

Original Article

The Extraction and Determination of Ellagic Acid Content in the Peels of Six Iranian Pomegranates Cultivars Using a New Miniaturized Matrix Solid-Phase Dispersion Method

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Abstract

Background and Aim: The peels of six Iranian pomegranates (*Punica granatum* L.) cultivars, as a traditional medicine, were treated with a new miniaturized matrix solid-phase dispersion (MSPD) method for the HPLC determination of ellagic acid (EA).

Materials and Methods: In the proposed method, only 10mg of the sample powder was ground and blended with an equal amount of C18 sorbent in an agate mortar. The use of the agate mortar with smooth surface facilitated the sample transfer into a cartridge and reduced the required amount of sample and sorbent. Micro volumes of dichloromethane, n-hexane and methanol were used as modifier, washing and elution solvents, respectively. The eluate was injected into an HPLC-UV system for the analysis.

Results: Several factors such as the type and amount of dispersing sorbent, modifier, washing solvent and eluent were carefully studied and optimized. Six replicated analyses at the optimized conditions resulted in a recovery of 96.7% and a relative standard deviation of 5.87%. The proposed method was successfully applied to the extraction and determination of EA in the peels samples.

Conclusion: According to the ultimate results, the MSPD method is an efficient technique for the quantitative extraction of EA from the peels of pomegranate. Malas cultivar has the highest amount (18.1 g kg⁻¹) of ellagic acid content compared to the other studied pomegranate cultivars.

Keywords: Matrix solid-phase dispersion, HPLC, Ellagic acid, Pomegranate

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Introduction

Punica granatum L. belongs to *Punicaceae* family. It is native to Iran (1) and has been widely distributed in many regions over the world including Iran, California, Turkey, Egypt, Italy, India, Chile and Spain (2). Pomegranate is an important nutritious-medicinal plant which has been reported to have

potent anti-inflammatory, anticancer, antiviral, antioxidant and anti-atherosclerotic activities attributed to its polyphenolic content (3, 4). Different parts of pomegranate such as fruits, peels, seeds and leaves have been commonly employed in herbal remedies by local healers in many countries (5). Pomegranate contains several important polyphenols including punicalagin, ellagic acid and other

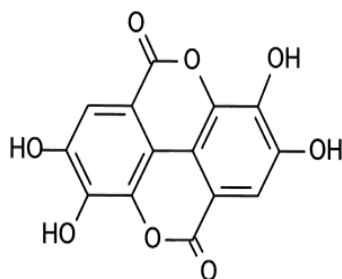


Figure 1. Structure of ellagic acid.

ellagitannins (2, 3, 6). Ellagic acid is one of the important polyphenolic compounds in pomegranate peel. Structure of ellagic acid (EA) has been shown in Figure 1. Li *et al.* in 2006 reported that pomegranate peel has the highest antioxidant activity among the peels, pulp and seed fractions of 28 kinds of fruits commonly consumed in China (7).

Matrix solid phase dispersion (MSPD) was introduced in 1989 by Barker *et al.* (8). This method has found special applications for the preparation, extraction and isolation of a wide range of drugs, herbicides, pesticides and naturally occurring constituents from a wide variety of solid, semi-solid or highly viscous samples such as animal tissues, fruits, vegetables and other matrices (9-13). This method involves several simple principles of chemistry and physics. After mechanical blending of a solid support material with sample, the sample is dispersed over the surface of the solid support by grinding forces (14). Classical sample preparation methods to prepare solid or semi-solid samples usually consist of multi and complex steps. These preparation techniques need a large amount of sample, sorbent, organic solvents, and are time consuming. MSPD can eliminate these problems and is simpler, more flexible and faster than classical methods for the pretreatment of solid, semi-solid and highly viscous samples (15-17).

In this study, a miniaturized MSPD method using agate mortar for grinding and octadecylsilane (C18) as sorbent was developed to extract ellagic acid from pomegranate peel before its determination by high performance liquid chromatography (HPLC). The amount of ellagic acid in six Iranian commercial pomegranate (*punica granatum L.*) cultivars were determined and compared using the proposed MSPD-HPLC-UV method.

