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Changes in hydrolysable and condensed tannins of pomegranate (*Punica granatum* L., *cv. Hicaznar*) juices from sacs and whole fruits during production and their relation with antioxidant activity

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ABSTRACT

The effects of clarification and pasteurization on hydrolysable tannins (HTs), condensed tannins (CTs) and antioxidant activities of pomegranate juices extracted from whole fruits and sacs were investigated. Tannin contents (53–85%) and antioxidant activities (18%) in juice from whole fruits (JFWF) were found higher than those in juice from sacs (JFS). In both juice samples, clarification led to significant decreases (15–74%) in total polyphenol, HT and CT contents as well as antioxidant activities, whereas pasteurization (1–52%) led to the increases, except for the CT contents. Similar to pasteurization, HT contents also increased ($r = 0.956$) as the pressure and time increased during pressing. Punicalin, α-punicalagin, β-punicalagin and ellagic acid hexoside were the major HTs in pomegranate juices. Among HTs, ellagic acid hexoside was the major HT in JFWF, whereas β-punicalagin was the major one in JFS. β-punicalagin was the most susceptible HT to clarification. Strong correlations were found between antioxidant activity values with total polyphenol contents ($r = 0.824–0.926$), and total HTs contents ($r = 0.775–0.994$) and CT contents ($r = 0.592–0.956$). Antioxidant activity of JFWF resulted mainly from HTs ($r = 0.994$), while that of JFS resulted mainly from CTs ($r = 0.956$).

1. Introduction

Pomegranate juice is produced by pressing either the whole fruits or the previously separated juicy sacs. Once the whole fruit is pressed, the high molecular weight phenolics called as tannins pass from the rinds into juice, causing astringency and bitterness in the juice (De Simon, Perez-İlzarbe, Hernandez, Gomez-Cordoves, & Estrella, 1992). Moreover, these tannins also lead to the formation of cloudy appearance in pomegranate juice during storage (Spanos & Wrolstad, 1992).

The contents and profiles of polyphenols in the pomegranate juices show differences depending on juice extraction procedure since different parts of pomegranate contain different polyphenol groups (Wang et al., 2004). The main difference between the contents and profiles of polyphenols in juice from whole fruits (JFWF) and juice from sacs (JFS) results from the polyphenol-rich pomegranate rinds. The pomegranate rind and membrane are characterized by the presence of HTs [e.g. punicalagin, punicalin, tellimagrandin, pedunculagin, corilagin and casuarinin (Wang, Ding, Liu, Xiang, & Du, 2010)], phenolic acids [(gallic acid (Gil, Tomas-Barberan, Hess-Pierce, Holcroft, & Kader, 2000), ellagic acid (Wang et al., 2004)], flavonoids [(e.g. catechin, epicatechin, kaempferol, luteolin, rutin, quercetin and naringin (Lansky & Newman, 2007)] and the absence of typical pomegranate anthocyanins (Gil et al., 2000). On the other hand, the sacs of pomegranates contain galloylglucose, HTs and anthocyanins (Gil et al., 2000).

Fischer, Dettmann, Carle, and Kammerer (2011) recently reported the polyphenol profiles (anthocyanins, gallotannins, ellagitannins, gallagyl esters, hydroxycinnamic acids, hydroxybenzoic acids and dihydroxy flavonols) of JFWF and JFS, and the effects of different processing steps (clarification, pressing pressure and storage) on the contents and profiles of these juice samples. The study conducted by Fischer et al. (2011) is the only study in the literature, showing the effects of juice extraction procedure and clarification on polyphenol profiles of JFWF and JFS. Although the dependence of polyphenol content and profile on variety is well-

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known (Mousavinejad, Emam-Diomeh, Rezaei, & Khodaparast, 2009), pomegranate variety in their study was unknown. Another significant difference between our and Fischer's studies was the clarification methods (cold and hot-clarification, respectively) used in these studies. Although the usage of pectinase and bentonite is not necessary for the clarification of pomegranate juice since pomegranates contain insignificant amounts of pectin (0.2–0.4 g/kg) and protein (Benk, 1971), pomegranate juice samples in their study were hot-clarified with gelatin, bentonite and silica sol after enzymatic treatment. Since the primary compounds causing turbidity in pomegranate juices are the polyphenols, more specifically high molecular weight tannins, the removal of these tannins needs to be targeted during the clarification of pomegranate juices, especially JFWF. For the removal of tannins, cold-clarification of pomegranate juices with only gelatin is sufficient (Özkan, Yemenicioğlu, Asefi, & Cemeroğlu, 2002).

Hot-clarification (ca. 50 °C) may lead to the differences in polyphenol profiles and contents of pomegranate juices. For example, anthocyanins are degraded at temperatures above 40 °C and degradation products of anthocyanins (e.g. protocatechuic acid, 2,4-dihydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid etc.) are formed (Seeram, Bourquin, & Nair, 2001). Since the degradation products of anthocyanins are the polyphenols, we cannot know whether the polyphenols are originally present in the pomegranate juices or formed during hot-clarification. In fact, in Fischer's study, although some polyphenols (gallotannins, hydroxycinnamic acids, dihydroflavonoids) were not detected in unclarified juice, they were detected and quantified in hot-clarified juice. This showed that hot-clarification might lead to the difference in polyphenol profile and content. In the present study, pomegranate juices from Hicaznar variety, which is highly preferred variety by Turkish fruit juice industry, were cold-clarified (using only gelatin), preventing the anthocyanin degradation due to low temperature (5 °C) applied during cold-clarification.

