Effect of Cultivar and Cold Storage of Pomegranate (*Punica granatum* L.) Juices on Organic Acid Composition

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Abstract: The purpose of this study was to identify and quantify organic acids of 25 Iranian pomegranate (*Punica granatum* L.) cultivars using HPLC. The juices (of three selected cultivars) were evaluated by individual calculation from the peak areas based on standard curves of each organic acid type during a period of 60 days of cold storage at 4°C. The major acids identified were citric (0.0 - 3763.6), malic (2.3 - 366.3), succinic (0.0 - 134.4), tartaric (25.7 - 106.8), acetic (0.0 - 76.1), oxalic (10.3 - 55.1), shikimik (0.0 - 47.4), maleic (0.04 - 19.20), fumaric (0.0 - 15.39), and ascorbic (0.0 - 12.0) mg/100g. Citric acid predominated in most cultivars and in the others tartaric and malic acid were predominant. After cold storage of three selected pomegranate juices for 60 days, results showed that the total amounts of organic acids decreased, but malic and maleic acids content increased. Initially, the amounts of some acids such as oxalic and citric increased and then decreased and contents of shikimik, succinic, ascorbic and fumaric acid decreased.

Keywords: Pomegranate (P*unica granatum* L.), organic acids, HPLC, storage, fruit juice

INTRODUCTION

The pomegranate, *Punica granatum* L. belongs to the punicaceae family and is one of the oldest known edible fruits (Singh, 1997), grown in many subtropical countries especially in the Mediterranean region and also grown extensively in Iran, India, Pakistan, Afghanistan, and Saudi Arabia and in the subtropical areas of South America (Elyatem and Kader, 1984). Iran is one of the most important pomegranate producer and exporter in the world with an annual production of 670000 ton (Anonymous, 2004). The edible part of pomegranate is consumed fresh and also used in the preparation of fresh juice, canned beverage, jelly, jam, paste and for flavoring and coloring drinks (Fadavi *et al.*, 2005). In folk medicine, pomegranate preparations, especially of dried pericarp and the roots, barks of the tree and roots and the juice of the fruit, are used as per arum medication in the treatment of colic, colitis, and dysentery (Schubert *et al.*, 1999). The pomegranate juice has anticancer activities (Jeune Louise *et al.*, 2005), antioxidant activity (Gil *et al.*, 2000) and it has been demonstrated that, in humans, consumption of this juice decreases the susceptibility of LDL to aggregation and retention and increases the activity of serum paraoxanase (Aviram *et al.*, 2000). The chemical composition of fruit differs depending on the cultivar, growing region, climate, maturity, cultural practice and storage conditions (Melgarejo *et al.*, 2000; Nanda *et al.*, 2001; Barzegar *et al.*, 2004; Miguel *et al.*, 2004b; Fadavi *et al.*, 2005).

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Organic acids are widely distributed in fruits and vegetables. More than 36 acids have been reported to occur in fruits (Hulme, 1970). The nature and level of the organic acids present in pomegranate may provide information concerning the origin of the fruit, microbiological growth and even processing techniques. In some cases, it is necessary to determine organic acids to assess whether an expensive juice has been illegally adulterated with a cheaper juice because organic acid profiles are distinct for each type of fruit juice. Organic acid profiles can also determine juice flavor, freshness or spoilage and are important for their contribution to sensory attributes, as well as for their potential health benefits in fruits and vegetables (Morales *et al.*, 1998; Shui and Leong, 2002). Data on the organic acids in foods are increasingly required by the food industries for quality control to meet legal requirements and as labeling information. Nutritionists, biochemists, and food scientists also require similar data during research on the metabolism and function of organic acids. Some physicochemical properties of 25 Iranian pomegranate cultivars (cvs) have previously been studied by us (Barzegar *et al.*, 2004; Fadavi *et al.*, 2005; Fadavi *et al.*, 2006). However, no studies have previously been done on the organic acid composition of these pomegranate cvs, so the present study was performed to identify and quantify the organic acids of 25 Iranian pomegranates (*Punica granatum* L.) cultivars using high-performance liquid chromatography (HPLC) with UV-Vis detector at 205 nm. In addition, effect of storage time on the organic acid compositions of juices (three cultivars), during a period of 60 days of cold storage at 4°C, were evaluated using HPLC.

