Herbal Medicines Journal **Spring 2017; Vol. 2, No. 2:71-9**
DOI: https://doi.org/10.22087/hmj.v0i0.615

Original Article

The Efficient Extraction of Phenolic Compounds from Oak Gall Using a Miniaturized Matrix Solid-Phase Dispersion Method before their HPLC Determination

Ahmad Daneshfar[1], Payman Hashemi[1*], Bahram Delfan[2], Majid Tavakkoli[3], Parivash Mollaei Rashno[1]

[1] Department of Chemistry, Lorestan University, Khorramabad, Iran
[2] Razi Herbal Medicines Research Center, Lorestan University (Medical Sciences), Khorramabad, Iran
[3] Agriculture and Natural Resource Research Center, Khorramabad, Iran

Received: 17.2.2017; Accepted: 22.04.2017

Abstract

Background and Aim: Several gall varieties are found in Lorestan Province, Iran, on *Quercus infectoria* oak trees, which contain important phenolic compounds. In this work, a miniaturized matrix solid-phase dispersion (MSPD) extraction method has been developed for quantitative extraction and HPLC/UV determination of them.

Materials and Methods: In the MSPD method, 10 mg of sample and 50 mg of silica gel adsorbent were transferred into an agate mortar. The mixture was finely pulverized after adding 40 µL dichloromethane as disperser solvent. It was then transferred into a cartridge, eluted by 350 µL of methanol, and the eluate was subsequently injected into HPLC for analysisn.

Results: The extractions were quantitative with mean recoveries of 103.0±6.8% and 99.5±7.3% for ellagic acid (EA) and gallic acid (GA) in six replicated extractions, respectively. The detection limit of the method was 0.05-0.06 mg g^{-1}. The method was successfully applied to the extraction and HPLC determination of the phenolic compounds in five gall species.

Conclusion: The proposed technique is simple and fast. It substantially reduced the amounts of sample, sorbent and organic solvents required for the extraction. The maximum amounts of the phenolic compounds were found in Qalqaf and Bramazu galls.

Keywords: Phenolic compounds, Oak gall, Matrix solid-phase dispersion, *Quercus infectoria*, HPLC

***Corresponding Author:** Payman Hashemi, Department of Chemistry, Faculty of Science, Lorestan University, 6713817133, Khorramabad, Iran. Tel/Fax: (+98) 66 33120611; Email: payman_hashemi@yahoo.com.

Please cite this article as: Daneshfar A, Hashemi P, Delfan B, Tavakkoli M, Mollaei Rashno P. The Efficient Extraction of Phenolic Compounds in Oak Gall Using Miniaturized Matrix Solid-Phase Dispersion Method before HPLC Determination. Herb. Med. J. 2017;2(2):71-9.

Introduction

Phenolic compounds are the most abundant and at once one of the most important groups of compounds in plants. An aromatic ring, with one or more hydroxyl groups, exists in all phenolic compounds. Different categories for these compounds are described. For example, a commonly used classification is based on

the number of carbon atoms conjugated with phenolic structures. The compounds act as the protection factors against UV radiation and prevent oxidation of food. Phenolic compounds have significant antimicrobial effects. Moreover, they are heart-protective, anti-allergic and anti-inflammatory (1).

Gallic acid (GA) is a phenolic acid (3, 4, 5 tri-hydroxy benzoic acid) which is usually found in two forms of free and hydrolysable tannins. GA is found in grapes, sumac, tea leaves, hops, oak and some other plants. Gallic acid is used as a standard in Folin-Ciocalteu method for the assay of total phenolic compounds. It is also commonly used in the pharmaceutical industry and for making paints and inks (2). This phenolic compound has been applied in the treatment of diabetes and albuminuria (3). GA has shown anti-viral, anti-fungal and antioxidant properties. It protects human cells against oxidative damage and acts as an astringent in cases of internal bleeding. GA has been also used as a flavoring spice in food (2). Pharmacological researches have indicated that this material has several biological properties such as antimicrobial (3), anti-inflammatory (4), antioxidant (5, 6), anti-cancer (7-10) and anti-mutagenic (6, 8) effects.