Materials and Methods

Reagents and chemicals

Ellagic acid standard was purchased from Sigma Co. HPLC grade acetonitrile, ethyl acetate; cyclohexane, methanol, ethanol, acetone, dichloromethane (DCM), n-hexane, orthophosphoric acid, and Chromobondoctadecylsilane (C18) adsorbent were prepared from Merck (Darmstadt, Germany). Double-distilled water was used throughout. Diatomaceous Earth, DE, (95% SiO₂) was prepared from Aldrich Chemical Co.

Apparatus

HPLC analysis of the samples was conducted using a Shimadzu (Model L-10AD) instrument consisting of two reciprocating pumps, a DGU-14A in-line degasser, a Model CT10-10AC oven, a high-pressure manual injection valve (20μL injection loop) and a UV/VIS (Model SPD-10A) detector. The software used for the data acquisition and processing was Class-VP V.R 6.1. The analytical column was a 25cm×4.6mm i.d. RP-18 column (Shim-Pack CLC-C18) packed with 5μm particles and equipped with a 1-cm guard column (C18-B197) packed with 10 μm particles of the same type. A 25μL HPLC microsyringe (F-LC, SGE, and Australia) was used for to samples withdrawal and injection.

Plant materials

The pomegranate samples were collected from the pomegranate collection of a research garden in Saveh (Shahvar, Siah, Abdanan, Yousofkhani and Malas cultivars) and Ardestan collection (Bidaneh cultivar), Iran, at ripening stage in the autumn of 2013. Approximately 200g of the pomegranate peels were washed twice with distilled water and air-dried at room temperature (20-25°C). Dried pomegranate peels were chopped and powdered using a household blender and stored in refrigerator until being used for analysis.

Analysis of samples by the MSPD method

In the optimized conditions, 10mg of a peel samples were accurately weighted and mixed with 10mg C18 and ground in an agate mortar with an agate pestle for 5 min to obtain an apparent homogeneous blend. The use of an agate mortar with smooth surface reduces the samples carry-over and facilitates transferring of the material to the cartridge. Before transferring the

mixture into a cartridge, 40 μ L DCM as modifier was added to be mixed with the blend. After evaporation of DCM, the mixture was transferred into an empty cartridge and compressed with a plunger. Then, 150 μ L of n-hexane as washing solvent was passed through the cartridge. Finally, the analyte was eluted by 350 μ L methanol and the eluate was collected into a microvial to be injected into HPLC after being filtered through a 0.45 μ m syringe filter. A matrix-matched calibration, using methanol as solvent, was used for the quantification of the extracts.

For HPLC separation of compounds, a gradient elution with a mixture of solvents A (5% methanol and 95% aqueous solution of 0.1% phosphoric acid) and B (50% methanol and 50% aqueous solution of 0.1% phosphoric acid) with a flow rate of 0.8 mL min⁻¹ was used. The temperature of column was 40°C. The absorption of compounds was detected at a wavelength of 254 nm. Table 1 indicates the elution program used.

A one-at-a-time method was applied for optimization of the effects of different parameters affecting the extraction by the MSPD method. The studied and optimized parameters were the natures and amounts of sorbent, modifier, washing solvent and eluent. The Malas pomegranate cultivar was used for optimization of the parameters.

Ultrasonic extraction

In order to estimate the analytic recovery in the MSPD method, the EA concentration in the sample was also evaluated by an ultrasonic assisted solvent extraction method. For the ultrasonic extraction, 1.0g of each pomegranate peel sample powder was dispersed in 20 mL of methanol in a centrifugation tube placed in an ultrasonic bath for 25 min. The mixture was then subjected to centrifugation at 4000 rpm for 5 min. The supernatant solution was filtered through a 0.45 μ m syringe filter and analyzed by HPLC.

Experimental design and statistical analysis

The dried and powdered pomegranate peels were homogenized and three weighed increments of it were separately extracted and analyzed by the MSPD-HPLC-UV method. For the comparison of different cultivars, a one way ANOVA method was applied using Minitab (version 17.1.0) software. For calculation of standard deviations and calibration

curve data usual statistical method were used. The detection limit of the method was calculated from three times of the standard deviation of 20 noise signals divided by the slope of the calibration curve.