MATERIALS AND METHODS

Plant Samples

Twenty-five important pomegranate cvs were obtained from mature fruits growing in Markazi province (10 cvs) and Yazd province (15 cvs) in Iran from the Agricultural Research Center of Saveh and Yazd. These two provinces (Markazi and Yazd) besides Khorasan and Fars provinces represent more than 25% of total production among 28 provinces (Anonymous, 2001). Commercially ripe fresh fruits were harvested during September and November from different mature trees randomly selected to represent the population of the plantation. Fruits were transported by ventilated car to the laboratory, where pomegranates with defects (sunburns, cracks, cuts and bruises in husk) were discarded.

Approximately 2 kg (n = 10) of pomegranates at harvesting maturity was sampled for each cultivars, from which cvs composites were prepared. The various cvs selected for this study collected from Yazd province were *Sefeede Robi Avale Brojen* (SRAB, No. 1), *Toghe Gardan* (TG, 2), *Zaghe Yazdy* (ZY, 3), *Mesrie Torshe Kazeron* (MTK, 4), *Tab-o-Larze Mehrmahi* (TLM, 5), *Ardestany Torshe Semnan* (ATS, 6), *Khoram Dizin Torshe Gorgan* (KDTG, 7), *Gorch Shahvare Yazdy* (GSY, 8), *Post Syahe Yazdy* (PSY, 9), *Malase Yazdy* (MY, 10), *Shirine Shahsavare Yazdy* (SSY, 11), *Vahshi Kane Tehran* (VKT, 12), *Torshe Mamooly Lasjar* (TML, 13), *Jangaly Post Ghermeze Rodbare Torsh* (JPGRT, 14), *Malase Porbarij Estahban* (MPE, 15). Those collected from Markazi province were *Bihastehe Post Zard* (BPZ, 16), *Alake Torsh* (AT, 17), *Malase Torsh* (MT, 18), *Pust Sefeede Shirin* (PSS, 19), *Malase Shirin* (MS, 20), *Tabestani* (T, 21), *Shirin Hastehe Bafgh* (SHB, 22), *Post Sorkhe Ravar* (PSR, 23), *Post Sefeede Torsh* (PST, 24), and *Agha Mohamad Ali Shirin* (AMAS, 25).

Chemicals

Phosphoric acid and water (HPLC grade) were purchased from Merck (Germany) and Caledon (Canada), respectively. Organic acid standards including oxalic, tartaric, malic, shikimik, ascorbic, acetic, maleic, succinic, citric, and fumaric acid were purchased from Supelco (USA). All other chemicals used were analytical reagent grade.

Juice Extraction, Storage, and Organic Acids Analysis

Following peeling out, the skins covering seeds were removed. The remaining seeds were placed in a hand press and the juices were extracted. The juice samples were centrifuged (2 min at 12000 rpm), filtered through a Sep–Pak C18 cartridge (Millipore, Milford, USA) and 0.45–μm Millipore filter to remove interferences and particles (AOAC, 1990). At the start of the study, the organic acid compositions of all cvs were determined by HPLC. Then, three cultivars (PSS, PGY, and KDTG) were selected, based on the amount of production, industrial applications and fresh consumption, and their juices were heated at 96°C for 1 min to inactivate enzymes (placed in an incubator (Irankhodsaz, Co. ltd, Tehran, Iran)). The pomegranate juices were then filled in sterile glass bottles. Samples were kept in an incubator at 4°C for 60 days. Organic acids in juices were determined by HPLC using a Waters HPLC system with an Empower software, a pump (Waters 600), a Rheodyne 7125i six-way injector with 20 l sample loop, and a UV-Vis detector (Waters model 2487). A column (Prontosil 120-3-C_{18} AQ, 250 46 mm, dp 3m, from Knauer (Germany)) was used for the separation.