Ellagic acid (EA) is one of the poly-phenolic compounds and at the same time a dimer derivative of GA. It has been studied extensively due to its effects on the astringent activity of hemorrhoids and its impact on skin whitening. Moreover, some reports published about the anti-cancer effects of EA in the last decade (11, 12) have demonstrated its anti-tumor properties and free radicals scavenging activity. It has also strong antioxidant effects (13). Polyphenolic compounds are found at high levels in many plants used as herbal medicines. The existence of EA in 46 types of fruits including berries, blackberries, blueberries, pomegranates, grapes, nuts and oak galls has been reported (11, 12, 14, 15).

Gall has been used for various purposes even as food in the East. It is still used in the leather industry (for tanning and leather processing) and making some commercial ink colors (16). Oak galls contain large amounts of tannin and smaller amounts of GA and EA (17, 18). Galls exhibit antioxidant effects and have shown medicinal value as a topical anesthesia, antipyretic and anti-Parkinson (19).

Matrix solid phase dispersion (MSPD) is a simple and inexpensive sample preparation method which is used for solid, semi-solid and viscous other samples. In this method, a sample is pulverized mechanically with a sorbent in a mortar before being transferred into a cartridge. The analyte is then eluted from the cartridge using an appropriate solvent. The common sorbents used in SPE columns are C18 (20), C8, alumina, silica gel, magnesium silicate, sand (21) and molecularly imprinted polymers (22, 23). MSPD can take advantages of high-speed extraction, low consumption of organic solvents, simplicity, efficiency and safety. A disadvantage of MSPD could be the relatively high consumption of adsorbents which are discarded after each single use. The use of agate mortars with smooth surface can substantially reduce the total amount of sample and adsorbent required for the extraction. It can also miniaturize the system.

In the present study, a miniaturized MSPD method is developed by the use of an agate mortar for the extraction of phenolic compounds in gall samples prior to their determination by high performance liquid chromatography (HPLC). The method is applied to the study of EA and GA content of some *Quercus infectoria* oak gall species.

Materials and Methods

Chemicals

Ellagic acid, Gallic acid and Tannic acid were purchased from Sigma company. Methanol, ethanol, acetone, dichloromethane (DCM), 1,4-Dioxane, n-hexane, ethyl acetate, phosphoric acid, hydrochloric acid, dimethyl sulfoxide, diethyl ether and acetonitrile with analytical purity were purchased from Merck and used without additional purification. Diatomaceous earth (DE) was obtained from Aldrich Chemical Co. and silica gel (15-40 μm) and C18 were purchased from Merck.

Stock standard solutions (1000 $mg.L^{-1}$) of ellagic acid and gallic acid were prepared in methanol solvent. The working standards were prepared by the appropriate dilution of the stocks by double distilled water.

Instrumentation

HPLC system with a Shimadzu SCL-10ASP Model via C18 column model Wakocia II 5C18R length 25cm, 4.6 mm diameter and particle size of 5 μm, was

used to analyze the compounds. The apparatus was equipped with two reciprocating pumps, a model CT10-10AC oven, a continuous degasser model DGU-14A, a 20 µL sample loop and a UV/Vis. detector, SPD-10AVP model, equipped with a 8 µL quartz cell. Class-VP VR 6.1 software and 25 µL micro injector manufactured by Hamilton Company (USA) were used to inject into the HPLC system. An electric grinder, Honeywell ELs305E model, was used for grinding the gall samples. A spectrophotometer apparatus model UV-160 (Shimadzu) was used for absorbance measurements. An accurate digital scale, made by a Japanese Shimadzu Model AX 200 balance, with the accuracy of 0.0001 g was used to measure weight. The extracted samples were filtered by 0.45 µm Porafil membrane filter before analysis. A variable pipette (20-1000 µL), manufactured by Orange Scientific company (Belgium), was used for dispensing solutions, and an agate mortar and pestle were used for blending the sample and sorbent in the MSPD procedure.

