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ORIGINAL PAPER

Anthocyanins characterization of 15 Iranian pomegranate (*Punica granatum* L.) varieties and their variation after cold storage and pasteurization

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Abstract Anthocyanins (ACs) are phenolic compounds that are distributed widely in fruits and vegetables. Apart from imparting color to plants, ACs also have an array of health-promoting benefits. In this research, the amounts of major ACs of 15 pomegranate (*Punica granatum* L.) varieties obtained from Yazd province were determined. The major ACs detected in the studied varieties were as follows: delphinidin 3-glucoside (2.19–16.29 mg/L), delphinidin 3,5-diglucoside (2.36–63.07 mg/L), pelargonidin 3-glucoside (0.26–1.36 mg/L), pelargonidin 3,5-diglucoside (0.01–8.11 mg/L), cyanidin 3-glucoside (5.78–30.38 mg/L), and cyanidin 3,5-diglucoside (4.39–166.32 mg/L). The effect of storage time of unprocessed and pasteurized juices on ACs content of four selected varieties was also studied. Average degradation percentage of each AC was between 23.0 and 83.0% during 10 days at 4 °C. Moreover, in pasteurized juices average degradation of ACs was 42.8 ± 0.5% after 10 weeks storage at 4 °C.

Keywords Anthocyanin · Pomegranate · *Punica granatum* L. · HPLC · Juice

Introduction

Pomegranate (*Punica granatum* L.) is one of the oldest known edible fruits and is extensively cultivated in Afghanistan, China, India, Iran, Japan, Mediterranean countries, Russia, and USA [1]. In Iran, pomegranate is eaten fresh and is also processed for jams, jellies, syrups, and pomegranate juice products. This fruit is one of the most important commercial fruits in Iran and its total production in 2005 was ~670,000 tons [2]. Pomegranate juice, which is a rich source of polyphenols such as anthocyanins (ACs), was recently shown to possess antioxidative properties [3]. Among different plants, the total AC content varies considerably, affected by genetic make-up, light, temperature, and agronomic factors [1].

Anthocyanins are glycosylated derivatives of the 3,5,7,3′-tetrahydroxyflavylium cation and are accepted as an important group of water-soluble pigments in nature. Anthocyanins are polyphenolic compounds that are responsible for the red, blue, and purple colors of most flowers and fruits that are classified as flavonoids [4]. Anthocyanins act as phytochemical antioxidants with potential health related benefits. Some positive therapeutic effects of ACs are more or less related to the antioxidant mechanisms [5].

The most common ACs in the edible parts of plants are the glycosides of cyanidin, pelargonidin, peonidin, delphinidin, petunidin, and malvidin [5]. Based on some researches, pomegranate juice is known to be a major source of phenolic compounds, where ACs are the most important ones, especially the 3-glucosides and 3,5-diglucosides of delphinidin, cyanidin, and pelargonidin [6]. The main ACs responsible for the pigmentation of pomegranate juice were isolated and identified as delphinidin 3,5-diglucoside (Dp35dG), delphinidin 3-glucoside (Dp3G), cyanidin 3,5-diglucoside (Cy35dG), cyanidin 3-glucoside (Cy3G), pelargonidin 3,5-diglucoside (Pg35dG), and pelargonidin 3-glucoside (Pg3G), which confirm previous reports on the other pomegranate cultivars [3, 6, 7].

Maintaining the pomegranate juice color is an important issue during processing and storage. The storage of pomegranate juice in a lower temperature such as 5 °C rather than 25 °C reduced the rate of AC degradation, but ascorbic

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acid addition to pomegranate juices increased the rate of AC degradation at both temperatures [3]. In another research, the influence of packaging material on pomegranate juice color and bioactive compounds (ACs, ellagic acid, and other non-colored phenols) was assessed after pasteurization during storage. They reported that the permeability of packaging to oxygen should be considered as a more important factor than light [8]. Miguel et al. [7] evaluated the effect of two methods of pomegranate juice extraction (centrifugation and squeezing) on ACs stability. The anthocyanin content of extracted juices (in both extraction methods) was similar during 72 h of cold storage at 4 °C.

