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Effect of sonication on anthocyanins, total phenolic content, and antioxidant capacity of pomegranate juices

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Abstract

In this study, the effect of ultrasonic treatment on physicochemical properties of juices extracted from two pomegranate parts (whole pomegranate and arils alone) was studied. The juices were continuously sonicated at different amplitudes (50, 75 and 100%) and times (0, 3, 6 and 9 min) at 25 ± 1°C. The results showed that different intensities and times had no significant effect on pH, acidity, °Brix. The degradation percentage of total anthocyanin content was 0.38-9.75%, while its total amount increased 0.44-7.32% at some amplitude levels and times. In addition, total phenolic content in some juices increased about 5.40-42.52% at 100% intensities and 9 min. Furthermore, the antioxidant activity of all juices compared to the control samples showed no significant difference ($p < 0.01$).

Introduction

Pomegranate fruit (*Punica granatum* L.) and its products have been used for centuries as a rich source of bioactive compounds such as anthocyanins, ellagitannins, (mainly punicalagins), ellagic acid, and punicalins (Gil *et al*., 2000) and in recent years, it has been recognized as one of the new superfoods having health promoting effects (Johanningsmeier and Harris, 2011).

Total production of pomegranate in the world was approximately 1,500,000 tons in 2007, and Iran accounted for 47% of world production and the rest (i.e. 53%) was produced by other countries including India, Afghanistan, Turkey, Egypt, Spain, United States and so on. Pomegranate is consumed as fresh fruit, juice, jams and jellies, as well as pomegranate supplements worldwide (Codex Alimentarius, 2009), and it is mainly consumed as fresh fruit and traditionally, ready to-drink pomegranate juice (as similar as possible to those produced at home) is sold in grocery stores in Iran and it contains no preservatives and offered to the customers without any processing, even though such products have a limited shelf-life.

The serious concern about unprocessed fruit juices is microbial contamination with acid-tolerant bacteria, fungi (yeasts and moulds), and pathogenic bacteria, that leads to the deterioration of nutritional and sensorial properties such as functional ingredients, colour, flavor, and odor, as well as food-borne diseases due to the pathogenic bacteria or toxigenic fungi (Tournas *et al.*, 2006).

It should be noted that thermal processing, alone or in combination with chemical or biochemical preservation techniques, is the most effective method for inactivation of microorganisms and enzymes and to increase the shelf-life in the food industry. However, thermal pasteurisation and sterilisation techniques decrease the organoleptic quality and freshness of foods. Because of the increasing health conscious of consumers, the demand for minimally-processed foods has been increasing, which resulted in the development and application of non-thermal technologies for juice processing (Adekunte *et al.*, 2010), compared with the thermal treatments have less destructive effects on nutritional and sensory properties of foods. Some of the non-thermal technologies with potential to replace thermal processing of foods include membrane filtration, osmotic dehydration, pulse electric field, ultrasound, irradiation, high pressure, active packaging and ozone treatment (Cheng *et al.*, 2007).

Ultrasound technology has shown important advances in food processing in the last few years, and its application in food industry has been reviewed recently (Knorr *et al*., 2004). The potential of ultrasound for inactivation of microorganisms and enzymes has been reported in various researches (Knorr *et al*., 2004; Tiwari *et al*., 2008).

Pomegranate juice is a rich source of functional ingredients such as phenolic compounds and

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anthocyanins, so thermal processing has a significant effect on these compounds. In recent years, the effect of ultrasound on the inactivation of microorganisms, increasing the shelf life and improving the organoleptic properties of some food products such as red wine (Masuzawa *et al*., 2000), orange juice (Valero *et al*., 2007), blackberry juice (Tiwari *et al*., 2009a; Wong *et al*., 2010), strawberry juice (Tiwari *et al*., 2009b), red grape juice (Tiwari *et al*., 2010) and kasturi lime juice (Bhat *et al*., 2011) has been studied. Based on these researches, the ultrasonic process has less destructive effect on functional compounds and sensory characteristics of foods. Although the effects of ultrasonic on various fruit juices and vegetables have been reported by several authors, no information is available about pomegranate juice sonication. Therefore, the aim of this study was to investigate the effect of sonication on functional properties (such as total and individual anthocyanins, total phenolic content and antioxidant capacity) of pomegranate juices.