To date, no study on the changes of total polyphenols, hydrolysable tannins (HTs), condensed tannins (CTs) and antioxidant activity values of pomegranate juices from Hicaznar variety during processing has been reported. The primary purpose of this study was to determine the changes in those parameters during clarification and pasteurization of JFS and JFWF. Additionally, the effects of different yields (33.2 and 39.2%) and pressing programs (namely 120–480 kPa for 25 min and 120–240 kPa for 15 min) on the profile and the content of HTs were investigated. The secondary purpose was to identify the HT profile of this major Turkish pomegranate variety.

2. Materials and methods

2.1. Chemicals and reagents

Standards of gallic acid and punicalagin, and Sephadex LH-20 were purchased from Sigma (St. Louis, MO, USA). Standard of punicalin was purchased from Biopurify Phytochemicals Ltd. (Sichuan, China). Standard of ellagic acid was purchased from Fluka (Seelze, Germany). Gelatin (A type, 80–100 Bloom strength) was obtained from Döhler (Geisenheim, Germany). All reagents used for liquid chromatography were HPLC grade and purchased from Merck (Darmstad, Germany). All other reagents were analytical grade and obtained from Merck.

2.2. Samples

2.2.1. Juices from whole fruits (JFWF) and sacs (JFS)

Pomegranates (*Punica granatum* L., cv. Hicaznar) were obtained from Alata Horticultural Research Institute (Erdemli, Mersin, Turkey) and immediately processed into juice in the fruit juice pilot plant at Ankara University as reported previously (Turfan, Türkyılmaz, Yemiş, & Özkan, 2011). A flow diagram of pomegranate juice processing is shown in Fig. 1.

In the present study, the pomegranate juice produced from only sacs was called as "juice from sacs, JFS." The pomegranates (168 kg) with rinds were cut into quarters and pressed on a-rack-and-cloth-press (Bucher-Guyer, Niederweningen, Switzerland) in the fruit juice pilot plant by gradually increasing the pressure up to a maximum of 1.1 MPa within 30 min resulting in 59.25 kg of pomegranate juice corresponding to 35.2% yield. The resulting juice was filtered through muslin cloth to remove the particles and this juice was called as "juice from whole fruits, JFWF."

The resulting cloudy juices from sacs and whole fruits were clarified and pasteurized as described by Turfan et al. (2011). After pasteurization, the juice samples were immediately cooled to room temperature.

2.2.2. Juices produced with different yields and pressing programs

For each pressing program, 26.5 kg of the pomegranates with rinds were cut into four pieces and processed into juice as reported by Türkyılmaz, Tağı, Dereli, and Özkan (2013). Two different pressing programs were applied, namely 120–480 kPa for 25 min and 120–240 kPa for 15 min (Table 1). Juice yield (%) was calculated by the following equation:

$$\text{Juice yield}\,(\%) = \frac{\text{Weight of unclarified pomegranate juice}}{\text{Weight of pomegranates with rinds}}(100)$$

Respective juice yields were 39.2 and 33.2% (Table 1).

2.2.3. Concentrates

Pomegranate juice concentrates were obtained from clarified and unclarified JFWF. They were concentrated to 69 °Brix by a rotary low-pressure evaporator (Heidolph Laborota 4003, Schwabach, Germany) at 40 °C and 20 mm-Hg pressure. Then, the resulting concentrate was heated up to 70 °C to prevent microbial spoilage during storage, and the hot concentrate was aseptically transferred into autoclaved sterile glass jars (30–35 g concentrate for each jar) which were immediately closed with air tight lids and the jars containing concentrate samples were then cooled immediately to room temperature in an iced-water bath.

2.3. Compositional analysis

The total soluble content (°Brix) of pomegranate juice samples was determined by an automatic digital refractometer (Atago Rx-7000α, Tokyo, Japan). pH was measured potentiometrically with a pH meter (WTW Inolab Level 1, Weilheim, Germany). Titratable acidity was determined according to the method outlined by IFU (1968) and expressed as "*g anhydrous citric acid/100 mL or g sample*."

2.4. Total polyphenol content

Polyphenol content of the samples was determined by the Folin-Ciocalteu method as reported previously (Türkyılmaz et al., 2013). Results were calculated and expressed as "*mg of gallic acid equivalent per L juice*." Total polyphenol measurements were replicated two times.

2.5. CT content

CTs (proanthocyanidins) were measured using the vanillin assay described by Tanner and Brunner (1979). The analysis was performed as explained by Türkyılmaz et al. (2013). The content of the