Sample Preparation

Quercus infectoria is an oak species in Lorestan province, Iran. *Q. infectoria* subsp. *boissieri* is a hygroscopic substance native to the area of Qalaei, Lorestan, Iran. The climate of this area is semi-humid Mediterranean with a minimum annual rainfall of 550-650 mm which is suitable for this species. Gal samples of *Andricus quercustozae* (qalqaf), *Andricus grossulariae* (tiqi or pantaly), *Andricus moreae* (kharnuk) and *Andricus sternlichti* (yellow mazoj) on Q. infectoria were collected from Qalaei region and *Aphelonyx persica* (bramazu) gall was obtained from Nojian region (Lorestan, Iran) in autumn 2012 (Fig. 1). The gall samples have been authenticated and deposited in the following institutions: Hungarian Natural History Museum (HNHM) Budapest, Hungary (curator S. Csosz); Collection of the Systematic Parasitoid Laboratory (SPL), Tanakajd, Hungary (curator G. Melika) and Research Institute of Forests and Rangelands (RIFR), Tehran, Iran (curator E. Sadeghi). The voucher numbers of the plant samples in the herbarium of RIFR are 95050 and 91433, respectively. The samples were shade-dried at room temperature and ground using an electric grinder.

MSPD procedure

In the optimized procedure, 10 mg of a milled gall sample, 50 mg of silica gel adsorbent and 40 µL of DCM solvent were mixed, and then the mixture was pulverized in an agate mortar for a few minutes. After being homogenized, the mixture was carefully transferred into a cartridge with a filter disc at bottom using a spatula. The analytes were then eluted from the cartridge using 350 µL of methanol as elution solvent.

Chromatography Method

Identification of the chromatographic peaks was performed by comparing their retention times with the values obtained for their individual standards. For compounds quantification, 20 µL of standard solutions with various concentrations were injected into the HPLC system, and the calibration curve was drawn according to the standards' peak areas.

HPLC analysis of the real samples was performed using a flow rate of 0.8 mL min^{-1}, an oven temperature of 40 °C and a detector wavelength of 254 nm. For the elution, a gradient program comprised of solvent A, 5% methanol/water solution containing phosphoric acid 0.1% and solvent B, 50% methanol/water solution containing phosphoric acid 0.1% were used. The elution program was as follows: 0 – 18 min, 10 – 64% B; 18 – 22 min, 64 – 100% B; 22 – 25 min, 100 – 64% B and 25 – 35 min, 64 – 10% B.

Results and Discussion

Sorbent Selection

Selecting a suitable adsorbent is an important step in a MSPD overall optimization, because this factor has a direct impact on the efficiency of extraction. Three sorbents of silica gel, diatomaceous earth (DE) and C18 were used for the extraction of the analytes via MSPD method. In this step, 10 mg sorbent with 10 mg of a ground gall sample (1:1) were mixed for the extraction. Dioxan (150 µL) and methanol (300 µL) were used as the disperser and elution solvents in this step. The results indicated that with regard to the number of components that appeared in the chromatogram, the number of silica gel sorbent with larger peaks for GA and EA was more when compared to the other sorbents (Fig. 2). Therefore, this sorbent was used in subsequent experiments.

Selection of the Elution Solvent

An elution solvent should be able to elute the analytes

Table 1: The results of the analysis of five gall samples by the MSPD-HPLC/UV method under the optimized conditions.

No	Gall species	Local name	EA (mg g^{-1})	GA (mg g^{-1})
1	*Aphelonyx persica*	*Bramazu*	15.2 (±0.2)*	47.4 (±0. 4)
2	*Andricus quercustozae*	*Qalqaf*	3.0 (±0.1)	65.5 (±1.5)
3	*Andricus grossulariae*	*Pentali*	10.2 (±0.10)	6.9 (±0.10)
4	*Andricus moreae*	*Kharnook*	< LOD	14.4 (±0.8)
5	*Andricus sternlichti*	*Mazooj-e-Zard*	< LOD	16.1 (±0.2)

* The figures within parentheses are standard deviations for three replicates.

Figure 1. Photographs of five gall species studied in this work.