Materials and Methods

Chemicals

Anthocyanin standards were purchased from Apin Chemicals Co. Ltd. (Oxfordshire, UK). Methanol and formic acid (HPLC grade), Folin-Ciocalteu's phenol reagent, tween-20 were bought from Merck (Darmstadt, Germany). The ABTS [2,20-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)], DPPH radical (1,1-diphenyl-2- picrylhydrazyl) and β-carotene were purchased from Sigma–Aldrich Chemical Company (St. Louis, USA) and linoleic acid was obtained from Applichem (Darmstadt, Germany). Ultrapure water was prepared with the Purise system (Seoul, South Korea). Potassium peroxodisulfate, sodium carbonate, sodium acetate, potassium chloride were purchased from Rankem (New Delhi, India). Other chemicals, reagents (analytical grade) and solvents (HPLC grade) were purchased from Merck (Darmstadt, Germany).

Preparation of pomegranate juice

Fresh pomegranate cultivars of Malase Momtaze Saveh (MMS) and Alak Saveh (AS) at commercial maturity were purchased from Agricultural Research Center of Saveh, Iran. After discarding defective pomegranate fruits, each fruit was washed, drained, peeled, and then cut into pieces to separate the arils manually. The whole pomegranate cultivars and their arils were juiced using a manual juicer. The juices of whole Malase Momtaze Saveh pomegranate (MMSW) and arils (MMSA); whole Alak Saveh pomegranate (ASW) and arils (ASA) were immediately centrifuged at 10,000 rpm for 2 min at 4°C with a refrigerated centrifuge (Sigma 3-30K, Osterode am Harz, Germany). The juices obtained were immediately frozen at –80°C till analysis.

Ultrasonic equipment and treatments

The pomegranate juice (100 mL) were sonicated using an ultrasonic liquid processor (Misonix, Inc., NY, USA) supplied with a 19 mm diameter probe, power density 400 W/cm^2, amplitude level (24.4–61 μm) at constant frequency of 20 kHz. Ultrasonic treatment was carried out in a 150 mL double wall cylindrical vessel pyrex glass (60 mm inner diameter, 80 mm outer diameter, 65 mm outer height, 55 mm inner height) connected to a recirculating refrigerated water bath (Cooling thermostat: Lauda Alpha RA 8, Lauda-Königshofen, Germany) to attain a constant temperature in the juice sample during sonication. Ethylene glycol with a flow rate of 0.5 L/min was used as the refrigerant to remove the heat generated during sonication in order to maintain the sample temperature constant at 25±1°C. The ultrasound probe was held in a depth of 25 mm in the pomegranate juice to continuously sonicate at different wave amplitudes (50, 75 and 100%) and times (0, 3, 6 and 9 min).

Measurement of total titratable acidity (TA), pH and soluble solids content (SSC)

pH, TA and SSC (°Brix) of treated and untreated pomegranate juice (PJ) samples were measured using methods described earlier (Alighourchi and Barzegar, 2009).

Determination of total phenolic content (TPC)

Total phenolic content was measured using Folin–Ciocalteu method described by Tezcan *et al.* (2009). The final result was expressed as milligram of gallic acid equivalents per 100 mL of pomegranate juice.

Determination of total monomeric anthocyanin pigment content (TMAC)

Total anthocyanin contents were estimated with a pH-differential method using two buffer systems: 0.025 M potassium chloride buffer at pH 1.0 and 0.4 M sodium acetate buffer at pH 4.5 (Wrolstad *et al.*, 2005). The absorbance of samples was recorded at 510 and 700 nm according to the following equation:

$$A = (Abs_{\lambda vis-max} - Abs_{700\,nm})pH_{1.0} - (Abs_{\lambda vis-max} - Abs_{700\,nm})pH_{4.5}$$

The results were expressed as cyanidin-3-glucoside equivalents for pomegranate juice using a molar absorptive coefficient (ε) of 26900 L/mol.

cm, molecular weight (MW) of 449.2 g/mol, dilution factor (DF), and absorption value (A), according to the following equation:

$$\text{Monomeric anthocyanin pigment (mg/liter)} = (A \times MW \times DF \times 1000)/(\varepsilon \times 1)$$

Determination of individual anthocyanins with HPLC

Separation of anthocyanins was conducted 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 Nucleodur C18 Gravity (4.6 ×250 mm, dp 5 µm) from Macherey Nagel (Düren, Germany) was used for the separation.