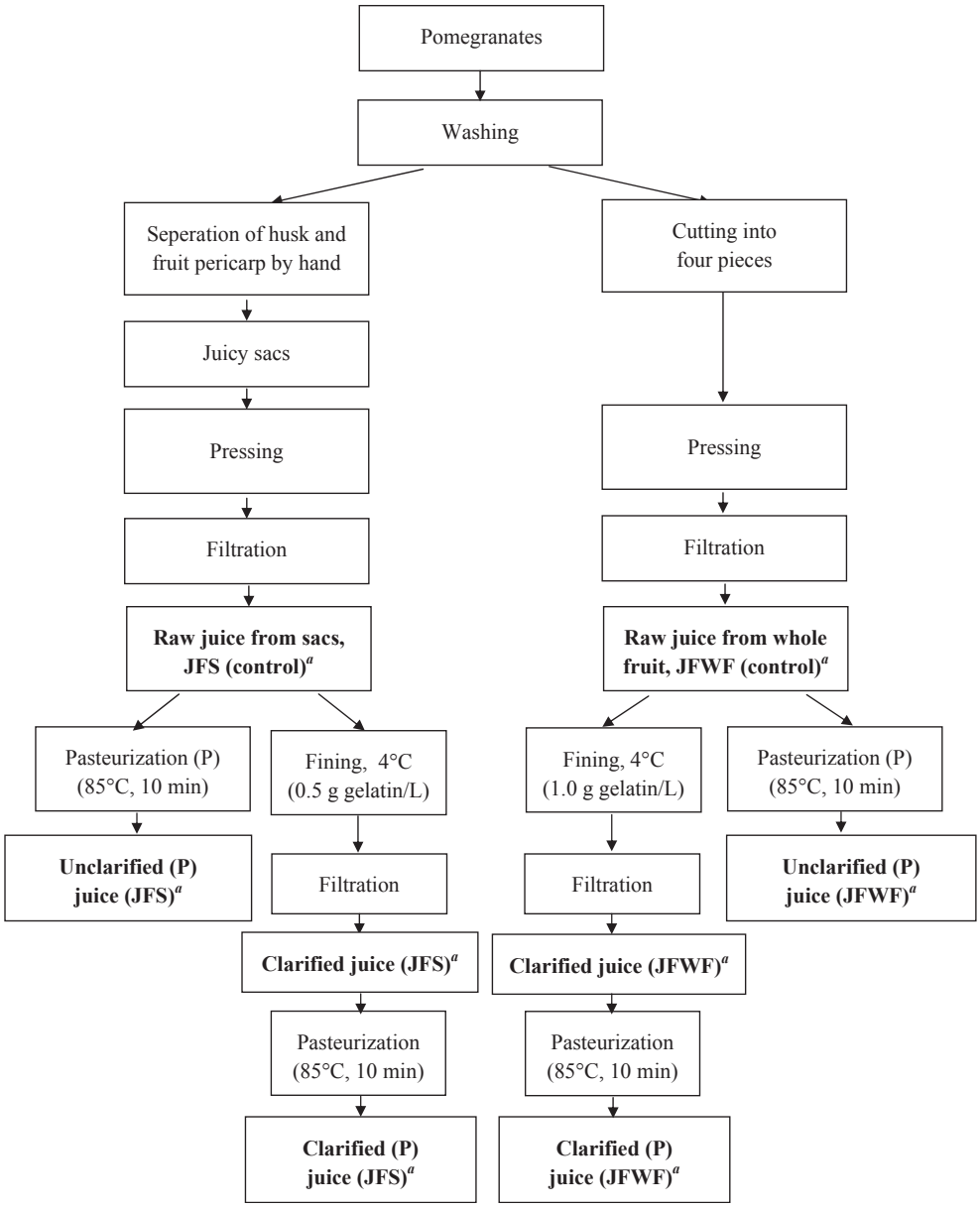

Fig. 1. Flow diagram for the processing of pomegranate juice samples. [a]Samples taken for analysis.

Table 1
Pressure applied on table surface (press pressure) and changes in yield (%) of pomegranate juice depending on pressure and time.

Pomegranate juice samples	Press pressure (kPa)[a]	Time (min)	Pomegranate juice amount (kg)	Yield (%)
P11	120	5.0	5.0	18.9
P12	180	5.0	2.2	8.3
P13	240	5.0	1.6	6.0
Y1	–	15.0	8.8	**33.2**
P21	120	5.0	4.5	17.0
P22	180	5.0	2.2	8.3
P23	240	5.0	1.6	6.0
P24	300	5.0	1.0	3.8
P25	480	5.0	1.1	4.1
Y2	–	25.0	10.4	**39.2**

[a] Pressure on table surface.

total CTs was expressed as "*mg of (+) catechin per L juice*." All samples were analyzed in two replications.

2.6. Antioxidant activity

Antioxidant activity was measured according to ABTS method described by Arts, Haenen, Voss, and Bast (2001). The detail of the analysis was as reported by Türkyılmaz et al. (2013). Antioxidant activity of samples was expressed as "*mmol Trolox per mL juice*."

2.7. HPLC separation of HTs

2.7.1. HT purification

HTs were isolated by using Sephadex LH-20 (Sigma–Aldrich, St. Louis, MO, USA) according to the methods outlined by Spanos and Wrolstad (1990) with a slight modification in column size, amount of column matrix and sample. An amount of 0.6 g of Sephadex LH-

20 was swollen in 10 mL water for overnight at 4 °C, and then the slurry was packed into a glass column (2.5 × 10 cm Kontes Flex-column, Fischer Scientific, Pittsburg, PA, USA) by elution with water giving a approximate height of 2.5 cm chromatographic media. The gel bed was rinsed with 5 mL of water, and then 2 mL of sample is loaded onto the column. The column was then washed with 30 mL aqueous methanol (80:20, v/v) to remove impurities, i.e., the sugars and low molecular weight phenols (polymeric compounds and phenolic acids). HTs were eluted from the column with 15 mL methanol. Flow of aliquots in column was faciliated with a peristaltic pump (H.R. Flow Inducer, Watson-Marlow Ltd., Cornwall, England) attached to the top reservoir of the column to obtain a flow rate of 0.3–0.4 mL/min. The solvent from the HTs fraction was removed in a vacuum rotary evaporator (Heidolph Laborota 4003) set at 35 °C, and the dry residue was dissolved in 2 mL of HPLC grade water. Finally, the sample was filtered through a 0.22 μm PVDF (polyvinylidene fluoride) filter (Sartorious AG, Goettingen, Germany) into an amber-colored auto-sampler vial and immediately used for HPLC analyses.