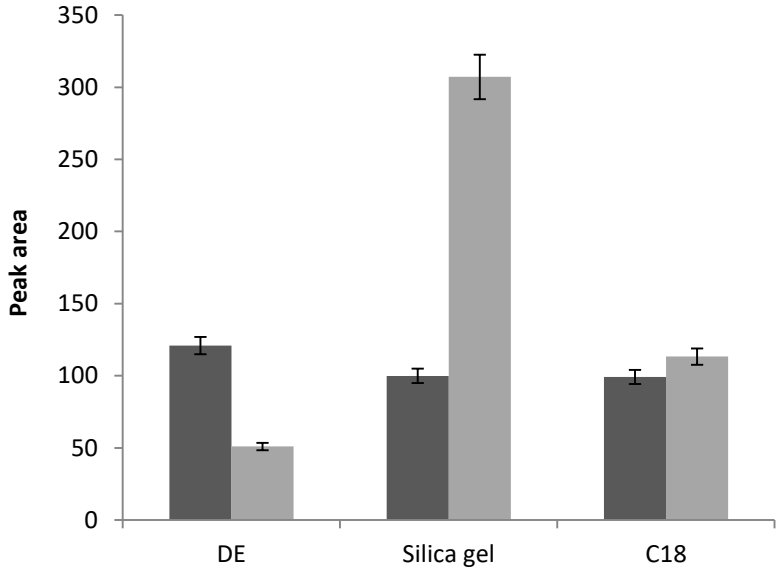

Figure 2. Comparison of different sorbents for the extraction of EA (■) and GA (■) by the MSPD method. Experimental conditions: 10 mg sample, 10 mg sorbent, 120 µL dioxane and 300 µL methanol (eluent).

with the highest efficiency and minimum volume. Due to the polarity of phenolic compounds, solvents with sufficient polarity were used to desorb the analytes with no harm to the sorbent.

For this purpose, methanol, ethanol (70%), distilled water, acetone, acetonitrile and dimethyl sulfoxide were studied as elution solvents. Among the solvents listed, methanol showed the highest extraction

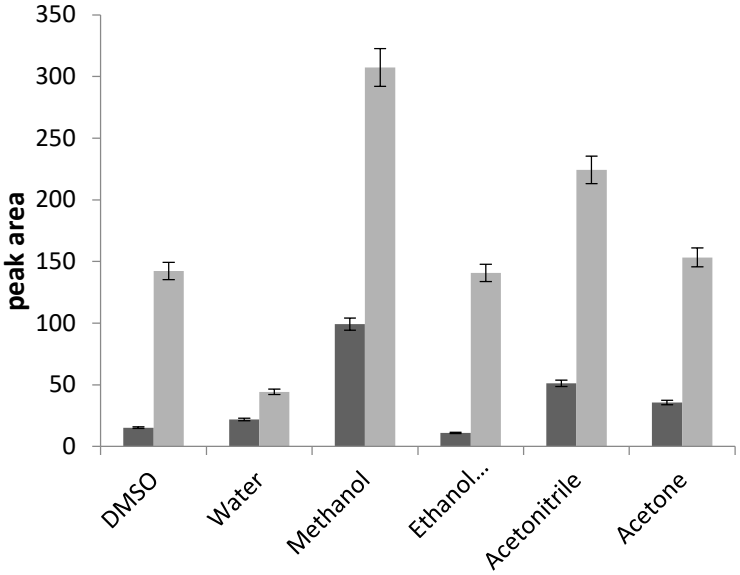

Figure 3. Comparison of different elution solvents for the extraction of EA (■) and GA (■) by the MSPD method. Experimental conditions: 10 mg sample, 10 mg silica gel, 120 µL dioxane and 300 µL eluent..

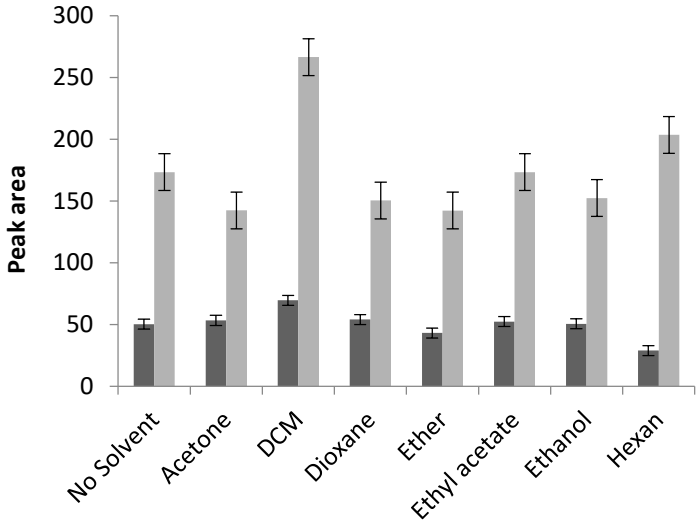

Figure 4. Comparison of different dispersion solvents for the extraction of EA (■) and GA (■) by the MSPD method. Experimental conditions: 10 mg sample, 10 mg silica gel, 120 µL dispersion solvent and 300 µL methanol (eluent).

efficiency (Fig. 3). In addition, methanol was a major constituent of the mobile phases in HPLC, and therefore, it was more compatible with the mobile phase and resulted in more symmetric peaks.

