



## Identification and quantification of phenolic compounds and their effects on antioxidant activity in pomegranate juices of eight Iranian cultivars

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### ARTICLE INFO

#### Article history:

Received 15 August 2008

Received in revised form 2 December 2008

Accepted 19 January 2009

#### Keywords:

Antioxidant activity

Cultivar

DPPH

HPLC

Phenolic compositions

Pomegranate juice

### ABSTRACT

Important health-promoting compounds, including six types of anthocyanins, phytoestrogenic flavonoids and ellagic acid were determined individually in pomegranate juices (*Punica granatum* L.) of eight Iranian cultivars by high performance liquid chromatography coupled to UV–vis detector (HPLC–UV) using individual calculation from the peak area based on standard curves of each component. Total phenolics and antioxidant activities were determined by Folin–Ciocalteu and 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods, respectively, and compared among the cultivars. The predominant anthocyanins were delphinidin 3,5-diglucoside (372–5301 mg/l) followed by cyanidin 3,5-diglucoside (242–2361 mg/l), delphinidin 3-glucoside (49–1042 mg/l) and pelargonidin 3,5-diglucoside (7–90 mg/l), respectively. The highest level of total tannins was found in Sweet Alak cultivar (3 mg/l). Saveh Black Leather showed the highest level of ellagic acid (160 mg/l). Antioxidant activity varied among the cultivars (18–42 Trolox equivalents antioxidant capacity) and was directly related to the total phenolics in each type of juice.

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### 1. Introduction

Pomegranate fruit (*Punica granatum* L.) is one of the most popular fruits native to Iran. However, it is now widely grown in many tropical and subtropical countries. The total production of pomegranate in Iran was ~670,000 tons in 2005 (Anonymous, 2005). The edible parts of pomegranate fruits can be consumed fresh or used for the preparation of fresh juice, canned beverages, jelly, jam and paste and also for flavouring and colouring beverage products. Despite the fact that a large number of pomegranate varieties can be found in Iran (more than 760 original, wild and decorative cultivars), only a few scientific studies have been reported on this area (Abbasi, Rezaei, Emamdjomeh, & Ebrahimzadeh Mousavi, 2007; Behzadi Shahrehabaki, 1998; Mirdehghan & Rahemi, 2007).

Pomegranate fruit has valuable compounds in different parts of the fruit whose functional and medicinal effects such as antioxidant, anticancer and anti-atherosclerotic effects have been confirmed (Mertens-Talcott, Jilma-Stohlawetz, Rios, Hingorani, & Derendorf, 2006; Perze-Vicente, Izquierdo, & Garcia-Viguera, 2002). Pomegranate juice is nutritionally an important beverage since it is consumed frequently for its phenolic compounds (such as anthocyanins, ellagic acid, phytoestrogenic flavonoids and tannins). Gil, Tomas-Barberan, Hess-Pierce, Holcroft, and Kader

(2000) evaluated the antioxidant activity of pomegranate juice and its relationship with phenolic composition in one cultivar (wonderful). They also quantified anthocyanins and ellagic acid by means of high performance liquid chromatography (HPLC) and reported higher antioxidant activity levels for commercial pomegranate juice compared to red wine and green tea. Miguel, Fontes, Antunes, Neves, and Martins (2004) monitored the anthocyanin concentrations of “Assaria” pomegranate fruit during different cold storage conditions. They used HPLC with a UV–vis detector for the quantification of anthocyanins. Kulkarni and Aradhya (2005) evaluated the changes of total anthocyanin and antioxidant activity in pomegranate arils during fruit development. Ellagic acid has also been identified and quantified in many fruits and fruit juices (Amakura, Okada, Tsuji, & Tonogai, 2000a, 2000b; Conde, Cadahia, Garcia-Vallejo, Simon, & Adrados, 1997; Gil, Holcroft, & Kadar, 1997; Rommel & Wrolstad, 1993). For example, Amakura et al. (2000a, 2000b) were able to find ellagic acid in fresh and processed fruits and phenolic acids (such as gallic, chlorogenic, caffeic and ferulic acids) in fruit juices. Phytoestrogenic flavonoids have also been found in pomegranate and other fruits by using different methods of chromatography (Elswijk, Schobel, Lansky, Irth, & Greef, 2003; Justesen, Kunthsen, & Leth, 1997).

