Journal of Research in Pharmacy

Research Article

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The extraction and determination of ellagic acid and resveratrol in blueberry species by HPLC-DAD and LC-MS/MS

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Received: 14 December 2022 / Revised: 20 December 2022 / Accepted: 21 December 2022

ABSTRACT: Ellagic acid (EA) and resveratrol (RES) are phenolic compounds naturally found in fruits. However, a significant amount of RES and EA is present in berries and their effective isolation enables the production of functional foods. In this study, EA and RES found in four highbush (*Vaccinium corymbosum*) blueberry varieties (Bluecrop, Brigitta, Darrow and Bluejay) were analyzed simultaneously for the first time. A chromatographic method was developed for EA and RES by HPLC-DAD and LC- MS/MS. Furthermore, pressurized liquid extraction (PLE) method was performed for the extraction of EA and RES from blueberry samples. LOD (limit of detection) and LOQ (limit of quantification) were determined as $0.1898x10^{-6}$ mol.L^{-1} and $0.5694x10^{-6}$ mol.L^{-1} for RES, $0.1271x10^{-6}$ mol.L^{-1} and $0.3814x10^{-6}$ mol.L^{-1} for EA, respectively. All the results were reevaluated according to 91.85% and 84.97% recovery value, for EA and RES, respectively. The amount of EA and RES were found out in the range of 1.65 - 9.16 mg/kg and 2.95 - 9.31 mg/kg in the lyophilized blueberry varieties.

KEYWORDS: Blueberry; ellagic acid; resveratrol; LC-MS/MS; HPLC

1. INTRODUCTION

Polyphenols are secondary plant metabolites. They are naturally occurring compounds found in many foods [1]. Polyphenolic compounds now represent possible key treatments for many diseases. Indeed, the anticancer, anti-aging, antibacterial, antioxidant, anti-inflammatory and antiviral activities of polyphenolics have been demonstrated in many studies [1,2]. For many years, berries have been using an important source of food and pharmaceutical ingredients [3]. Vaccinium berries such as blueberries (*Vaccinium corymbosum L.*) contain high amounts of sugars and acids as well as phenolic compounds. Due to their high phenolic content, blueberries have a strong antioxidant property and consequently potential health-promoting effects [4-6]. EA (phenolic acid group) and RES (stilbene group) are naturally occurring compounds belonging to one of the group of phenolic compound found in grapes, berries and fruit juice [7-12]. Several research studies have associated EA with beneficial pharmacological activities, above all against in diabetes, cancer, neurodegenerative disorders and cardiovascular diseases, and it has attracted great interest recently [13-14]. Many research studies have demonstrated the many biological functions of RES, including antioxidant, anti-inflammation, anti-cancer, anti-diabetes, anti-obesity, anti-microbial, anti-aging, cardio protection, and neuroprotection [15].

The amount of EA in the blueberry species [3,7,16-20] and in different types of fruit was determined simultaneously with certain phenolic compounds by HPLC method [8,9,21]. Studies on resveratrol have focused on particularly grape and wine [10,11,21-24], the amount of RES in blueberries studied using various analysis methods such as HPLC-FL detector, LC-MS/MS and GC-MS [5,12,17,25].

The quality of phenolic compound extracts is dependent on the extraction process, the solvent used and the raw material. The extraction techniques and the solvents used must be carefully chosen to obtain maximizing yields and selectivity. Extraction of fruits with different solvents and techniques also showed variable results.

How to cite this article: Seyhan S, Yalcin G, Ayaz Seyhan S. The extraction and determination of ellagic acid and resveratrol in blueberry species by HPLC-DAD and LC-MS/MS. J Res Pharm. 2023; 27(1): 311-319.