Selection of the Disperser Solvent

Addition of a disperser (modifier) solvent to the sample-sorbent mixture during the blending step of MSPD may improve the extraction efficiency (20). This solvent wets the mixture and facilitates the blending of the sample and sorbent and also contributes to the isolation of the analytes from the sample matrix. To select an appropriate disperser solvent for the desired extraction, the seven solvents of acetone, diethyl ether, 1,4-dioxane, hexane, ethanol, dichloromethane (DCM) and ethyl acetate were used. An experiment also was performed using no disperser solvent for comparison. According to the results shown in Fig. 4, DCM was the best solvent and was used as the disperser (modifier) solvent in subsequent experiments.

Optimizing the Amount of Disperser Solvent

To optimize the disperser solvent volume, different volumes of DCM (20 - 120 µL) were tested. According to the results shown in Fig. 5, 40 µL DCM

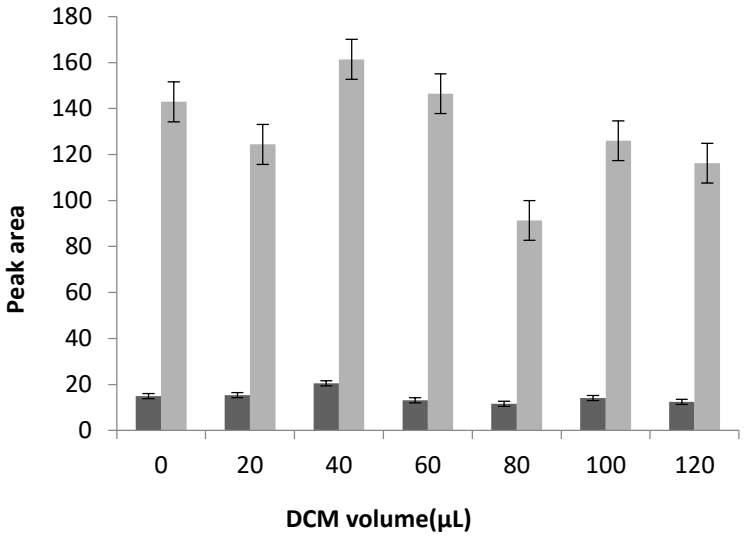

Figure 5. Effect of dispersion solvent (DCM) volumes on the extraction of EA (■) and GA (■)by the MSPD method. Experimental conditions: 10 mg sample, 10 mg silica gel and 300 µL methanol (eluent)**.**

was the most appropriate volume to be used in the extraction method.

Optimizing the Sample to Sorbent Ratio

Another important factor in MSPD is the ratio of the amount of sample to sorbent. Different sample to sorbent ratios were examined from 1:1 to 1:7 for this purpose using the optimal parameters of the previous steps. Fig. 6 shows that the 1:5 ratio provides the highest extraction efficiency of target analytes from the gall sample. So, this ratio was used in subsequent experiments.

Optimizing the Elution Solvent Volume

Elution solvent volume is an effective parameter that influences both recovery and enrichment factors in MSPD method. This volume should be enough high to desorb efficiently the analytes from the cartridge but not so high to reduce the preconcentration factor. For optimization, different methanol volumes of 300 - 600 µL were used. According to the results shown in Fig. 7, 350 µL was selected as the optimal methanol volume as eluent.