Although the major ACs of pomegranate juices have been well documented, only few studies have been carried out on identification and quantification of ACs content in Iranian pomegranate varieties. Therefore, the objectives of the current study were (1) to quantify the major ACs content present in juices of different pomegranate varieties grown in Iran (2) to study their changes during 10 days storage at 4 °C, and (3) to determine the stability of ACs in pasteurized pomegranate juices after thermal treatment and storage at 4 °C for 10 weeks.

Materials and methods

Samples

Fifteen important pomegranate varieties were selected from ripe fruits growing in the agricultural research center of Yazd in the southwest of Iran. Commercially ripe fresh fruits were harvested during the September of 2006 from different mature trees randomly selected to represent the population of the plantation. The fruits were transported by a ventilated car to the laboratory, where pomegranates with defects (sunburns, cracks, cuts, and bruises in husk) were discarded. The different varieties selected for this study are: *Shirin Shahvare Yazd* (SSY), *Gorche Shahvar Yazdi* (GSY), *Malase Yazdi* (MY), *Vahshe Kane Tehran* (VKT), *Mesri Torshe Kazeron* (MTK), *Jangali Pust Germeze Rodbare Torsh* (JPGRT), *Torshe Mamoli Lasjer* (TML), *Ardestani Torshe Semnan* (ATS), *Khoram Dizin Torshe Gorgan* (KDTG), *Toghe Gardan* (TG), *Zaghe Yazdi* (ZY), *Tabo Larze Mehr Mahi* (TLMM), *Sefeede Robi Aval Brojen* (SRAB), *Pust Syahe Yazd* (PSY), and *Malase Porbarij Stahban* (MPS). Approximately 2 kg ($n = 10$) of pomegranates at harvesting maturity were sampled for each variety. Some analytical properties of studied varieties are displayed in Table 1.

Table 1 Some analytical properties of studied pomegranate juices

SSC (°Brix)	TA (g/L)	pH	Juice (%)	Variety
15.20	0.54	4.06	53.00	SSY
14.83	4.14	4.11	46.53	GSY
14.10	3.53	3.61	49.17	MY
16.97	1.22	3.66	53.00	VKT
12.10	3.14	3.16	51.73	MTK
18.33	3.25	3.24	51.40	JPGRT
18.20	4.11	4.07	41.50	TML
14.33	3.71	3.76	42.50	ATS
16.30	3.57	3.64	51.57	KDTG
15.23	1.13	3.64	53.17	TG
14.77	1.58	3.41	55.50	ZY
14.33	0.45	4.06	47.10	TLMM
16.30	3.47	3.04	40.83	SRAB
13.20	4.04	4.05	40.50	PSY
16.10	3.50	3.51	54.47	MPS

Values are mean of triplicate measurements

Chemicals

Delphinidin 3,5-diglucoside, Dp3G, Cy35dG, Cy3G, Pg35dG, and Pg3G standards were purchased from Apin Chemicals Co. Ltd. (Oxon, UK). Methanol (HPLC grade) was purchased from Caledon laboratories (Ontario, Canada). Formic acid was purchased from Merck (Darmstadt, Germany) and ultra pure water was prepared with the Purise system (Seoul, South Korea).

Preparation of raw pomegranate juice

Each pomegranate variety was washed in cold tap water and drained. They were manually cut-open and the outer leathery skin, which encloses hundreds of fleshy sacs, was removed. The arils were manually separated from the fruits and juices were obtained by pressing the arils. The juice samples (50 mL) were centrifuged (2 min at 10,000 rpm at −4 °C), and then divided into small vials and kept frozen at −18 °C upon analysis.

