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ORIGINAL ARTICLE

Organic Acids and Phenolic Compounds in Pomegranates (*Punica granatum* L.) Grown in Turkey

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Thirteen pomegranate varieties obtained from four different growing regions of Turkey were analyzed for their individual organic acids and phenolic compounds in freshly prepared pomegranate juices. Total titratable acidity ranged between 4.58 and 17.30 g/L (average of 9.82 g/L), and total sugars ranged between 139.6 and 160.6 g/L (average of 148.75 g/L) in pomegranate juices. According to the results of the *z*-test for comparison of means, intervarietal differences in total titratable acidity and sugar content of pomegranate varieties were found to be significant within the confidence interval of 95%.

Organic acids such as citric, L-malic, tartaric, oxalic, (−)−quinic and succinic acids were individually detected and quantitated. On average, citric acid was predominant with a range of 0.33–8.96 g/L (overall mean of 4.85 ± 2.83 g/L). L-Malic acid was the second most abundant, with a range of 0.56–6.86 g/L (overall mean of 1.75 ± 1.59 g/L). Tartaric, oxalic, (−)−quinic and succinic acids ranged between 0.28–2.83, 0.02–6.72, 0.00–0.82 and 0.00–1.54 g/L, respectively. Phenolic compounds identified in freshly prepared pomegranate juices were gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, ferulic acid, *o*- and *p*-coumaric acids, catechin, phloridzin and quercetin.

Key Words: pomegranate; *Punica granatum* L; organic acids; phenolic compounds; oxalic acid.

INTRODUCTION

The pomegranate (*Punica granatum*, Punicaceae) is one of the oldest known edible fruits. The fruit is consumed fresh or can be processed into juice, a syrup (grenadine), jams, or a type of wine. There is an increased concern in the fruit juice industry about the availability of high juice yielding pomegranate cultivars with suitable juice composition. The juice content of pomegranate fruit accounts for about 45–65% of the whole fruit or 76–85% of the arils. The number of pomegranate varieties grown in the Mediterranean region of Turkey has been examined for juice extraction yield,

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serum-to-pulp ratio and sugar–acid balance. The average juice yield of pomegranates has been reported as 40% and pomegranates have been classified as sweet, sour-sweet and sour according to their sugar and acid compositions (Cemeroğlu *et al.*, 1992).

The chemical composition of fruits differs depending on the cultivar, growing region, climate, maturity, cultural practice and storage. Significant variations in organic acids, phenolic compounds, sugars, water-soluble vitamins and minerals composition of pomegranates have been reported over the years by various researchers (Cemeroğlu *et al.*, 1988, 1992; Ünal *et al.*, 1995; Melgarejo *et al.*, 2000). Among the different compounds that could serve as unequivocal markers in a fruit juice product, organic acids and phenolic compounds are potentially the most useful because of their ubiquity, specificity and multiplicity. Much of the work on the separation of phenolic compounds has been done by using thin layer chromatography (TLC) (Cemeroğlu, 1977) and recently high-performance liquid chromatography (HPLC) (Artık *et al.*, 1998).

Both organic acids and phenolic compounds are important for their contribution to sensory attributes, as well as for their potential health benefits in fruits and vegetables. The effects of phenolic compounds on low-density lipoproteins and aggregation of platelets are beneficial because they reduce some of the major risk factors for coronary heart disease.

Although the major acids in pomegranate juices have been well documented, few studies dealt with the identification and quantitation of minor organic acids such as lactic, acetic and fumaric acids in pomegranates (Melgarejo *et al.*, 2000). The objective of this study was to quantify both minor and major organic acids and phenolic compounds present in different pomegranate varieties grown in Turkey. The analytical separation and determination of phenolic compounds and organic acids were performed using reversed phase HPLC with photodiode array detector. Some other analytical properties of pomegranates, including pH, titratable acidity, total and reducing sugars, formol number and Hunter color scores, were also determined by International Federation of Fruit Juice Union (IFJU) methods.

