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Determination of the Major Phenolic Compounds in Pomegranate Juices by HPLC−DAD−ESI-MS

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Supporting Information

ABSTRACT: Traditionally, pomegranate (*Punica granatum* L.) has been consumed as fresh fruit or as pomegranate juice. In this study, the main phenolic compounds of 12 pomegranate varieties and 5 pomegranate clones were determined by HPLC−DAD−ESI-MS. Two chromatographic methods with a fused-core C18 column and a classical HPLC system were developed. Thirteen anthocyanins and fourteen other phenolic compounds were determined in the pomegranate juices. As far as we are concerned, a new flavonol-glycoside, phellatin or its isomer amurensin, has been tentatively identified for the first time in pomegranate juices. Total phenolic content ranged from 580.8 to 2551.3 mg/L of pomegranate juice. Anthocyanins varied between 20 to 82% of total phenolic content. Flavonoids were 1.6−23.6% of total phenolic compounds, while phenolic acids and ellagitannins were in the range 16.4−65.8%. The five clones reported a phenolic content comparable with that of the other pomegranate samples.

KEYWORDS: *pomegranate, phenolic compounds, anthocyanins, HPLC−DAD−ESI-qTOF-MS, fused-core column*

INTRODUCTION

Pomegranate is native from the zones comprised between Iran and the Himalayas in Northern India, and it has been cultivated and naturalized over the whole Mediterranean region since ancient times. In addition, today it is widely cultivated throughout India and the drier parts of Southeast Asia, Malaya, the East Indies, and tropical Africa.[1,2] Many studies show that pomegranate is one of the most powerful foods for overall good health,[3] and pomegranate juice is also touted for its health benefits because, in some clinical studies, it has been demonstrated to produce significant benefits.[4] A clinical trial on healthy male volunteers developed by Aviram et al.[5] revealed that, in humans, pomegranate juice consumption decreased LDL susceptibility to aggregation and retention, and increased the activity of serum paraoxonase. The results obtained by Pérez-Vicente et al.[6] indicate that anthocyanins and other phenolic compounds and vitamin C can be bioavailable after digestion and might contribute to protecting humans from several diseases. Furthermore, recently, Afaq et al.[7] has reported that pomegranate extract could be a potent antitumor-promoting agent because it inhibits several biomarkers of TPA-induced tumor promotion in an in vivo animal model.

Some of the beneficial health effects of pomegranate are due to the phenolic compounds contained in this fruit. Pomegranate is rich in antioxidants such as phenolic acids, tannins, flavonols, and anthocyanins.[8] Hydrolyzable tannins including ellagitannins and gallotannins constitute the most prevalent compounds present in pomegranate, and the predominant hydrolyzable tannin in pomegranates is known as punicalagin.[1,9−12] Reddy and co-workers[13] demonstrated the antimalarial and antioxidant activities of pomegranate tannin fraction, and particularly, punicalagin has been shown to possess remarkable pharmacological activities. However, tannins are not absorbed by humans and, thus, they are hydrolyzed to ellagic acid. This ellagic acid is also very poorly absorbed, but it is mainly metabolized by gut microbiota to yield urolithins,[14,15] which are one of the main compounds responsible of the healthy benefits likely together with other phenolic metabolites.

Some researchers have reported that pomegranate juice is one of the major sources of anthocyanins, especially 3-glucosides and 3,5-diglucosides of delphinidin, cyanidin, and pelargonidin.[1,12,16]

Recently, several authors[1,17] identified different gallic and ellagic derivatives in pomegranate fruit and juices with or without a previous hydrolysis of samples. Moreover, the characterization and quantitation of phytoestrogenic lignans from pomegranate fruits and fruit-derived products was reported by Fischer et al.[18] However, the phenolic content and composition of pomegranate juices are strongly influenced

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by the cultivar, agronomical and climate conditions, harvest time, and juice extraction methods.[2,9,19−22]

Gil and co-worker[12] underlined that the activity of phenolic compounds depends on the phenolic structure. Therefore, the main objectives of the present study were the establishment of LC−DAD methods to quantify the individual pomegranate phenolic compounds. Moreover, the LC system was coupled with an electrospray ionization (ESI)-quadrupole-time-of-flight (qTOF)-mass spectrometer to identify the principal phenolic compounds in different pomegranate juices proceeding from 12 varieties and 5 pomegranate clones.

MATERIALS AND METHODS

Samples. Fruits of the pomegranate varieties Wonderful 1 and Wonderful 2, Akko and Hershkovitz (imported from Israel), Mollar and Valenciana (imported from Spain), Hijaz (imported from Turkey), Shiraz (from Iran), and G1 and G2 (from Tunisia) were collected during the second and third decade of September. Dente di Cavallo (provided from the Department of Scienze Agro Ambientali e Territoriali of the University of Bari) was collected in the second week of October. Moreover, fruits of other clones were harvested from noncommercial trees and identified with experimental nicknames: Ecotipo 1 and Ecotipo 2, harvested near Naples (approx 40°40′N, 14°45′E); Ravenna, Radisa and Veneti harvested near Ravenna (44°25′N, 12°12′E). Clone fruits were collected in the second week of September.

Intact arils were manually obtained from whole fruits, and the juices were obtained by squeezing them.

Before the analysis, the juices were centrifuged at 14000*g* for 5 min at room temperature and filtered with a 0.2 μm RC filter (Phenomenex, Torrance, CA, USA). The juices were stored at 4 °C and were analyzed within one week of juice production.

Chemicals. All the solvents and reagents were purchased from Merck (Darmstadt, Germany). The following phenolic standards were supplied by Sigma-Aldrich (St. Louis, MO, USA): ellagic acid, punicalagin 98%, rutin, cyanidin-chloride 90%, catechin and phlorizin hydrate.

Determination of Phenolic Compounds by HPLC−DAD−ESI-qTOF-MS. A liquid chromatography apparatus HP 1100 series from Agilent Technologies, including a degasser, a binary pump delivery system, and an automatic liquid sampler, was used and coupled to diode array and mass spectrometer detectors.