The pH, titrable acidity, and soluble solid content

The pH measurements were performed using a Metrohm model 692 pH meter (Switzerland) at 20 °C. Total titrable acidity (TA) was determined potentiometrically using 0.1 M NaOH to the end point of pH 8.1 and expressed as grams of citric acid per litre. The soluble solid content (SSC) was measured by refractive index as °Brix with an Atago N1 refractometer (Tokyo, Japan) at 20 °C.

Storage conditions

After the separation and determination of ACs in the 15 varieties studied, 4 pomegranate varieties were selected (VKT, MTK, JPGRT, and TML) based on the total

Table 2 Linear calibration equations of individual anthocyanin standards

Compound	t_R (min)	Linear range (mg/L)	Linear equation	r
Dp35dG	9.9	1–100	$A = 18718C - 41455$	0.9995
Cy35dG	10.6	1–500	$A = 18720C - 57351$	0.9998
Pg35dG	11.2	0.5–50	$A = 23444C + 431$	1.00
Dp3G	11.9	1–250	$A = 22933C - 47821$	0.9999
Cy3G	12.7	1–250	$A = 76950C - 439615$	0.9989
Pg3G	13.5	0.02–50	$A = 66468C - 17320$	0.9995

A peak area, *C* concentration (mg/L)

production, industrial applications, and fresh consumption. The pomegranate juices were filled in sterile brown glass bottles and placed in an incubator (Irankhodsaz Co. Ltd., Tehran, Iran). The samples were kept in an incubator at 4 °C for 10 days.

Thermal treatment

The selected pomegranate juices (MY, VKT, JPGRT, and TML), 10 mL of each variety juices, were completely sealed in Pyrex tubes and then pasteurized in a thermostatic waterbath (Memmert, Germany) at 85 °C for 5 min before storage. The samples were rapidly cooled in an ice bath and stored in an incubator at 4 °C for 10 weeks.

Anthocyanin analysis

Anthocyanins in juices were determined by HPLC using a Waters HPLC system equipped with an Empower software, a pump (Waters 600), a Rheodyne 7125i six-way injector with 20 µL sample loop, and a UV–Vis detector (Waters model 2487). A column μBondapack™ C_{18} (4.6 × 250 mm, dp 10 µm) from Waters (Ireland) was used for the separation.

Twenty microliters of clarified juice was injected onto the HPLC. The elution was carried out at room temperature using 5% formic acid (A) and methanol (B) in a linear gradient from 15 to 35% B for 15 min, followed by an isocratic run until 20 min. The flow rate was 1 mL/min with UV-Vis detector at 510 nm. Calculation of the concentrations was based on the external standard method and ACs were identified by comparison of their retention times with those of pure standards (linear calibration equations of standards are presented in Table 2). 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

Attractive color is one of the most important sensory characteristics of fruits and pomegranate arils and juice products. The AC profiles of some food products derived from red fruits were used to verify the authenticity and control the quality of these products [4].

In this study, the AC composition of 15 Iranian pomegranate varieties was determined by HPLC. A representative chromatogram of the ACs of pomegranate juice (MTK variety) is shown in Fig. 1. Delphinidin 3,5-diglucoside, Dp3G, Cy35dG, Cy3G, Pg35dG, and Pg3G were identified in freshly prepared juices by comparing their retention times with those of authentic standards (and by spiking), which confirm previous reports on the other pomegranate varieties [3, 6–11].

As it can be seen in Fig. 1, the applied chromatographic conditions successfully separated both the major and minor ACs of pomegranate juice. Table 3 also shows the AC contents of the studied varieties. The individual and total AC contents of all the studied varieties were significantly different ($p < 0.05$). The TML variety had the highest (252.22 mg/L) whereas ATS, PSY, TG, TLMM, and ZY had the lowest AC content. The ACs identified in our study were similar to those previously isolated and identified for