2.7.2. Instrumentation and chromatography

Separation and quantification of HTs were performed on a high performance liquid chromatography (Agilent 1200 series, Waldbronn, Germany) with a binary pump, a photo diode array (PDA) detector, a thermostatted auto sampler, a degasser and a thermostatted column compartment. The chromatographic data were recorded and processed on an Agilent 1200 series ChemStation rev.B.02.01 software. HTs were separated on a C_{18} (5 μm) column (250 × 4.6 mm) (Phenomenex Inc., Los Angeles, CA, USA) with a C_{18} (5 μm) guard column (4 × 3 mm, 5 μm) (Phenomenex Inc.). The eluents used were (A) 9.5 g/L KH_2PO_4 (pH 2.5) and (B) methanol with a flow rate of 1 mL min^{-1}. Separation was performed with gradient elution using a modification of the elution profile described by Spanos and Wrolstad (1990). The linear gradient program for the separation of pomegranate HTs was as follows: from 0% to 10% B in 5 min, from 10% to 20% in 5 min, holding at 20% B for 10 min, from 20% to 25% B in 10 min, from 25% to 40% B in 10 min, holding at 40% B for 20 min. Sample injection volume was 50 μL, column temperature was 25 °C, and the detector was set at 280 and 320 nm.

Identification of punicalin and punicalagins were carried out by comparing retention times and absorption spectras of unknown peaks with external reference standards. Quantification of HTs was carried out using the calibration curves of external reference standards; punicalin ($R^2 = 0.998$), α-punicalagin ($R^2 = 0.999$) and β-punicalagin ($R^2 = 0.999$). The calibration curves for each HT standard contained 6 data. Quantification of total HTs by HPLC was calculated based on ellagic acid.

As mentioned above, 3 out of 4 HTs in pomegranate juice samples were identified by comparing retention times and absorption spectras of unknown peaks with external reference standards. However, since the commercial standard for ellagic acid hexoside was not available at the time of analysis, mass spectrophotometric detection was carried out for ellagic acid hexoside. The mass/charge (m/z) ratio of 463 that was previously determined for ellagic acid hexoside by Gil et al. (2000) was used. Mass experiments were performed on a Waters NanoAcquity UPLC system with a Waters Xevo TQ triple quadrupole tandem mass spectrometer (Waters Co., Milford, MA, USA) equipped by an electrospray ionization source (ESI). The mobile phase consisted of solvent A including of 1 g/L formic acid in water-acetonitrile (90:10, v/v) and solvent B including of 1 g/L formic in acetonitrile. A 10-min linear gradient elution program was applied using a UPLC BEH analytical column (C18, particle size 1.7 μm, i.d. 100 μm, length 100 mm, Waters Co.) at a constant temperature of 40 °C. The flow rate was 0.4 mL/min, and injection volume was 10 μL. The gradient elution profile was as follows: 0–0.5 min 100% A; 0.5–7 min linear increase to 100% B; 7–8 min 100% B; 8–8.01 min linear decrease to 0% B; 8.1–10 min 100% A. ESI-MS/MS experiments were carried out in the negative mode and the following optimum conditions were used: cone voltage, 35 V; capillary voltage, 2 kV; desolvation gas flow, 800 L/h; desolvation temperature, 400 °C; source temperature, 150 °C and the scan range, 120–2000 m/z.

2.8. Statistical analyses

Results from polyphenol contents were analyzed using the Minitab statistical software, version 14 (Minitab Inc., State College, PA, USA). Juice processing (JFS and JFWF), clarification, pasteurization, pressing programs and yields were considered as the main effects. Statistical differences among means were determined by the Duncan's multiple range test at the 1% significance level.

3. Results and discussion

3.1. Effects of juice extraction and concentration processes on some properties

The initial values of soluble solid contents (°Brix), pH and titratable acidity for unclarified JFWF and JFS were 16.39–16.46 (g/100 g), 3.35–3.39 and 1.10–1.10 (g/100 mL), respectively. Clarification and pasteurization had no significant effect ($p > 0.01$) on these parameters. The initial values of soluble solid contents (°Brix), pH and titratable acidity for pomegranate juice concentrates obtained from unclarified and clarified JFWF were 64.31–65.47 (g/100 g), 3.36–3.37, and 4.54–4.56 (g/100 mL), respectively. There were no significant ($p > 0.01$) differences between these parameters of pomegranate juice concentrates produced from unclarified or clarified juices.

3.2. Effect of juice extraction process on polyphenols

Total polyphenol, HT and CT contents of JFWF were 2773, 1671 and 127 mg/L, respectively (Table 2), while these values in JFS were 2073, 255 and 60 mg/L, respectively (Table 2). Total polyphenol, HT and CT contents of JFWF were 25, 85 and 53%, respectively, higher than those of JFS ($p < 0.01$). These differences are due to the passing of various forms of polyphenols from pomegranate rinds into juice

Table 2
Changes in polyphenol compounds and antioxidant activities of JFWF and JFS during processing.