Results and Discussion

Effect of dispersing sorbent

In the MSPD procedure, the sorbent is used as an adsorption separation material that is dispersed into the sample matrix and closely interacts with its constituents by the grinding procedure. Silica gel, C18 and DE were examined in order to find the most suitable dispersing adsorbent using methanol as the eluting solvent. The results have been shown in Figure 2. C18 was selected as dispersing sorbent because of its higher extraction efficiency. C18 is a suitable sorbent for the extraction of non-polar and moderately polar compounds and is the most widely used sorbent in SPE applications.

Effect of dispersing sorbent to sample ratio

To obtain a successful extraction, the ratio between the dispersing sorbent and sample must be optimized. Five different ratios of C18 to sample mass, i.e., 1:1, 2:1, 3:1, 4:1 and 5:1 were tested. The results in Figure 2, indicate that the 1:1 mass ratio produced a higher recovery than the other ratios. It seems that in this ratio, the sample is homogenized and dispersed more efficiently in the sorbent and as a result an appropriate contact between the analyte and sorbent is achieved. Thus, this ratio was selected for the subsequent experiments.

Table 1: HPLC gradient elution; solvents A (methanol solution 5% and phosphoric acid 0.1%) and B (phosphoric acid 0.1% and methanol 50%), flow rate 0.8 mL min⁻¹ and wavelength was 254 nm.

Step	Time (min)	Solvent A (%)	Solvent B (%)
1	0.01	90	10
2	18	36	64
3	22	0	100
4	25	36	64
5	35	90	10