The HPLC column was a fused-core Poroshell 120, SB-C18 (3.0 × 100 mm, 2.7 μm) from Agilent Technologies (Agilent Technologies, Palo Alto, CA, USA). Separation was carried out with different gradient elution programs depending on the phenolic classes.

a. Determination of Anthocyanins. The mobile phases consisted of water plus 5% formic acid (A) and acetonitrile (B). The following multistep linear gradient was applied: 0 min, 5% B; 2 min, 7% B; 4 min, 9% B; 6 min, 12% B; 8 min, 15% B; 9 min, 16% B; 10 min, 17% B; 11 min, 17.5% B; 12 min, 18% B; 13 min, 100% B; 17 min, 100% B; 18 min, 5% B. The initial conditions were maintained for 5 min. The flow rate was set at 0.80 mL/min throughout the gradient. The injection volume in the HPLC system was 2.5 μL. UV spectra were recorded from 210 to 600 nm, while the chromatograms were registered at 280 nm for flavanol−anthocyanin adducts and 520 nm for anthocyanins. Separation was carried out at 25 °C. Calibration curves of cyanidin-chloride and catechin were arranged from 1 to 500 mg/L. MS analysis was carried out using electrospray ionization (ESI) interface in positive ionization mode. The HPLC analysis was replicated three times for samples and calibration points (n = 3).

b. Determination of Other Phenolic Compounds. The mobile phases consisted of water plus 1% acetic acid (A) and acetonitrile (B). The following multistep linear gradient was applied: 0 min, 5% B; 2 min, 7% B; 4 min, 9% B; 6 min, 12% B; 8 min, 15% B; 9 min, 16% B; 10 min, 17% B; 11 min, 17.5% B; 12 min, 18% B; 14 min, 20% B; 16 min, 28% B; 18 min, 100% B; 22 min, 100% B; 23 min, 5% B. The initial conditions were maintained for 5 min. The flow rate was set at 0.80 mL/min throughout the gradient. The injection volume in the HPLC system was 2.5 μL. UV spectra were recorded from 210 to 600 nm, while the chromatograms were registered at 280 and 360 nm. The calibration curve of punicalagin was arranged from 5 to 500 mg/L at 360 nm. The calibration curves of ellagic acid, gallic acid, and rutin were arranged from 1 to 500 mg/L, at six concentration levels, plotting peak area vs analyte concentration at 280 nm for gallic acid and phlorizin hydrate, and at 360 nm for rutin and ellagic acid. Separation was carried out at 23 °C. MS analysis was carried out using an electrospray ionization (ESI) interface in negative ionization mode. The HPLC analysis was replicated three times for each extract and calibration point (n = 3).

The HPLC system was coupled to a micrOTOF-Q II mass spectrometer (Bruker Daltoniks, Bremen, Germany) equipped with an ESI interface (Bruker Daltoniks, Bremen, Germany) operating in negative and positive ion modes using a capillary voltage of +4 kV. To establish the best MS conditions a flow injection analysis (FIA) method was used to set up the MS method. The optimum values of the ESI-qTOF-MS parameters were drying gas temperature, 210 °C; drying gas flow, 8 L/min; and nebulizing gas pressure, 2 bar. Detection was carried out within a mass range of 50−1100 m/z. The MS/MS analyses were acquired by automatic fragmentation where the three most intense mass peaks were fragmented. Collision energy values for MS/MS experiments were adjusted as follows: m/z 100, 20 eV; m/z 500, 30 eV; m/z 1000, 35 eV. Nitrogen was used as drying, nebulizing, and collision gas.

The accurate mass data of the molecular ions were processed using DataAnalysis 4.0 software (Bruker Daltoniks), which provided a list of possible elemental formulas via the SmartFormula Editor. The SmartFormula Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring plus double bond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (Sigma Value) for increased confidence in the suggested molecular formula. The widely accepted accuracy threshold for confirmation of elemental compositions was established at 8 ppm.[23] During the development of the HPLC method, the instrument was calibrated externally with a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL, USA) directly connected to the interface and injected with a sodium acetate cluster solution containing 5 mM sodium hydroxide and 0.2% acetic acid in water:isopropanol (1:1, v/v). The calibration solution was injected at the beginning of each run, and all the spectra were calibrated prior to compound identification. By using this method, an exact calibration curve based on numerous cluster masses, each differing by 82 Da ($NaC_2H_3O_2$), was obtained. Due to the compensation of temperature drift in the micrOTOF-Q II, this external calibration provided accurate mass values of better than 8 ppm for a complete run without the need for a dual sprayer setup for internal mass calibration.

Statistical Analysis. Tukey's honest significant difference multiple comparison (one-way ANOVA), $p < 0.05$ level, was evaluated using Statistica 8.0 software (2007, StatSoft, Tulsa, OK, USA).

RESULTS AND DISCUSSION

The optimization of two new methods, one for the determination of anthocyanins and the other for phenolic compounds in pomegranate juices, was established. To this purpose, a fused-core column was used to develop fast methods using a standard HPLC system (maximum pressure lower than 400 bar). The selection of the mobile phases was done on the basis of previous experiments reported in the bibliography, which show that acetonitrile presents a system back pressure much lower than other solvents such as methanol.[24] This fact allowed us to work at higher flow rates and to obtain faster analysis times with a standard HPLC system compared to literature.[22,25,26]

Separation and Identification of Anthocyanins. To determine the anthocyanins, different mixtures of water