Twenty μl of clarified juice was injected onto the HPLC. The elution was carried out at room temperature using H_3PO_4 (50 mM) at a flow rate of 0.7 ml/min as the mobile phase with UV-Vis detector at 205 nm. Calculation of the concentrations was based on the external standard method (multiple points calibration curve) and organic acids were identified by comparison of their retention times with those of pure standards and spiking some samples. For each sampling point, there were three replicates.

Statistical Analysis

One-way analysis of variance was used to analyze the data. A p value of 0.05 or less was considered significant (using SAS software).

RESULTS AND DISCUSSION

In this study, the organic acid compositions of 25 Iranian pomegranate cultivars were determined by HPLC. A representative chromatogram of organic acids of pomegranate juices (TLM cv) is shown in Figure 1. Oxalic, tartaric, malic, shikimik, ascorbic, maleic, succinic, citric, and fumaric acids were identified in freshly prepared juices by comparing their retention times with those of authentic standards. As seen from Figure 1, the applied chromatographic conditions have successfully separated both major and minor organic acids of pomegranate juice. Table 1 shows the contents of organic acids of studied cultivars. Pomegranate juice contains a variety of organic acids such as citric, malic, oxalic, acetic, fumaric, tartaric, ascorbic, quinic, and succinic acids (Legua *et al.*, 2000; Melgarejo *et al.*, 2000; Poyrazoglu *et al.*, 2002).

The highest organic acids content, 3959.3 mg/100g, was found in the SRAB cultivar and the lowest, 105.4 mg/100g in the TML cv (Table 1). Citric acid was determined to be the predominant organic acid in 17 pomegranate cultivars (No. 1 - 9, 11 - 13, 15 - 17, 21, and 22). Its concentration ranged between 13.6 ± 0.8 (TML) and 3763.6 ± 144.8 mg/100g (SRAB). The average value of citric acid content for the entire 25 pomegranate cvs was 631.2 mg/100g (Table 1) which is in agreement with the value reported by Melgarejo *et al.* (2000) for some Spanish pomegranate cultivars. The highest value reported in this study (3763.6 mg/100g, SRAB) is higher than the value obtained by others (Melgarejo *et al.*, 2000; Poyrazoglu *et al.*, 2002). The values of MY, BPZS and TLM are higher than the values reported by Khodade *et al.* (1990).

Malic and tartaric acids were determined to be the second most abundant organic acid in the cultivars No. 18 - 20, and 25 and cvs No. 10, 14, 23, and 24, respectively. Malic and tartaric acids concentrations ranged between (0.0 - 366.3 mg/100g) and (0.0 - 106.8 mg/100g), respectively. The mean values of these