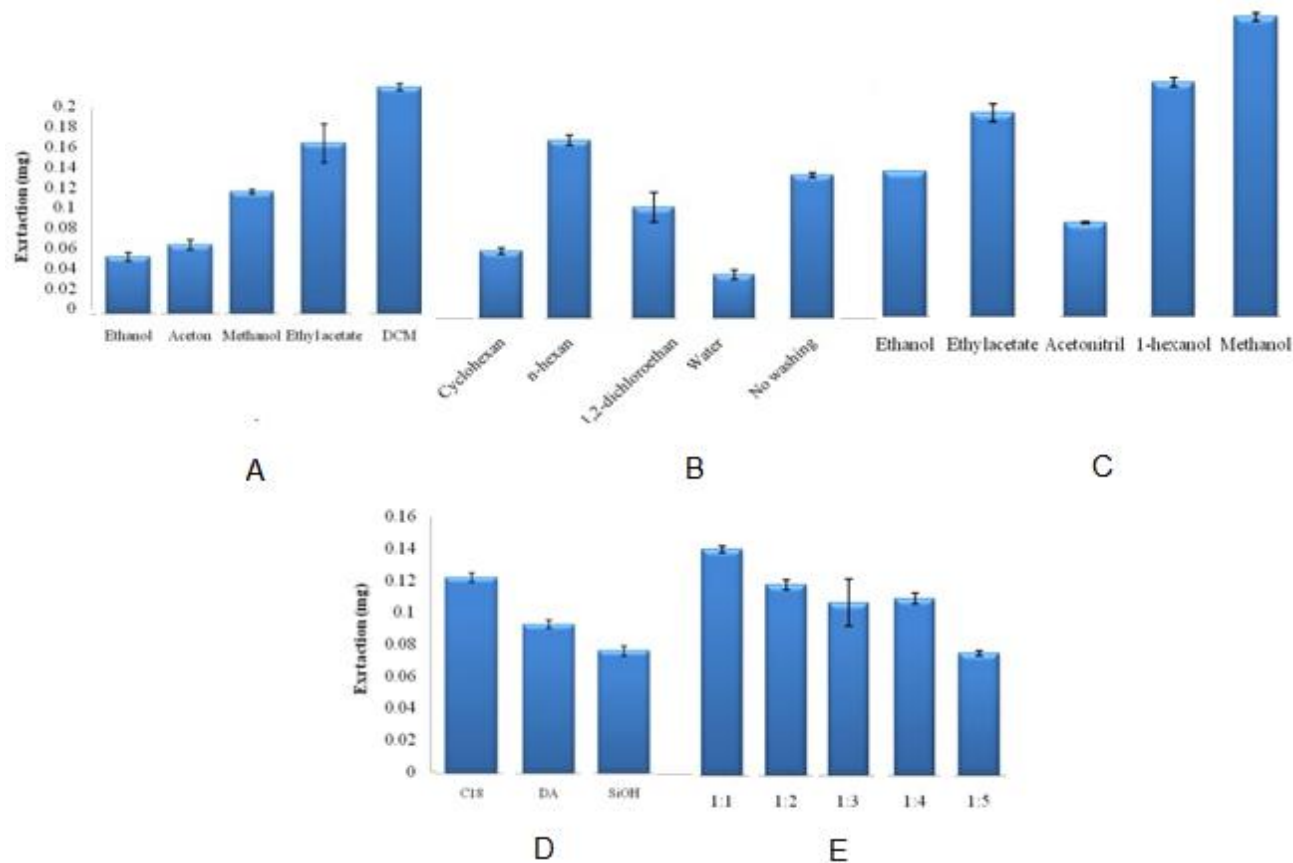


Figure 2. Effect of modifier solvent (A) washing solvent (B), eluent type (C), sorbent type (D) and sample-sorbent ratio (E) on the extraction amount of EA by the MSPD method. Conditions: sorbent, C18; sample-sorbent ratio, 1:1; modifier solvent volume, 40µL; eluent solvent, methanol (350µL). Vertical bars indicate standard deviations for three replicates.

Effect of modifier solvent and its volume

The next factor which was studied and optimized was the effect of addition of a modifier solvent on the extraction procedure. Although modifier solvents have not been commonly used in the MSPD method, it has been shown that addition of an appropriate modifier, also called disperser solvent, increases the contact between sample matrix and sorbent and therefore improves the recovery of analyte (18, 19). Some organic solvents such as ethyl acetate, ethanol, methanol, acetone and DCM were tested as modifier. Among them DCM increased the extracted amount of EA. So, this solvent was selected as the most appropriate modifier (Figure 2). In order to optimize the volume of the modifier, different volumes of DCM, i.e., 5, 10, 20, 40 and 60µL were mixed with the sample and it was found that the extracted amount of EA increases by increasing the amount of DCM up to 40µL, with no further increase in higher volumes.

Effect of washing solvent

Washing solvent is often used in extraction methods in order to remove potential interferences to obtain a clean matrix for the analyte. Furthermore, the C18 chains tend to coil up in the dry form. Treatment with an organic solvent causes these chains to uncoil and a good surface contact between the analyte and solid phase is obtained that increases the recovery. For

Table 2: Concentration of EA (C_{EA}) in six pomegranate cultivars determined by the MSPD-HPLC method.

No.	Cultivar	C_{EA} (g Kg ⁻¹) ^a
1	Shirin shahvar	1.3 (±0.09)
2	Siah	2.5 (±0.24)
3	Abdandan	6.1 (±0.47)
4	Bidaneh	3.4 (±0.28)
5	Yousofkhani	7.2 (±0.64)
6	Malas Saveh	18.1 (±0.72)

^aAverage recoveries (± standard deviations for three replicates).

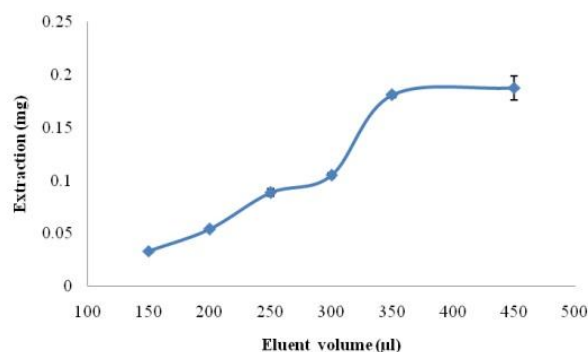


Figure 3. Effect of eluent volume on the extraction amount of EA by the MSPD method. Conditions: sorbent, C18; sample-sorbent ratio, 1:1; modifier solvent, dichloromethane (40µL); washing solvent, n-hexane; eluent solvent, methanol. Vertical bars indicate standard deviations for three replicates.

evaluation of suitable washing solvents, some solvents such as cyclohexane, n-hexane, 1,2-dichloroethane and water were tested. As shown in Figure 2, when n-hexane was used as washing solvent, a higher recovery was obtained for the analyte. Washing with n-hexane possibly removes the non-polar interferences. Various volumes of the washing solvent were also tested and the maximum recovery was obtained for a volume of 150µL that was selected as optimal.