Therefore, 10 mg of sample, 50 mg of silica gel adsorbent, 40 µL DCM as dispersive solvent and 350 µL methanol as elution solvent were selected as the

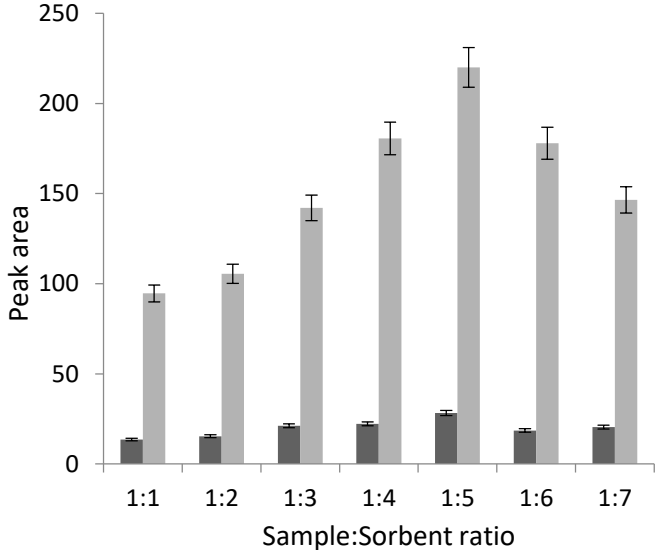

Figure 6. Effect of the sample: adsorbent ratio on EA (■) and GA (■) extraction by the MSPD method. Experimental conditions: 10 mg sample, different amounts of silica gel, 40 µL DCM (disperser) and 300 µL methanol (eluent).

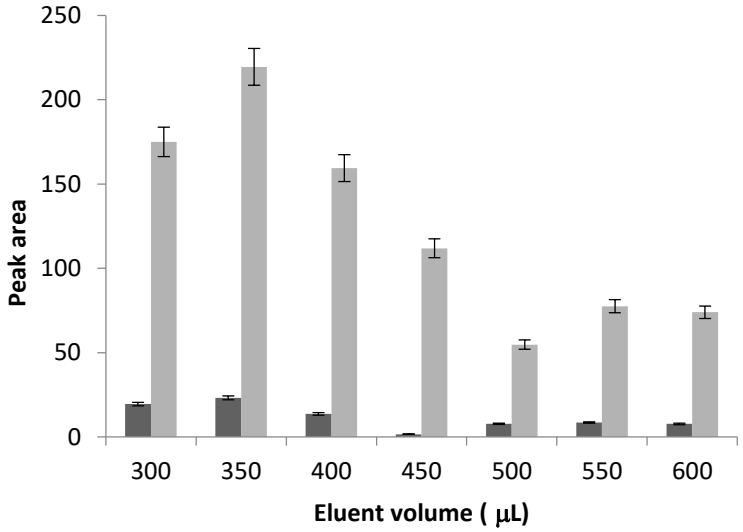

Figure 7. Effect of the elution solvent volume on EA (■) and GA (■) extraction by the MSPD method. Experimental conditions: 10 mg sample, 50 mg silica gel, 40 µL DCM (disperser) and different volumes of methanol (eluent).

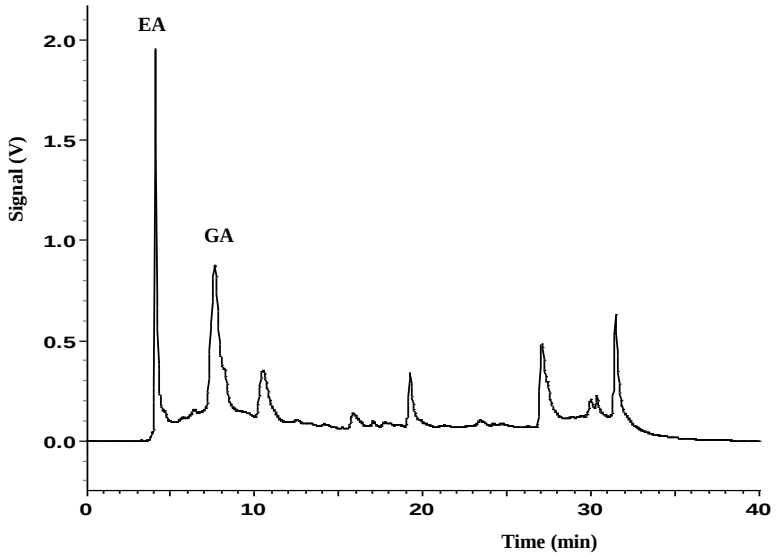

Figure 8. A chromatogram obtained from *Aphelonyx persica* gall after extraction by the MSPD method under the optimized conditions.

optimized conditions for GA and EA extraction from gall samples by the miniaturized MSPD method.