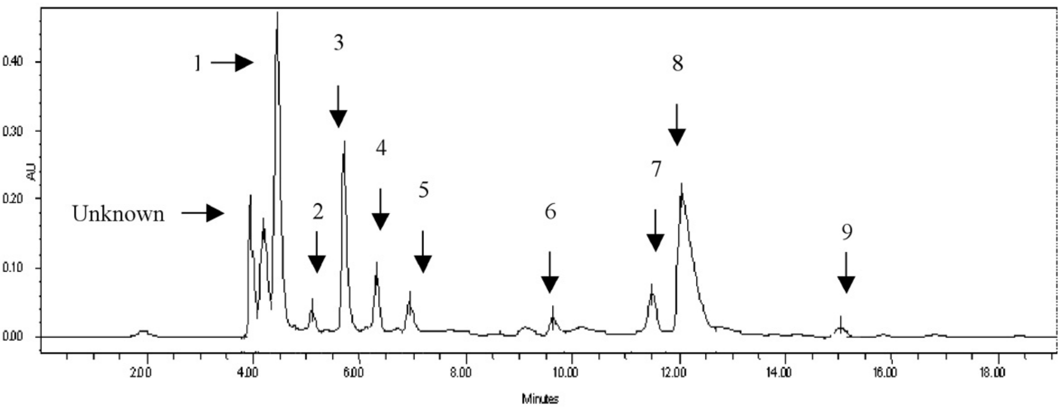

Figure 1: HPLC chromatogram of organic acids in pomegranate juice. Peaks for acids: 1= oxalic; 2= tartaric; 3= malic; 4= shikimik; 5= ascorbic; 6= maleic; 7= succinic; 8= citric; 9= fumaric.

acids contents of all the 25 cvs studied were 54.94 and 56.75 mg/100g, respectively. Malic acid content below 100 mg/100g in Turkish cvs has been reported (Cemeroglu *et al.*, 1992), but Legua *et al.* (2000) has reported values higher than 200 mg/100g for clones ME5, ME17 and MO16. These results are in agreement with values previously obtained for the varieties 01-N-01 (88.0 ± 15.0 mg/100g) and 01-N-07 (56.0 ± 8.0 mg/100g) which were grown in Adana, Turkey (Poyrazoglu *et al.*, 2002). In comparison to existing literature, tartaric acid results were similar to the findings of Poyrazoglu *et al.* (2002). On the contrary, Legua *et al.* (2000) could not detect tartaric acid in their studied clones, but Melgarejo *et al.* (2000) detected and determined tartaric acid over the range 0.0-51.0 mg/100g, which is lower than values reported in this study.

Oxalic acid was observed in all pomegranate cvs over the range 10.3 ± 0.8 (MPS) to 55.1 ± 4.5 (SRAB) mg/100g. The overall mean concentration of oxalic acid was found to be 28.18 mg/100g. The results of this study was lower than the value reported by Poyrazoglu *et al.* (2002) (116.0 mg/100g) and higher than amounts obtained by Legua *et al.* (2000). Results reported in this study, for oxalic acid, were similar to the findings of others (Melgarejo *et al.*, 2000).

In this study, shikimik acid was observed in 12 cvs ranging from 0.0 to 47.4 ± 1.4 mg/100g (MY cv). On average, the shikimik acid content was 3.64 mg/100g, which belongs to the minor organic acids.

Acetic acid is both volatile and odorous; it has not been previously reported in pomegranate until 2000 when Melgarejo *et al.* reported the presence of acetic acid in three cultivars BA1, BB1, and ADO4 (with average content 15.0 mg/100g). Legua *et al.* (2000) also found acetic acid in pomegranate juice over the range 6.0 to 76.0 mg/100g. In this study, acetic acid was observed in nine cvs (No. 7- 9, 11, 14, 16, and 22-24). The concentration ranged between 0.0 and 76.1 ± 4.6 mg/100g (JPGR cv), with overall mean content 13.84 mg/100g, which is in agreement with previously published results (Legua *et al.*, 2000; Melgarejo *et al.*, 2000).

Ascorbic acid (AA) was determined in 17 pomegranate cultivars. Its amounts ranged between 0.0-12.0 ± 2.6 mg/100g (SRAB cv). The average content of AA for 17 cvs was 3.43 mg/100g (overall mean value of 25 cvs was 2.33 mg/100g). The results of this study are lower than, and in some cases similar, to amounts previously reported (Legua *et al.*, 2000).