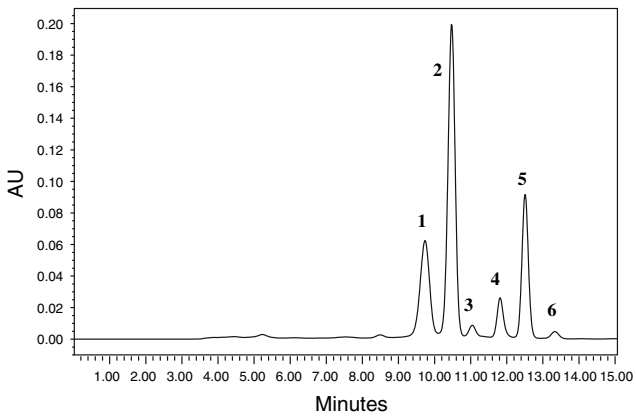

Fig. 1 A representative chromatogram of the separated ACs (MTK variety). *1* Dp35dG, *2* Cy35dG, *3* Pg35dG, *4* Dp3G, *5* Cy3G, *6* Pg3G

Table 3 Anthocyanin composition of 15 Iranian pomegranate varieties (unprocessed juices, mg/L)

Variety	Dp3G	Dp35dG	Pg3G	Pg35dG	Cy3G	Cy35dG	Total
SSY	3.84 ± 0.05f	5.66 ± 0.10gh	0.30 ± 0.01fg	0.13 ± 0.03gf	7.57 ± 0.20g	13.15 ± 1.42g	30.65
GSY	3.62 ± 0.01g	5.28 ± 0.08h	0.32 ± 0.03f	0.34 ± 0.02ef	7.45 ± 0.00g	20.22 ± 0.07f	37.23
MY	3.63 ± 0.01g	8.14 ± 0.14e	0.29 ± 0.11hfg	0.07 ± .0.00gf	6.89 ± 0.04h	12.66 ± 0.36g	31.68
VKT	3.42 ± 0.05h	8.50 ± 0.25e	0.39 ± 0.00d	0.49 ± 0.01e	9.06 ± 0.02d	27.51 ± 0.43d	49.37
MTK	16.29 ± 0.02a	63.07 ± 0.22a	1.31 ± 0.01b	5.38 ± 0.11b	20.63 ± 0.04c	143.78 ± 0.43c	250.46
JPGRT	9.62 ± 0.07c	47.61 ± 0.24b	0.86 ± 0.07c	8.11 ± 0.34a	30.38 ± 0.17a	152.55 ± 0.71b	249.13
TML	10.84 ± 0.04b	40.52 ± 0.83c	1.36 ± 0.02a	6.49 ± 0.07c	26.69 ± 0.03b	166.32 ± 1.94a	252.22
ATS	2.46 ± 0.11j	2.50 ± 0.23j	0.32 ± 0.03f	1.38 ± 0.01d	6.02 ± 0.07ij	8.70 ± 0.06h	21.38
KDTG	4.48 ± 0.01e	9.62 ± .0.28d	0.38 ± 0.02de	0.64 ± 0.09e	8.34 ± 0.00e	24.59 ± 0.32e	48.05
TG	2.19 ± 0.04l	2.36 ± 0.02j	0.26 ± 0.03h	0.03 ± 0.02g	5.78 ± 0.02k	4.39 ± 0.09j	15.01
ZY	2.42 ± 0.01jk	2.76 ± 0.00ij	0.27 ± 0.05gh	0.04 ± 0.00g	6.18 ± 0.00ij	7.78 ± 0.06hi	19.45
TLMM	2.29 ± 0.00lk	3.34 ± 0.02i	0.27 ± 0.01gh	0.09 ± 0.01gf	5.99 ± 0.02jk	6.46 ± 0.32i	18.44
SRAB	2.75 ± 0.05i	6.42 ± 0.16fg	0.27 ± 0.02gh	0.01 ± 0.00gf	6.22 ± 0.02i	12.61 ± 0.22g	28.28
PSY	5.34 ± 0.02d	4.90 ± 0.05h	0.36 ± 0.04e	0.07 ± 0.01gf	7.92 ± 0.01f	6.32 ± 0.04ji	24.91
MPS	3.44 ± 0.00h	6.69 ± 0.03f	0.28 ± 0.01hfg	0.06 ± 0.01gf	6.74 ± 0.00h	13.53 ± 0.03g	30.74