Analytical Performance

Linear calibration curves were obtained within the studied range of 5 - 2000 mg L^{-1} for the HPLC analysis of EA and GA with the equations of $S = 38433C + 2 \times 10^6$ and $S = 42623C - 7.9 \times 10^5$ and R^2 values of 0.993 and 0.994, respectively.

The repeatability of the MSPD method with six replicated measurements of a gall sample was studied under the optimized conditions (i.e. 1:5 sample to sorbent ratio, 40 µL DCM as disperser solvent and 350 µL methanol as elution solvent). Relative standard deviations of 6.81 and 7.31 were obtained for GA and EA with mean recoveries of 103% and 99.5%, respectively. The recoveries were calculated through comparing the results with an ultrasonic assisted solvent extraction method using 0.2 g of sample and 10 mL methanol: water (50% v/v) as solvent. The limit of detection (LOD) was evaluated based on 3 times of the standard deviation for 20 blank measurements. The calculated LOD was 0.05 mg g^{-1} for GA and 0.06 mg g^{-1} for EA.

Analysis of Gall Samples

EA and GA compounds were extracted and determined by the MSPD method in the five gall species of *Andricus phelonyx persica*, *Andricus quercustozae*, *Andricus grossulariae*, *Andricus moreae*, and *Andricus sternlichti*. The results have been listed in Table I. Based on the results, the two first gall species with the local names of *bramazv* and *qalqaf* contain the highest amounts of EA and GA compared to the other species.

A typical chromatogram of a gall extract obtained under the optimized conditions has been shown in Fig. 8. As specified in the chromatogram, the EA and GA peaks have appeared at 4.1 and 8.8 min, respectively.

Different parameters influencing the extraction efficiency of the phenolic compounds by the MSPD procedure were studied and optimized in this research. The optimization was carried out using a one-variable-at-a–time method. Silica gel was the most efficient sorbent for the extraction. Probably, the silanol groups (Si-OH) of silica gel are responsible for the more efficient extraction of the phenolic compounds.

The study showed that the utilization of a disperser (or modifier) solvent may be helpful for the better extraction of the components in MSPD method. Here, using 40 µL DCM proved to be the best choice among the studied solvents. It seems that the modifier acts as an intermediate phase that influences inside the texture of the plant material and releases the target compounds from it. The released compounds are then adsorbed by the silanol groups of the sorbent particles.

Our effort was to use the minimum possible amounts of sample and sorbent weights. For this purpose, an agate mortar with smooth surface was used to minimize the sample or sorbent loses during their grinding and transferring steps. The utilization of agate mortars diminished the amount of sample to only 10 mg. However, a 1:5 ratio of sample to sorbent was the most efficient model for the extraction and therefore, 50 mg of sorbent was required in each run.

The method quantitatively extracted the target compounds under the optimized conditions. Therefore, the proposed method may replace the classical techniques such as solvent extraction method. Study of oak gall samples by the proposed method indicated that there was a significant difference between the amounts of EA and GA in different varieties of gall samples.

Conclusion

It may be concluded that the proposed miniaturized MSPD method is simple, quick and inexpensive for the efficient extraction of phenolic compounds such as EA and GA in gall samples. The MSPD method requires small amounts of sample and organic solvents compared to other extraction methods. Hence, it is considered a green technique. The study indicated that there was a significant variation in the amounts of the phenolic compounds in different gall species. The qalqaf and bramazu galls contained relatively higher amounts of EA and GA. Therefore, they may be used as sources of these valuable products.