Samples	Total phenolic content (mg/L)	Hydrolysable tannins content (mg/L)	Condensed tannins contents (mg/L)	Antioxidant activity (mmol Trolox/mL)
JFWF				
Control	2773 ± 31[a]	1671 ± 218	127 ± 6	26.8 ± 0.0
Clarified	1726 ± 2	432 ± 70	108 ± 3	18.2 ± 0.2
Unclarified-pasteurized	3023 ± 35	1525 ± 52	90 ± 16	25.0 ± 0.2
Clarified-pasteurized	2023 ± 127	554 ± 53	39 ± 6	18.1 ± 0.1
JFS				
Control	2073 ± 8	255 ± 14	60 ± 2	22.0 ± 0.2
Clarified	1590 ± 20	76 ± 6	40 ± 1	14.1 ± 0.3
Unclarified-pasteurized	2393 ± 14	432 ± 108	52 ± 1	20.0 ± 0.2
Clarified-pasteurized	1740 ± 16	106 ± 5	35 ± 6	15.1 ± 0.5

[a] Values are means ± standard deviation of two determinations.

during pressing of the fruits with rinds. Pomegranate rinds contained much higher amounts of HTs than the sacs. Similar to our results, Fischer et al. (2011) also reported that total polyphenol and HT contents of JFWF were higher (ca. 95%) than those of JFS. In their study, although total polyphenol and HT contents of JFWF (8707 and 8539 mg/L, respectively) were found 3–5 times higher than those in our study, total polyphenol content of JFS was found 4 times lower that in our study. As known, variety has a profound effect on the polyphenol contents of fruits and vegetables. Therefore, the large differences between the polyphenol contents of pomegranate juices investigated in both studies could be attributable to the varietial differences.

Tannins in both juice samples consisted mainly of HTs. Four major HTs in JFWF and JFS were identified (in the HPLC elution order) as punicalin, α-punicalagin, β-punicalagin and ellagic acid hexoside (Fig. 2). Although HT profiles of JFWF and JFS were similar, predominant HT and individual HT contents of these samples were quite different. While the predominant HT of JFWF was ellagic acid hexoside (797 mg/L, 48% of total peak area), that of JFS was β-punicalagin (221 mg/L, 70% of total peak area). This finding agrees with that of Fischer et al. (2011). On the other hand, the predominant HT in JWFW form Wonderful variety was determined as punicalagin isomer (Gil et al. 2000). In addition, Gil et al. (2000) found that ellagic acid content of JFWF was found very low (37.9 mg/L, 2.8% of total HT content). Thus, the major HT and HT contents of pomegranate parts also significantly depend on the variety. Besides variety, juice extraction process also affected the individual HT contents in pomegranate juices. Punicalin, α-punicalagin, β-punicalagin and ellagic acid hexoside contents of JFWF were 10.0, 14.6, 2.5 and 18.1 times higher than those of JFS, respectively (Table 3).

3.3. Changes in polyphenol composition during clarification with gelatin and pasteurization

Total polyphenol, HT and CT contents of clarified JFWF were 1726, 432 and 108 mg/L, respectively, whereas the respective values in clarified JFS were 1590, 76 and 40 mg/L (Table 2). Clarification with gelatin caused substantial decreases in total polyphenols (38%, from 2773 to 1726 mg GAE/L), in HTs (74%, from 1671 to 432 mg/L) and in CTs (15%, from 127 to 108 mg/L) of JFWF, respectively. Similar reductions for JFS were detected in total polyphenols (23%, from 2073 to 1590 mg/L), in HTS (70%, from 255 to 76 mg/L) and in CTs (33%, from 60 to 40 mg/L). Clarification with gelatin significantly reduced total polyphenol, HT and CT contents of juice samples ($p < 0.01$). The decreases in these polyphenol groups occured as a result of the interaction between the positively and negatively charged gelatin and polyphenols, respectively, and this interaction led to flocculation.

The effect of clarification on the polyphenol groups of JFWF and JFS was different from each other. Reductions in total polyphenol and HT contents of JFWF were higher than those in JFS. This was attributable to the use of gelatin at different concentrations to clarify JFWF and JFS samples. In this study, we aimed to reach the similar turbidity levels in both juice samples after the clarification with gelatin. Therefore, preliminary tests were carried out with different gelatin concentrations, and similar turbidity values in JFWF (1.7 NTU) and JFS (2.2 NTU) were obtained by the use of gelatin at 1 and 0.5 g/L concentrations, respectively.

Interactions between tannins and gelatin are complex and many factors (relative amounts of gelatin and tannin, pH, and salt concentration) affect this interaction as well as the following precipitation (Calderon, Van Buren, & Robinson, 1968). Among the factors, the mass ratio of tannins to gelatin plays very important role in this precipitation, since the ability of tannins to precipitate with gelatin increases with the increased amount of tannins (Yi, Cheng, & Xing, 2006). To form the precipitate, the mass ratio of tannins to gelatin should be over 0.8 (Yi et al., 2006). In the present study, these ratios for JFWF and JFS were 1.671 (1.671:1) and 0.510 (0.255:0.5), respectively. Thus, the precipitate was mainly formed in JFWF and the precipitation led to the removal of not only HTs but also other polyphenols from the samples. Therefore, although reductions in total HT contents of JFWF (74%) and JFS (70%) were similar, reduction in total polyphenol content of JFWF (38%) was much higher than that of JFS (23%).