Table 1. Anthocyanins Data Obtained by HPLC−DAD−ESI-qTOF-MS

	assignment	t_r (min)	UV data λ_{max} (nm)	$[M]^+$ calcd	$[M]^+$ exptl	error (ppm)	mSigma	MS^2	molecular formula
A1	delphinidin-3,5-diglycoside	4.4	519, 277	627.5248	627.5253	3.0	17.6	465.1031 303.0493	$C_{27}H_{31}O_{17}$
A2	cyanidin-3,5-diglycoside	5.8	513, 277	611.1607	611.1611	2.9	10.7	449.1079 287.0558	$C_{27}H_{31}O_{16}$
A3	pelargonidin-3,5-diglycoside	6.5	499, 274	595.1657	595.1662	1.5	13.1	433.1071 271.0981	$C_{27}H_{31}O_{15}$
A4	delphinidin-3-glycoside	7.0	522, 277	465.1061	465.1074	1.2	10.4	303.0499	$C_{21}H_{21}O_{12}$
A5	cyanidin-pentoside-hexoside	7.8	516, 273	581.1510	581.1501	4.0	29.0	449.1160 419.1173 287.0100	$C_{26}H_{27}O_{15}$
A6	(epi) afzelchin-delphinidin-3-hexoside	8.4	282, 326, 528	737.1713	737.1729	3.9	19.5	575.2124 557.2683 439.1009	$C_{36}H_{33}O_{17}$
A7	(epi) gallocatechin-pelargonidin-3-hexoside	8.6	272, 530	737.1715	737.1724	4.7	16.4	575.1247 449.3128 407.3410	$C_{36}H_{33}O_{17}$
A8	(epi) afzelchin-delphinidin-3-hexoside	8.8	286, 330, 526	737.1713	737.1731	5.0	22.6	575.2121 557.2679 439.1014	$C_{36}H_{33}O_{17}$
A9	cyanidin-3-glycoside	9.0	516, 280	449.1157	449.1162	3.5	28.4	287.0996	$C_{21}H_{21}O_{11}$
A10	(epi) gallocatechin -cyanidin-3-hexoside	9.3	276, 532	753.1661	753.1675	4.1	20.1	591.3590 573.2471 423.3174	$C_{36}H_{33}O_{18}$
A11	cyanidin-3-rutinoside	9.8	503, 274	595.1739	595.1736	1.1	13.8	449.1151 287.0998	$C_{27}H_{31}O_{15}$
A12	pelargonidin-3-glycoside	10.1	503, 274	433.1210	433.1207	2.7	35.5	271.3212	$C_{21}H_{21}O_{10}$
A13	(epi) catechin-cyanidin-3-hexoside	13.4	286, 328, 534	737.1715	737.1789	4.4	28.7	575.1256 557.3160 423.3169	$C_{36}H_{33}O_{17}$

acidified with diverse concentrations of acetic and formic acid were used as mobile phase A. The concentrations of acid used varied from 1 to 5%. The best results were obtained using water with 5% of formic acid. Concentrations of formic acid lower than 5% or the use of water with 1 to 5% of acetic acid reported the coelution of some anthocyanin compounds. After that, other parameters were optimized to allow the best separation. First, different temperatures (from 20 to 40 °C) were checked to reduce the viscosity of the solvents and to obtain faster analysis. The change of the temperature did not cause a considerable decrease of the analysis time. However, the anthocyanins showed a high variation in retention time which led to an overlapping. Basically, temperatures higher than 30 °C caused the coelution of three anthocyanins. At 25 °C, all the peaks were well separated. The flow rate was changed from 0.4 to 1 mL/min, and the best results were obtained at 0.8 mL/min.

Table 1 shows the UV and MS data of anthocyanins identified in Akko pomegranate juice (Figure S1, Supporting Information). All the identified compounds showed a typical UV maximum of absorption between 499 and 519 nm and other maximum of absorption in the range 272−280 nm. According to Gil and co-workers,[12] these typical absorptions can be attributed to anthocyanin compounds. MS data confirmed this affirmation. Compounds **A1** and **A4** showed an ion at 303 m/z that can be assigned to delphinidin. Particularly, compound **A1** reported a molecular ion at 627 m/z and molecular formula $C_{27}H_{31}O_{17}$ and two fragment ions at 465 and 303 m/z corresponding to two hexose losses. Compound **A4** showed a molecular ion at 465 m/z (molecular formula $C_{21}H_{21}O_{12}$) and a fragment ion at 303 m/z (−162 Da). According to Fischer and co-workers[1] these compounds were identified as delphinidin-3,5-diglycoside and delphinidin-3-glycoside, respectively.

Compounds **A2**, **A5**, **A9**, and **A11** showed a common ion at m/z 287 corresponding to cyanidin. A molecular formula $C_{27}H_{31}O_{16}$ and a molecular ion at 611 m/z were obtained for compound **A2**. MS^2 data reported two fragment ions at m/z 449 and 287 corresponding to sugar losses. According to Fischer and co-workers[1] this compound was assigned as cyanidin-3,5-diglycoside. A molecular ion at m/z 581 and molecular formula $C_{26}H_{27}O_{15}$ was extrapolated for anthocyanin **A5**. MS^2 data showed two major fragments corresponding to losses of hexoside and pentoside (419 and 449 m/z, respectively), and another one corresponding to cyanidin aglycon (287 m/z). According to the literature,[1] this compound was identified as cyanidin-pentoside-hexoside. Compound **A9** exhibited a molecular formula $C_{21}H_{21}O_{11}$ and a fragment at 287 m/z that was obtained by a sugar loss; this compound was identified as cyanidin-3-glycoside. Compound **A11** reported a molecular formula $C_{27}H_{31}O_{15}$. The peak presented a mass for the molecular ion at m/z 595. The MS/MS of m/z 595 produced fragment ions at m/z 449, which corresponded to a loss of rhamnose, and at m/z 287, a common aglycon cation. The fragmentation pattern of this anthocyanin matched with cyanidin 3-rutinoside.

Compounds **A3** and **A12** showed a common fragment at 271 m/z which corresponded to pelargonidin. Compound **A3** had a molecular ion (M^+) at m/z 595 and two fragment ions at m/z 433 and 287 (loss of two glycoside groups); on the basis of this evidence, it was concluded that this compound was pelargonidin-3,5-diglycoside. Finally, compound **A12** yielded a molecular ion at m/z 433 and it presented a molecular formula $C_{21}H_{21}O_{10}$; MS/MS analysis gave a base peak at m/z 271 (pelargonidin) (M − 162, loss a glycosyl group), and therefore, according to Borges and Crozier,[26] it was identified as pelargonidin-3-glycoside.

