulp and peel samples, respectively. Results show that extracts prepared from the peel have a phenolic content from twenty to forty-five times higher than the corresponding extracts obtained from the pulp. Obtained results were found to be in accordance with the literature reporting TPC values of the same order of magnitude for pomegranate peel and pulp samples [17, 19, 36]. Results of TEAC and DPPH scavenging assays were in accordance and significant differences among samples are evident (Table 4). In general, as observed for TPC, peel extracts showed higher values of TEAC and DPPH than pulp extracts for all analysed samples. These results are in agreement with previously published data [19]. TEAC values ranged between 1291 and 3998 μmol TE/g and between 41 and 97 μmol TE/g for peel and pulp, respectively. While DPPH values ranged 660 and 2191 μmol TE/g and between 37 and 93 μmol TE/g for peel and pulp, respectively. Among analysed samples peel extracts from Gaeta 3 (B) variety showed the highest value of TPC and antioxidant activity while for pulp samples the highest values were obtained for Gaeta 1 (A) variety. Pearson correlation between TPC and antioxidant activity values was evaluated. Results show a good and significant correlation between TPC and antioxidant activity (TPC vs. TEAC r^2 between 0.873 and 0.986; TCP vs DPPH r^2 between 0.713 and 0.984) and between antioxidant activities

measured with the two assays (TEAC vs. DPPH r^2 between 0.887 and 0.972) for all the studied matrices. The highest Pearson r^2 values were obtained for pulp extract samples.

It is evident that as the total phenolic concentration increases the antiradical activity against DPPH and ABTS radicals raises independently of the matrix. This means that detected phenolic compounds in the samples are those exhibiting antiradical properties.

Conclusions

A method for the analysis of pomegranate phenolic compounds belonging to different classes have been developed and validated. The analyses were performed by HPLC-PDA/ESI-MS directly after simple centrifugation for juice and after solvent extraction for two important pomegranate by-products juice production like peel and pulp. The method was fully validated and applied to the analysis of phenolic compounds in samples from six different Italian pomegranate varieties and one well-known international called Wonderful. The method allowed qualitative and quantitative analysis of the principal phenolic compounds in the different parts of pomegranate fruit. Differences in phenolic compounds profile and concentration can be evidenced allowing distinguishing among different pomegranate fruit parts based on concentration of compounds of specific phenolic classes.

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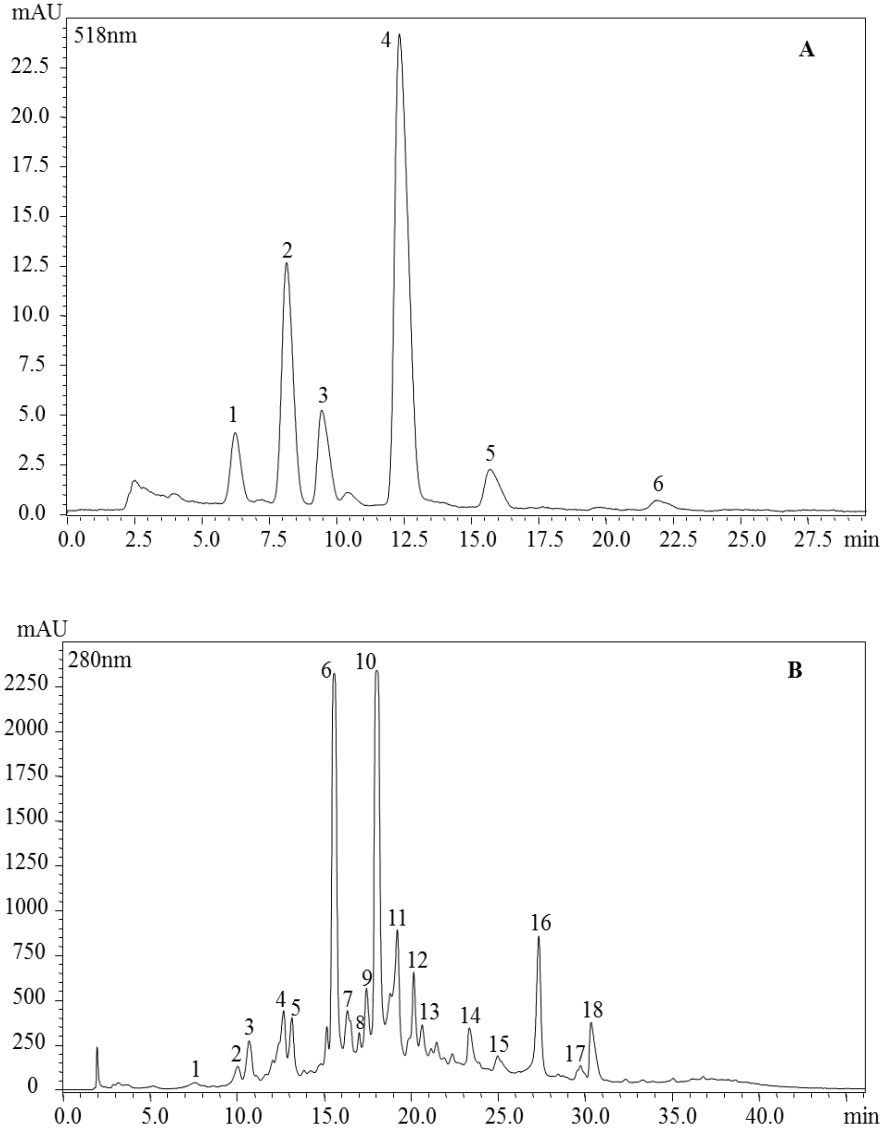
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Figure 1