Acknowledgment

The financial support of Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences is gratefully acknowledged.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Hurtado-Fernández E, Gómez-Romero M, Carrasco-Pancorbo A, Fernández-Gutiérrez A. Application and potential of capillary electroseparation methods to determine antioxidant phenolic compounds from plant food material. J Pharmaceut Biomed. 2010;53(5):1130-60.
2. Abbasi S, Daneshfar A, Hamdghadareh S, Farmany A. Quantification of Sub-Nanomolar Levels of Gallic Acid by Adsorptive Stripping Voltammetry. Int J Electrochem Sci. 2011;6:4843-52.
3. José-Yacamán M, Martín-Gil J, Ramos-Sánchez MC, Martín-Gil FJ. Chemical Composition of a Fountain Pen Ink. J Chem Edu. 2006;83(10):1476.
4. de Mejia EG, Chandra S, Ramirez-Mares M, Wang W. Catalytic inhibition of human DNA topoisomerase by phenolic compounds in Ardisia compressa extracts and their effect on human colon cancer cells. Food Chem Toxicol. 2006;44(8):1191-203.
5. Hsieh CL, Lin YC, Yen GC, Chen HY. Preventive effects of guava (Psidium guajava L.) leaves and its active compounds against α-dicarbonyl compounds-induced blood coagulation. Food Chem. 2007;103(2):528-35.
6. Limpisophon K, Schleining G. Use of Gallic Acid to Enhance the Antioxidant and Mechanical Properties of Active Fish Gelatin

Film. J Food Sci. 2017;82(1):80-9.
7. Kubola J, Siriamornpun S. Phenolic contents and antioxidant activities of bitter gourd (Momordica charantia L.) leaf, stem and fruit fraction extracts in vitro. Food Chem. 2008;110(4):881-90.
8. Hurrell RF, Reddy M, Cook JD. Inhibition of non-haem iron absorption in man by polyphenolic-containing beverages. Br J Nutr. 1999;81(4):289-95.
9. Cook JD, Reddy MB, Hurrell RF. The effect of red and white wines on nonheme-iron absorption in humans. Am J Clin Nutr. 1995;61(4):800-4.
10. Min Z, Colin WB, Lee AH. Cancer Epidemiol. Biomakets Prev. 2002;11:713.
11. Vekiari SA, Gordon MH, García-Macías P, Labrinea H. Extraction and determination of ellagic acid contentin chestnut bark and fruit. Food Chem. 2008;110(4):1007-11.
12. Amakura Y, Okada M, Tsuji S, Tonogai Y. High-performance liquid chromatographic determination with photodiode array detection of ellagic acid in fresh and processed fruits. J Chromatogr A. 2000;896(1–2):87-93.
13. Dalvi LT, Moreira DC, Andrade R, Ginani J, Alonso A, Hermes-Lima M; Ellagic acid inhibits iron-mediated free radical formation. Spectrochim Acta A. 2017; 173:910-917.
14. da Silva Pinto M, Lajolo FM, Genovese MI. Bioactive compounds and quantification of total ellagic acid in strawberries (Fragaria x ananassa Duch.). Food Chem. 2008;107(4):1629-35.
15. Mousavinejad G, Emam-Djomeh Z, Rezaei K, Khodaparast M. Identification and quantification of phenolic compounds and their effects on antioxidant activity in pomegranate juices of eight Iranian cultivars. Food Chem. 2009;115(4):1274-8.
16. Sadeghi Seyed A, Asareh M. Oak gall wasps. Research Institute of Forests and Rangelands. 2009.
17. Ikram M, Nowshad F. Constituents of Quercus infectoria. Planta Medica. 1977;31(03):286-7.
18. Bate-Smith E, Swain T. Flavonoid compounds. Comp Biochem. 1962;3:705-809.
19. Sanjay P. Antioxidant potential of galls of Quercus infectoria. J. Pharmacol. 2008;5(2):1531-41.
20. Dawidowicz AL, Rado E. Matrix solid-phase dispersion (MSPD) in chromatographic analysis of essential oils in herbs. J Pharmaceut Biomed Anal. 2010;52(1):79-85.
21. Barker SA. Matrix solid phase dispersion (MSPD). J Biochem Biophys Methods. 2007;70(2):151-62.
22. Pena M, Casais MC, Mejuto MC, Cela R. Development of a matrix solid-phase dispersion method for the determination of polycyclic aromatic hydrocarbons in sewage sludge samples. Anal Chim Acta. 2008;626(2):155-65.
23. Shi X, Jin Y, Liu J. Matrix solid phase dispersion extraction of ginsenosides in the leaves of Panax ginseng CM Mey. Food Chem. 2011;129(3):1253-7.