MATERIALS AND METHODS

Pomegranate Varieties

Thirteen different pomegranate varieties were obtained from four different provinces located in the Mediterranean region of Turkey. The selection of these varieties was based on the results of earlier studies performed on the compositional characterization of Turkish pomegranate varieties (Cemeroğlu *et al.*, 1988, 1992). Approximately 1 kg of pomegranates at harvesting maturity was sampled for each variety. Each sampling was repeated five times for the determination of organic acids and three times for the determination of phenolic compounds compositions of pomegranates for each variety.

Preparation of Raw Pomegranate Juice

Following peeling out, the skins covering seeds were removed. The remaining pomegranate seeds were placed in a pilot plant press and the juice was extracted by applying a gauge pressure of 2.8 kg/cm^2. Raw juices were kept frozen at –18°C upon analyses.

Analytical Measurements

HPLC instrument. The analytical equipment consisted of a Varian 9010 solvent delivery system, a Hewlett-Packard 1040A photodiode array detector interfaced with an AC/DC signal converter and HP ChemStation software, a Rheodyne 7125 six-way injector with 10 μL sample loop, and a HiChrom reversed phase C18 (5 μm, 25 cm × 4 mm i.d.) analytical column. Organic acids and phenolic compounds were analyzed using two different C18 HPLC columns, separately.

Analysis of individual organic acids by HPLC. Raw pomegranate juice (1 mL) was diluted with 3 mL of 0.01 M KH_2PO_4 (pH 8.0 NaOH) solution. A C18 solid-phase extraction cartridge was conditioned by eluting 3 mL of methanol, 10 mL distilled water and 10 mL air sequentially.The diluted sample (1 mL) was eluted through a pre-conditioned C18 cartridge. The eluate was collected and recorded as eluate A. The cartridge was then eluted with 1 mL of 0.01 M KH_2PO_4 (pH 8.0 NaOH) solution. The eluate was collected and recorded as eluate B. Eluates A and B were combined and 10 μL of combined eluates was injected onto HPLC. HPLC elution was carried out at room temperature using 0.2 M KH_2PO_4 (pH 2.4 adjusted with H_3PO_4) at a flow rate of 0.5 mL/min as the mobile phase. The chromatogram was monitored simultaneously at 210 nm with 2 nm bandwidth, with spectra taken continuously throughout the elution. Calculation of concentrations was based on the external standard method. Dilutions 1:0, 1:1, 1:2 and 1:4 of an aqueous solution containing 1 g/L of each of the organic acid standards (citric, malic, tartaric, oxalic, (−) − quinic and succinic acids) were used to fit a standard curve (peak area versus concentration in mg/L) with linear regression for each individual compound.

Analysis of individual phenolic compounds by HPLC. Extracted pomegranate juice was diluted (1:1) by distilled water and filtered through 0.45 μm Millipore filter and injected onto HPLC. HPLC elution was carried out at room temperature and utilized as solvent A, the mixture of formic acid and water (5:95, v/v), and as solvent B, methanol. The elution profile was, at a flow rate of 1.0 mL/min, 15% solvent B isocratic for 5 min followed by a 15–30% linear gradient for 15 min and 30–50% linear gradient for 10 min with solvent B and holding with 50% solvent B for an additional 10 min, and finally followed by a 50–15% linear gradient with solvent B for 10 min. The chromatogram was monitored simultaneously at 280 and 320 nm with 2 nm bandwidth, with spectra taken continuously throughout the elution. Calculation of concentrations was based on the external standard method. Dilutions 1:0, 1:1, 1:2 and 1:4 of an aqueous solution containing 30 mg/L of each of the phenolic standards (gallic, protocatechuic, chlorogenic, *o*- and *p*-coumaric, ferulic and caffeic acids, catechin, quercetin and phloridzin) were used to fit a standard curve (peak area versus concentration in mg/L) with linear regression for each individual compound.

pH and total titratable acidity. pH measurements were performed using a NEL pH 890 model pH meter at 20°C. Total titratable acidity was determined potentiometrically using 0.1 N NaOH to the titration end point of pH 8.1 and expressed as g citric acid per liter.