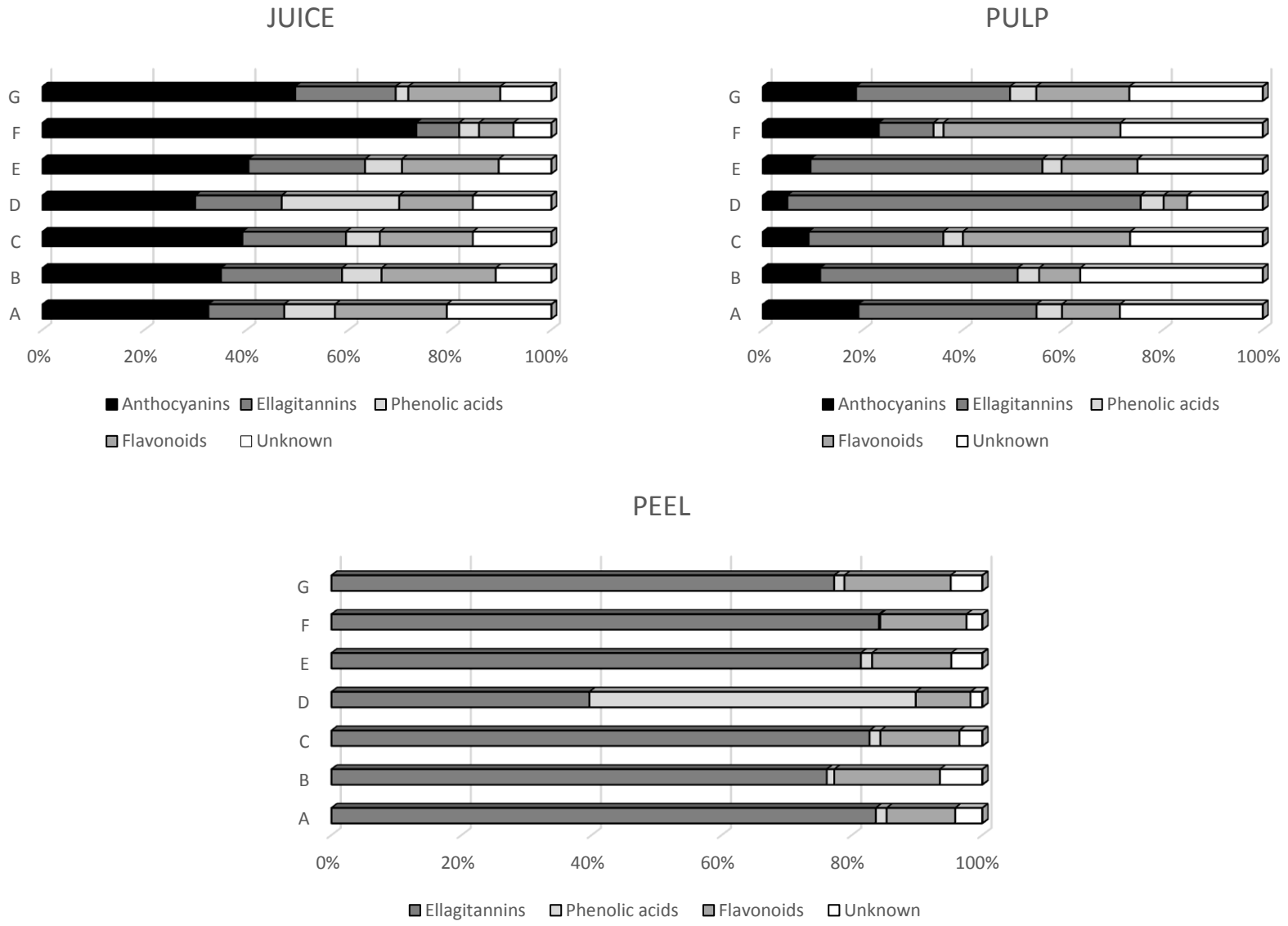
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Figure 2