The aim of this work was to quantify the phenolic compounds among eight different Iranian pomegranate cultivars, by using HPLC, and to evaluate the antioxidant activity of each cultivar by the DPPH (2,2-diphenyl-1-picrylhydrazyl) method, in order to

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compare and differentiate cultivars as valuable sources of antioxidant compounds.

## 2. Materials and methods

### 2.1. Sample preparation

Eight pomegranate cultivars (Sweet Aalak, Sooleghan, Malase (sweet and sour) Ardestan, Saveh Sweet White Leather, Malase Ashkezar, Saveh Black Leather, Ardestan Black Leather and Ostokhania Tabas) were selected from mature fruits grown in the collection of the Agricultural Research Center of Saveh, Iran. Ten kg of each cultivar were picked at maturity. According to Mirdehghan and Rahemi (2007), the harvest maturity for pomegranate is achieved in September, when the arils' weight is greater than that of the peel. Fruits were transferred to a 4 °C store room on the same day as they were harvested. To avoid possible contamination of the juices with the metabolites produced by microorganisms, fruits with cracks, cuts, sunburn and other defects in husk were disposed of and only healthy fruits of uniform size and appearance were arranged in one row in wooden boxes containing packing material during the experiments. Fruits for each cultivar were manually peeled and, by using a manual device with a pedal for pressing the arils, the juice passed through a perforated plate and the seeds and pulp remained on the plate.

### 2.2. Chemicals

The anthocyanin standards were obtained from Apin Chemicals Ltd. (Abingdon, England), Flavone, flavonols (kaempferol and quercetin) and ellagic acid standards, and also DPPH were from Sigma Aldrich (Steinheim, Germany). All the standards were HPLC-grade. Methanol, acetic acid, formic acid, hydrochloric acid, *p*-coumaric acid and water were also HPLC-grade and all other chemicals used in this study were obtained from Merck Chemical Company (Darmstadt, Germany).

### 2.3. HPLC analysis

The chromatographic analysis was carried out on a Knauer HPLC system (Berlin, Germany) equipped with a Triathlon autosampler, a K-1001 pump and a UV-vis detector (K-2600). Analysis of anthocyanins and ellagic acid was performed according to the method of Gil et al. (2000). Before injection, each juice was centrifuged in an eppendorf tube (4 min at 5000 rpm) and the centrifuged supernatant was allowed to pass through a 0.45 µm PTFE filter (Chromafil CA-45/25 S, Duren, Germany). Injection volume was 50 µl. An RP C<sub>18</sub> Nucleosil 100 (12.5 cm × 5.0 mm × 5.0 µm) column was used for the separation of sample components. Mobile phase consisted of solvent A (2.5%, v/v, solution of acetic acid in water) and solvent B (2.5%, v/v, solution of acetic acid in methanol) at different ratios, the gradient profile was 100% A at 0–5 min, 90% A at 15 min, 50% A at 45 min and 100% A at 55 min. Flow rate was 1.0 ml/min. Chromatograms were recorded at 510 nm.

To analyse phytoestrogenic flavonoids, each juice was prepared according to Justesen et al. (1997), for the extraction of flavones and flavonols from pomegranate juices. Final extracts were filtered through a 0.45 µm PTFE filter (Chromafil CA-45/25 S). A Knauer Lichrospher 100 RP C<sub>18</sub> (25.0 cm × 5.0 mm × 5.0 µm) column was used for the analysis of the compounds. Injection volume was 50 µl. Flow rate was set at 1.0 ml/min. Mobile phase consisted of solvent A (a 1.0%, v/v, solution of formic acid in methanol) and solvent B (a 1.0%, v/v, solution of formic acid in a methanol:water mixture, 30:70, v/v). Formic acid was added to increase peak resolution in each case. The gradient profile was 5% A at 0 min, 25% A at

10–15 min, 90% A at 40 min and 5% A at 50 min. Flow rate was set at 1.0 ml/min. Chromatograms were recorded at 365 nm. Data were recorded and processed using ChromGate software (Version 3.1, build 3.1.0.2384, Knauer, Berlin, Germany).