Fumaric and maleic acids were also determined in relatively lower amounts in

Table 1
Organic acid composition of 25 Iranian pomegranate cultivars (mg/100g)*

No.	Cultivar	Oxalic	Tartaric	Malic	Shikimik	Ascorbic	Acetic	Maleic	Succinic	Citric	Fumaric	Total
1	SRAB	55.1±4.5	106.8±24.0	20.8±4.2	0.41±0.06	12.0±2.6	ND	0.14±0.00	ND	3763.6±144.8	0.4±0.0	3959.3
2	ZY	30.2±1.8	64.8 ± 5.6	31.5±5.3	ND	ND	ND	0.17±0.01	78.4±6.1	1164.3±31.8	0.24±0.04	1369.6
3	MTK	25.5±2.0	61.6±2.7	19.3±2.1	ND	3.0±0.0	ND	0.15±0.00	59.3±3.0	1611.3±54.1	0.55±0.01	1780.7
4	ATS	34.6±1.9	74.5±8.0	37.3±4.0	ND	2.96±0.18	ND	0.22±0.01	118.4±10.9	920.5±17.9	ND	1188.5
5	KDTG	25.7±0.8	53.6±5.4	19.4±0.7	1.89±0.12	8.78±0.95	ND	0.08±0.00	38.8±3.85	747.4±14.0	0.28±0.00	895.9
6	GSY	36.7±1.9	105.1±8.4	7.1±0.9	ND	ND	ND	0.10±0.00	134.4±10.4	398.5±11.4	0.44±0.01	682.3
7	PSY	20.9±0.9	66.5±8.0	46.6±4.4	ND	1.91±0.08	30.8±1.1	0.24±0.00	92.6±9.3	316.5±6.8	0.28±0.00	576.3
8	MY	23.3±1.5	46.6±4.6	ND	47.40±1.40	ND	43.6±2.1	0.04±0.01	ND	140.8±5.2	ND	301.7
9	VKT	17.2±1.6	ND	ND	ND	5.82±0.46	31.5±1.8	0.08±0.10	46.9±1.7	1193.2±64.9	ND	1294.7
10	TML	23.5±2.2	51.1±5.0	13.9±1.3	2.82±0.40	ND	ND	0.15±0.00	ND	13.6±0.8	0.37±0.00	105.4
11	JPGR	33.2±1.7	78.7±5.5	48.4±7.7	2.89±0.08	ND	76.1±4.6	19.2±0.8	ND	1114±6.5	0.65±0.02	1373.1
12	MPS	10.3±0.8	ND	2.3±0.7	0.38±0.01	ND	ND	0.37±0.02	13.4±3.6	278.6±8.9	ND	305.6
13	AT	37.7±2.2	ND	178.8±3.3	ND	2.00±0.18	ND	0.54±0.03	28.6±1.6	1239.6±84.6	11.37±1.48	1498.6
14	T	35.5±2.7	83.6±2.9	31.1±0.7	7.00±1.40	1.72±0.19	42.0±1.3	0.11±0.01	20.2±6.6	38.5±3.8	11.56±0.23	271.3
15	SHRB	29.2±3.0	67.3±2.8	23.1±2.1	3.12±1.0	2.29±0.16	ND	0.22±0.01	60.9±6.5	1881.9±76.6	6.41±0.14	2074.4
16	PST	29.4±0.6	ND	15.6±1.6	5.00±0.60	1.18±0.33	42.9±2.7	0.39±0.02	12.4±2.6	311.8±45.1	11.59±0.24	430.3
17	TG	23.4±0.6	75.6±12.7	21.3±4.9	ND	2.6±0.5	ND	0.19±0.01	80.3±12.6	667.1±12.9	0.35±0.03	870.4
18	BPZS	23.2±2.2	37.4±9.6	162.7±8.6	ND	0.36±0.07	ND	0.31±0.01	ND	155.6±6.4	8.04±0.06	891.4
19	TM	45.3±7.5	86.0±4.6	366.3±6.0	ND	1.11±0.23	ND	0.99±0.04	88.4±2.3	50.7±7.1	11.67±1.55	650.5
20	SP	22.4±1.9	37.9±1.9	97.0±11.4	4.20±0.30	2.47±0.14	ND	ND	ND	ND	7.42±0.40	171.4
21	TLM	14.6±0.7	25.7±2.6	45.3±3.5	ND	2.25±0.09	ND	0.09±0.00	13.4±3.6	128.5±11.8j	0.38±0.00	230.2
22	SSY	17.3±1.1	69.4±5.2	35.5±3.0	ND	ND	0.81±0.02	0.36±0.02	68.2±1.4	394.2±25.6	ND	585.8
23	PSS	34.8±0.8	62.7±0.5	24.0±2.5	11.40±0.30	5.11±0.12	38.5±6.0	0.47±0.04	53.5±11.5	28.5±2.0	15.39±0.29	274.4
24	SM	38.6±2.3	66.0±5.7	25.5±0.8	ND	2.71±0.39	39.7±3.8	0.25±0.01	18.8±1.3	14.4±1.16	6.87±0.94	212.8
25	AMAS	17.2±0.7	97.8±3.1	100.3±20.3	4.20±0.20	ND	ND	0.26±0.01	46.6±3.4	27.1±3.5	7.53±0.38	301.0
Average		**28.2**	**56.8**	**54.9**	**3.64**	**2.33**	**13.8**	**1.01**	**42.6**	**631.2**	**4.07**	**838.9**