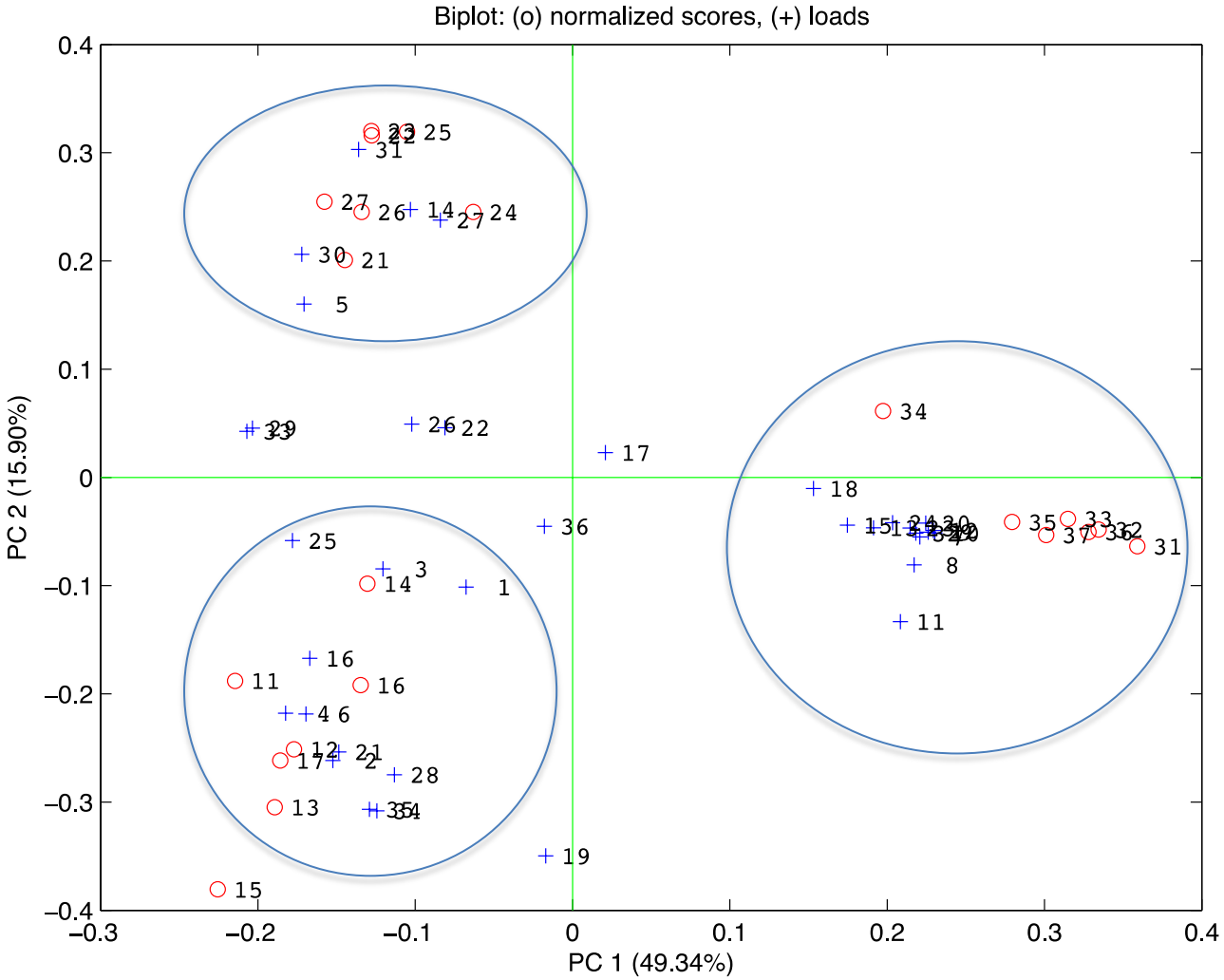
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Figure 3

