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Sugar and anthocyanin characterization of four Iranian pomegranate (*Punica granatum* L.) varieties using HPLC System

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Abstract

Among all types of fruit, pomegranate has been known as an excellent fruit due to its high valued nutraceutical components; i.e. anthocyanins, phenolic compounds and organic acids. This research aimed to measure anthocyanins and sugars in different varieties of pomegranate. Four cultivars were Vahshe Kane Tehran, Gorche Shahvar Yazdi, Shirin Shahvare Yazd and Pust sefid. High performance liquid chromatography (HPLC) was used in order to analyze the samples for measuring their components. Cyanidin 3-glucoside, cyanidin 3,5-diglucoside, delphinidin 3-glucoside, pelargonidin 3-glucoside and pelargonidin 3,5-diglucoside were detected in all samples as major anthocyanins. However, their quantities were different such that cyanidin 3,5-diglucoside was the main pigment, followed by cyanidin 3-glucoside and delphinidin 3-glucoside, while pelargonidin derivatives were always present in small amounts. Pust Sefid and Shirin Shahvare Yazd cultivars contained more anthocyanin compared to Gorche Shahvar Yazdi and Vahshe Kane Tehran. Results showed Fructose, glucose and sucrose were major sugars of the cultivars. Vahshe Kane Tehran had the highest fructose and sucrose content, while Gorche Shahvar Yazdi had the highest glucose content. Sugar content in Gorche Shahvar Yazdi and Vahshe Kane Tehran was more than that in Pust sefid and Shirin Shahvare Yazd cultivars.

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Introduction

Iran commercially covers a large number types of fruit especially pomegranate that its total production is 650,000 – 680,000 tons per annum (Mousavinejad *et al*., 2009). Pomegranate is a rich source of functional ingredients involving polyphenols, carotenoids, diet fibers, pectin and sugars (Mousavinejad *et al*., 2000; Gil MI *et al*, 2000), Its juice is known to be a major source of phenolic compounds, where anthocyanins are the most important, especially 3-glucosides and 3,5-diglucosides of delphinidin, cyanidin, and pelargonidin (Du *et al*., 1975). Anthocyanins (ACs) are phenolic compounds that are widely distributed in fruit and vegetables. Anthocyanins have a single circular structure that is called cyanidin and all of them are derived from this molecule through the addition and removal of hydroxyl groups, methylation of hydroxyl groups or sugar concentrations (Hulme, 1970). Anthocyanins are imparting color to plant (Hulme, 1970), and have an array of health–promoting benefits (Mertens-Talcott *et al*., 2006). Anthocyanin acts as phytochemical antioxidant with anticancer and anti–atherosclerotic effects (Kong et al., 2003). Some positive therapeutic effects of anthocyanins are somewhat related to the antioxidant properties (Miguel et al., 2004). Pomegranate juice is nutritionally an important beverage due to its phenolic compounds such as ACs, ellagic acid and tannins (Mousavinejad *et al*., 2009). Three types of Anthocyanins; Cyanidin (causing red or dark red), Delphinidin (causing purple or violet) and Pelargonidin (causing bright red to orange red), and their derivatives were the dominant pigment in various parts of pomegranate, especially its juice (Hulme, 1970). Gil *et al* (2000) measured the concentration of anthocyanins in a pomegranate variety named Wonderful by means of high–performance liquid chromatography (Gil *et al*., 2000). Miguel et al (2004) evaluated the anthocyanin concentration in a pomegranate cultivar named Assaria (Sepúlveda *et al*., 2010). Sepúlveda *et al* (2010) evaluated the type and amount of sugar, type and amount of anthocyanins, antioxidant activity and color of pomegranate juice of a cultivar named Chilean.

Sugars had an important role in creating various tastes of pomegranate juice through interaction with organic acids. Hulme's results show that Glucose was the most common sugar in pomegranate juice, and other sugars were Arabinose, Galactose, Fructose, Sucrose and Rhamnose (Hulme, 1970). This study was conducted for identification and quantitative determination of sugars and anthocyanins compounds in four commercial cultivars of pomegranate juice by HPLC analysis.