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ISSN: 2630-6344

http://dx.doi.org/10.29228/jrp.313

Extraction with pressurized liquids uses solvents at high temperatures to accelerate the extraction of compounds from solid samples. Because extractions can be carried out protected from the light and under an inert atmosphere, it is a good alternative to the classic methods of extraction; degradations by oxidations or by light can be completely avoided. Assuming the complex nature of the fruits, if the pressurized liquid extraction (PLE) technique is combined with solid phase extraction (SPE), an effective technique with high selectivity may be present [24].

In recent years in Turkey, there are an increasing demand for blueberry cultivation, sales and production. An effective extraction and purification method combined with a sensitive detection technique is essential to discover the application of value-added by-products of blueberry. The aim of the present study, a method is developed for determining EA and RES in blueberries samples by means of the pressurized liquid extraction (PLE) technique use combined with solid phase extraction and determined by HPLC-DAD and LC/MS/MS.

2. RESULTS and DISCUSSION

Blueberry extracts obtained by PLE and PLE-SPE were analyzed by using HPLC and LC-MS/MS. Peak identification was performed on basis of their relative retention time values, their absorption spectra in UV-visible region, their mass spectra obtained by ESI-MS/MS, and by using the information previously reported in the literature [26-28].

2.1. Pressurized liquid extraction (PLE)

Two different extraction methods (PLE-PLE/SPE) were tried for determination and optimization of the method applicable for the EA and RES extraction from the blueberry samples. Based on the PLE studies carried out by Palma et al. [29] on different phenolic compounds, the blueberry samples underwent 100% methanol extraction repeated for 3 times at 60°C and 90°C on the lyophilized blueberry samples taken at equivalent quantities. Furthermore, the combined solid-phase extraction accelerated solvent extraction (PLE/SPE) method, which is a new method observed in some recent studies, was tried at two different temperatures, e.g. 60°C and 90°C. Table 1 reveals that the best results were obtained under PLE 60°C conditions. Generally, it is expected that the extraction selectivity is increased by the PLE-SPE method. Also, the increase of temperature enhances the efficiency of extraction. However, a previous study carried out by Palma et al. [29] have been reported that the gallic acid had a low recovery rate in grape matrix and our studies showed that a similar result for EA in our samples (Table 2). Also, no significant increase was observed in the peak area values for RES as a result of the increased temperature. Therefore, the PLE SPE method and 90°C was not used in this study. As a result, 60°C working temperature was determined in PLE method for extraction.

Table 1. Extraction optimization for EA and RES

Extraction Methods	EA			RES		
	Retention Time ($R_{t,min.}$)	Peak Area	Peak Height	Retention Time ($R_{t,min.}$)	Peak Area	Peak Height
PLE 60°C	**17.07**	**154.80**	**4.00**	**32.82**	**59.72**	**7.91**
PLE 90°C	16.96	119.20	3.20	32.82	61.02	6.97
PLE / SPE-60°C	17.03	49.45	1.89	32.81	24.26	2.99
PLE /SPE- 90°C	17.21	108.80	3.14	32.80	41.43	5.35

Table 2. Statistical evaluation of the results obtained from the recovery percentages for ellagic acid and resveratrol for the lyophilized blueberry samples.

Statistical values	EA	RES
Recovery % for lyophilized blueberry	91.85	84.97
S	1.26	2.26
RSD	1.37	2.65
n	3	3
$\pm txS/(n)^{1/2}$	3.13	5.61
Confidence interval (95%)	88.72-94.98	79.36-90.58