Moreover, five principal anthocyanin−flavanol adducts were identified. Their presence in pomegranate was described by Sentandreu and co-workers.[27−29]

Compounds **A6** and **A8** showed a molecular ion at 737 m/z and a molecular formula $C_{36}H_{33}O_{17}$, and MS/MS analysis produced three major fragment ions at 575, 557, and 439 m/z; according to Sentandreu et al.[28] these compounds were tentatively identified as (epi) afzelchin-delphinidin-3-hexoside. Compound **A7** at m/z 737 reported three principal fragment ions at 575, 449, and 407 m/z; Sentandreu et al.[28] reported the same fragmentation pattern for (epi) gallocatechin-pelargonidin-3-hexoside. Compound **A10** showed the same molecular and fragment ions reported by Sentandreu et al.[27,28] for the

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Table 2. Phenolic Compounds Data Obtained by HPLC−DAD−ESI-qTOF-MS

	assignment	t_r (min)	UV data λ_{max} (nm)	$[M - H]^-$ exptl	$[M - H]^-$ calcd	error (ppm)	mSigma	MS^2	molecular formula
1	galloyl-glucose	1.2	276	331.0657	331.0671	4.0	3.5	271.0444, 169.0135, 125.0233	$C_{13}H_{16}O_{10}$
2	punicalagin α	3.6	258, 378	1083.0593	1083.0503	8.1	37.9	781.6071, 601.3680, 301.4796	$C_{48}H_{28}O_{30}$
3	peduncalagin I	4.8	253, 377	783.0686	783.0657	3.8	37.6	481.0516, 300,9975	$C_{34}H_{24}O_{22}$
4	punicalagin β	6.5	258, 378	1083.0593	1083.0503	8.0	37.8	781.6071, 601.3680, 301.4796	$C_{48}H_{28}O_{30}$
5	galloyl-HHDP-hexose	8.3	266, 365	633.0733	633.0702	4.9	5.4	615.0388, 463.0477, 300.9995	$C_{27}H_{22}O_{18}$
6	ellagic acid glucoside	10.0	252, 361	463.0518	463.0498	4.4	10.5	300.9970	$C_{20}H_{16}O_{13}$
7	granatin B	10.4	274, 365	951.0745	951.0683	6.6	5.5	933.0604, 613,2044, 300.9980	$C_{41}H_{28}O_{27}$
8	punigluconin	10.6	268, 375	801.0792	801.0773	2.4	17.1	649.1016, 300.9973	$C_{34}H_{26}O_{23}$
9	ellagic acid deoxyhexose	12.3	360	447.0569	447.0568	4.7	12.6	301.9998, 300.9969, 299.9895	$C_{20}H_{16}O_{12}$
10	ellagic acid pentoside	12.7	255, 360	433.0412	433.0387	5.8	7.0	300.9965, 299.9899	$C_{19}H_{14}O_{12}$
11	ellagic acid	12.9	275, 367	300.9990	300.9975	4.9	3.4	257.0049, 185.0208	$C_{14}H_6O_8$
12	syringetin-hexoside	15.6	272	507.1144	507.1111	6.3	3.8	312.0562, 295.0800	$C_{23}H_{24}O_{13}$
13	phellatin or amurensin	15.8	284, 330	533.1637	533.1664	5.1	6.9	515.1506, 447.0930, 371.1111	$C_{26}H_{30}O_{12}$
14	phlorizin	17.2	280	435.1297	435.1280	2.8	13.3	297.0770, 273.0747, 167.0337	$C_{21}H_{24}O_{10}$

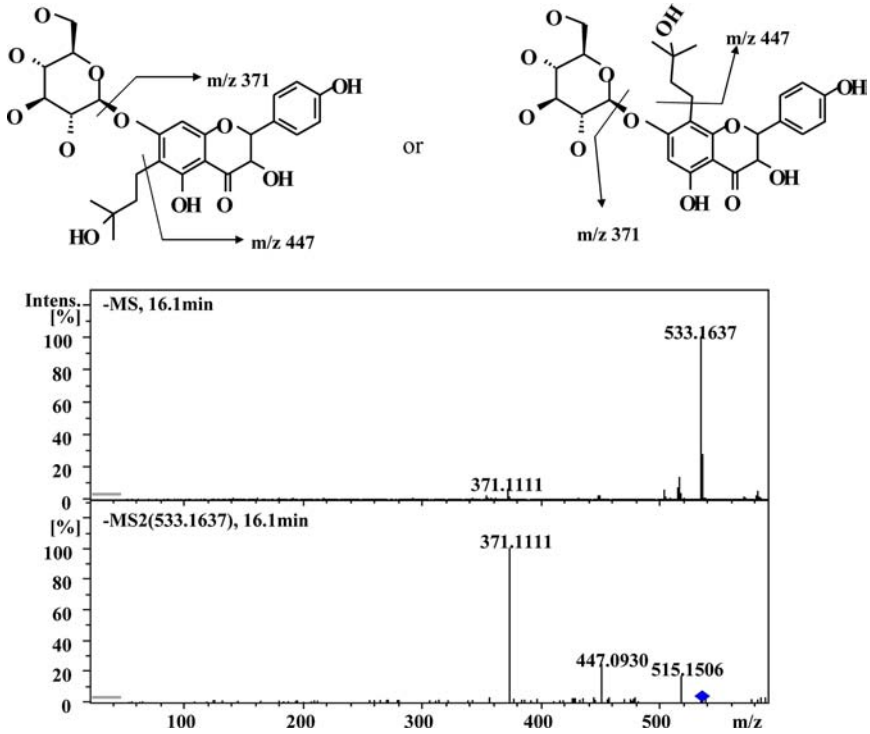

Figure 1. Mass spectra (MS and MS^2) of compound **13** and possible fragmentation pathway.

