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Research Article

Determination of phenolic acids in pomegranate juices by HPLC-DAD

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Abstract

In this study, five phenolic acids, gallic, chlorogenic, caffeic, p-coumaric and ferulic acid were determined in freshly squeezed pomegranate juice, commercial pomegranate juice and pomegranate juice concentrate beverages. For this purpose, an analytical method using high-performance liquid chromatography (HPLC), with a combination of photodiode array detection (DAD), was developed for the characterization and quantification of phenolic acid in pomegranate juices. Then the analyses of pomegranate juices were performed. The limit of detection (LOD, S/N=3) of individual compounds was gallic acid 0.1 mg/L, chlorogenic acid 0.01 mg/L, caffeic acid 0.03mg/L, p-coumaric acid 0.02 mg/L and ferulic acid 0.04 mg/L. The limit of quantitation (LOQ, S/N=10) of individual compounds was gallic acid 0.4 mg/L, chlorogenic acid 0.05 mg/L, caffeic acid 0.1 mg/L, p-coumaric acid 0.1 mg/L and ferulic acid 0.1 mg/L. The relative standard deviation (% RSD) in commercial pomegranate juice was found to be gallic acid 0.08%, chlorogenic acid 0.32%, caffeic acid 0.21%, p-coumaric acid 0.08% and ferulic acid 0.38%. The relative standard deviation (% RSD) in freshly squeezed pomegranate juice was found to be gallic acid 0.01%, chlorogenic acid 0.46%, caffeic acid 0.17%, p-coumaric acid 0.04% and ferulic acid 0.8%. The relative standard deviation (% RSD) in pomegranate concentrate juice was found to be gallic acid 0.78%, chlorogenic acid 0.25%, caffeic acid 0.49%, p-coumaric acid 0.36% and ferulic acid 0.17%.

Keywords: HPLC; gallic acid; chlorogenic acid; caffeic acid; p-coumaric acid; ferulic acid.

1. Introduction

Phenolic acids are widely distributed in the plant cause tailing and broadening of their peaks. In this kingdom and are present in, e.g. tea, red wine, fruits, paper, we report the results of a simple and rapid beverages and various medicinal plants [1].

The Pomegranate juice was reported to be effective in the prevention of at herosclerosis, low-density lipoprotein oxidation, prostate cancer, platelet aggregation and various cardiovascular diseases [2]. Pomegranate fruit juice has potential chemopreventive agents for prostate cancer [3]. Sumner et al. showed that daily consumption of pomegranate juice for 3 months may decrease myocardial is chemia and improve myocardial perfusionin patients who have is chemic coronary heart disease [4]. Anoosha et al. conclude that consumption of pomegranate juice may be proven to be beneficial in attenuation of atherosclerosis development [5].

Pomegranate juice has gained lots of attention in the last years due to its nutritional value and antioxidant properties. Pomegranate fruit has three-fold higher antioxidant activity than that of red wine or green tea [6] and two-, six- and eight-fold higher levels than grape/cranberry, grape fruit, and orange juice, respectively [7]. Pomegranate (Punica granatum) is an important source of bioactive compounds. These compounds are primarily phenolic or polyphenolic, flavonoids (flavonois, flavoneflavanones, catechins and isoflavones) and related compounds (phenolic acids, chalcones and isoflavones) [8].

The term "phenolic acids" refers to phenols that have one carboxylic acid functionality. But, when describing plant metabolites, it refers to a distinct group of organic acids. In the view of the importance of these substances for health, accurate analysis methods for their determination in foodstuffs are required. In the literature on the determination of phenolic acids in foodstuffs, including UV spectrophotometry, gas chromatography, thin-layer chromatography, high-performance liquid chromatography (HPLC), liquid chromatography mass spectrometry and capillary electrophoresis systems are used [9].

(Punica granatum) has gainedlots ofattention for its health benefits in the past couple years.

Pomegranate (Punica granatum Linn.) is a very rich source of anthocyanins (cyanidin 3,5-di and 3-O-glucoside, delphinidin 3,5-di and 3-O-glucoside, pelargonidin 3,5-di and 3-O-glucoside), punicalagin isomers, different flavanols (catechins ascatechins

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and epicatechin, and gallocatechins as gallocatechinandepigallocatechin).