Anthocyanins were determined according to the procedure of Del Carpio Jiménez *et al*. (2011) with some modification. Twenty microliters of the clarified juice were injected onto the HPLC. The elution was carried out at room temperature at flow rate of 0.8 mL/min using 10% formic acid aqueous solution (A) and acetonitrile (B) in a linear gradient from 95% A and 5% B for 0–1.67 min; 90% A and 10% B for 3.34 min; 80% A and 20% B for 20 min; 95% A and 5% B for 25 min. The detection of anthocyanins was monitored at 520 nm wavelength and their concentrations were measured based on the external standard method and finally, anthocyanins (ACs) were identified by comparison of their retention times with those of pure standards.

Antioxidant activity evaluation

Three methods were used to analyze the antioxidant activity of pomegranate juice. The first technique was the ABTS• assay which is based on the methodology described by Çam *et al.* (2009). The calibration curve was plotted using different concentrations of L-ascorbic acid (between 0 and 18 µM). The results were expressed as mg L-ascorbic acid/100 mL of PJ. The DPPH• assay was used as the second method which uses the free radical 2,2 diphenyl-1-picrylhydrazyl (DPPH) according to the method of Tezcan *et al*. (2009) and radical scavenging activity was expressed as the inhibition percentage. The final procedure was the β-carotene bleaching method which used to assay the antioxidant capacities of the PJ with β-carotene–linoleic acid (linoleate) system (Çam *et al*., 2009).

Statistical analysis

All analyses were performed using SAS statistical software, version 9.2 (SAS Institute, Inc). A value of $P < 0.01$ was taken to be statistically significant. All experiments were conducted in triplicate and their means were reported as the results.

Results and Discussion

Changes in pH, TA and SSC of pomegranate juices

The results of some physicochemical characteristics of the studied pomegranate juices are presented in Table 1. The analysis of variance indicated that there was a significant difference in terms of pH, TA and SSC between different juices. The SCC and TA of pomegranate juices of whole fruit and pomegranate arils showed a significant difference, but in terms of pH no significant difference observed between them ($P < 0.01$). In comparison with MMSA and ASA juices, SSC increased 8.38% and 9.88% and TA also increased 16.45% and 3.73% in MMSW and ASW juices, respectively which could be due to the entrance of soluble solids and acids existed in their skins. It should be noted that various intensities of ultrasound and processing times had no significant effect on pH, TA and SSC of pomegranate juices, which is in accordance with previously published findings (Ugarte-Romero *et al*., 2006; Cheng *et al.*, 2007; Tiwari *et al*., 2008; Tiwari *et al.*, 2009a; Adekunte *et al*., 2010; Tiwari *et al*., 2010). They indicated that ultrasound treatment did not influence TA, pH, and SSC of the cider, guava juice, orange juice, blackberry juice, red grape juice and tomato juice. However, Yuan *et al*. (2009) reported that the acidity of apple juice significantly rose by increasing the ultrasonic treatment time, which could be due to ultrasound-induced heat generation (in this study, temperature was constant (25 ± 1°C)).

Table 1. Main physicochemical quality parameters of untreated pomegranate juices obtained from: Malase Momtaze Saveh arils (MMSA); Alak Saveh arils (ASA); whole Malase Momtaze Saveh pomegranate (MMSW); and whole Alak Saveh pomegranate (ASW)

	MMSA*	ASA	MMSW	ASW
SSC** (°Brix)	16.7±0.1d	17.2±0.1c	18.1±0.1b	18.9±0.1a
pH	3.56±0.01a	3.09±0.02b	3.54±0.01a	3.05±0.01b
TA (g/100mL)	0.81±0.01d	1.61±0.00b	0.93±0.01c	1.67±0.02a
TMA (mg/L)	375.5±12.9b	409.4±6.1a	338.7±3.3d	355.8±4.1c
TPC (mg/100mL)	204.6±6.9c	234.9±4.6b	278.3±11.0a	290.7±6.8a

* Values with different letters within a similar row are significantly different ($P<0.01$). ** Soluble solids content (SSC); total titratable acidity (TA); total phenolic content (TPC); total monomeric anthocyanin pigment content (TMAC)