2.2. Quantification of ellagic acid and resveratrol by HPLC assay

Several HPLC analysis method for EA and RES has already been reported in the literature. In preliminary works isocratic method was performed according to the method of by Amakura et al. (1). But due to very long retention times especially for RES, the isocratic method was modified by gradient elution method and developed. An evaluation of the results of the developed method revealed that the peak shapes were symmetric and repeatable results were obtained in terms of retention times (Figure 1). EA and RES peaks were efficiently separated from the other peaks in the chromatogram of sample. On the other hand, low pH value of the mobile phase has been positive effect on the retention time, selectivity and peak shapes For EA, linearity was obtained in the concentration range of 0.4963 – 11.5815 x 10^{-6} mol.L^{-1}, y=40.733x+ 1.9204 (r^2=0.9963); limit of detection (LOD) and limit of quantification (LOQ) were determined as 0.1271 x 10^{-6} mol.L^{-1} and 0.3814 x 10^{-6} mol.L^{-1}, respectively. The linearity for RES was obtained in the concentration range of 0.6571 – 10.9530 x 10^{-6} mol.L^{-1}, y=35.105x+ 9.2686 (r^2=0.9963); limit of detection (LOD) and limit of quantification (LOQ) were determined as 0.1898 x 10^{-6} mol.L^{-1} and 0.5694 x 10^{-6} mol.L^{-1}, respectively. All the results were reevaluated according to 91.85% and 84.97% recovery value, for EA and RES, respectively. Based on the developed HPLC and the extraction methods, the quantification results for four different species of blueberry were reviewed in comparison with the rates of recovery obtained in the accuracy test (Table 3).

Table 3. The quantification results of EA and RES by HPLC and LC-MS/MS

Blueberry varieties	EA (mg/kg) HPLC	RES (mg/kg) HPLC	EA (mg/kg) LC-MS/MS	RES (mg/kg) LC-MS/MS
Bluecrop	1.650±0.010	3.456±0.020	1.662±0.001	3.696±0.002
Brigitta	4.202±0.020	9.311±0.010	4.291±0.002	10.091±0.003
Darrow	9.161±0.020	2.953±0.010	9.353±0.003	3.190±0.001
Bluejay	1.739±0.010	n.d	1.834±0.001	1.080±0.001

*n.d. not detected

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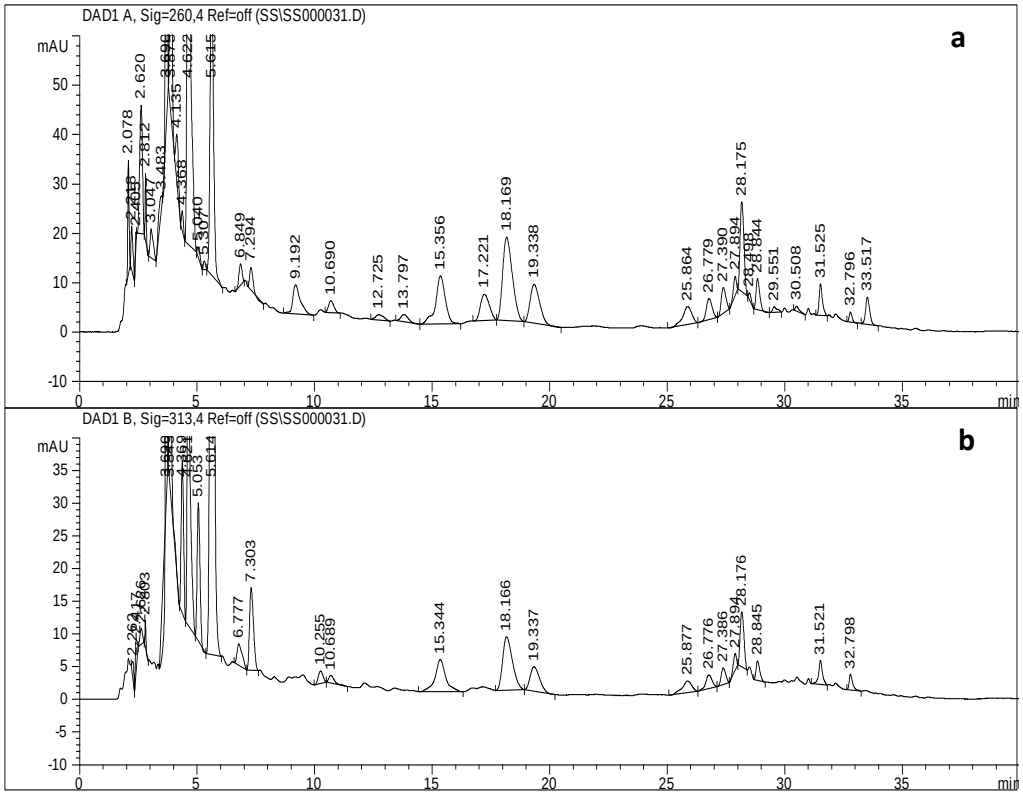