In this study, a direct method to determine simultaneously caffeic, chlorogenic, p-coumaric, ferulic and gallic acids in various juices by reversed-phase HPLC using DAD detection was performed. Then freshly squeezed pomegranate juices, commercial pomegranate juices and pomegranate juices from concentrate were analyzed for the quantitative determination of free and bound phenolic acids in juices with the methods and their health benefits.

2. Materials and Methods

2.1. Materials

Samples of non-concentrated pomegranate juice, were prepared using pomegranates harvested in 2013 season in Denizli region. The processing steps of the raw material were washing, filtering, grinding, pressing, filtration, and bottling respectively.

Samples of concentrated pomegranate juice, were prepared from variety pomegranate of Denizli region in 2013 season. Production process of concentrated pomegranate juice was similar to the non-concentered one expect for the decoction step after filtration. All juices were frozen at −20 0C until their analytical analysis.

Pomegranate juice from the local market is trademarked. All companies state that their juice is 100% pomegranate, which they claim on their packages of fruit juices and none of them contain any extra-added ingredients.

The brix degree of concentrated and non-concentrated pomegranate juices was adjusted to 11.2o brix with deionized water before analysis. Samples were filtered through a 0.45 µm pore size membrane filter before injection.

Caffeic, chlorogenic, p-coumaric, ferulic and gallic acids standard solutions were supplied from ACS.

Methanol, Acetic acid and hydrochloric acid (Merck) were used as pH modifiers. Solvents and all standards were of HPLC grade. Water was purified in a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chemicals were purchased from Fluka (Neu-Ulm, Germany). The standards were dissolved in methanol. The dilutions of the standards ranged from 0.5 to 100 µg ml -1 [12].

2.2. HPLC-DAD instrumentation and condition

A Surveyor HPLC system (Thermo Technologies, United States) operated by Windows NT based Chrome Quest software was used. The HPLC equipment was used with a diode array detector (DAD). System consisted of a binary pump, degasser and autosampler. The column was a Thermo Hypersil ODS : 4.6 mm×250 mm, 5 µm equipped.

The mobile phase consisted of two solvents: Solvent A, water/acetic acid (99.5: 0.5; v/v) and Solvent B, methanol. Phenolic compounds were eluted under the following conditions: 0.8 mL min−1 flow rate and the temperature was set at 30°. 0 to 2 with 0% B, from 0% to 10% B in 10 min, from 10% to 40% B in 30 min, from 40% to 0% B in 37 min, in 2 min, followed by washing and reconditioning the column. The ultra-violet-visible spectra (scanning from 200 nm to 600 nm) were recorded for all peaks. Triplicate analyses were performed for each sample. The identification of phenolic compounds was obtained by using authentic standards and by comparing the retention times and ultra-violet-visible spectra with those found in the literature [10], while quantification was performed by external calibration with standards. The DAD detection was performed at two different wavelengths, 280 nm and 320 nm, volume injected, 10 µl [11].

Individual retention time and maximum wavelengths were summarized in Table 1.

Table 1. *Retention Time and maximum wavelengths.*

Phenolic Acids	Retention Time/min	Maximum Wavelengths/nm
Gallic Acid	8.90	320
Chlorogenic Acid	13.3	280
Caffeic Acid	14.89	280
p-Coumaric Acid	17.3	280
Ferulic Acid	17.9	280

2.3. Sample preparation for HPLC–DAD

The fruit juice samples (5 g) were diluted with purified water to 15 ml, and 25 ml of methanol, which contained 2 g/ l tert-butylhydroxyquinone (TBHQ), were added. Moreover, 10 ml of 6 M HCl was added (final HCl concentration, 1.2 M). The mixture (in a 100-ml round-bottomed bottle) was refluxed for 2 h at 85 0C . The extract was allowed to cool and was then filtered. A 15 ml portion of the filtrate was evaporated to dryness using a rotary evaporator and a 35 0C water bath. The residue was dissolved in 1.5 ml of methanol and filtered through a 0.45 µm filter that was compatible with organic solvents (cellulose acetate, Lida, USA) prior to injection into the HPLC–DAD system [13].

2.4. Recovery Studies

Recovery experiments were performed in order to study the accuracy of the method described. Known amounts of pure standards gallic acid; chlorogenic acid; caffeic acid; p-coumaric acid; ferulic acid were added to a variety of samples and the resulting spiked samples were subjected to the entire analytical sequence, including the extraction step. The recoveries of phenolic acid were found within the range of 96.03 -99.78 % by this method.