Among individual HTs, β-punicalagin was the most susceptible to the clarification with gelatin, while punicalin in JFS was the most stable. The differences between the stabilities of individual HTs may be due to differences in the number of polyphenol hydroxyl groups in HT molecules. As soon as gelatin is added to fruit juice for clarification, tannins in the juice attack to bind of the gelatin molecules and the interaction occurs between these two molecules (Yi et al., 2006). The structure of tannins enables the hydrogen binding through the interaction of polar carboxylic groups in gelatin with the free hydroxyl group in tannin molecule. As the number of free hydroxyl groups increases in tannins, the possibility of tannins to interact with gelatin molecules will also increase.

Since HTs contain a lot of free hydroxyl groups, they are very good candidates for hydrogen binding. In fact, punicalagin and punicalin, i.e., the highest amount of the tannins present in pomegranate juice, contain 17 and 13 free hydroxyl groups, respectively. Since the number of free hydroxyl groups in punicalagin was greater than that of punicalin, the possibility of punicalagin to form complexes with gelatin will be higher than punicalin due to the greater number of its free hydroxyl groups. As a result of this interaction, higher decreases in β-punicalagin will occur during the clarification of pomegranate juice due to the higher interaction of β-punicalagin with gelatin as compared to that of punicalin.

Unlike clarification effect, the amounts of total polyphenols (8–15%) and most of the individual HTs (1–52%) in JFWF and JFS increased after pasteurization, whereas CT contents decreased (12–64%). The results from previous studies (Dawes & Keene, 1999; Sentandreu, Navarro, & Sendra, 2007) indicated that the effect of pasteurization on polyphenol content showed differences depending on processing and food matrix. For example, while pasteurization (90 °C for 30 s) of orange juice has negligible effects on the polyphenol content (Sentandreu et al., 2007), an ultra pasteurized (high-temperature short-time) kiwi juice contained

Table 3
Changes in individual HTs of JFWF and JFS during processing.

Samples	Punicalin (mg/L)	α-punicalagin (mg/L)	β-punicalagin (mg/L)	Ellagic acid hexoside (mmol Trolox/mL)
JFWF				
Control	42 ± 7.4[a]	171 ± 22	550 ± 84	797 ± 92
Clarified	9.3 ± 0.1	54 ± 9.4	118 ± 27	209 ± 27
Unclarified-pasteurized	42 ± 3.9	141 ± 19	465 ± 3	788 ± 3
Clarified-pasteurized	19 ± 1.7	71 ± 13	131 ± 17	274 ± 11
JFS				
Control	4.2 ± 0.0	11.7 ± 1.2	221 ± 11	44 ± 2.9
Clarified	2.9 ± 1.5	7.7 ± 0.4	46 ± 1	21 ± 3.9
Unclarified-pasteurized	7.5 ± 0.3	24.5 ± 4.7	341 ± 90	89 ± 21
Clarified-pasteurized	3.6 ± 0.3	10.1 ± 0.7	64 ± 4	30 ± 0.1

[a] Values are means ± standard deviation of two determinations

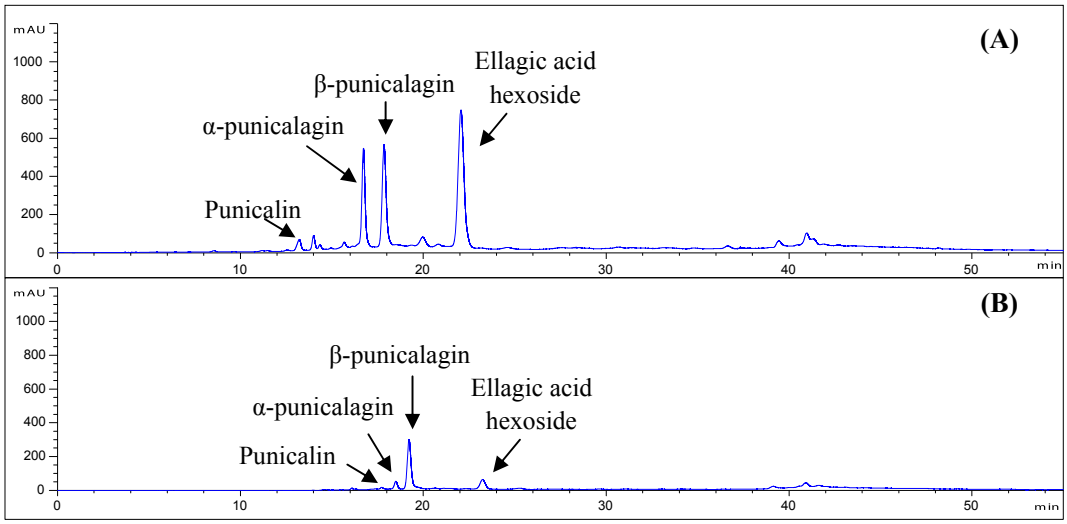

Fig. 2. HPLC chromatograms of HTs. **A:** JFWF, **B:** JFS.

higher levels of phenolic acids in comparison to the fresh juice (Dawes & Keene, 1999).