(epi) gallocatechin-cyanidin-3-hexoside. Finally, the compound **A13** with a molecular ion at 737 m/z and fragment ions at 557 and 423 m/z was tentatively identify as (epi) catechin-cyanidin-3-hexoside according to Sentandreu et al.[29]

The repeatability was assessed for Akko juice. The sample was injected 4 times on the same day (intraday precision) and for three consecutive days (interday precision, n = 12). The percent relative standard deviations (% RSD) of the peak areas (UV detection) and retention times were determined for each peak detected. The intraday repeatability (expressed as % RSDs) of the retention times was in the range from 0.16 to 2.41%, whereas the interday repeatability was from 1.02 to 2.85%. The intraday repeatability (expressed as % RSDs) of the total peak area was 0.37−0.54%, whereas the interday repeatability was 0.99−1.15% (Table S1, Supporting Information).

LOD and LOQ calculated for cyanidin-chloride and catechin standards were 7.07 and 23.6 ng/mL, and 175 and 584 ng/mL, respectively.

Separation and Identification of Phenolic Acids, Flavonoids, and Tannins. To set up a separation method for the determination of phenolic acids, flavonoids, and tannins in pomegranate juice, a method proposed by Qu et al.[10] was used and subsequently changed. The best separation conditions are reported in Materials and Methods (Figure S2, Supporting Information). The established method permitted the determination of 14 phenolic compounds in 23 min (Table 2).

Peak 1 at 1.2 min and molecular formula $C_{13}H_{16}O_{10}$ showed a molecular ion at 331 m/z and three major fragment ions at

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Table 3. Anthocyanin Content in Pomegranate Juices[a]

samples	A1[b]	A2[b]	A3[b]	A4[b]	A5[b]	A6[b]	A7[b]	A8[b]	A9[b]	A10[b]	A11[b]	A12[b]	A13[b]	total
Akko	690.50 b	57.70 g	4.14 m	369.64 a	3.57 f	8.50 d	<LOQ	7.82 e	<LOQ	1.03 e	0.16 d	<LOQ	6.16 a	1149.23 e
Dente di Cavallo	90.71 i	<LOQ	10.22 i,l	226.46 d	5.35 f	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	332.74 i
Ecotipo 1	84.80 i	320.43 c	97.28 e	2.49 i	534.17 a	4.44 e	<LOQ	6.67 e	14.14 b	8.10 b	<LOQ	<LOQ	2.07 c	1078.75 f
Ecotipo 2	189.21 h	2.97 l	141.76 d	93.78 f	2.22 f,g	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.63 c	4.16 a	<LOQ	430.35 h
G1	76.46 i	116.21 e	1.32 n	<LOQ	3.94 f	24.45 a	40.73 b	34.95 a	<LOQ	13.98 a	<LOQ	<LOQ	4.12 b	316.15 i
G2	92.93 i	14.64 h	91.26 f	<LOQ	<LOQ	2.01 f	1.86 f	1.14	<LOQ	<LOQ	<LOQ	<LOQ	1.57 c	205.43 l
Hejaz	81.86 i	510.41 b	700.26 a	95.56 f	531.40 a	9.00 d	3.10 e	6.39 e	6.08 c	10.75 a	<LOQ	1.59 b	1.63 c	1956.35 a
Hershkovitz	469.05 c	603.07 a	60.26 h	5.18 h	141.91 d	8.55 d	52.12 a	4.79 f	<LOQ	7.03 b	<LOQ	<LOQ	0.28 d	1382.67 d
Mollar 1	327.19 e	9.88 i	7.66 l	240.84 c,d	12.78 e	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	598.40 g
Mollar 2	235.52 g	84.38 f	290.20 b	38.38 g	128.69 d	0.38 g	<LOQ	1.10 h	220.46 a	<LOQ	8.17 a	<LOQ	<LOQ	1006.81 f
Radisa	214.16 g,h	298.48 c	71.06 g	160.91 e	178.12 c	11.59 c	9.50 c	9.64 d	2.23 d,e	5.86 c	<LOQ	<LOQ	<LOQ	962.16 f
Ravenna	273.24 f	10.77 i	15.79 i	<LOQ	<LOQ	21.55 a	6.78 d	20.51 c	<LOQ	0.22 f	<LOQ	<LOQ	<LOQ	348.86 i
Shiraz	403.52 d	15.71 h	93.25 e,f	46.20 g	<LOQ	18.28 b	7.48 d	25.86 b	<LOQ	0.36 f	<LOQ	<LOQ	<LOQ	610.66 g
Valenciana	102.08 i	1.46 l	<LOQ	7.34 h	<LOQ	0.96 g	1.52 f	2.06 g	<LOQ	2.03 d	<LOQ	<LOQ	<LOQ	117.45 m
Veneti	15.52 l	202.22 d	69.47 g	238.00 c,d	3.09 f	8.78 d	1.23 f	8.53 d	<LOQ	12.73 a	<LOQ	<LOQ	1.51 c	561.01 g
Wonderful 1	327.65 e	537.43 b	15.68 i	290.77 b	333.26 b	3.61 e	5.54 d	0.33 i	3.08 d	8.26 b	1.79 b	1.39 b	0.23 d	1528.94 c
Wonderful 2	1119.47 a	94.02 e,f	283.84 c	266.95 b,c	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.96 e	<LOQ	<LOQ	<LOQ	1766.51 b

[a]Different letters in the same column indicate significant differences ($p < 0.05$). [b]**A1**, **A2**, **A3**, **A4**, **A5**, **A9**, **A11**, and **A12** compounds are expressed as mg cyanidin chloride/L juice; **A6**, **A7**, **A8**, **A10**, and **A13** compounds are expressed as mg catechin/L juice. The compound numbers are the same as those reported in Table 1.