The recoveries were calculated using the following formula:

Recovery= concentration $_{\text{spiked sample}}$ − concentration $_{\text{nonspiked sample}}$ / concentration

$_{\text{spiking solution}}$.

$_{\text{spiked sample}}$ − concentration $_{\text{nonspiked sample}}$ / concentration $_{\text{spiking solution}}$..

3. Results and Discussion

Simultaneous separation of some of these compounds using gradient elution has been reported [10]. In this work, a study was carried out to determine caffeic, chlorogenic, ferulic, p-coumaric

and gallic acids with isocratic elution. The mobile phase composition was optimized to obtain a good resolution between the different peaks detected for juice samples by HPLC-DAD.

In this study, we tried to determine the optimum isocratic HPLC conditions for determination of the five phenolic acids. As a consequence, the sample solution was chromatographed on an column ODS2 with a mobile phase water:acetic acid (99.5:0.5)-methanol at a flow-rate of 0.8 ml/min. Using these proposed HPLC conditions, a well resolved chromatogram between the different peaks detected for the standard by HPLC was obtained (Figure 1).

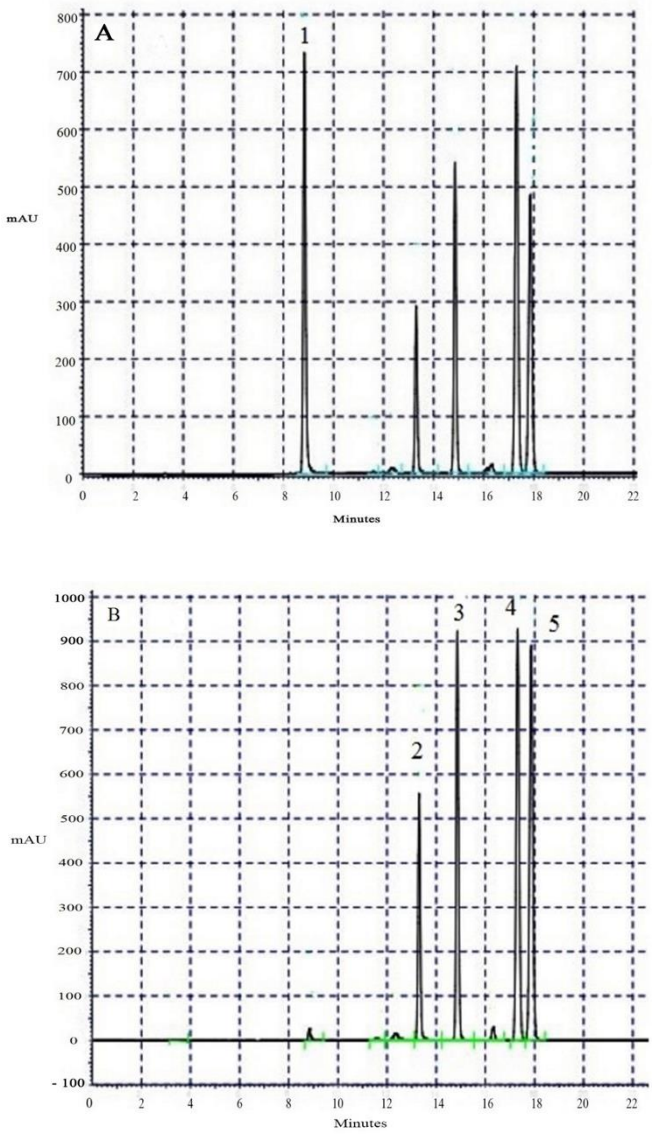

Figure 1.*Chromatograms of HPLC analyses of standards using DAD at 280 nm (A) and 320 nm (B). Peaks: (1) gallic acid; (2) chlorogenic acid; (3) caffeicacid;(4) p-coumaric acid; (5) ferulic acid.*

The within-day repeatability (n=3) and between day precision (n=3) of retention times were within 0.01 and 0.86 % relative standard deviation (RSD), drink respectively. RSD values are given in Table 2. The limit of detection (LOD, S/N=3) of individual compounds at 280 nm and 320 nm are given in Table 3.