There may be few reasons for the increasing effect of pasteurization on polyphenols. The logical answer would be the inactivation of PPO during pasteurization, which prevented further loss of polyphenol compounds. In fact, much higher individual HT contents in unclarified pasteurized juices than those in unclarified non-pasteurized (control) juices also confirmed this assumption. Individual HT contents of all samples rose after pasteurization, except for unclarified JFWF whose individual HT contents showed no significant differences after pasteurization. Among the individual HTs, α-punicalagin content showed the highest increase in unclarified JFWF (17%) and JFS (52%) after pasteurization, whereas β-punicalagin (15% in JFWF; 35% in JFS) and punicalin (0% in JFWF; 44% in JFS) showed relatively lower changes.

Another reason may be the biochemical reactions which caused the formation of new polyphenol compounds during pasteurization. During processing, some reactions (i.e., hydroxylation, methylation, isoprenylation, dimerization and/or glycosylation) can induce the modifications among the different polyphenol compounds (Rice-Evans, Miller, & Paganga, 1997). Moreover, polyphenols can also be formed by the partial degradation of the combined forms during processing of fruits or by loosing the moieties between phenols and sugars (Dugo et al., 2005). Despite significant decrease in CT content and slight decrease in total HT of unclarified JFWF after pasteurization, increase in total polyphenol content of this sample confirmed this assumption.

3.4. Effect of juice yield on HT profile and content

Much higher total HT in JFWF than that in JFS clearly showed that HTs at high concentration passed from rinds to juice during pressing. High level of HT in JFWF, more specifically in rinds, inclined us to study the effects of different yields (33.2 and 39.2%) and pressing programs (namely 120–480 kPa for 25 min and for 15 min) on individual HTs during the production of JFWF. There were no significant differences in total HT contents (219.1–244.8 mg/L juice) of JFWF samples produced with different yields ($p > 0.01$). This was probably because total HT contents of JFWF samples in the first two steps (120 kPa for 5 min and 180 kPa for 5 min) of pressing programs were similar (Table 4). In addition, a substantial portion (64–82%) of JFWF was obtained from these two steps of pressing programs (Table 1).

The total HT contents of JFWF samples obtained from each step of pressing programs ranged from 121.7 to 287.0 mg/L juice ($p < 0.01$). Thus, HT contents of JFWF depended on the pressure applied during pressing (Table 4). A linear correlation was found between pressing pressure and total HT contents ($y = 44.843\ x + 81.415$, $r = 0.956$). Increase in pressing pressure by 120 kPa resulted in 19% increase in total HT contents. Similar to our results, substantial increase in total HT contents were observed as a result of elevated pressures during pressing of pomegranate juice, ranging from 741 mg/L (1 MPa) to 16 113 mg/L (15 MPa) (Fischer et al., 2011). The increase in total HT contents by higher pressing pressure is mainly attributed to the extraction of the water-soluble HTs called

Table 4
Changes in individual HTs of pomegranate juice samples depending on pressing pressure-time.

Samples	Punicalin (mg/L)	α-punicalagin (mg/L)	β-punicalagin (mg/L)	Ellagic acid hexoside (mmol Trolox/mL)	Total HTs (mg/L)
P11	12.7 ± 0.4[a]	52.5 ± 0.4[a]	221.0 ± 24[a]	195.1 ± 2.9[a]	123.5 ± 3.8[a]
P12	14.8 ± 1.4	80.8 ± 0.8	166.6 ± 13.1	305.6 ± 1.3	154.4 ± 3.9
P13	18.6 ± 3.2	109.6 ± 7.9	165.3 ± 19.5	437.1 ± 30.8	204.9 ± 1.7
Y1	15.7 ± 0.3	91.9 ± 0.7	199.5 ± 1.2	318.1 ± 1.7	219.1 ± 42.2
P21	11.4 ± 1.7	52.1 ± 1.3	222.9 ± 18.4	188.7 ± 6.3	121.7 ± 5.8
P22	17.5 ± 0.3	90.1 ± 0.7	206.8 ± 15.2	331.4 ± 3.4	176.3 ± 4.3
P23	20.2 ± 1.0	89.9 ± 2.6	155.4 ± 1.3	354.5 ± 62	172.1 ± 2.4
P24	27.8 ± 0.5	130.3 ± 5.4	153.9 ± 0.7	538.5 ± 6.4	241.8 ± 4.5
P25	30.5 ± 1.0	153.9 ± 2.9	150.6 ± 7.3	667.6 ± 0.2	287.0 ± 0.3
Y2	87.9 ± 0.9	115.9 ± 0.4	229.5 ± 0.9	319.1 ± 1.9	244.8 ± 5.5

[a] Values are means ± standard deviation of two determinations.

ellagitannins (Gil et al., 2000) from the rinds and membranes, resulting them to pass into pomegranate juice in the proportion to the force applied during pressing.

The pressing pressure-time had different effects on individual HTs. As pressing pressure-time increased, punicalin (9–35%), α-punicalagin (15–42%) and ellagic acid hexoside (6–43%) contents increased, while β-punicalagin (7–25%) content decreased. Substantial amount of β-punicalagin in rind passed to juice in the first step of pressing programmes. Although much lower amount of β-punicalagin passed to juice after the first steps of pressing, even this low amount of β-punicalagin was higher than punicalin and α-punicalagin contents. Strong correlations were also found between punicalin ($y = 5.2311\ x + 7.6698$, $r = 0.932$), α-punicalagin ($y = 27.129\ x + 31.64$, $r = 0.947$), β-punicalagin ($y = -19.822\ x + 230.25$, $r = 0.800$) and ellagic acid hexoside ($y = 131.12\ x + 69.989$, $r = 0.965$) with pressing pressure-time.