Total soluble solids. Total soluble solids (%) were measured by using a Bausch–Lomb refractometer at 20°C.

Formol number. Formol number was expressed as mL of 0.1 N NaOH per 100 mL sample required to maintain a pH of 8.1 after the addition of formaldehyde into pomegranate juice.

Total and reducing sugars. Total and reducing sugars were determined by the Lane–Eynon method and expressed as g sugar per liter.

Color. Color measurements were performed by a Minolta CR200 model chromometer as described by Batu *et al*. (1997).

Statistical analysis. The statistical examinations of the data were performed by using the MiniTab software package. Mean concentrations of each analyte in different varieties were compared using the *z*-test.

RESULTS AND DISCUSSION

Some analytical properties of pomegranate juices prepared using 13 different varieties are given in Table 1. The mean pH and soluble solid content of raw pomegranate juices were 3.55 ± 0.21 and $17.16 \pm 1.01\%$, respectively. The predominant constituent of total soluble solids of pomegranate juice extract was sugars at amounts ranging between 139.6 and 160.6 g/L with a cv. of 4.57%. The most significant variation was determined in titratable acidities with a cv. of 35.16%. Titratable acidity of the samples varied from 4.58 to 17.3 g/L expressed as citric acid. Previous studies have also reported varying ranges of titratable acidity and total sugar in pomegranate juices (Cemeroğlu *et al*., 1992). Gabbasova and Abdurazakova (1969) have reported the titratable acidity and total sugar content of former Soviet Union originated pomegranate juices as 0.52–1.6 and 15.2–20.5%, respectively. Veres (1976) has also reported the titratable acidity and total sugar content of Macedonian originated pomegranate juices as 0.37–2.80 and 8.4–13.2%, respectively. In an earlier attempt to determine the chemical composition of pomegranates grown in Turkey, total acidity of 1.47% and total sugars of 13.9% have been reported by Cemeroğlu (1977). Parallel to the improvements in analytical instrumentation, recent studies have focused on the composition of

TABLE 1

Some analytical properties of pomegranate juices ($n = 65$)

Analytical property		Variation limits				
		Min	Max	Mean	S.D.	cv.(%)
pH		3.29	3.93	3.55	0.21	5.970
Titratable acidity, g/L		4.58	17.30	9.82	3.45	35.16
Soluble solids, (%)		16.00	19.00	17.16	1.01	5.79
Reducing sugars, (g/L)		125.00	160.20	146.45	9.55	6.52
Total sugars, g/L		139.60	160.60	148.75	6.80	4.57
Formol number, (mL 0.1 N NaOH/100 mL)		9.00	15.00	11.00	2.17	19.29
Hunter	*L*	13.07	24.01	19.01	3.98	20.93
	a	+14.08	+27.46	+19.09	3.92	20.55
	b	+1.70	+7.79	+4.50	1.95	43.20