Table 1

Variety	Fruit fresh weight (g)	Equatorial diameter (mm)	Longitudinal length (mm)	Fruit shape	Total arils fresh weight (g)	Percent arils
A	363.4 a	88.3 a	76.5 ab	0.87 ns	229.7 a	63.2 a
B	291.6 ab	81.2 b	70.6 bc	0.85 ns	168.1 b	57.8 a
C	274.7 bc	81.2 b	70.8 bc	0.87 ns	166.1 b	60.6 a
D	348.9 a	89.3 a	74.3 ab	0.83 ns	162.9 b	46.7b
E	262.2 bc	79.8 b	70.1 bc	0.88 ns	156.3 b	59.6 a
F	355.8 a	89.1 a	81.1 a	0.91ns	179.4 ab	50.4 b
G	228.5 c	75.2 b	67.2 c	0.89ns	142.4 b	62.3 a

Table 2

Variety	TSS (°Brix)	pH	TA (%)	MI
A	14.29 b	3.59 a	0.73 b	19.58 a
B	13.92 b	3.61 a	0.64 b	21.75 a
C	14.15 b	3.53 ab	0.71	19.93 a
D	14.24 b	3.26 c	2.42 a	5.88 c
E	14.25 b	3.52 ab	0.56 b	25.45 a
F	16.12 a	3.15 c	2.14 a	7.53 c
G	15,82 a	3.41 bc	0.68 b	23.26 a

Table 3

Class	N	Compound	*m/z* ([M-H])⁻	Juice (mg/L)		Pulp (mg/Kg)		Peel (mg/Kg)	
				range	*average*	*range*	*average*	*range*	*average*
Anthocyanins									
	1	Cyanidin-3,5- diglucoside[a]	611	1.6 – 284.3	50.7	7.5 – 118.0	28.2	-	-
	2	Pelargonidin-3,5- diglucoside[a]	595	28.7 – 209.2	61.7	17.1 – 156.6	45.9	-	-
	3	Delphinidin-3,5- diglucoside[a]	627	0.9 – 137.2	31.8	17.2 – 121.7	42.2	-	-
	4	Cyanidin-3- glucoside	449	44.2 – 124.1	72.6	51.4 – 159.7	92.4	< LOD	-
	5	Pelargonidin-3- glucoside[a]	433	1.2 – 4.8	2.3	7.2 – 35.2	16.6	-	-
	6	Delphinidin-3- glucoside[a]	465	3.2 – 9.1	6.1	4.9 – 29.4	11.2	-	-
		All		*92.3 – 765.9*	*225.2*	*133.8 – 586.7*	*264.3*	-	-
Ellagitannins									
	7	Granatin A[b]	784	-	-	-	-	294.0 – 3618.8	2495.0
	8	Punicalagin α	1084	0.1 – 7.3	3.3	< LOD	-	500.9 – 23092.7	17089.5
	9	Peduncalagin I[b]	784	-	-	-	-	673.8 – 3436.4	2459.2
	10	Ellagic acid glucoside[c]	464	-	-	-	-	4518.5 – 33054.0	18326.5
	11	Punicalagin β	1084	6.9 – 15.0	12.0	< LOD	-	491.0 – 24455.1	17818.3
	12	Ellagic acid glucoside[c]	464	-	-	-	-	4608.5 - 20854	15224.7
	13	Ellagic acid pentoside[c]	434	-	-	-	-	1535.6 – 18407.0	7315.6
	14	Ellagic acid	302	42.2 – 77.8	57.3	277.8 – 1942.2	733.3	56.3 – 16520.3	8397.6
	15	Punigluconin[b]	802	-	-	-	-	556.4 – 12916.6	6181.8
		All		*52.3 – 92.1*	*72.6*	*277.8 – 1942.2*	*733.3*	*20829.4 – 128133.4*	*94425.0*
Phenolic acids									
	16	Gallic acid	170	2.6 – 34.1	20.5	20.8 – 61.1	34.3	0.0 – 904.2	212.6
	17	Galloyl glucose[d]	332	3.9 – 91.1	19.4	24.9 – 82.8	43.1	50.5 – 24327.0	4144.1
	18	Galloyl-HHDP-hexose[d]	634	-	-	-	-	81.7 – 1781.8	929.0
		All		*9.2 – 125.2*	*39.9*	*50.2 – 126.6*	*77.4*	*196.6 – 26350.5*	*5285.6*
Flavonoids									
	19	Gallocatechin[e]	306	11.2 – 31.1	19.2	-	-	497.1 – 6332.7	3430.1
	20	Phellatin[f]	534	-	-	-	-	1406.5 – 5221.7	3058.0
	21	Phlorizin[f]	436	0.5 – 3.8	1.2	< LOD – 4.1	1.6	-	-
	22	Catechin	290	12.2 – 41.3	22.3	15.4 – 238.4	102.7	893.9 – 11766.8	4540.9
	23	Prunin[e]	434	-	-	-	-	826.5 – 4492.0	3058.6