Juices made from fruits of Iranian pomegranate (*Punica granatum* L.) accessions of pomegranate were studied for their organic acids, sugars, and anthocyanin contents, using high performance liquid chromatography.

The aim of this research is study of sugar and anthocyanin characterization of four Iranian pomegranate (*Punica granatum* L.) varieties using HPLC System.

Material and methods

Sample preparation

Four Commercial pomegranate cultivars including Shirin Shahvare Yazd (SSY), Vahshe Kane Tehran (VKT), Pust Sefid (PS) and Gorche Shahvar Yazdi (GSY) were selected in November 2012 of the Research Center of Yazd Province, Iran. Thirty Kg of each variety was purchased and all of them transferred to a 4°C cold storage on the same day as they were harvested. Defected pomegranates (scratched, cracked, cut and sunburnt) were discarded before the experiments and the healthy ones selected for extracting juice. After Pomegranate varieties were manually peeled by a hand-held device; juice was extracted under pressure affect and pulp and seeds remained on the screen mesh. Pomegranate juice was passed through the plastic filter to achieve the lowest impurities, then it was

stored in dark glasses in freezer (24°C) in order to protect against the light and biochemical changes.

Chemicals

Five standard anthocyanins (Cyanidin 3-glucoside (Cy3), Cyanidin 3,5-di glucoside (Cy3,5), Delphynidin 3-glucoside (Dp3), Pelargonidin 3-glucoside (Pg3) and Pelargonidin 3,5-diglucoside (Pg3,5)) were purchased from Apin Chemicals Ltd. (Abingdon, England). Also three sugar Standards which found in pomegranate juice (sucrose, glucose and fructose) were purchased from Sigma Aldrich (Steinheim, Germany). All the standards were HPLC-grade. Other chemicals used in this study were obtained from Merck Company (Darmstadt, Germany).

HPLC analysis

Anthocyanin and sugar analyses were performed according to the method of Gil et al. (2000). Knauer HPLC system (Berlin, Germany) equipped with a UV–vis detector (K-2600), and a Triathlon auto sampler and a K-1001 pump used for chromatographic qualitative and quantitative analysis. Column used for separation of anthocyanins was RP C18 Nucleosil 100 (12.5 cm × 5.0 mm ×5.0 µm). After centrifuging in an eppendorf tube (4 min at 5000 rpm) and allowing supernatant to pass through a 0.45µm PTFE filter (Chromafil CA-45/25 S, Duren, Germany) juice was injected into the column HPLC system. The mobile phase was included in: A solvent (2.5% v/v, acetic acid in water) and B solvent (2.5% v/v, acetic acid in methanol). Gradient profile used for these two solvents were considered as: 100% A solvent at 0-5 min, 90% A for 15 min, 50% A for 45 min, 100% A in 55 minutes. Flow rate was 1 mm/min and chromatograms were recorded at 510 nm. Column used for separation of sugars was Eurokat H (300mm × 8 mm × 10 µm). Pomegranate juices were prepared as mentioned conditions; Chromatograms were recorded with the RI detector (Refract Index). Mobile phase included in water with constant flow rate of 0.5 mm/min. Injection volumes were 50 µl for anthocyanins and 20 µl for sugars. In order to draw standard curves of the anthocyanins and sugars, various concentrations of pure samples of each compound were injected in column similar to original sample injection conditions in the HPLC system. Prepared concentrations were in the range of 0.01-0.04 mg/100µl for anthocyanins (Cy3, Cy3,5, Dp3, Dp3,5, Pg3 and Pg3,5). These ranges were 0.47-2.04% for glucose and fructose, and 0.235-1.5% for sucrose. Each compound was quantified by comparing its peak area with standard curve of that.