Different letters in the same column present significant difference at $p < 0.05$

Mollar, *Assaria*, and *Wonderful* cultivars [3, 7–9]. Except for PSY variety, in terms of quantity, the main ACs in most of the varieties were Cy35dG (4.39–166.32 mg/L), followed by Dp35dG (2.36–63.07 mg/L), Cy3G (5.78–30.38 mg/L), Dp3G (2.19–16.29 mg/L), Pg3G (0.26–1.36 mg/L), and Pg35dG (0.01–8.11 mg/L). In contrast, the amount of Cy3G in PSY was higher than other ACs. But, in *Assaria* cultivar the main AC was Dp3G, followed by Dp35dG, Cy35dG, Cy3G, Pg35dG, and Pg3G [7]. In addition, another study showed that the percentage of individual ACs in *Mollar* variety were Dp35dG (6%), Cy35dG (38%), Pg35dG (4%), Dp3G (5%), Cy3G (40%), and Pg3G (7%) and the total AC content was 250.87 mg/L [8]. Gil et al. [3] reported that the total AC content in juices from fresh arils, frozen arils, single-strength commercial juice, and commercial juice were over the range of 161.9–387.4 mg/L. The main pigment in the juice of the *Wonderful* variety was Cy3G (59.5–128.3 mg/L). Other identified ACs were as follows: Dp3G (23.6–95.2 mg/L), Cy35dG (31.4–71.4 mg/L), Dp35dG (21.1–61.1 mg/L), and Pg3G (3.9–8.5 mg/L). They reported that the AC content on single-strength commercial *Wonderful* pomegranate juice was 387.4 mg/L, and for juice from fresh arils was 306.0 mg/L. Therefore, the total AC content of *Wonderful* variety is higher than the values reported by the present study on Iranian varieties.

Figure 2 shows that the AC content of unprocessed juices decrease significantly during storage time. During the first 48 h of storage (at 4 °C), a slight increase and subsequently (until 10 days) a decreasing trend was observed in the amounts of ACs of some varieties. This behavior has previously been reported by Miguel et al. [7] In addition, the fruit juice showed a pronounced decrease in Dp35dG mainly after 48 h of storage (Fig. 2). It was previously reported that in the *Wonderful* pomegranate juice, the diglucoside ACs were more stable than the monoglucosides [12]. Therefore, the pronounced reduction of Dp35dG observed in our experiment was unexpected. However, these results were in accordance with result of *Assaria* pomegranate that was reported by Miguel et al. [7]. But, they reported higher decrease in Cy35dG instead of Dp35dG. Also, in another study, Pg35dG followed by Cy35dG, Pg3G, Cy3G, and Dp35dG had the highest and the Dp3G had the lowest stability. They found that the total AC content was reduced to 20% after storage of the juice at 5 °C for 5 months [9].

The HPLC analysis showed that after 10 days, the degradation percentage of ACs content in studied varieties was as follows: Dp35dG (79.0–85.7%), Dp3G (57.1–77.0%), Pg3G (32.1–74.5%), Pg35dG (1.1–79.3%), Cy3G (9.3–38.7 %), and Cy35dG (0.7–48.5%). Table 4 shows the average degradation of ACs in selected pomegranate varieties during 10 days of storage. Therefore, the stability of Cy35dG was higher than the others. The higher stability of the diglucosides can be explained if it is considered that glycosyl substitution at C5 reduces the nucleophilic character of the C6 and C8 positions; thus anthocyanin 3,5-diglucosides are less prone to electrophilic attack than 3-glucosides [13]. There was a considerable reduction in the individual AC content during the first 48 h. These decreases were gradually milder after 48 h and finally reached a steady state. This research showed that stability of Cy was more than Dp and Pg, and the most unstable AC was Dp during storage. It seems that the instability of ACs is probably depended on the biological as well as environmental conditions.