Table 2. *RSD values*

	%RSD (HPLC-DAD)		
	commercial pomegranate juice	freshly squeezed pomegranate juice	pomegranate concentrate juice
Caffeic acid	0.08	0.01	0.78
p-coumaric acid	0.32	0.46	0.25
Ferulic acid	0.21	0.17	0.49
Gallic acid	0.08	0.04	0.36
Chlorogenic acid	0.38	0.8	0.17

Table 3. *The limit of detection (LOD), the limit of quantitation (LOQ), Coefficient*

	LOD (mg/L)	LOQ (mg/L)	Correlation Coefficient
Gallic acid	0.1	0.4	0.9997
Chloro genic acid	0.01	0.05	0.9996
Caffeic acid	0.03	0.1	0.9999
p-coumar ic acid	0.02	0.1	0.9999
Ferulic acid	0.04	0.1	0.9999

a Correlation coefficients of the regression equation y = a +bx, where x is the phenolic compound concentration (ppm) and y the peak area.

Table 3, where a, b and r were the coefficients of the regression equation y=ax+b x being the concentration of the phenolic compound (mg/kg), y the peak area, and r the correlation coefficient of the equation. All the phenolic compounds showed good linearity (r2 ≥0.9996) and obeyed Beer's law in the concentration range investigated [16].

In this work, phenolic compounds of freshly squeezed pomegranate juice, commercial pomegranate juice and pomegranate juice concentrate were determined by using HPLC-DAD.

Phenolic compounds identified in freshly squeezed pomegranate juice, commercial pomegranate juice and pomegranate juice concentrate were gallic, chlorogenic, caffeic, p-coumaric and ferulic acid.

73.3mg/L of caffeic acid, 30.8 mg/L of p-coumaric acid, 27.9 mg/L of ferulic acid, 20.1 of mg/L gallic acid and 15.2 mg/L of chlorogenic acid were quantified in commercial pomegranate juice by using HPLC-DAD (Figure 2).

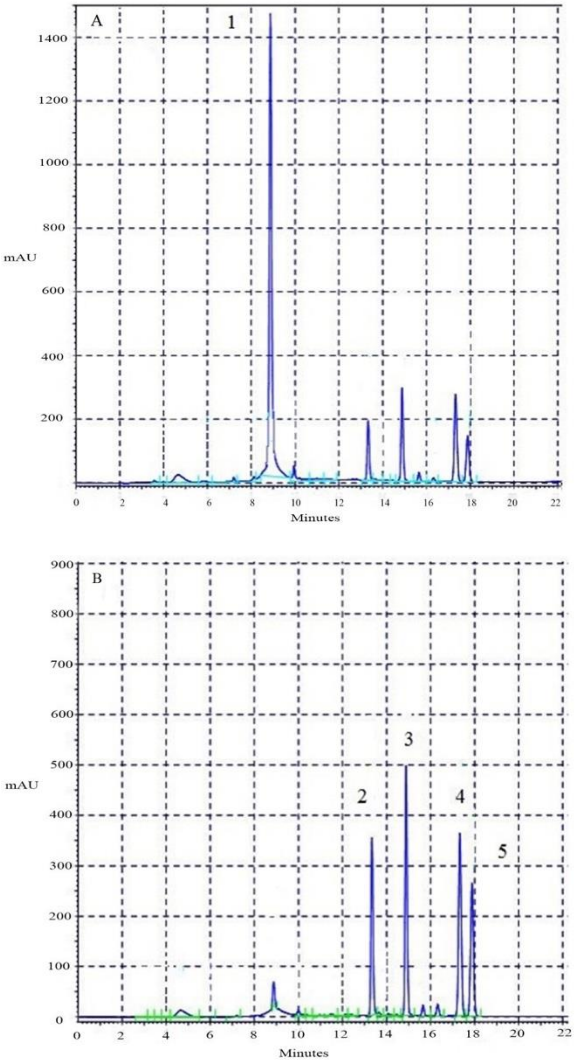

Figure 2. *Chromatograms of HPLC analyses of commercial pomegranate juice using DAD at 280 nm (A) and 320 nm (B). Peaks: (1) gallic acid; (2) chlorogenic acid; (3) caffeic acid; (4) p-coumaric acid; (5) ferulic acid.*

422.8 mg/L of caffeic acid, 45.5 mg/L of p-coumaric acid, 34.7 mg/L of ferulic acid, 24.3 of mg/L gallic acid and 17.1 mg/L of chlorogenic acid were quantified in freshly squeezed pomegranate juice by using HPLC-DAD (Figure 3).