3.5. Changes in antioxidant activity during clarification with gelatin and pasteurization

Differences between antioxidant activity values of JFWF and JFS, and changes in antioxidant activity of both juice samples during various stages of pomegranate juice processing were given in Table 2. Antioxidant activity of unclarified JFWF (26.8 mmol trolox/mL) was 1.2 times higher than that of JFS (22.0 mmol trolox/mL). This could be attributable to 1.3 times higher polyphenol contents of JFWF. Clarification and pasteurization caused to similar changes in antioxidant activity of both juice samples. Clarification led to significant reduction (32–36%) in antioxidant activity values of juice samples ($p < 0.01$), while pasteurization (7–9% changes) showed no significant effect ($p > 0.01$). Similar to our results, slight decreases in antioxidant activity as evaluated by the ORAC assay were observed after pasteurization of unclarified (7%) and clarified (1%) blueberry juices (Brownmiller, Howard, & Prior, 2008).

These changes in antioxidant activity values could result from the changes in total polyphenol, HT and CT contents. In fact, there were mostly strong correlations between antioxidant activity values with total polyphenol contents ($r = 0.926$ for JFWF, $r = 0.824$ for JFS), total HT contents ($r = 0.994$ for JFWF, $r = 0.775$ for JFS) and CT contents ($r = 0.592$ for JFWF, $r = 0.956$ for JFS). The correlation coefficients indicated that antioxidant activity of JFWF resulted mainly from HTs ($r = 0.994$), while that of JFS resulted mainly from CTs ($r = 0.956$). Although the total polyphenol content of JFWF was only 1.3 times higher than that of JFS, total HT content of JFWF was 6.5 times higher. This finding indicated that the non-colored polyphenol compounds of JFWF consisted mainly of HTs, whereas those of JFS did not contain HTs. Therefore, there may not be very strong correlation ($r = 0.592$) between antioxidant activity and total HT content of JFS.

Of the HTs, β-punicalagin showed the strongest correlation with antioxidant activity values of JFWF ($r = 0.999$) and JFS ($r = 0.833$). Seeram et al. (2005) found the antioxidant activity values (TEAC assay) of total polyphenols, punicalagin and ellagic acid in pomegranate juice were found as 100, 90 and 40 μmol trolox equivalent, respectively. Similar to our results, this study also showed punicalagin had the highest antioxidant activity among polyphenols of pomegranate juice. While there were also strong correlations between punicalin ($r = 0.958$), α-punicalagin ($r = 0.990$) and ellagic acid hexoside contents ($r = 0.985$) with antioxidant activity values of JWFW, no strong correlations ($r = 0.544$–0.637) were found between the individual HTs and antioxidant activity values of JFS. These weak correlations between antioxidant activity values and the individual HTs in JFS, except for β-punicalagin (46–341 mg/L), may be due to very low concentration (2.9–89 mg/L) of these compounds in JFS.

Table 5
Changes in polyphenol compounds and antioxidant activities of pomegranate juice concentrate samples during processing.

Analyzed parameters	Unclarified juice concentrate	Clarified juice concentrate
Phenolic contents (mg/L)		
Total phenolics	12061 ± 122[a]	8256 ± 53
Hydrolysable tannins (HTs)	1369 ± 45	650 ± 28
Condensed tannins (CTs)	516 ± 18	311 ± 18
Individual HT contents (mg/L)		
Punicalin	112 ± 39	80 ± 11
α-punicalagin	499 ± 175	289 ± 35
β-punicalagin	1414 ± 470	442 ± 109
Ellagic acid hexoside	2107 ± 700	1088 ± 75
Antioxidant activity (mmol Trolox/mL)	117 ± 11	84 ± 11

[a] Values are means ± standard deviation of two determinations.

3.6. Effect of clarification with gelatin on polyphenols and antioxidant activities of pomegranate juice concentrates

There were significant differences between total polyphenol, HT and CT contents of the concentrate samples ($p < 0.01$). The respective values of total polyphenol, HT and CT in unclarified juice concentrate were 12061, 1369 and 516 mg/L (Table 5), while these values in clarified juice concentrate were 8256, 650 and 311 mg/L, respectively. Total polyphenol, HT and CT contents of unclarified juice concentrate were 31, 52 and 40% higher than those of clarified juice concentrate. These differences are due to the interaction between gelatin and polyphenols.

Similar to pomegranate juice samples, four major HTs in unclarified and clarified concentrates were identified (in the HPLC elution order) as punicalin, α-punicalagin, β-punicalagin and ellagic acid hexoside. The predominant HT of juice concentrates was ellagic acid hexoside. Although the HT profiles of concentrate samples were the same, individual HT contents in these samples were different. Punicalin, α-punicalagin, β-punicalagin and ellagic acid hexoside contents in unclarified juice concentrate were 1.4, 1.7, 3.2 and 1.9 times higher than those of clarified juice concentrate, respectively. The most stable HT to the clarification with gelatin was found to be punicalin, followed by α-punicalagin, ellagic acid hexoside and β-punicalagin. Antioxidant activity value of concentrate sample obtained from clarified juice also decreased by 28% (Table 5) as compared to concentrate sample obtained from unclarified juice. This finding was parallel to the reduction in the polyphenol contents of concentrate sample obtained from clarified juice.

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