Figure 1. Chromatogram of the blueberry sample. Rt(EA): 17.22, Rt(RES): 32.79 (a. λ=260 nm, b. λ=313 nm)

2.3. Quantification of ellagic acid and resveratrol by LC-MS/MS assay

LC-MS/MS apparatus was used to verify the results obtained by the HPLC method for EA and RES quantities. The specimen substances were determined only on the basis of their retention times in the standard HPLC method, while the molecules ionized through the LC-MS/MS method were evaluated with both the essential molecular ions and fragmented ions for qualitative and quantitative detections. EA and RES compounds were checked if they were ionized in Q3 or Q1 scanning mode by means of the ESI (Electrospray Ionization) method. The scanning revealed that EA easily underwent deprotonization in negative polarity due to the carboxylic acid (-COOH) functional group, while RES was protonated due to the hydroxylic group (OH-). Having a molecular weight of 302.1 g/mol, the ellagic acid was monitored with [M-H] = 301.1 m/z molecular ion value in negative polarity (Figure 2a). The molecular ion value was however monitored as $[M+H]^+$ = 229.1 m/z in positive polarity for RES having a molecular weight of 228.2 g/mol (Figure 2b). The collision energies (CE) for the molecular ion fragmented by the fission gas were automatically determined. Thereafter, the third quadrapole selected the fission ions of high sensitivity to be used for quantitative and qualitative purposes. In MRM (multiple reaction monitoring) mode, the m/z values, Q1, Q3 and collision energies (CE) were determined for the main ion and fission ions monitored in Q3. Though the precursor molecular ion of the EA compound was sent to the second quadrapole through the high collision energy (301.1 m/z), it released very low characteristic fission ions at 284, 184 and 150 m/z (Figure 3). As the structure was considerably consistent, the fission products were very weakly monitored. When the precursor molecular ion (229.1 m/z) of RES compound was sent to the second quadrupole by means of 23V collision energy, it was observed to have been converted into 107.0, 119.1 and 135.0 characteristic fission ions with the aid of the fission gas (Figure 4). The results revealed that the EA and RES amounts obtained through the both methods were parallel. Furthermore, the RES content was found to be 1.08 mg/kg through LC-MS/MS, as it could not be detected by the HPLC method in Bluejay species of the Blueberry samples.