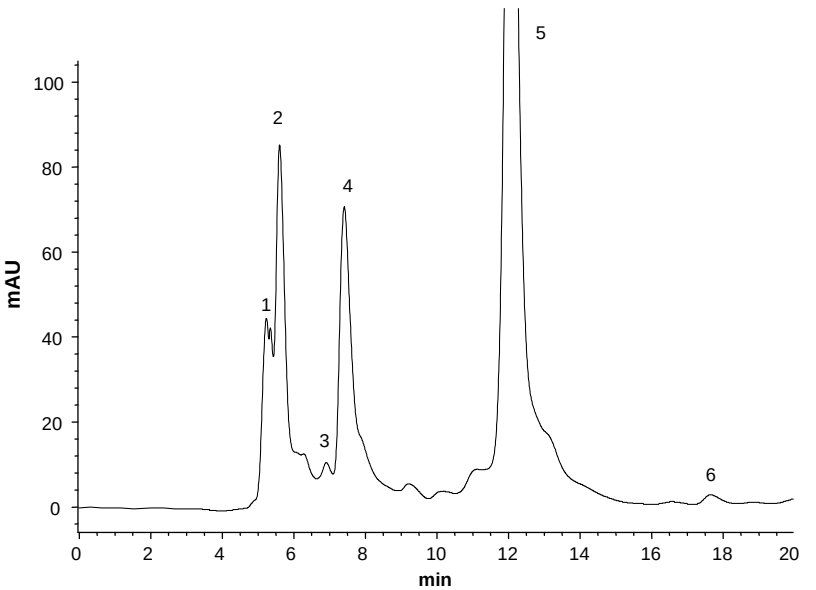

FIGURE 1. Chromatogram of organic acids in raw pomegranate juice. Peaks: 1, oxalic acid, 2, tartaric acid, 3, (−)−quinic acid, 4, L-Malic acid, 5, citric acid, 6, succinic acid.

pomegranate juices in more detail (Ünal *et al.*, 1995; Artık *et al.*, 1998; Melgarejo *et al.*, 2000).

The chromatogram illustrating the individual organic acids in raw pomegranate juice is shown in Figure 1. The chromatographic conditions applied successfully resolved both major and minor organic acids in pomegranate juices. However, baseline separation of oxalic acid, which eluted between solvent front and tartaric acid, could not be achieved properly, particularly for the samples which contained relatively lower amounts of oxalic acid. Citric, L-malic, (−)−quinic, succinic, tartaric and oxalic acids were identified and quantitated in freshly prepared pomegranate juices.

Individual organic acid concentrations of pomegranate varieties are given in Table 2. Citric acid was determined to be the predominant organic acid in 10 pomegranate varieties. Its concentration ranged between 0.33 and 8.96 g/L with an overall mean concentration of 4.85 ± 2.83 g/L. L-Malic acid was determined to be the second most abundant organic acid in these samples, but in higher amounts than citric acid in two other pomegranate samples. L-Malic acid concentration ranged between 0.56 and 6.86 g/L with an overall mean concentration of 1.75 ± 1.59 g/L. Oxalic and tartaric acids were determined in considerable amounts in all pomegranate varieties. The overall mean concentrations of oxalic and tartaric acids were found as 1.16 ± 2.07 and 0.87 ± 0.75 g/L, respectively. In one of the pomegranate varieties, the level of oxalic acid was found to be as high as 1.59 mg/L, which was higher than both citric and L-malic acid levels of that variety. The maximum concentrations of these organic acids were 2.96 g/L for oxalic acid and 2.83 g/L for tartaric acid. (−)−Quinic and succinic acids were also determined in relatively lower amounts in most of the pomegranate varieties. Only four varieties were found to be free of (−)−quinic acid and three varieties were free of succinic acid. The respective maximum and overall mean concentrations of these organic