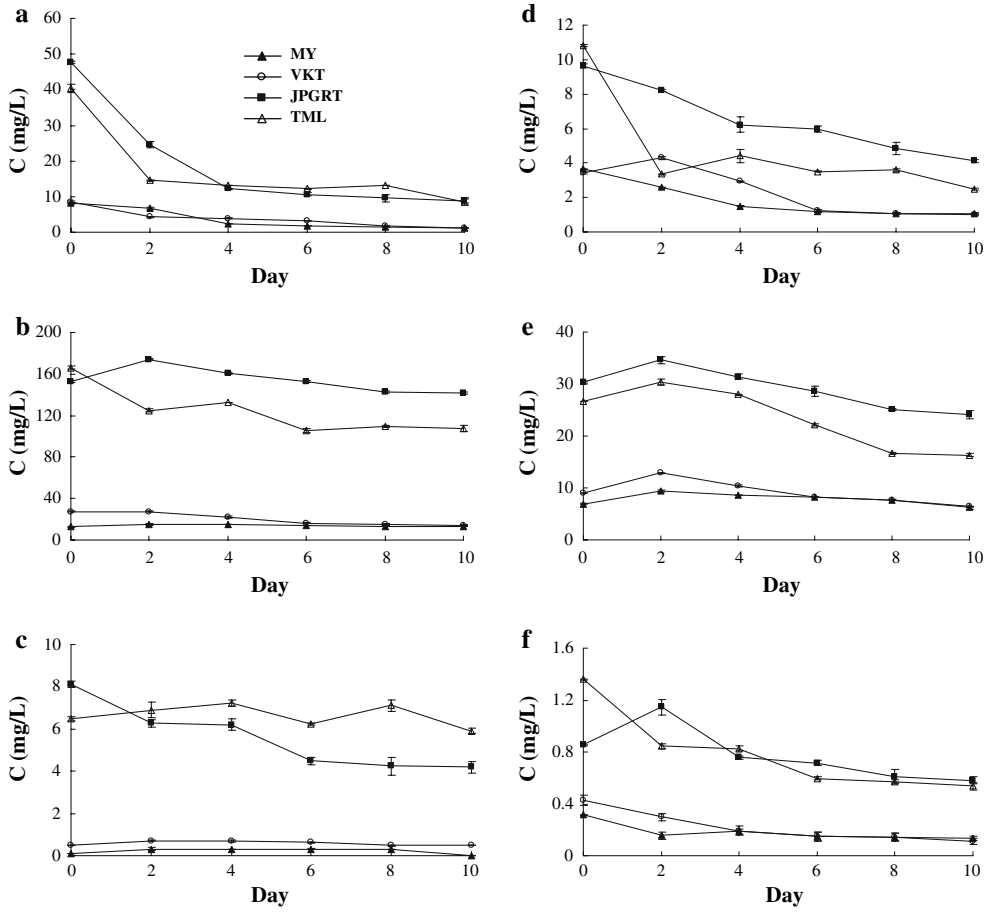

Fig. 2 Evolution of: **a** Dp35dG, **b** Cy35dG, **c** Pg35dG, **d** Dp3G, **e** Cy3G, and **f** Pg3G concentrations in selected pomegranate varieties (unprocessed) during storage at 4 °C for 10 days