Each compound was quantified by comparing its peak area against the standard curve obtained specifically for the reference solutions containing that compound. To obtain the standard curves, five different concentrations of ellagic acid (0.0012–0.01 mg/100 µl) and four different concentrations (0.01–0.04 mg/100 µl) of each anthocyanin type (cyanidin 3-glucoside (Cy3), cyanidin 3,5-diglucoside (Cy3,5), delphinidin 3-glucoside (Dp3), delphinidin 3,5-diglucoside (Dp3,5), pelargonidin 3-glucoside (Pg3), pelargonidin 3,5-diglucoside (Pg3,5)) were injected. Sum of the means from all the measured anthocyanins was reported as total amount of anthocyanins. Total tannin expressed in 100 g sample was measured by titration with a known concentration of potassium permanganate in the presence of indigo carmine as an indicator (Anonymous, 2000; Tabasum, Ahmad, Akhlaq, & Rahman, 2001). A 50 g sample from Malas Ashkezar and Ostokhania Tabas cultivars, which have light colour and 25 g samples from other cultivars (with dark colours) were titrated in two stages and then by considering that 1 ml potassium permanganate (0.1 N) is equivalent to 0.0035 g tannin (according to a local standard for pomegranate), total tannin was calculated and expressed per 100 g sample.

### 2.4. Total phenolics and antioxidant activity evaluation

To determine total phenolics, pomegranate juices at 1:10 dilution levels were studied by applying the Folin-Ciocalteu method (Singleton & Rossi, 1965) using a UV-vis instrument at 660 nm. Dilutions were made in duplicate and total phenolics were measured against a calibration curve obtained with *p*-coumaric acid.

DPPH method was used to measure the antioxidant activities of pomegranate juices based on the evaluation of the free radical-scavenging capacities of the juices. The results were expressed as Trolox equivalent antioxidant capacity (TEAC) (Cano, Hernandez-Ruiz, Gracia-Canovas, Acosta, & Arnao, 1998; Cao, Russell, Lischner, & Prior, 1998; Gil et al., 2000). The same methods were applied by Gil et al. (2000) for the evaluation of total phenolics and antioxidant activity in the juice of one pomegranate cultivar (wonderful).

### 2.5. Statistical analysis

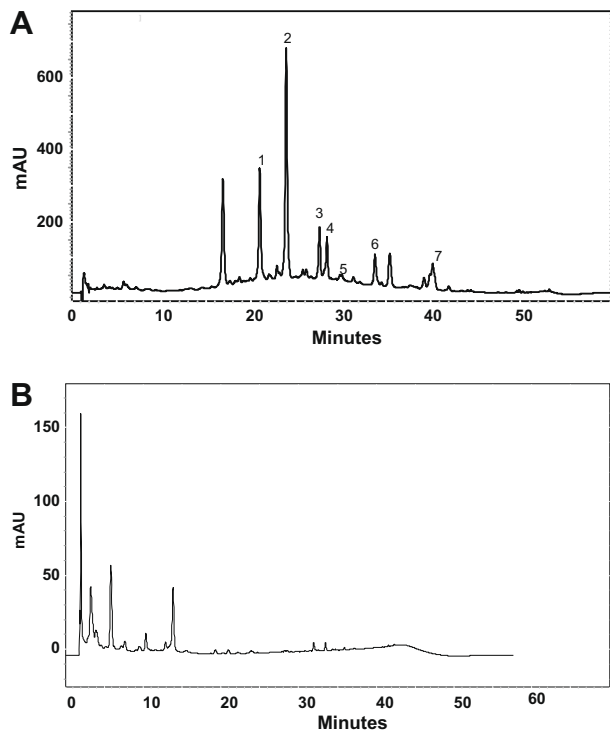
Triplicate experiments were performed and means were compared using SPSS statistical software (SPSS version 10.0.0; SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) and Tukey's *post-hoc* tests with  $\alpha$  set at 0.05, was applied to compare means at the 95% confidence level.

## 3. Results and discussion

### 3.1. Anthocyanins

Fig. 1 shows a typical HPLC chromatogram recorded at 510 nm, which showed better sensitivity compared to other wavelengths. Anthocyanin profiles and their elution order were similar in all eight cultivars, but the peak areas varied significantly among the studied cultivars. As shown, the most polar anthocyanin among the eight cultivars is Cy3,5, which eluted first, followed, in order, by Dp3,5, Dp3, Pg3,5, Cy3 and Pg3. Monoglucoside anthocyanins were less polar and eluted after diglucoside types.