*Values are mean ± standard deviation of triplicate determinations. ND: not detected.

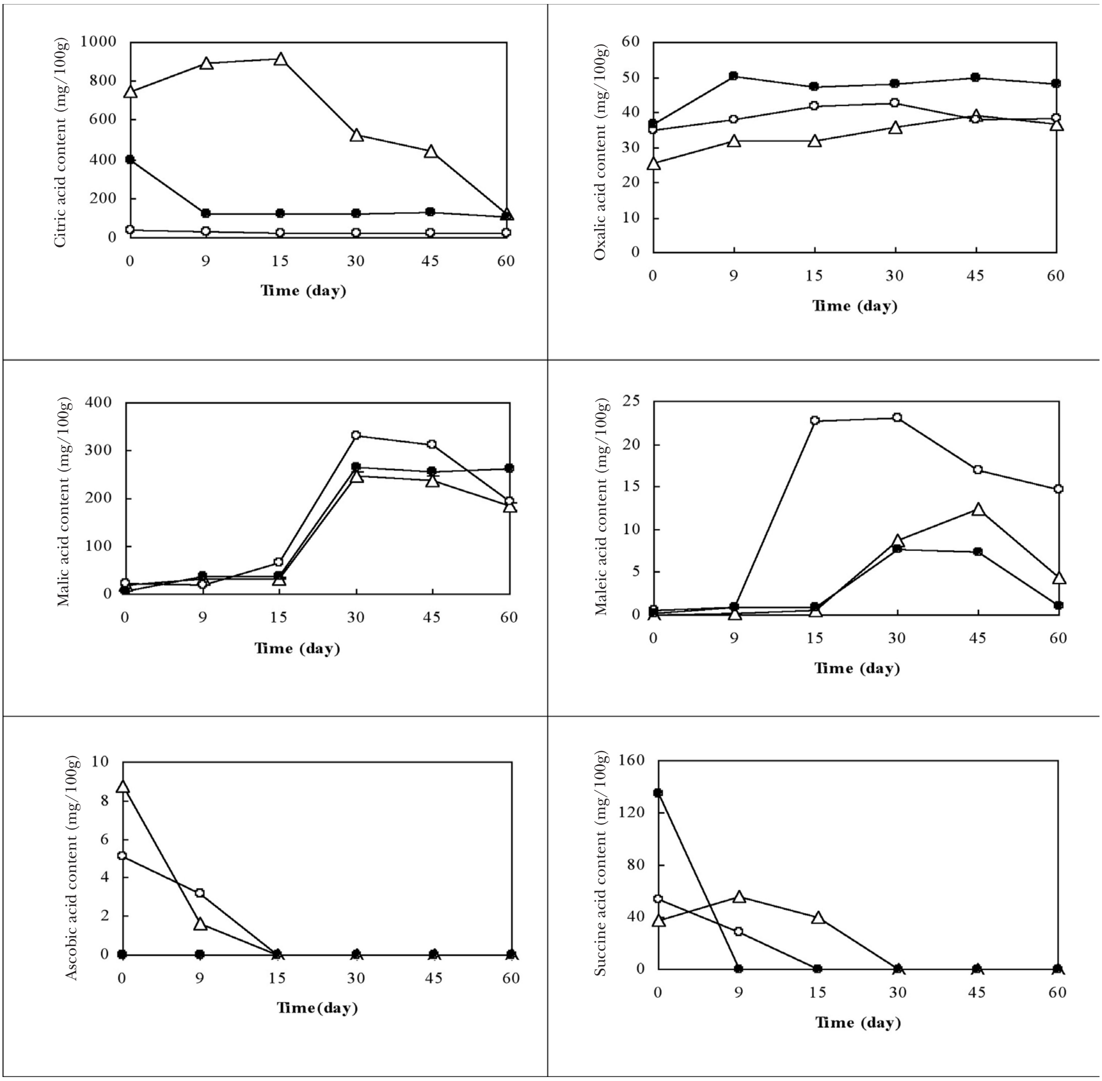

Figure 2: Changes in organic acid content of three pomegranate cultivars during storage at 4°C for 60 days. Open circles, Post Seffide Shirin (PSS) cv; full circles, Gorch Shahvare yazdi (GSY) cv; open triangles, Khoram Dizin Torshe Gorgan (KDTG) cv.

most of the pomegranate cultivars. Their concentration range were 0.0 to 15.39 ± 0.29 mg/100g (PSS) and 0.0 (SP cv) to 19.2 ± 0.8 mg/100g (No. JPGR cv), respectively. The respective overall mean amounts of these organic acids were 4.07 and 1.01 mg/100g. Other researchers could not find fumaric acid in pomegranates that were studied (Legua *et al.*, 2000; Poyrazoglu *et al.*, 2002), but Melgarejo *et al.* (2000) and Miguel *et al.* (2004a) have reported relatively lower amounts of fumaric acid, in some pomegranate cvs, than those reported in this study. Miguel *et al.* (2004a) found maleic acid in pomegranate juices extracted by two different extraction methods over the range 50-90 mg/L that is higher than the values reported for this study.

The highest succinic acid content was found in GSY cultivar (134.4 ± 10.4 mg/100g), and it was not detected in cultivars No. 1, 8, 10, 11, 18, and 20. The overall mean content of succinic acid in all the 25 pomegranate cvs was 42.6 mg/100g which is slightly similar to overall means reported by others (Poyrazoglu *et al.*, 2002). However, other researchers could not detect succinic acid in pomegranate fruit (Legua *et al.*, 2000; Melgarejo *et al.*, 2000). We could not confirm the presence of quinic acid that has been found in some Turkish pomegranate cultivars (Poyrazoglu *et al.*, 2002). In addition, lactic acid was not detected and so not quantified in any cvs.

Effect of Cold Storage on Organic Acid Composition

The organic acid compositions of the three pomegranate cultivars, KDTG, PSS, and GSY, monitored over 60 days of storage at 4°C, are presented in Figure 2. It shows that each cultivar behaved differently during storage and so varying changes were observed in individual organic acid content.

After storing GSY cv for 9 days, its citric acid content decreased to 122.6 mg/100g and then remained constant. As shown in Figure 2 (KDTG cv), the concentration of citric acid reached a maximum (917.7 mg/100g) after 15 days and then an apparent decrease occured, while the citric acid content of PSS cultivar remained constant during 60 days storage. In addition, a similar pattern was observed for fumaric acid contents of these cultivars and after 60 days, their fumaric acid contents reached about zero (graph not shown).