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271, 169, and 125 m/z. Moreover, it presented a UV spectrum with a maximum at 276 nm. According to the literature,[1,9,12] this compound was identified as galloyl-glucose. Peaks 2 and 4 (3.6 and 6.5 min) reported the same UV and MS data. They showed the same UV spectra with maxima at 258 and 378 nm (characteristic of gallagic acid derivatives)[12] and a molecular ion at 1083 m/z. In the HPLC−MS spectrum, some principal fragments corresponding to the loss of ellagic acid (781 m/z) and for the gallagic acid (601 m/z) and ellagic acid (301 m/z) residues were observed. According to some authors,[1,12] these compounds were identified as punicalagin α and punicalagin β.

Peak 3 at 4.8 min demonstrated a base peak at 783 m/z and two fragments at m/z 481 (loss of ellagic acid) and m/z 301 (corresponding to ellagic acid); according to Fischer and co-workers,[1] this fragmentation pattern was assigned to peduncalagin I.

The compound at 8.3 min with 633 m/z (peak **5**) showed three fragments at m/z 615 ($[M - H - H_2O]^-$), 463 (ellagic acid-hexoside) and 301 (ellagic acid). This compound was identified as galloyl-HHDP-hexose.[1] Similarly, peak **6** with molecular formula $C_{20}H_{16}O_{13}$, molecular ion at 463 m/z, and fragment ion 301 m/z was identified as ellagic acid glucoside. A compound with molecular formula $C_{34}H_{26}O_{23}$ was detected at 11.4 min. It reported a molecular ion at 951 m/z and fragments at 933, 613, and 301 m/z; the same fragmentation pattern was described by Fischer et al.[1] for granatin B. Compound **8** displayed a $[M - H]^-$ at m/z 801 and showed MS^2 fragment ions, which were in accordance with those reported for tentative identification of punigluconin.[1] Peak **9** at m/z 447 reported three fragment ions at 302, 301, and 300 m/z; Fischer and co-workers[1] identified this compound as ellagic acid deoxyhexose. According to the same authors, peak **10** at m/z 433 (fragment ions 301 and 300 m/z) and molecular formula $C_{19}H_{14}O_{12}$ was assigned to ellagic acid pentoside. The UV and MS data of compound **11**, the coelution of the standard solution, and the molecular formula obtained by TOF-MS analysis and the research by Gil and co-workers[12] permitted the identification of ellagic acid. Compound **12** produced a $[M - H]^-$ ion at m/z 507 and a fragment at m/z 312 and 295; these results and the absorption maximum at 270 nm were reported by Fischer et al.[11] for syringetin hexoside. Peak **13** reported a maximum absorption at 284 and 336 nm. MS data revealed a molecular ion at 533 m/z, and MS^2 data showed three fragment ions at m/z 515 $[M - H - H_2O]^-$, 447, and 371 $[M - H - Glu]^-$ (Figure 1). This compound with molecular formula $C_{26}H_{30}O_{12}$ was tentatively identified as phellatin or its isomer amurensin, and, to the best of our knowledge, it has not previously been described in pomegranate. This compound is a *tert*-amyl alcohol derivative of kaempferol-7-*O*-glucoside. Because of that, the presence of compound **13** in pomegranate can be justified because it could be formed in the same kaempferol-7-*O*-glucoside metabolic pathway.[30]

Finally, compound **14** produced a molecular ion at m/z 435 and molecular formula $C_{21}H_{24}O_{10}$; moreover, the fragmentation pattern confirmed the presence of phlorizin as reported by Mena et al.[17]

The repeatability of the injection was assessed for Akko juice. The sample was injected 4 times on the same day (intraday precision) and for three consecutive days (interday precision, $n = 12$). The percent relative standard deviations (% RSD) of the peak areas (UV detection) and retention times were determined for each peak detected. The intraday repeatability (expressed as % RSDs) of the retention times was from 0.22 to 2.38%, whereas the interday repeatability was from 1.38 to 2.96%. The intraday repeatability (expressed as % RSDs) of the total peak area was 0.49−0.81%, whereas the interday repeatability was 1.32−1.99% (Table S2, Supporting Information).

LOD and LOQ calculated for gallic acid, ellagic acid, punicalagin, phlorizin hydrate, and rutin standards were 0.15 and 0.5, 0.32 and 1.07, 0.48 and 1.6, 0.21 and 0.70, and 0.60 and 2.0 mg/L, respectively.

Quantification of Phenolic Content in Pomegranate Juices. The proposed methods were used to determine the anthocyanins, phenolic acids, flavonoids, and tannins in seventeen juice samples.

Table 3 shows the anthocyanin content in the samples.

Only the sample Wonderful 1 reported all the identified anthocyanins in a concentration higher than LOQ.

High variability was found among the samples (117.4−1956.3 mg/L). Hejaz, Wonderful 2, Wonderful 1, Hershkovitz, Akko, Ecotipo 1, and Mollar 2 showed a total anthocyanin content higher than 1000 mg/L.

Samples Akko, Ecotipo 2, G2, Mollar 1, Ravenna, Shiraz, Valenciana, and Wonderful 2 reported the delphinidin-3,5-diglycoside as first compound, and this compound represented from 44 to 87% of total anthocyanin compounds. The samples Hejaz and Ecotipo 1 showed cyanidin-3,5-diglycoside, pelargonidin-3,5-diglycoside, and cyanidin-pentoside-hexoside as first, second, and third anthocyanin, respectively. G2 and Ravenna samples contained only three major anthocyanins, delphinidin-3,5-diglycoside, pelargonidin-3,5-diglycoside, and cyanidin-3,5-diglycoside. Moreover, samples G1, Hershkovitz, and Radisa were characterized by the presence of cyanidin-3,5-diglycoside, delphinidin-3,5-diglycoside, and cyanidin-pentoside-hexoside as major anthocyanins.