The effect of cultivar on anthocyanin contents is shown on Fig. 2. The major anthocyanin in these cultivars was Dp3,5 followed by Cy3,5 and Pg3,5. In all pomegranate juice samples studied here, diglucoside type anthocyanins were found at higher



**Fig. 1.** (A) Typical HPLC chromatogram recorded at 510 nm showing the anthocyanins and ellagic acid peaks of pomegranate juice (Malase Ashkezar cultivar) using HPLC column RP C<sub>18</sub> Nucleosil 100 (12.5 cm × 5.0 mm × 5.0 μm). 1: Cyanidin 3,5-diglucoside, 2: delphinidin 3,5-diglucoside, 3: delphinidin 3-glucoside, 4: pelargonidin 3,5-diglucoside, 5: cyanidin 3-glucoside, 6: pelargonidin 3-glucoside. (B) Typical HPLC chromatogram recorded at 365 nm showing the flavonoid peaks of pomegranate juice (Ostokhani Tabas) using HPLC column Lichrospher 100 RP C<sub>18</sub> (25.0 cm × 5.0 mm × 5.0 μm).

concentration levels than monoglucosides. The amount of Cy3 varied among all the cultivars studied, whereas Pg3 was found at similar levels among the studied cultivars.

The anthocyanin profile of only one cultivar of pomegranate has been reported previously (Gil et al., 2000). In the current study, the amount of each anthocyanin varied among different cultivars of pomegranate and there was a high genetic heterogeneity within the Iranian studied cultivars. The levels of anthocyanins obtained in the current study are greater than those reported by Gil et al.

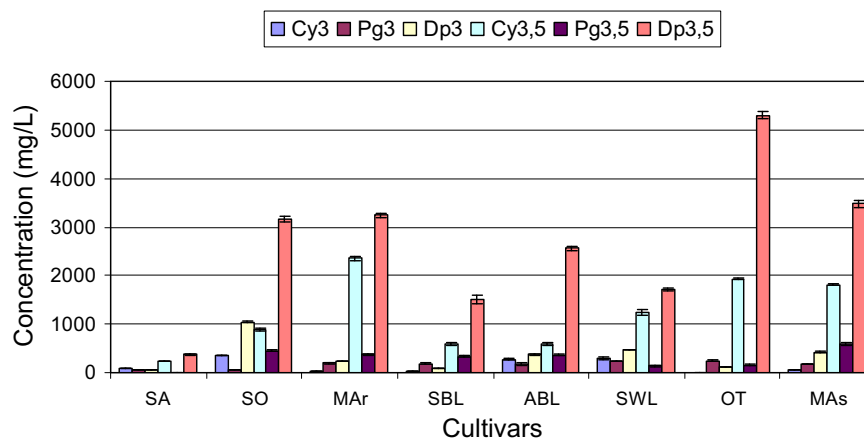
(2000) on Wonderful cultivar. They also reported the amounts of Pg3, Dp3, Cy3,5, Dp3,5 and Cy3 at 0.30, 3.80, 2.65, 2.14 and 6.40 μg/50 μl, respectively. However, in eight cultivars studied here anthocyanins were found at 2.5–12.2 μg/50 μl for Pg3, 2.5–2.1 μg/50 μl for Dp3, 12.1–118.0 μg/50 μl for Cy3,5 and 18.6–265.1 μg/50 μl for Dp3,5. For Cy3, the results were varied between cultivars (0.1–17.9 μg/50 μl). This can be related to the differences in the cultivars studied, harvesting time and the time of performing the experiments, which influence the anthocyanin content significantly (Miguel et al., 2004; Mirdehghan & Rahemi, 2007). Data clearly showed that the anthocyanin fingerprints among pomegranate cultivars were quite different at harvest, which can be used as a means to differentiate them for different application.

### 3.2. Ellagic acid

Ellagic acid, which is an important phenolic acid with high antioxidant activity, has been reported in some fruit juices (Amakura et al., 2000a,2000b) and was also detected in pomegranate juice in the current study. Due to the similarity with anthocyanins, ellagic acid eluted immediately after anthocyanins on the chromatograms (retention time: 40.3 min). The ellagic acid concentrations of the studied pomegranate cultivars are shown in Fig. 3. Significant differences were found among the ellagic acid levels of different cultivars. Saveh Black Leather showed the highest level of ellagic acid (160 mg/l) and Sweet Alak contained the least amount of ellagic acid (7 mg/l).