Table 5 shows the changes in total and individual ACs of unprocessed and processed (thermal pasteurization) selected juices. The amount of ACs in selected pasteurized juices was determined during storage for 10 weeks at 4 °C. In JPGRT and TML variety the amount of individual and total ACs decreased after pasteurization. These results were in accordance with the previous report of Pérez-Vicente et al. [8] in which they investigated the degradation percentage of bioactive compounds (ACs, ellagic acid, and other non-colored phenols) in pomegranate juices during the storage. After pasteurization, the amount of total ACs was decreased to about 14% and after that, during 180 days storage at 24/18 °C, a decreasing trend was observed (ACs content was decreased to about 70%) [8]. But, in MY and VKT varieties (with pinkish arils), the total amount of ACs increased after pasteurization. This may be due to the consequence of native copigments and self-association of ACs that improve color stability of the juices. The conversion of leucoanthocyanin to AC could also happen, but probably at a slower rate of thermal degradation. The effect of heat on some plant varieties that causes pinkish discoloration is attributed to the conversion of colorless leucoanthocyanin to red ACs which has been subjected to an acid-catalyzed dehydration by oxidation [14]. These results were in accordance with the results of Lee et al. [15] which reported that pasteurized blueberry juice had higher amount of ACs than initial pressed juice. The reduction rates of ACs in pasteurized juices during storage were lower than stored raw juices due to thermal inactivation of polyphenol oxidase (PPO). The ACs content (especially diglucoside ACs) of some juices increased after pasteurization. Also, after two weeks storage,a minor increase in some ACs content was observed

Table 4 Average degradation of ACs in selected pomegranate juices (unprocessed) during cold storage at 4 °C (%)

Anthocyanins	Variety				Average
	MY	VKT	JPGRT	TML	
Dp35dG	85.5	85.7	81.4	79.0	83.0
Dp3G	71.4	69.8	57.1	77.0	68.7
Pg3G	58.0	74.5	32.1	60.7	56.2
Pg35dG	79.3	1.1	48.4	8.8	34.4
Cy3G	9.3	29.4	20.6	38.7	24.5
Cy35dG	0.7	48.5	7.4	35.3	23.0

Table 5 Anthocyanin content of unprocessed juice (UJ), pasteurized juice (PJ), and stored pasteurized juice (SJ) for 10 weeks (mg/L)

Variety	Treatment	Anthocyanins						Total
		Dp35dG	Cy35dG	Pg35dG	Dp3G	Cy3G	Pg3G	
MY	UJ	8.0b	12.7b	0.1b	3.5b	6.9a	0.3a	31.7b
	PJ	13.9a	19.1a	0.3a	3.2a	5.3b	0.2b	42.0a
	SJ	6.4c	13.8b	0.0b	2.1b	2.6c	0.1c	25.0c
VKT	UJ	8.4b	27.4b	0.5c	3.3a	9.1a	0.4a	49.4b
	PJ	11.2a	31.3a	1.0a	2.8a	6.0b	0.3b	52.6a
	SJ	5.9c	23.3c	0.7b	1.6b	2.8c	0.1c	34.4c
JPGRT	UJ	47.5a	152.6a	8.0a	9.5a	30.4a	0.9a	249.0a
	PJ	32.2b	129.7b	7.3b	6.4b	17.4b	0.4b	193.4b
	SJ	14.8c	75.8c	4.3c	3.5c	8.2c	0.1c	106.7c
TML	UJ	40.4a	166.2a	6.5b	10.7a	26.7a	1.4a	251.1a
	PJ	33.7b	135.3b	7.1a	8.8b	18.6b	1.2b	204.7b
	SJ	17.2c	79.6c	4.0c	4.5c	9.1c	0.6c	115.0c

Different letters in the same column present significant difference at $p < 0.05$

(Fig. 3). This phenomenon was probably caused by two concomitant events: the thermally induced extraction of AC molecules previously complexed or polymerized and the retention of active principles caused by the inactivation of the enzymes involved in their catabolism [16].

These differences among juices represented that in actual food systems, the relative stability of an AC is likely to be a function of its matrix, structural features, and the combined conditions of processing and storage [17–19]. Previous studies have shown that during thermal processing and storage, degradation of ACs presumably occurred due to formation of by-products from carbohydrate and organic acids degradation such as furfurals and other aldehyde compounds that can form condensation products with ACs and polyphenolics [17, 18].