TABLE 2

Organic acid composition of pomegranate juices

Growing region	Variety code	*n*	Organic acids in pomegranate juice (g/L)					
			Citric	L-Malic	Tartaric	Oxalic	(−)−Quinic	Succinic
Adana	01-N-01	5	4.40±0.33	0.88±0.15	0.28±0.04	0.06±0.01	0.05±0.01	0.50±0.04
	01-N-07	5	0.43±0.05	0.56±0.08	1.26±0.19	1.59±0.21	nd	nd
	Mean		2.42	0.72	0.77	0.83	0.03	0.25
	S.D.		2.81	0.23	0.69	1.10	0.04	0.36
Antalya	07-N-06	5	1.83±0.24	6.86±0.29	2.05±0.09	2.96±0.21	0.82±0.10	nd
	07-N-08	5	7.08±0.37	1.76±0.11	0.56±0.07	0.05±0.01	0.12±0.02	0.18±0.03
	Mean		4.45	4.31	1.31	1.50	0.47	0.09
	S.D.		3.71	3.61	1.05	2.06	0.49	0.12
Hatay	31-N-01	5	0.33±0.08	1.88±0.13	2.83±0.21	0.05±0.02	0.12±0.03	nd
	31-N-07	5	8.96±0.45	1.62±0.09	0.77±0.09	0.03±0.01	0.176±0.06	1.18±0.10
	Mean		4.65	1.75	1.80	0.04	0.15	0.59
	S.D.		6.11	0.19	1.46	0.02	0.04	0.83
İçel	33-N-11	5	5.38±0.39	1.29±0.09	0.62±0.10	0.03±0.01	nd	0.24±0.07
	33-N-12	5	6.53±0.27	1.12±0.15	0.42±0.04	0.03±0.00	nd	0.15±0.04
	33-N-15	5	8.82±1.01	1.06±0.11	0.43±0.06	0.03±0.02	nd	0.59±0.09
	33-N-20	5	4.07±0.40	1.30±0.19	0.48±0.09	0.02±0.01	0.11±0.03	1.10±0.02
	33-N-23	5	4.95±0.31	1.55±0.07	0.58±0.07	0.02±0.00	0.08±0.02	0.33±0.04
	33-N-24	5	6.72±0.56	1.85±0.21	0.51±0.04	6.72±0.31	0.08±0.02	0.71±0.10
	33-N-25	5	3.54±0.21	1.04±0.05	0.46±0.06	3.54±0.18	0.17±0.05	1.54±0.05
	Mean		5.72	1.32	0.50	1.49	0.11	0.67
	S.D.		1.80	0.30	0.07	2.65	0.04	0.51
	Overall mean		4.85	1.75	0.87	1.16	0.19	0.65
	S.D.		2.83	1.59	0.75	2.07	0.24	0.45

acids were 0.82 and 0.19±0.24 g/L for (−)−quinic acid and 1.54 and 0.65±0.45 mg/L for succinic acid.

Table 2 clearly shows the significance of variations in individual organic acid concentrations of different pomegranate varieties. The mean percentage contribution of each acid in total organic acids quantitated by HPLC were 55% (with a range of 6–79%) for citric acid, 20% (with a range of 13–47%) for L-Malic acid, 13% (with a range of 5–54%) for tartaric acid, 6% (with a range of 0–23%) for succinic acid, 5% (with a range of <1–41%) for oxalic acid and 1% (with a range of 0–6%) for (−)−quinic acid. Since these individual contributions vary significantly, the organic acid composition of pomegranates is not easily predictable and very largely dependent on the growing region, climate as well as the type of variety.

The averages of *L*, *a* and *b* values (Hunter color scores) were estimated as 19.0, +19.1 and +4.5, respectively. According to these results, "*L*" and "*b*" values were found to be extremely low for pomegranate juice in comparison to previous research. On the other hand, it was shown that "*a*" values were similar to the previous results (Bayındırlı *et al*., 1994).

In this study, three samples for each pomegranate variety were analyzed for the determination of their individual phenolic compounds composition. The chromatogram illustrating the phenolic compounds in raw pomegranate juice is shown in Figure 2. The chromatographic separation in combination with photodiode array detection (PDA) allowed identifying and quantitating minor phenolic compounds in