Some anthocyanin−flavanol adducts were identified and quantified in the juice samples. As reported by Santandreu and co-workers,[27,28] these compounds were present at very low concentrations and can coelute with main anthocyanins which absorb at the same wavelengths. Several authors[31−33] demonstrated that the anthocyanin−flavanol adducts showed a higher absorption at 280 nm than 520 nm (typical of anthocyanin compounds); according to the UV and MS data, five major adduct compounds were quantified as catechin derivatives at 280 nm. The results confirmed that these compounds are minor anthocyanin derivative compounds in pomegranate juices and the higher percentage was detected in G1 sample (1.7% of total anthocyanins). The other samples showed an anthocyanin−flavanol adduct content lower than 0.8% of total anthocyanin content.

These data confirmed the results of previous investigations,[9,34] demonstrating that it is not possible to determine an anthocyanin marker either in the same cultivar or in different cultivars. Also, Fischer and co-workers[11] demonstrated that some anthocyanin compounds lacked in some cultivars. However, all the samples, except Dente di Cavallo cultivar, showed a percentage of diglucosylated anthocyanins higher than 50%. As reported by Borochov-Neori and co-workers,[22] the degree of anthocyanin glucosylation was highly dependent on the climate conditions, and the proportion of diglucosylated anthocyanins increased with seasonal warming. In summer fruit most of the pigment molecules were diglucosylated, whereas the higher quantity of monoglucosylated anthocyanins was detected in Dente di Cavallo cultivar; this cultivar was collected in October, and thus, according to Borochov-Neori and co-

Table 4. Phenolic Content in Pomegranate Juices[a]

samples	1[b]	2[b]	3[b]	4[b]	5[b]	6[b]	7[b]	8[b]	9[b]	10[b]	11[b]	12[b]	13[b]	14[b]
Akko	50.98 e	11.61 d	14.43 m	13.51 f	32.45 g	47.53 d	7.59 c,d	25.15 c	53.05 a	7.88 d,e	437.14 c	41.91 b	150.48 c	33.68 b
Dente di Cavallo	53.53 c	<LOQ	33.15 h	<LOQ	38.93 f	27.53 e,f	<LOQ	20.94 d	<LOQ	17.51 a	207.92 h	6.62 n	32.81 o	34.71 a
Ecotipo 1	38.05 g	<LOQ	30.80 i	8.39 h	21.60 i	20.97 h	<LOQ	11.77 g,h	13.99 m	14.11 b	141.24 n	12.43 h	61.81 i	11.04 h
Ecotipo 2	52.25 d	<LOQ	6.24 p	15.94 e	16.07 m	5.08 m	<LOQ	5.93 l,m	42.47 b,c	4.06 h	163.86 m	10.73 l,m	19.05 q	14.96 g
G1	11.45 q	4.63 g	10.17 o	8.65 h	85.92 c	112.75 a	6.36 e,f	55.23 a	34.71 g	6.92 e,f	473.45 b	52.71 a	74.88 g	30.98 c
G2	25.76 l	<LOQ	49.25 e	5.97 i	58.24 d	18.12 i	<LOQ	7.54 i,l	20.47 l	<LOQ	168.08 l	10.93 i,l	35.65 n	24.09 d
Hejaz	17.55 n	14.30 c	12.90 n	31.49 d	22.89 h,i	25.14 f,g	34.99 a	16.13 e	39.81 d,e	8.61 c,d	253.24 e	15.01 f	95.91 d	6.96 n
Hershkovitz	74.18 a	24.71 b	7.08 p	44.31 a	18.65 l	27.12 e,f	26.83 b	13.59 f,g	43.28 b	9.67 c	229.84 g	13.83 g	91.37 f	6.48 o
Mollar 1	11.59 q	9.06 e	50.02 d	13.57 f	40.53 f	14.65 l	4.60 h	4.29 m	22.21 i,l	5.28 g	160.26 m	12.25 h	13.67 s	22.43 e
Mollar 2	43.29 f	8.57 e	19.30 l	13.03 f,g	24.59 h	14.54 l	6.39 e,f	5.77 l,m	37.82 e,f	6.22 f,g	208.21 h	12.31 h	17.55 r	24.40 d
Radisa	30.92 h	12.06 d	32.53 h	12.91 f,g	34.38 g	51.67 c	5.73 f,g	33.03 b	25.94 h	<LOQ	490.36 a	26.23 d	164.23 b	10.56 i
Ravenna	51.99 d	11.49 d	45.60 g	33.10 c	46.68 e	86.92 b	8.55 c	55.51 a	40.83 c,d	7.28 e,f	299.28 d	32.81 c	275.95 a	11.15 h
Shiraz	28.86 i	11.51 d	105.08 b	12.14 g	103.61 b	12.12 l	8.50 c	9.14 i	13.73 m	8.52 d	202.36 i	9.81 m	47.59 l	4.06 q
Valenciana	14.37 p	5.87 f	11.83 n	37.02 b	24.18 h	23.83 g	5.22 g,h	11.19 h	35.80 f,g	6.39 f,g	206.37 h	16.11 e	44.12 m	21.10 f
Veneti	18.55 m	6.09 f	115.04 a	8.55 h	120.28 a	29.17 e	8.66 c	20.21 d	15.64 m	17.99 a	244.90 f	11.88 h,i	94.71 e	5.38 p
Wonderful 1	16.70 o	4.49 g	68.42 c	6.59 i	56.60 d	20.40 h,i	7.18 d,e	14.48 e,f	24.02 h,i	<LOQ	244.30 f	12.27 h	68.10 h	8.48 l
Wonderful 2	56.01 b	34.52 a	46.98 f	12.56 f,g	18.72 l	13.76 l	5.64 f–h	6.58 l	15.59 m	2.88 i	139.73 n	7.49 n	20.47 p	7.56 m

[a]All phenolic compounds are expressed as mg/L juice. Different letters in the same column indicate significant differences ($p < 0.05$). [b]Compound numbers are the same than those reported in Table 2. Compounds **1** and **5** were quantified with gallic acid; compounds **12** and **13** were quantified with rutin; compounds **6** and **9**–**11** were quantified with ellagic acid; compounds **2**–**4**, **7**, and **8** were quantified with punicalagin standard; and phlorizin was quantified with phlorizin standard.