Selection of the elution solvent and its volume

Selection of an appropriate eluent which can efficiently elute the analyte with a minimum volume is critical in the MSPD extraction. For this purpose, some solvents with different polarities such as ethanol, ethyl acetate, acetonitrile, 1-hexanol and methanol were investigated. In general, phenolic compounds such as ellagic acid are polar compounds, which are best extracted with more polar solvents. As shown in Figure 2, methanol showed the most efficient elution of EA from the sorbent. Acetonitrile, with weak hydrogen bonds, showed the worse results. Therefore, methanol was used as elution solvent in subsequent experiment.

Effect of volume of methanol on the extraction of EA was also investigated. As shown in Figure 3, different volumes from 100 to 450µL of the elution solvent were tested. By increasing the volume, the extraction amount of the analyte was increased, as expected, reaching to a plateau at 350µL that was selected as optimum.

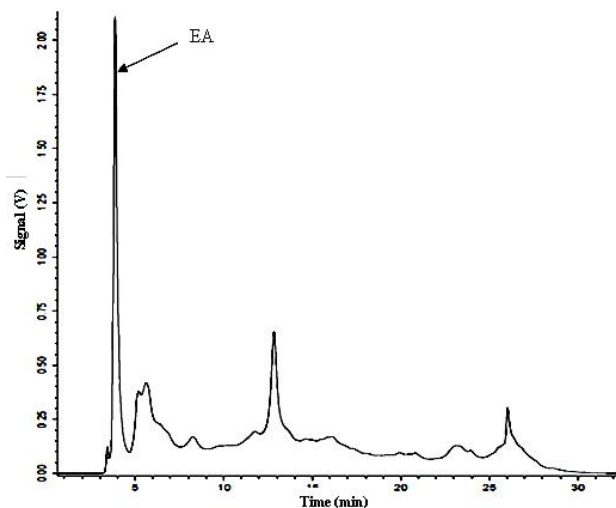


Figure 4. Chromatogram of the extract of EA under the optimized conditions for Malas Pomegranate Peel. See the text for experimental conditions.

Analytical performances

To investigate the analytical performances of the proposed MSPD-HPLC-UV method, six replicated analyses at the optimized conditions (10mg C18 as sorbent, 10 mg sample, 40µL DCM as modifier, 150µL n-hexane as washing solvent and 350µL methanol as eluent solvent) resulted in a recovery of 96.7% with a relative standard deviation (RSD) of 5.9%. The recovery was calculated by comparison of the MSPD results with the data obtained from an ultrasonic assisted extraction by methanol, as mentioned previously.

The detection limit of the method (3σ) was calculated to be 1.3 mg L^{-1} for the analyte. The calibration curve was linear over a range of 5–800 mg L^{-1} of EA, with an R^2 value of 0.994.

Figure 4 shows a chromatogram of the pomegranate peel after extraction under the optimized conditions. As shown in the chromatogram, the retention time of EA was about 4min.

Table The MSPD of pomegranate samples

The MSPD method was applied to the extraction of EA in the peels of six different pomegranate cultivars in Iran. Table 2 indicates the concentration of EA in the samples. The results indicate that the amount of EA in Malas cultivar is substantially higher than that of other pomegranate cultivars.

The amount of EA in the pomegranate peels have been rarely reported in the literature. Nasr *et al.* (1996) reported an EA content of 0.117 g Kg^{-1} for the peels of

Tunisian Chelfi variety pomegranate which are substantially lower than that of the studied pomegranate samples in this work (20).

In addition, generally times consuming solvent extraction methods with the use of large amounts of organic solvents have been used for the extraction of EA or other phenolic compounds from peel samples.

Conclusion

The study indicated that quantitative extraction of EA from pomegranate peel is feasible by the proposed MSPD method. The use of an agate mortar with smooth surface reduced the samples carry-over and facilitated transferring of the material to the cartridge. The miniaturized and the optimized MSPD method was quite efficient and appropriate for the extraction and HPLC analysis of the target compound using only 10mg of the sample and the C18 sorbent.

The method was simple, fast and inexpensive with minimum consumption of organic solvents compared to ordinary solid phase extraction and solvent extraction methods. In addition, good linearity, precision and reproducibility and a high recovery was obtained in the proposed method. The study also showed that there is a substantial amount of EA, as an important phenolic compound, in the studied pomegranate peels which its amount varies in different plant species.

Acknowledgment

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Conflict of Interest

The authors declare that they have no conflict of interest.

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