### 3.3. Phytoestrogenic flavonoids

Presence of phytoestrogenic flavonoids (luteolin, kaempferol and quercetin) were reported in pomegranate peel by Elswijk et al. (2003). By using HPLC–UV/Vis, presence of these phenolic compounds was studied in pomegranate juices of eight cultivars but they were not found in pomegranate juice of any of the cultivars in the current study. Fig. 1B shows the typical HPLC chromatogram for flavonoids recorded at 365 nm. No measurable peaks were found from 48 to 52 min on the chromatograms. Chemical composition of fruit juices can change significantly with the ripening stage and also during storage. Kulkarni and Aradhya (2005) monitored the chemical changes in pomegranate arils during fruit ripening. According to their study, the increase in the anthocyanin content was related to a decrease in the other phenolic contents during the ripening of the fruit.



**Fig. 2.** Anthocyanin profiles of different pomegranate cultivars. Cy3: cyanidin 3-glucoside, Cy3,5: cyanidin 3,5-diglucoside, Dp3: delphinidin 3-glucoside, Dp3,5: delphinidin 3,5-diglucoside, Pg3: pelargonidin 3-glucoside, Pg3,5: pelargonidin 3,5-diglucoside. Abbreviations used for the names of cultivars: SA: Sweet Alak, SO: Solehgan, MAr: Malase Ardestan, SBL: Saveh Black Leather, ABL: Ardestan Black Leather, SWL: Saveh Sweet White Leather, OT: Ostokhani Tabas, MAs: Malase Ashkezar.

### 3.4. Total tannins

Changes in the total tannin contents of the juices for eight pomegranate cultivars are shown in Fig 4. Sweet Alak cultivar showed the highest tannin level (32 mg/100 g juice). This value varied within 15–22 g/100 g in other cultivars. Because of antioxidant and antitumoral activities of pomegranate tannins, higher levels of total tannins in juices can be directly associated with higher functional properties of the juices.

Total tannin obtained in this study (150–320 mg/l) were somewhat less than that reported by Gil et al. (2000), who reported a to-

tal tannin content of 417–539 mg/l for experimental and commercial juices of one pomegranate cultivar (Wonderful). According to their reports, a higher level of tannins in the commercial juices was due to the presence of some compounds imparted from other parts (such as peel) of pomegranate fruit.

### 3.5. Total phenolics and antioxidant activity

Total anthocyanin and total tannin contents of the eight cultivars studied here are shown in Table 1. These compounds along with ellagic acid are the major phenolic compounds of

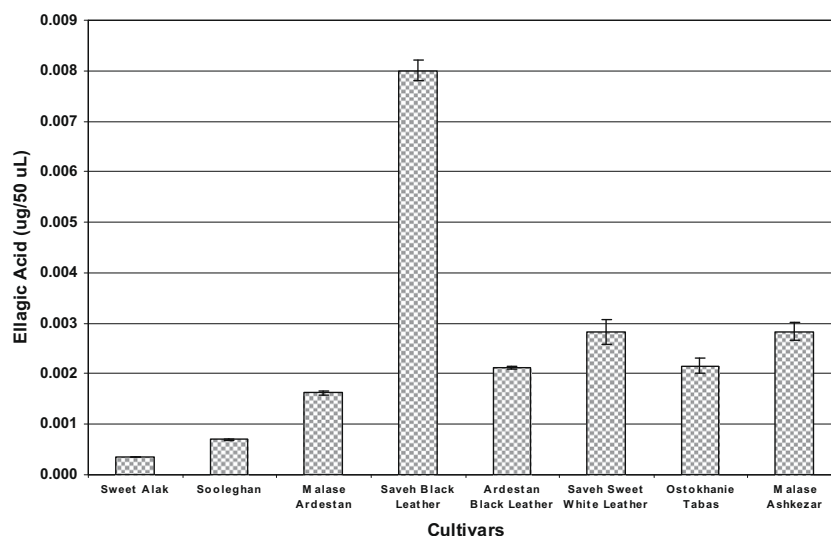


Fig. 3. Comparison of ellagic acid concentration among eight pomegranate juices.