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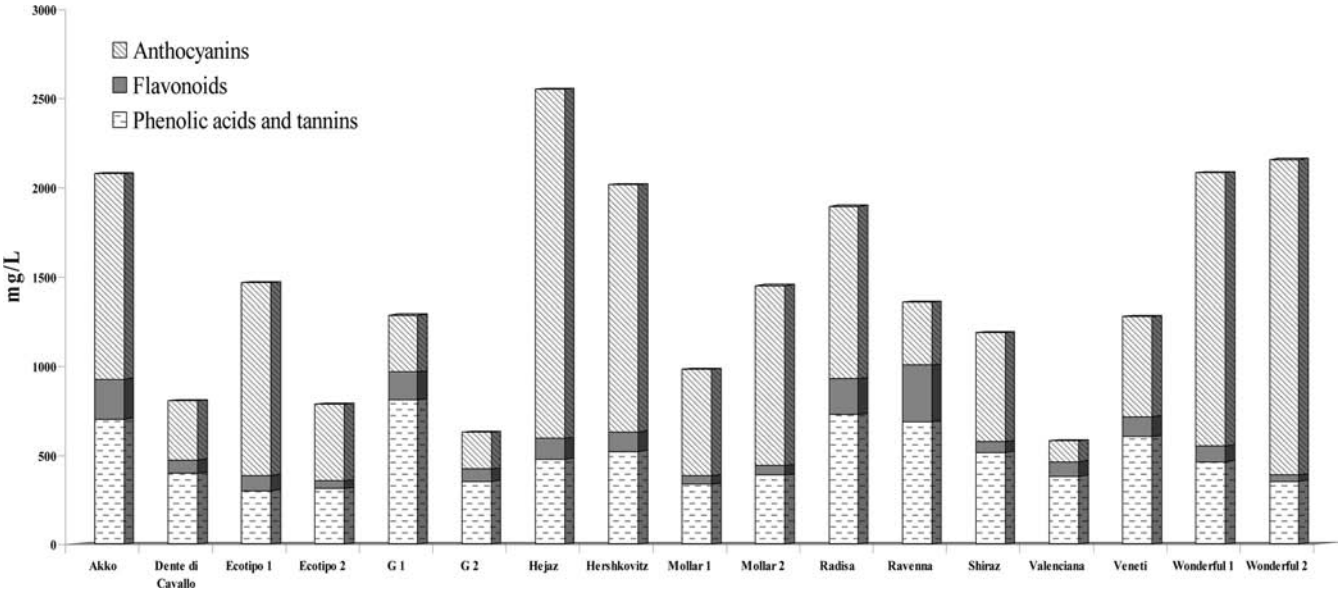

Figure 2. Total anthocyanin, flavonoid, and phenolic acid and ellagitannin concentrations (mg/L) in pomegranate juices.

workers,[22] the different seasonal temperature should explicate the different trend.

Table 4 shows the content of phenolic acids, ellagitannins, and flavonoids in the studied samples. Phenolic acids were gallic and ellagic derivatives. Gallic acid derivatives, constituted by galloyl-glucose and galloyl-HHDP-hexose, were 7−23% of total phenolic fraction (without anthocyanins). Ellagic acid was the first compound in all samples in the range 139.7−473.4 mg/L of juice, and it varied from 29.7 to 52.7% of total phenolic compounds (without anthocyanins). These data agree with the results reported by Fischer et al.[11] and Gil et al.[12] Other major compounds were peduncalagin I, syringetin-hexoside, phellatin/amurensin, and phlorizin.

Phlorizin content ranged between 3.4 and 34 mg/L. Gundogdu and Yilmaz[2] reported similar values in Turkish pomegranate juices.

Total phenolic content and the content of single classes of phenolic compounds are illustrated in Figure 2. Total phenolic content varied from 573.6 (Valenciana sample) to 2519.8 (Hejaz sample) mg/L. These results agreed with the literature.[9,19,20,35,36]

The content of different phenolic classes in the samples was diverse; content of phenolic acids and ellagitannins ranged between 300 and 810 mg/L, anthocyanins varied between 110 and 1925 mg/L, and flavonoids were comprised between 35 and 319 mg/L. These data accorded to the literature.[1,11,12,16,34,36,37]

As reported for the phenolic content, concerning the total content of phenolic compounds in the samples, the percentage of the different classes was very variable too. Effectively, the percentages of phenolic acids and ellagitannins, flavonoids, and anthocyanins were in the ranges 16.4−69.5%, 1.6−24.4%, and 17.0−82.0%, respectively. G1, G2, and Valenciana samples showed the highest percentages of phenolic acids and ellagitannins; flavonoids were 24% of total compounds in Ravenna sample. Besides, it was observed that Akko, Ecotipo 1, Hejaz, Hershkovitz, Mollar 1 and 2, and Wonderful 1 and 2 contained an anthocyanin percentage higher than 50%.

Lignan compounds were not identified in all the pomegranate juices; this result according to Fischer and co-workers[18] can be explained because the juices were obtained only from arils and dejuicing was carried out at low pressures, consequently, the analyzed samples did not contain lignans.

In conclusion, two different methods were set up for the determination of anthocyanins and other phenolic compounds (phenolic acids, ellagitannins, and flavonoids) in pomegranate juices. A new flavonoid compound was tentatively identified for the first time in pomegranate juices. The results confirmed that the pomegranate variety influences substantially the phenolic composition. The pomegranate clones showed a phenolic composition comparable with the other varieties.

The HPLC technique coupled to qTOF-MS detector permitted analysis of the major phenolic compounds of juices without a previous hydrolysis of the samples. The established methods may be used for routine analysis and may be useful to determine the evolution of these antioxidant compounds during the shelf life of fruits and juices using any classical HPLC−UV/DAD system.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Intraday and interday repeatability of anthocyanins (Table S1) and other phenolic compounds (Table S2). Chromatograms of anthocyanins (Figure S1) and other phenolic compounds (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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