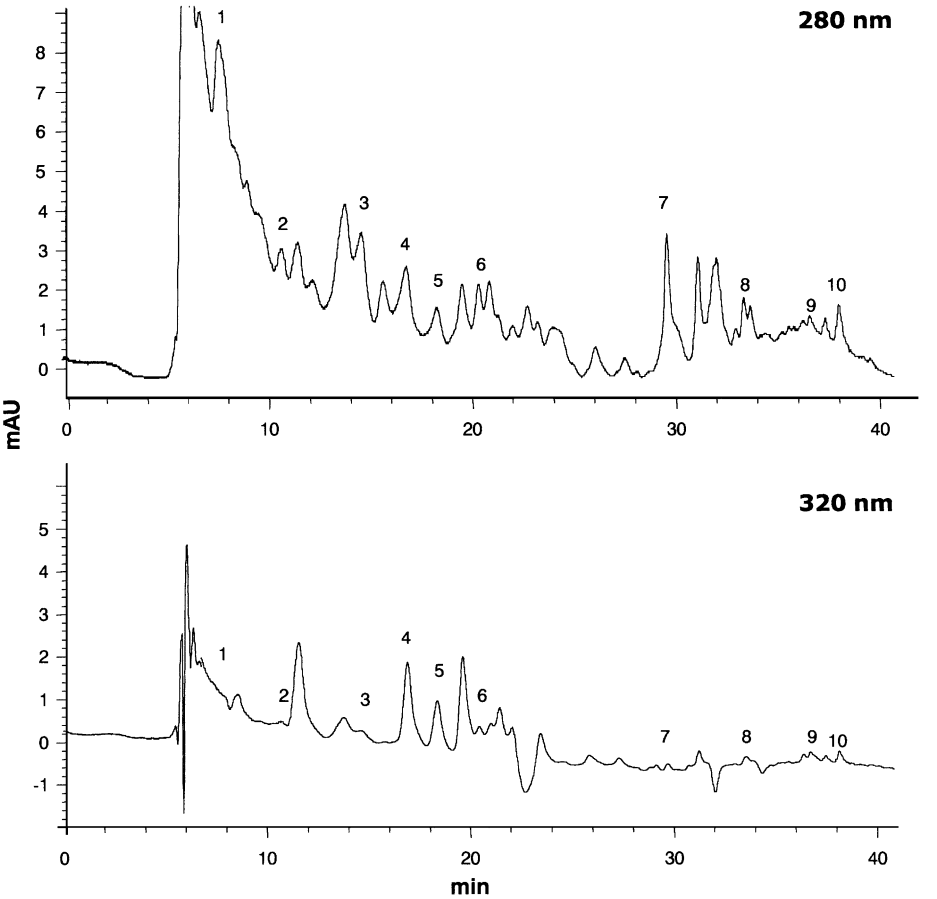

FIGURE 2. Chromatogram of phenolic compounds in pomegranate juice. Peaks: 1, gallic acid, 2, protocatechuic acid, 3, catechin, 4, chlorogenic acid, 5, ferulic acid, 6, caffeic acid, 7, *o*-coumaric acid, 8, *p*-coumaric acid, 9, phloridzin, 10, quercetin.

pomegranates. Table 3 gives the concentrations of individual phenolic compounds (mean $\pm$ S.D.) identified in pomegranate samples of different varieties. Intervarietal differences in the phenolic compounds compositions of pomegranate samples were also distinct as determined in organic acid compositions. A total of 10 phenolic compounds, which were hydroxybenzoic acids such as gallic and protocatechuic acids, hydroxycinnamic acids such as chlorogenic, caffeic, ferulic, *o*- and *p*-coumaric acids, flavan-3-ols such as catechin, dihydrochalcones such as phloridzin and flavonols such as quercetin, were identified in raw pomegranate juices. Each phenolic compound identified in pomegranate juices was found in minor quantities. Overall mean concentrations of phenolic compounds were as follows: gallic acid 4.55 ± 8.55 mg/L, protocatechuic acid 0.84 ± 0.64 mg/L, catechin 3.72 ± 2.29 mg/L, chlorogenic acid 1.24 ± 1.42 mg/L, caffeic acid 0.78 ± 0.79 mg/L, *p*-coumaric acid 0.06 ± 0.07 mg/L, ferulic acid 0.01 ± 0.02 mg/L, *o*-coumaric acid 0.17 ± 0.08 mg/L, phloridzin 0.99 ± 1.47 mg/L, quercetin 2.50 ± 1.96 mg/L. The concentrations reported in this study represent only the free forms of phenolic compounds since