Changes in individual anthocyanins of sonicated pomegranate juices

In this study, the anthocyanins of six major pomegranate juices were identified by HPLC,

namely Delphinidin 3, 5-diglucoside (Dp 3, 5 dG), 3-glucoside (Dp 3 G), cyanidin 3, 5-diglucoside (Cy 3, 5 dG), 3-glucoside (Cy 3 G), pelargonidin 3, 5-diglucoside (Pg 3, 5 dG), and 3-glucoside (Pg 3 G). These results are in accordance with our previously reports on other pomegranate cultivars (Pérez-Vicente *et al*., 2004; Alighourchi *et al*., 2008). The individual and total anthocyanin contents of all studied cultivars were significantly different ($p < 0.01$). The ASA cultivar contained the highest (409.39 ± 6.07 mg/L) total anthocyanin content followed by MMSA (375.53 ±12.90 mg/L), ASW (355.85 ± 4.05 mg/L), and MMSW (338.73 ± 3.30 mg/L). A typical chromatogram of the detected ACs of pomegranate juice (ASA cultivar) is shown in Figure. 1. The main ACs in the studied juices were Cy 3, 5 dG (124.55-173.40 mg/L), followed by Cy 3 G (72.69-84.96 mg/L), Dp 3, 5 dG (42.282-77.42 mg/L), Dp 3 G (15.95-24.35 mg/L), Pg 3, 5 dG (11.22-13.38 mg/L), and Pg 3 G (4.20-5.64 mg/L) that are in agreement with our previous findings on the Yazd pomegranate cultivars (Alighourchi *et al*., 2008).

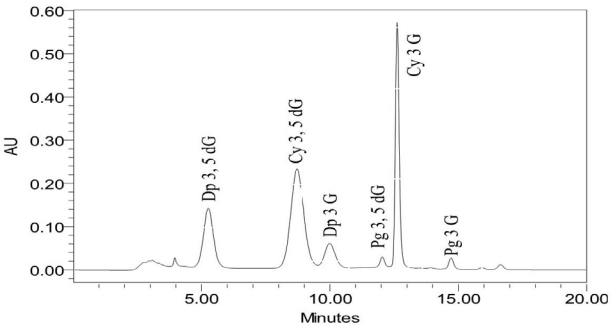

Figure 1. A representative chromatogram of the separated anthocyanins: Delphinidin 3, 5-diglucoside (Dp 3, 5 dG), 3-glucoside (Dp 3 G), cyanidin 3, 5-diglucoside (Cy 3, 5 dG), 3-glucoside (Cy 3), pelargonidin 3, 5-diglucoside (Pg 3, 5 dG), and 3-glucoside (Pg 3 G) (Alak Saveh arils juice (ASA)).

The amounts of anthocyanins in pomegranate juices extracted from whole fruits were less than juices from pomegranate arils in the same cultivar, which is due to the presence of non-edible parts of the fruit such as pieces of fleshy mesocarp, which prevents the exertion of the same pressure as pomegranate arils, and consequently reducing the extraction efficiency of pomegranate juice.

Variations of individual anthocyanins of sonicated pomegranate juices are shown in Figure 2. The contents of individual and total anthocyanins in various cultivars at different times and amplitude levels did not decrease substantially, and even they increased slightly at some amplitude levels and times. The degradation percentage of total anthocyanin content was between 0.67-8.41% and

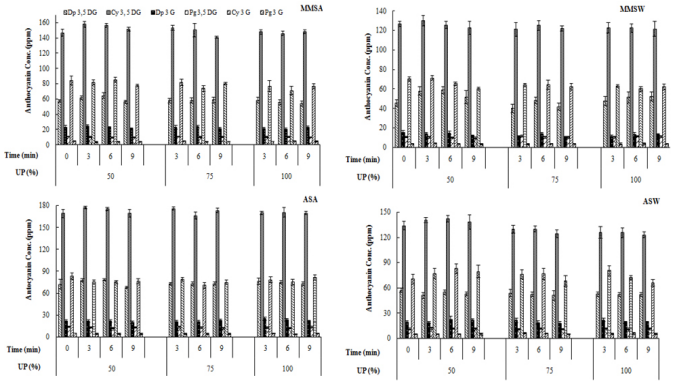

Figure 2. Evolution of individual anthocyanins in studied pomegranate juices obtained from: Malase Momtaze Saveh arils (MMSA); Alak Saveh arils (ASA); whole Malase Momtaze Saveh pomegranate (MMSW); and whole Alak Saveh pomegranate (ASW) after sonication at different amplitude levels and times (UP: percentage of ultrasonic power)

the highest decline occurred in ASW juice at 100% amplitude for 3 min. However, the amount of total anthocyanin content increased about 0.26-6.84% at some amplitude levels and times, especially at 50% amplitude level. The variation of diglucoside and monoglucoside anthocyanin could be due to the separation of sugar from diglucoside anthocyanins structure, degradation of polymeric anthocyanin or copigmentation reactions. Similarly, Tiwari *et al.* (2009b) reported a slight increase in the anthocyanin content of strawberry juice at lower amplitude levels and treatment times as a result of extraction of bound anthocyanins from the suspended pulp. Therefore, it can be concluded that ultrasound treatments had no undesired effects on anthocyanins content of juices. It should be noted that the changes in the amount of total and individual anthocyanins after sonication were significant compared to the fresh juices, which were almost less than 10%.