The oxalic acid levels increased gradually, reaching maximum values after 9, 30, and 45 days storage of GSY, PSS, and KDTG cvs, respectively and then remained stable. A similar trend was found during 72 h storage of pomegranate juice at 4°C (Miguel *et al.*, 2004a).

Malic and maleic acid contents changed significantly during storage (Figure 2). The levels of malic and maleic acids were low at the beginning of storage, and then the malic acid levels of all cultivars reached maximum values (after 30 days) and then the malic acid levels of KDTG, PSS, and GSY cvs decreased to 183.9, 194.0, and 263.3 mg/100g, respectively. As seen from Figure 2, the levels of maleic acid increased and reached maximum levels after 15, 30, and 45 days storage of PSS (23.0 mg/100g), GSY (7.8 mg/100g), and KDTG (12.5 mg/100g) cultivars and then their amounts decreased to 14.7, 1.1, and 4.4 mg/100g, respectively.

The influences of cold storage on ascorbic and succinic acids contents are shown in Figure 2. The rate of ascorbic acid (AA) loss in PSS and KDTG cultivars was high, fruit juice stored for 15 days had lost 100% of their initial AA contents, and the rate of AA loss of KDTG was higher than the PSS cultivar. Miguel *et al.* (2004a) found that after storing pomegranate juice for 3 days, its AA content remained quite stable independent of the extraction methods. Fresh fruits and vegetables present the highest content of ascorbic acid, although it varies among species and varieties. This compound, better known as vitamin C, is associated with health by most consumers so that it is an attractive index of product quality. Generally, fruits and vegetables show a gradual decrease in AA content as the storage temperature or duration increases (contrary to other organic acids) (Bode *et al.*, 1990; Angberg *et al.*, 1993), and thus considered an indication of freshness in fruits.

In addition, the content of succinic acid was highest at the beginning of juice storage (its levels for KDTG, PSS, and GSY cvs were 38.2, 53.5, and 134.5 mg/100g, respectively) and then decreased to zero. Shikimik acid contents of KDTG and PSSS cultivars decreased and reached zero after 9 days storage (graph not shown).

Tartaric acid content showed fluctuations in GSY and KDTG cvs and their levels at the beginning of storage were 105.1 and 53.7 mg/100g and after 60 days, reached 102.5 and 36.5 mg/100g, respectively. PSS cultivar had initial tartaric acid content 34.8 mg/100g and its level changed significantly during 15 days (reached to 103.2 mg/100g) and then its content decreased and reached zero after 45 days storage. Miguel *et al.* (2004a) observed similar changes of tartaric acid content of studied cv and amount of tartaric acid, after some fluctuations, remained constant.

At the beginning of storage, acetic acid contents of PSS, GSY and KDTG cultivars were 38.5, 0.0, and 0.0 mg/100g, respectively (graph not shown). The acetic acid content of KDTG cv remained constant without any changes, but PSS and GSY cultivars showed different changes and their acetic acid levels increased to a maximum of 85.9 and 99.0 mg/100g, respectively (after 30 and 45 days of storage). Then a continual decrease was observed during the last 15 days of storage, reaching 0.0 mg/100g.

CONCLUSIONS

In conclusion, there are significant differences in the levels of organic acids of the studied pomegranate cultivars. Pomegranate fruit contains important amounts of organic acids that might play a significant role in its flavor. Our results showed that citric acid is the main acid in most cvs and malic and tartaric acids are the second predominant acids of pomegranate. To our knowledge, this is the first time that shikimik acids has been determined and quantified in Iranian pomegranate cultivars. It is evident from the study that significant changes of organic acid composition that affect organoleptical properties occur during the cold storage of pomegranate juice. Finally, the composition of pomegranate juice depends on cultivar type, environmental and post harvest factors, and storage conditions.

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