	24	Rutin[f]	610	-	-	-	-	525.2 – 3817.1	2068.8
	25	Diosmetin glucoside[f]	462	3.6 – 16.0	9.0	8.9 – 102.6	29.8	-	-
	26	Acetyl prunin[e]	476	2.6 – 7.6	4.1	0.9 – 176.1	36.3	-	-
	27	Syringetin hexoside[f]	508	2.1 – 8.9	6.0	15.3 – 454.3	174.1	-	-
	28	Diosmetin glucoside[f]	462	3.2 – 14.0	7.1	-	-	-	-
		All		*43.8 – 89.0*	*68.9*	*106.3 – 897.2*	*344.4*	*4407.5 – 27302.7*	*16156.4*
Unknown polyphenols									
	29	Unknown a*	302	1.8 – 8.4	5.6	21.5 – 37.8	26.8	-	-
	30	Unknown *	647	1.4 – 6.0	2.9	20.0 – 33.1	24.9	-	-
	31	Unknown c*	610	1.8 – 54.0	26.6	198.0 – 609.2	382.5	-	-
	32	Unknown d*	325	-	-	-	-	943.4 – 10905.2	5256.7
	33	Unknown e*	464	7.9 – 17.5	13.8	38.8 – 78.3	58.8	-	-
	34	Unknown f*	464	2.9 – 6.8	4.7	-	-	-	-
	35	Unknown g*		1.9 – 4.3	2.8	-	-	-	-
		All		*23.9 – 84.7*	*56.5*	*309.6 – 722.9*	*492.9*	*943.4 – 10905.2*	*5256.7*
		BIOACTIVE MOLECULES		***228.8 – 1046.0***	***463.0***	***1157.9 – 2749.3***	***1912.3***	***52530.8 – 168275.8***	***121123.7***

For quantitative determination of all the identified polyphenolic compounds were calculated calibration curve of: [a]cyanidin-3-glucoside, [b]punicalagin α, [c]ellagic acid, [d]gallic acid, [e]catechin, [f]quercetin; *quantitative determination of unknown polyphenols were based on correction factor equal to 1.