Statistical analysis

Experiments were performed in Triplicate; Data analysed using SPSS statistical software (SPSS version 10.0.0; SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) were performed using Duncan's multiple range test with α set at 0.05 for comparing means at 95% confidence level ($P<0.05$).

Results and discussion

Anthocyanins

Anthocyanins in juices were measured by HPLC using a Knauer HPLC system. Elution of compounds in reversed phase HPLC depends on their polarity; more polar compounds are eluted earlier. HPLC chromatogram recorded at 510 nm for getting sharp peaks. Figure 1 shows chromatogram obtained by HPLC for analysis of anthocyanins in PS cultivar. Polarity levels of compounds are as follows: Cy3,5 > Dp3 > Cy3 > Pg3. Pg3 was the last peak recorded on the chromatogram, so it was a nonpolar compound among mentioned Anthocyanins. Anthocyanin profiles and their elution order were similar for all cultivars, but the peak areas varied significantly among studied cultivars which were in coincidence with results obtained by Mousavinejad *et al* (2009). As shown in figure 1, the most polar anthocyanin among the four cultivars was Cy3,5 and the others were respectively Dp3, Cy3, and Pg3. As it was showed in table1, P3,5 was not exist in any of the pomegranate juice and this result was similar to findings of Gill *et al* (2000). The most anthocyanins for SSY were cyanidin 3,5-diglucoside (2241.64 mg/l), followed by cyanidin 3-glucoside (816.31 mg/l) and for PS it was cyanidin 3-glucoside (1084.72 mg/l).

Diglucoside type of Anthocyanins for all cultivars were found at higher concentration levels than monoglucosides except GSY variety which was similar to results obtained by Mousavinejad et al. (2009). The identified anthocyanins were Cy3, Cy3,5, Dp3, Pg3 and Pg3,5 (trace) which their amounts were 261.43-1084.72 mg/l, 0–2241.64 mg/l, 0-231.77 mg/l and 0-194.02 mg/l, respectively. SSY (3308.76 mg/l) had the most amount of anthocyanin in pomegranate juice, followed by PS (3103.07 mg/l). As it showed in table2, the difference between amount of anthocyanins in all varieties were significant statistically with $p<0.05$.

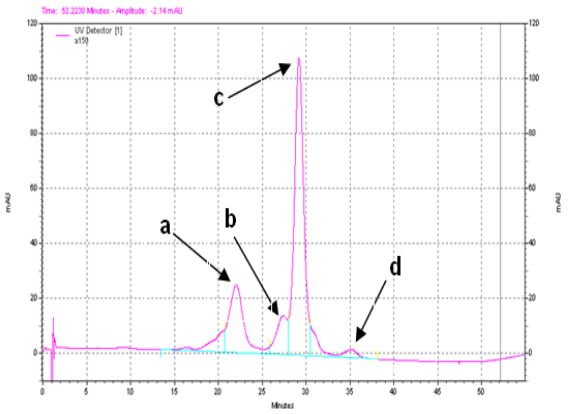

Fig.1. Typical HPLC chromatogram recorded at 510 nm showing the anthocyanins Peaks of pomegranate juice (SSY cultivar) using HPLC column RP C18 Nucleosil 100 (12.5 cm × 5.0 mm × 5.0 lm). a: Cy 3,5 b: Dp3, c: Cy3, d: Pg3

Table 1. Anthocyanin composition of 4 Iranian pomegranate varieties (mg/l)

variety	Cy3	Cy3,5	Pg3	Pg3,5	Dp3	Total
PS	1084.72±17.99a	1610.56±31.05a	194.02±4.75a	0.00	213.77±2.00a	3103.07±55.64a
SSY	816.31±8.39b	2241.64±27.47b	104.51±1.37b	0.00	146.31±2.87b	3308.76±40.04b
GSY	261.43±12.84c	0.00	0.00	0.00	0.00	261.43±12.84c
VKT	313.70±6.68d	576.02±11.63d	11.44±1.08d	0.00	0.00	901.16±17.25d