Anthocyanins are labile compounds and easily susceptible to degradation in various environmental conditions. The stability of AC color can be improved by copigmentation, where the AC molecule reacts with other natural plant components (such as some flavonoids, polyphenols, organic acids) directly or through weak interactions, resulting in an enhanced and stabilized color [19, 20]. The transformation

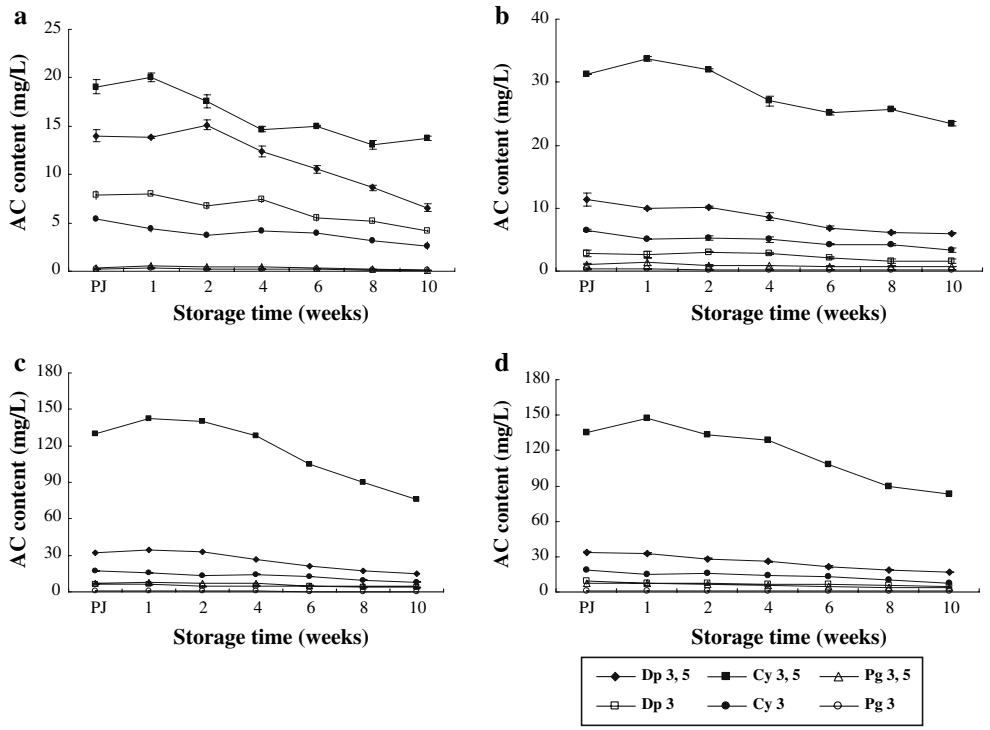

Fig. 3 Evolution of individual ACs in selected pomegranate juices: **a** MY, **b** VKT, **c** GPGRT, and **d** TML after pasteurization (pasteurized juice: PJ) and during storage at 4 °C for 10 weeks

of these pigments to other forms by enzymes (polyphenoloxidase, peroxidase, and glycosidase enzymes), oxidation, light, temperature, etc. during storage, causes color changes which has a negative impact on appearance of the product [21, 22].

Conclusion

Maintaining a strong and stable color in pomegranate juice is problematic during processing and storage. Based on our study, the amount of total ACs, in some variety, was lower than 25.0 mg/L, whilst the amount of ACs in varieties MTK, JPGRT, and TML were the highest (~250.0 mg/L). In our study, there was significant difference in AC levels amongst various varieties. In addition, storage of pomegranate juice and pasteurized juices resulted in significant reduction of ACs. In JPGRT and TML varieties, the amount of individual and total ACs decreased after pasteurization. But, in MY and VKT varieties, the total amount of ACs slightly increased after pasteurization. These differences among juices represented that in actual food systems, the relative stability of an AC is a likely function of its matrix, structural features, and the combined conditions of processing and storage.

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