TABLE 3

Phenolic compound composition of pomegranate juices

Growing region	Variety code	*n*	Phenolic compounds in pomegranate juice (g/L)									
			Gal	Pro	Cat	Chl	Caf	p-Cou	Fer	o-Cou	Phl	Que
Adana	01-N-01	3	0.51	0.28	0.13	0.47	0.10	0.04	0.06	0.08	0.06	4.58
	01-N-07	3	0.48	0.62	1.70	0.21	0.21	nd	nd	0.12	nd	3.69
	Mean		0.50	0.45	0.91	0.34	0.15	0.02	0.03	0.10	0.03	4.13
	S.D.		0.03	0.24	1.12	0.18	0.08	0.03	0.04	0.03	0.04	0.63
Antalya	07-N-06	3	1.49	0.85	3.05	0.09	0.09	nd	nd	0.21	nd	2.29
	07-N-08	3	0.37	0.61	3.14	0.09	0.33	nd	nd	0.30	4.93	4.97
	Mean		0.93	0.73	3.09	0.09	0.21			0.25	2.46	3.63
	S.D.		0.79	0.17	0.06	0.00	0.17			0.07	3.49	1.90
Hatay	31-N-01	3	0.34	0.34	5.30	nd	0.88	nd	nd	0.07	2.02	4.43
	31-N-07	3	1.10	1.38	7.25	2.97	1.57	0.05	nd	0.13	0.55	3.36
	Mean		0.72	0.86	6.28	1.49	1.23	0.03		0.10	1.28	3.89
	S.D.		0.54	0.73	1.38	2.10	0.49	0.04		0.04	1.04	0.75
İçel	33-N-11	3	1.63	2.09	3.50	1.78	0.14	0.21	nd	0.30	0.26	5.30
	33-N-12	3	1.51	0.12	3.63	4.72	0.88	0.15	nd	0.18	0.18	0.55
	33-N-15	3	0.69	0.41	2.09	2.29	2.89	0.12	nd	0.23	nd	0.23
	33-N-20	3	4.48	0.69	8.44	1.47	0.78	0.07	nd	0.27	0.23	1.51
	33-N-23	3	12.45	0.50	4.65	nd	0.49	nd	nd	0.15	0.20	0.23
	33-N-24	3	30.86	0.94	3.85	1.27	1.36	0.13	nd	0.15	1.96	0.26
	33-N-25	3	3.27	2.05	1.65	0.73	0.42	0.04	0.01	0.08	2.46	1.16
	Mean		7.84	0.97	3.97	1.75	1.00	0.10		0.19	0.75	1.32
	S.D.		10.90	0.79	2.23	1.50	0.92	0.07		0.08	1.01	1.83
	Overall mean		4.55	0.84	3.72	1.24	0.78	0.06	0.01	0.17	0.99	2.50
	S.D.		8.55	0.64	2.29	1.42	0.79	0.07	0.02	0.08	1.47	1.96

Note: Phenolic compounds: Gal: gallic acid; Pro: protocatechuic acid; Cat: catechin; chl: Chlorogenic acid; Caf: caffeic acid; *p*-Cou: *p*-coumaric acid; Fer: ferulic acid; *o*-Cou: *o*-coumaric acid; Phl: phloridzin; Que: quercetin.

no hydrolysis was applied to the samples before HPLC analysis. The presence of gallic acid, quercetin, catechin, chlorogenic acid and *o*-coumaric acid in pomegranate juices has also been reported previously by Artık *et al*. (1998). In this study, the peel and skin covering the seeds were also analyzed separately for their phenolic composition. The most significant phenolic compounds in peel and skin were found to be gallic acid and quercetin. A low amount of phloridzin was also determined in peels.

CONCLUSION

In this study 13 pomegranate varieties were analyzed for organic acid and phenolic compounds. It is easily concluded from the results that the analytical composition of pomegranate juice, such as acidity, sweetness and color, is not easily predictable due to intervarietal differences.

Organic acids are important for their contribution to sensory attributes in fruits and vegetables. Regarding organic acids, citric and L-Malic and oxalic acids were considered to be the major organic acids in pomegranates, while tartaric, succinic and (−)−quinic acids were usually in minor quantities. However, the levels of these minor acids were higher in some cases and exceeded the level of those major organic acids.

Phenolic compounds of pomegranates examined here were based on phenolic acids (gallic, protocatechuic, chlorogenic, caffeic, ferulic, *o*- and *p*-coumaric acids). Some flavonoids (catechin, quercetin and phloridzin) were also identified in pomegranates. Profiles of both previously reported and newly identified phenolic compounds create a better understanding of the compounds affecting the sensory and health aspects of pomegranates and their products.

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