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

Sugars

Sugars in juices were measured by HPLC system equipped with a column named Eurokat H. Means for total sugar content in four cultivars were 19.45 g/100g. Figure 2 shows a typical HPLC chromatogram for analysis of sugars in PS. Sugar profiles and their elution order were similar for all cultivars, but the peak areas varied significantly among studied cultivars which were in coincidence with results obtained by Hulme, A. C. (1970). Figure 3 shows concentration of glucose, fructose and sucrose in four commercial cultivars of pomegranate juice. Fructose (8.25-9.55 g/100g) and sucrose (2.25-5.55 g/100g) had highest and lowest values in the pomegranate juice; this finding were similar to results obtained by Melgarejo *et al* (2000). VKT had the highest sucrose (5.3 g/100g) and fructose (9.16 g/100g) while the highest glucose (7.14 g/100g) was found for the GSY cultivar. There was not found significant difference for amount of sugar between the VKT and GSY varieties but the difference between them with SSY and PS cultivars was significant statistically ($p<0.05$). Observed significant differences for amount of sugar between varieties could be due to the differences in harvesting time, rainfall in growing season, geographical condition and the type of cultivars. VKT and GSY were the best varieties in terms of sugar percentage in pomegranate juice. Results found by Fadavi *et al* (2005), shows higher levels of sugars in compare with present findings (Fadavi *et al.*, 2005). These differences may be result from different times of harvesting due to the fact that the amount of sugar is increased by the time of fruit maturity.

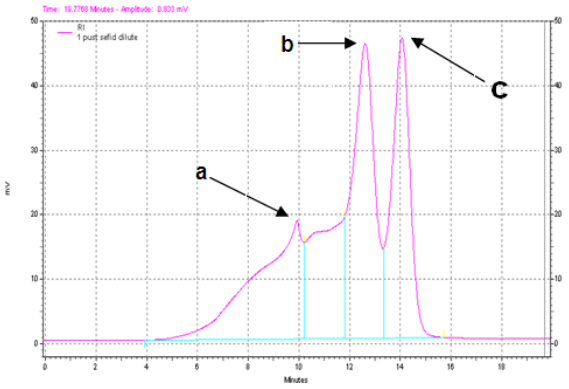

Fig.2. Typical HPLC chromatogram recorded at 365 nm showing the sugars peaks of pomegranate juice (SSY cultivar) using HPLC column Eurokat H (300mm × 8 mm × 10 µm), a : sucrose, b : glucose, c : fructose

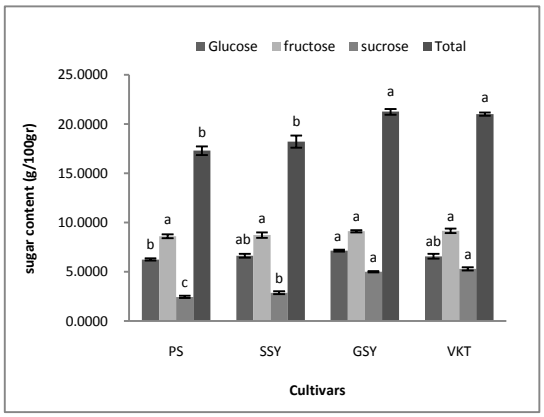

Fig. 3. Sugar profiles of different pomegranate cultivars, a-b: Different letters present significant difference at $p < 0.05$

Conclusion

Results indicated significant differences among different cultivars of pomegranate in terms of their anthocyanins and their sugar contents. PS and SSY cultivars contained higher amounts of anthocyanins compared to GSY and VKT. While, in contrast, GSY and VKT had higher content of sugars. Fructose was detected as the most common sugar and Cy3,5 as the most common anthocyanin in pomegranate juice. It is therefore recommended to use juices produced from a mixture of juices from all cultivars to get a functional beverage of pomegranate.

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