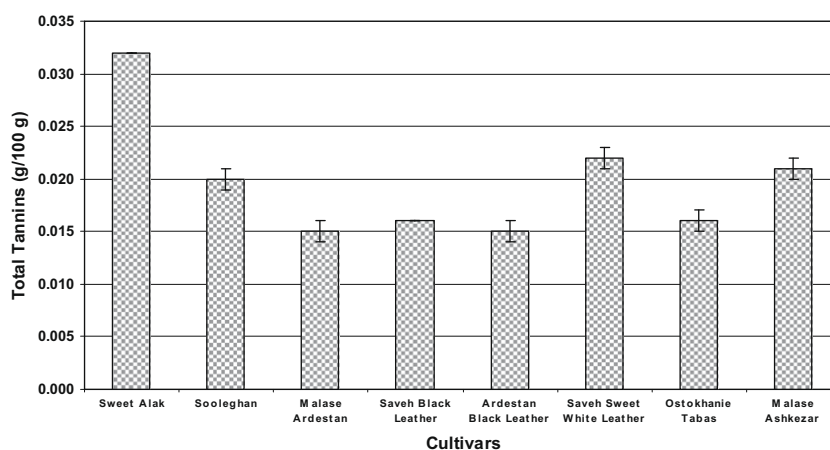


Fig. 4. Comparison of total tannin contents of pomegranate juices from different cultivars.

Table 1

Mean values of total phenolic (mg/l) and total anthocyanins (mg/l) and antioxidant activity (mM)<sup>a</sup> of juices from eight pomegranate cultivars (n = 3).

	Sweet Aalak	Sooleghan	Malase Ardestan	Saveh Black Leather	Ardestan Black Leather	Saveh Sweet White Leather	Ostokhani Tabas	Malase Ashkezar
Total phenolics	2380 ± 0.0	7440 ± 0.1	7920 ± 0.1	4200 ± 0.2	5820 ± 0.1	5490 ± 0.2	9300 ± 0.1	8130 ± 0.1
Total anthocyanin	815 ± 0.0	5980 ± 0.1	6430 ± 0.2	2750 ± 0.1	4330 ± 0.0	4070 ± 0.2	7760 ± 0.1	654830 ± 0.1
Antioxidant activity	18.6 ± 0.1	35.6 ± 0.1	36.6 ± 0.0	23.8 ± 0.1	29.2 ± 0.0	28.3 ± 0.1	42.8 ± 0.0	38.1 ± 0.0

<sup>a</sup> Antioxidant activity represents the Trolox equivalent antioxidant capacity (TEAC).

pomegranate juice. In order to determine all the phenolic compounds in pomegranate juice including phenolic acids, punicalagins, some ellagic acid derivatives, Folin–Ciocalteu method was applied. Results indicate large differences among the eight cultivars studied (2376–9304 mg/l). The level of total phenolics in one cultivar (Alake Shirin) was close to the results reported by Gil et al. (2000) for cultivar Wonderful. According to the current study, other Iranian cultivars have higher levels of phenolic compounds (2–4 times) and the amounts of these compounds are directly related with the antioxidant activities of pomegranate juices. Therefore, level of total phenolics in each pomegranate juice can be a good indication of its health benefits.

#### 4. Conclusions

Considering the importance of functional and high quality components of food and beverages in today's world, results of this study can propose some specific cultivars of pomegranate with higher levels of the above-mentioned compounds and as a result higher antioxidant activities.

Although some cultivars such as Saveh Black Leather have a high level of ellagic acid (160 mg/l), they may have lower levels of total phenolics (e.g., 4201 mg/l for Saveh Black Leather) and make it unsuitable for healthy drinks. Anthocyanins make a large contribution to the antioxidant activity and total phenolic content of each cultivar (more than 70%).

The highest level of total phenolics was present in Ostokhani Tabas (9304 mg/l) followed by Malase Ashkezar (8129 mg/l), Malase Ardestan (7923 mg/l) and Sooleghan (7438 mg/l).

#### Acknowledgements

Gratitude is expressed to the Council for Research at the Campus of Agriculture and Natural Resources of the University of Tehran and the Research Council of the University of Tehran for partial support of this project.

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