The results of this study at some amplitude levels were similar to those previously reported for blackberry juice (Tiwari *et al*., 2009a; Wong *et al*., 2010), strawberry juice (Tiwari *et al*., 2009b) and red grape juice (Tiwari *et al*., 2010). The content of anthocyanins in blackberry juices sonicated up to 32 min showed no significant differences for all treatment times (Wong *et al.*, 2010). The ultrasonicated blackberry juice retained a significant amount of anthocyanins (>94%) at 100% amplitude for 10 min (Tiwari *et al.*, 2009a). Also, at the highest acoustic energy densities (0.81 W/mL) and treatment time (10 min), the maximum reduction found in the strawberry juice anthocyanin after sonication, which was less than 5% (Tiwari *et al*., 2009b). Moreover, Tiwari *et al*. (2010) reported that the major grape juice anthocyanins (cyanidin, delphinidin and malvanidin)

were significantly influenced by ultrasound.

Intrinsic properties of the product and the process such as pH, temperature, light, oxygen, enzymes, ascorbic acids, sugars, enzymes, metal ions, co-pigmentation (intermolecular and intramolecular complex formations, self-association and metal complexation) as well as chemical structure and concentration of anthocyanins can affect anthocyanin stability (Rein, 2005; Patras *et al.*, 2009). It can be inferred from this study that anthocyanin stability in the sonicated juices, in addition to the effects of intrinsic properties of the product and the process, is mainly a function of the final processing temperature. According to the literature, thermal pasteurization is associated with degradation of anthocyanins and sensory properties (Pérez-Vicente *et al.*, 2004; Alighourchi *et al*., 2008). Therefore, there is a need for non-thermal preservation techniques such as ultrasonic to maintain the functional and nutritional compounds and sensory properties. The formation of hydroxyl radicals due to cavitational thermolysis or sonolysis of water leads to the chemical decomposition of anthocyanins by opening thier rings and formation of chalcone. This mechanism is mainly due to the extreme temperatures and pressure rise which occur during sonication (Tiwari *et al.*, 2010). It seems that there is no clear trend in the anthocyanin content in different amplitude levels and times of sonication, which could be due to the enhanced isomerization and polymerization/depolymerization, and co-pigmentation formation/decomposition reactions during sonication.

Changes in TMAC, TPC and antioxidant activity

The effect of ultrasonic on TMAC, TPC and antioxidant activities of ASA and ASW juices is shown in Table 2. Similar trends were observed for MMSA and MMSW samples (data not shown). The amount of TMAC slightly increased (about 0.44-7.32%) compared to the control sample at 50% amplitude level but it was not significant. The degradation percentages were 0.38-9.75% at different amplitudes and times with similar degradation trends of anthocyanins that mentioned in previous section.

The MMSW and ASW samples had the highest TPC content of 278.27 ± 11.03 and 290.67 ± 6.76 mg/100 mL gallic acid equivalent, respectively. In comparison with the control samples, TPC in MMSA and ASA juices at different amplitude levels and times showed no noticeable increasing or decreasing trend (<6%). However, the TPC of MMSW and ASW juices showed a significant increase between 0.02-42.53%, especially at higher amplitude level and times. The sonicated MMSW and ASW juices

Table 2. Evaluation of total monomeric anthocyanin (mg/L), total polyphenol content (mg/100 mL juice), assay of antioxidant activities based on ABTS (mg/100 mL juice), DPPH (%AA) and β-carotene (%ALPA) in pomegranate juices as a function of the ultrasonic amplitude levels and treatment times