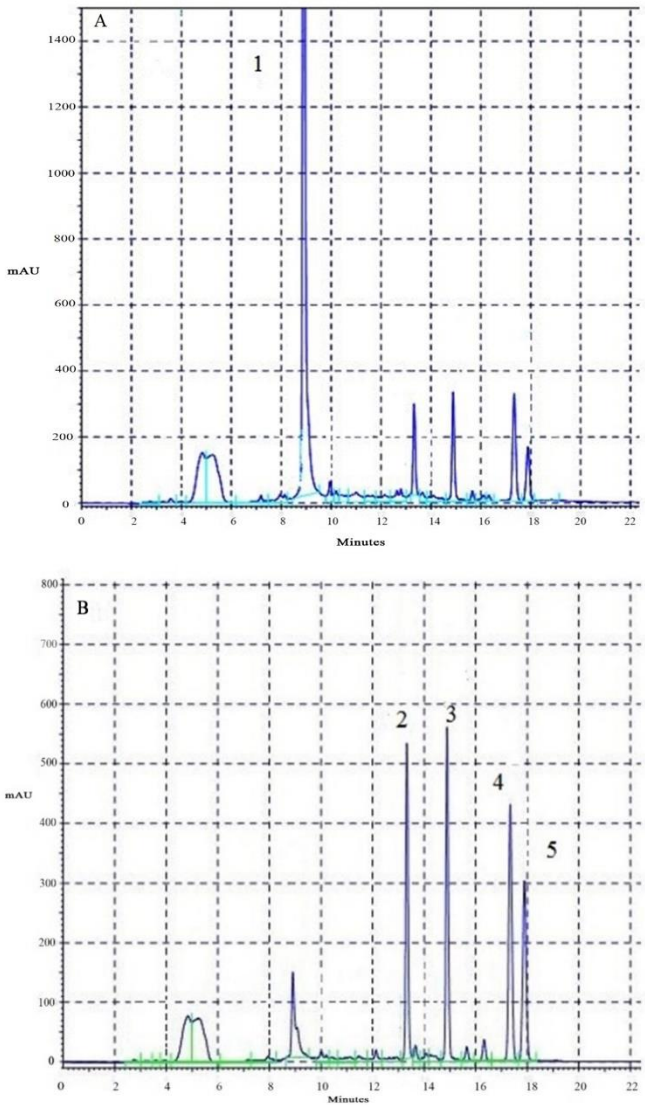

Figure 3. *Chromatograms of HPLC analyses of freshly squeezed pomegranate juice using DAD at 280 nm (A) and 320 nm (B). Peaks: (1) gallic acid; (2) chlorogenic acid; (3) caffeic acid; (4) p-coumaric acid; (5) ferulic acid.*

61 mg/L of caffeic acid, 21.4 mg/L of p-coumaric acid, 20.6 mg/L of ferulic acid, 15.9 of mg/L gallic acid and 11.5 mg/L of chlorogenic acid were quantified in pomegranate juice concentrate by using HPLC-DAD (Figure 4).