Table 4

Sample	Juice			Peel			Pulp		
	TPC[a] (mg GAE/mL)	TEAC[b] (μmol TE/mL)	DPPH[c] (μmol TE/mL)	TPC[a] (mg GAE/g FW)	TEAC[b] (μmol TE/g FW)	DPPH[c] (μmol TE/g FW)	TPC[a] (mg GAE/g FW)	TEAC[b] (μmol TE/g FW)	DPPH[c] (μmol TE/g FW)
A	$1.93 \pm 0.05_{a}$	$17.31 \pm 0.03_{a}$	$2.97 \pm 0.06_{a}$	$179.92 \pm 1.31_{a}$	$2785.51 \pm 26.83_{a}$	$1690.47 \pm 19.75_{a}$	$8.89 \pm 0.08_{a}$	$97.35 \pm 0.17_{a}$	$93.32 \pm 0.18_{a}$
B	$1.34 \pm 0.07_{b}$	$8.48 \pm 0.11_{b}$	$0.82 \pm 0.07_{b}$	$244.61 \pm 1.41_{b}$	$3998.05 \pm 2.62_{b}$	$2191.96 \pm 12.68_{b}$	$5.40 \pm 0.09_{b}$	$68.22 \pm 0.11_{b}$	$56.61 \pm 1.16_{b}$
C	$0.87 \pm 0.03_{c}$	$8.51 \pm 0.09_{b,c}$	$0.80 \pm 0.04_{b,c}$	$182.15 \pm 1.57_{a}$	$2561.84 \pm 1.38_{a}$	$1571.78 \pm 23.74_{c}$	$4.95 \pm 0.06_{c}$	$65.21 \pm 2.73_{b,c}$	$52.86 \pm 0.95_{c}$
D	$1.22 \pm 0.03_{b,d}$	$12.59 \pm 0.08_{d}$	$2.52 \pm 0.07_{d}$	$89.68 \pm 0.61_{c}$	$1291.03 \pm 18.98_{c}$	$660.00 \pm 34.96_{d}$	$3.19 \pm 0.07_{d}$	$41.12 \pm 0.30_{d}$	$37.37 \pm 1.88_{d}$
E	$1.20 \pm 0.05_{d,e}$	$11.91 \pm 0.02_{e}$	$0.91 \pm 0.07_{b,e}$	$141.14 \pm 0.45_{d}$	$2339.89 \pm 6.66_{a,d}$	$1509.72 \pm 12.32_{e}$	$6.11 \pm 0.07_{e}$	$70.83 \pm 0.20_{b,e}$	$57.98 \pm 0.65_{b,e}$
F	$1.58 \pm 0.02_{f}$	$16.88 \pm 0.16_{f}$	$4.43 \pm 0.13_{f}$	$137.28 \pm 1.19_{d,e}$	$2245.62 \pm 13.81_{a,d}$	$1514.21 \pm 5.76_{e,f}$	$6.14 \pm 0.03_{e,f}$	$73.70 \pm 0.99_{e,f}$	$66.17 \pm 0.49_{f}$
G	$1.08 \pm 0.06_{d,g}$	$10.37 \pm 0.04_{g}$	$0.77 \pm 0.13_{b,g}$	$191.59 \pm 3.38_{f}$	$3661.41 \pm 6.94_{a,d}$	$1778.50 \pm 5.94_{g}$	$5.32 \pm 0.05_{b,g}$	$63.96 \pm 0.08_{c,g}$	$54.70 \pm 1.51_{b,c,g}$

[a]TPC expressed as milligrams of gallic acid per mL of fresh juice and as milligrams of gallic acid per g of fresh weight (FW) of peel and pulp. [b]TEAC expressed as micromoles of trolox per mL of fresh juice and as micromoles of trolox per g of fresh weight (FW) of peel and pulp. [c]DPPH expressed as micromoles of trolox per mL of fresh juice and as micromoles of trolox per g of fresh weight (FW) of peel and pulp.

All values are expressed as mean ± SD (n = 3).

Different letters ($_{a}$ to $_{g}$) in columns present statistically significant differences ($P < 0.05$) among pomegranate species.

Supplementary material

Table S1. Linearity, limits of detection (LOD), limit of quantification (LOQ), retention time precision and recoveries of the HPLC-PDA method for the determination of phenolic compounds in pomegranate extracts.

Analyte	t_R (min)	UV/Vis λ max (nm)	Calibration curve	R^2	LOD (μg/mL)	LOQ (μg/mL)	Precision (RSD, %)	Recovery (%)
Gallic acid	7.8	280	y = 61271x – 39908	0.999	0.006	0.010	3.58	95.4 ± 2.06
Cyanidin-3-glucoside	12.7	518	y = 16005x – 3698.8	0.998	0.163	0.285	4.89	83.2 ± 8.03
Punicalagin α	15.6	283	y = 61020x + 37446	0.989	0.006	0.010	1.36	55.4 ± 3.44
Punicalagin β	18.2	283	y = 67615x – 12448	0.991	0.005	0.009	1.41	58.0 ± 4.25
Catechin	18.7	280	y = 34260x + 9610	0.999	0.011	0.017	1.11	94.6 ± 2.80
Caffeic acid	20.3	325	y = 12917x – 6846	0.999	0.028	0.046	1.39	95.2 ± 3.46
p-coumaric acid	26.0	325	y = 13039x + 18348	0.999	0.030	0.052	1.66	93.5 ± 0.68
Ellagic acid	31.5	280	y = 11242x – 38183	0.988	0.032	0.053	1.16	90.8 ± 4.06
Quercetin	41.9	325	y = 25087x – 2210	0.998	0.014	0.024	0.28	94.2 ± 2.72