Juice	Ultrasonic power (%)	T (min)	TMAC (mg/L)	TPC (mg/100mL)	ABTS (mg/100mL)	DPPH (AA%)	β-carotene (%ALPA)
ASA**	0	0	409±6ab	235±5ab	1330±67a	67±3a	79±5ab
	50	3	408±12ab	229±3ab	1231±44a	67±8a	77±2ab
		6	442±5a	229±7ab	1108±46a	68±5a	76±1b
		9	425±22ab	224±4b	1282±96a	64±3a	77±2ab
	75	3	385±5c	243±9ab	1219±39a	66±5a	84±5ab
		6	388±12bc	245±6ab	1243±128a	64±6a	79±0ab
		9	403±10bc	249±10a	1203±90a	65±5a	80±4ab
	100	3	381±10c	236±10ab	1220±102a	65±8a	81±3ab
		6	400±4bc	239±7ab	1288±80a	67±4a	85±1ab
		9	373±8c	235±6ab	1277±44a	66±3a	89±3a
ASW	0	0	356±4a	291±7b	1558±109a	72±5a	92±5a
	50	3	361±4a	312±10ab	1524±88a	73±8a	89±1a
		6	354±11a	294±15ab	1569±101a	73±2a	93±5a
		9	347±4a	315±17ab	1438±117a	74±7a	85±2a
	75	3	341±9a	285±14b	1539±96a	72±4a	89±2a
		6	339±4a	298±10ab	1498±106a	73±8a	93±0a
		9	344±7a	283±15b	1442±109a	73±4a	89±4a
	100	3	348±15a	340±15a	1482±67a	73±5a	88±3a
		6	343±8a	318±13a	1511±128a	72±3a	92±1a
		9	335±7a	306±8a	1423±74a	73±4a	89±2a

* Different letters in the same column for each cultivar present significant difference at $p < 0.01$. **The studied pomegranate juices obtained from: Alak Saveh arils (ASA); and whole Alak Saveh pomegranate (ASW).

(after 3-9 min at 100% power) had significantly higher TPC, which increased 38.59-42.52% and 5.40-16.82%, respectively. Somewhat expected results were observed compared to other studies in red wine (Masuzawa *et al.*, 2000), strawberry juice (Tiwari *et al.*, 2009b) and kasturi lime juice (Bhat *et al.*, 2011). The concentration of phenol in sonicated model system at 20 kHz did not show any significant change, but at higher frequencies (358 kHz and 1062 kHz) it decreased significantly (Ashokkumar *et al.*, 2008). Also, Ashokkumar *et al.* (2008) proposed that hydroxyl radicals generated by acoustic cavitation and food chemicals such as phenolic compounds can be sonochemically hydroxylated. Therefore, increasing trends in phenolic content at some amplitude levels and times can be probably due to the addition of hydroxyl radicals to the aromatic ring of phenolic compounds (Bhat *et al*., 2011).

The antioxidant activity of MMSW and ASW was higher than MMSA and ASA juices, showing the importance of juice extraction method. These results are in agreement with previously reported findings that determined hydrolysable tannins including

punicalagins as main compounds influencing the antioxidant capacity of pomegranate juices (Gil *et al*., 2000). On the other hands, the amounts of punicalagin derivatives in juices obtained from whole pomegranate were higher than the juices obtained from arils. The change of antioxidant activities was measured with three different methods, which showed no significant difference between treated and untreated juices, while the TPC in the studied juices at different amplitude levels and times significantly changed. These results indicated the non-destructive effects of ultrasound on the compounds with antioxidant properties. The sonochemically generated hydroxyl radicals lead to the hydroxylation of aromatic ring of the phenolic compounds in the ortho-, meta- and para-positions. This reaction has been suggested as a reason for increasing the antioxidant properties of flavonoids and other antioxidant molecules that can be extracted from food materials (Wanasundara *et al*., 1997; Ashokkumar *et al.*, 2008). Moreover, the antioxidant activities of phenolic acids are based on free radical acceptors and chain breakers mechanisms (Wanasundara *et al*., 1997). However, Pearson correlation showed a good correlation ($r > 0.90$) between the β-carotene and DPPH results as well as previously published paper (Çam *et al*., 2009).

Conclusions

Pomegranate is a rich source of functional ingredients and thermal processing significantly affects these functional compounds. During sonication no significant differences ($p < 0.01$) in pH, TA, SSC were observed. The observed differences between the results of our study and other researches may be due to the temperature control (25 ± 1°C) during sonication, because most previous researches were conducted at moderate temperatures as a consequence of the simultaneous effect of ultrasound and ultrasound-induced heat generation. Also, degradation percentage of total and individual anthocyanins, total phenolic content, antioxidant activities of juices and colour parameters were not considerable. These results indicated the non-destructive effects of ultrasound on visual and chemical properties of pomegranate juice.

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