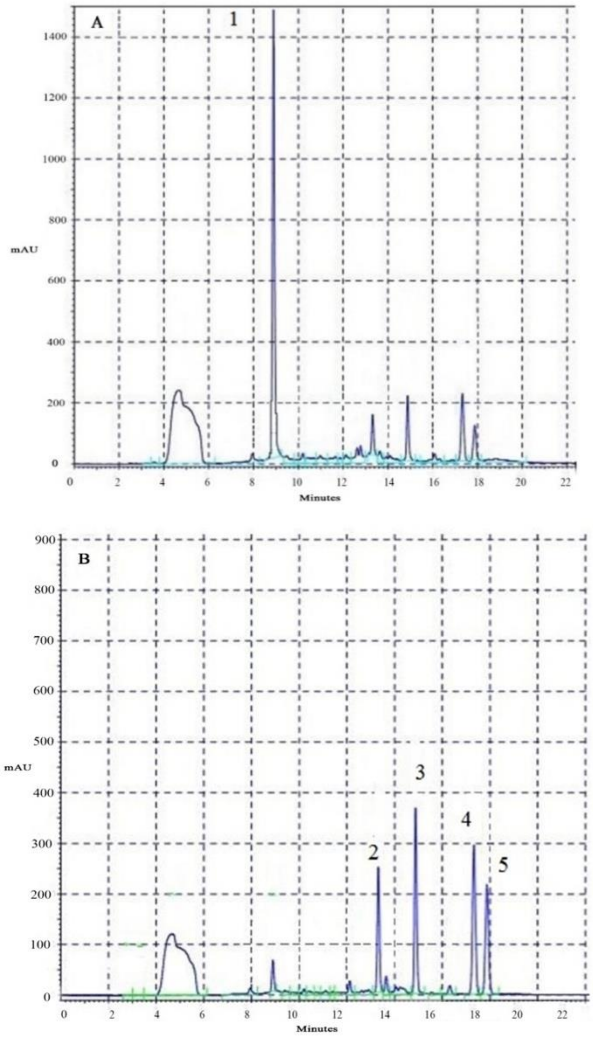

Figure 4. *Chromatograms of HPLC analyses of pomegranate juice concentrate using DAD at 280 nm (A) and 320 nm (B): Peaks: (1) gallic acid; (2) chlorogenic acid; (3) caffeic acid; (4) p-coumaric acid; (5) ferulic acid.*

The combination of DAD has been shown to be a powerful technique for the fast finger printing characterization of fruit extracts [14], food-based products [15], food plant sand food industry by-products [17].

We analyzed three juices using this method and the experimental result indicated that freshly squeezed pomegranate juice contained an especially high concentration of caffeic acid (422.8 mg/L) in 5 of its phenolic acids. It was also shown that the main acid of the other juices was caffeic acid (73.3 mg/L) in the commercial pomegranate juice, caffeic acid (61 mg/L) in the pomegranate juice concentrate.

The experimental results indicated that freshly squeezed pomegranate juice contained a high concentration of caffeic (422.8 mg/L), which can be hydrolyzed to caffeic acid. It was shown also that the phenolic acids occur mainly in bound forms in commercial pomegranate juice and pomegranate juice and that their bound caffeic acid contents were high (73.3 and 61 mg/L, respectively) compared to those of the other phenolic acids.

The phenolic acids, components of human foods, have shown interesting activities as inhibitors of mutagenic and carcinogenic processes. However, these studies were performed with pure crystalline substances, while in foods the substances are usually present as bound phenolic acids and little is known about the fate of most plant phenolics after ingestion. In order to utilize the anticarcinogenic properties of the phenolic acids for reduction of risk of human cancer, further studies are necessary. However, we know that chlorogenic acid, a bound caffeic acid, is similar to caffeic acid, an inhibitor of carcinogenesis in animal studies and in vitro [12].

Polyphenolic compounds in fruit been analyzed by using liquid-liquid extraction (LLE) followed by high performance liquid chromatography with ultraviolet detection [18].

Phenolic acid contents of pomegranate juices are given in Table 4.

Table 4. *Phenolic acid contents of pomegranate juices*

Phenolic Acid (mg/L)	Commercial pomegranate juice	Freshly squeezed pomegranate juice	Pomegranate juice concentrate
Caffeic acid	73.3±0.06	422.8±0.06	61±0.05
p-coumaric acid	30.8±0.1	45.5±0.2	21.4±0.05
Ferulic acid	27.9±0.06	34.7±0.06	20.6±0.1
Gallic acid	20.1±0.02	24.3±0.01	15.9±0.06
Chlorogenic acid	15.2±0.06	17.1±0.1	11.5±0.02

4. Conclusion

A convenient and rapid DAD-HPLC method for the identification and quantification of the main phenolic compounds in pomegranate in a single chromatographic run was developed, validated and successfully performed.

In this study, for the first time, phenolic contents of freshly squeezed pomegranate juice and pomegranate juice concentrate obtained from the Denizli in Turkey were examined. The results indicated that phenolic contents of freshly squeezed pomegranate juice were higher than that of pomegranate juice concentrate. Phenolic compounds of boiled and pasteurized juices were decreased. The methanol extraction with posterior acid hydrolysis which was practiced has shown to be suitable both for phenolic standards and pomegranate extracts. A methanol-based mobile phase was employed, and the convenience of this organic solvent can substitute acetonitrile as the most commonly used solvent. An exhaustive method validation was achieved, and it showed that the proposed method is linear in the studied concentration range, sensible, precise, and accurate. The phenolic contents obtained for three pomegranate juices from Turkey was in agreement with those reported in literature.

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