http://dx.doi.org/10.29228/jrp.313
J Res Pharm 2023; 27(1): 311-319

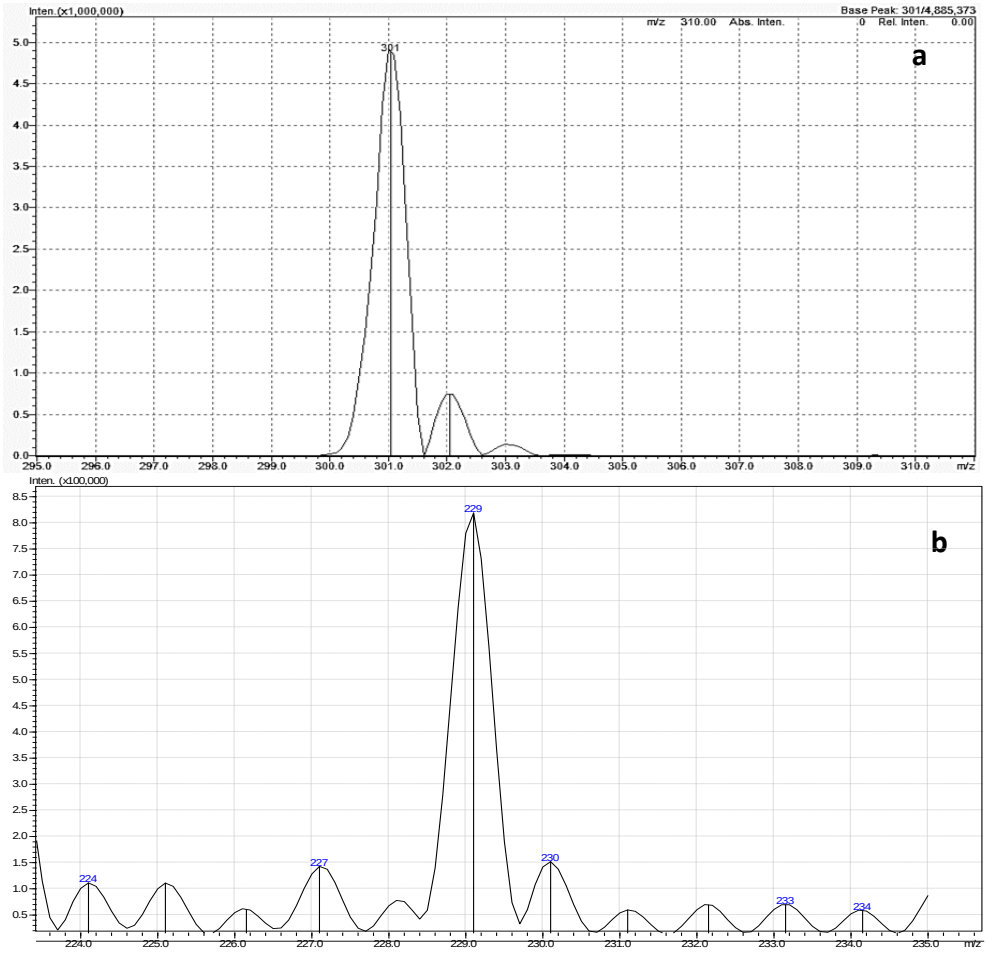

Figure 2. a) $[M+H]^+$= 229.1 m/z in positive polarity molecular ion spectrum of RES, **b)** $[M+H]^+$= 229.1 m/z molecular ion chromatogram of RES.

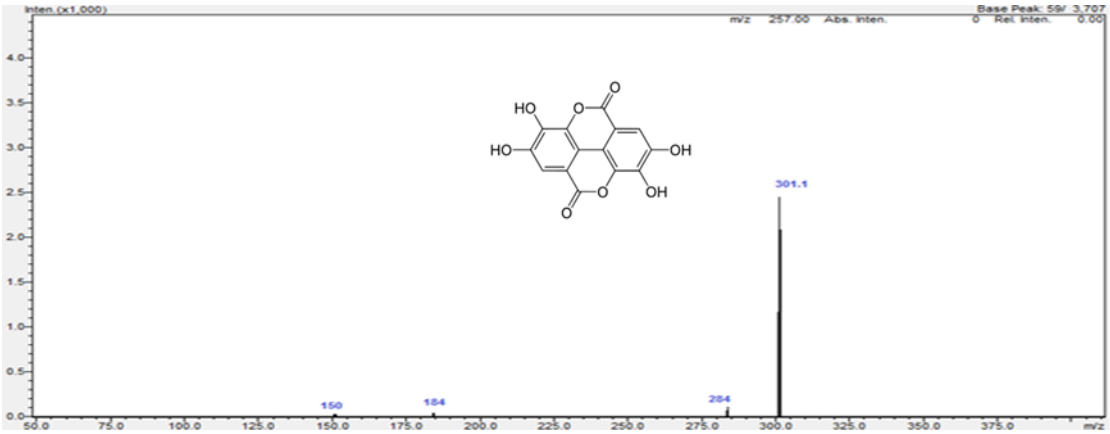

Figure 3. The main ion and fission ions of EA

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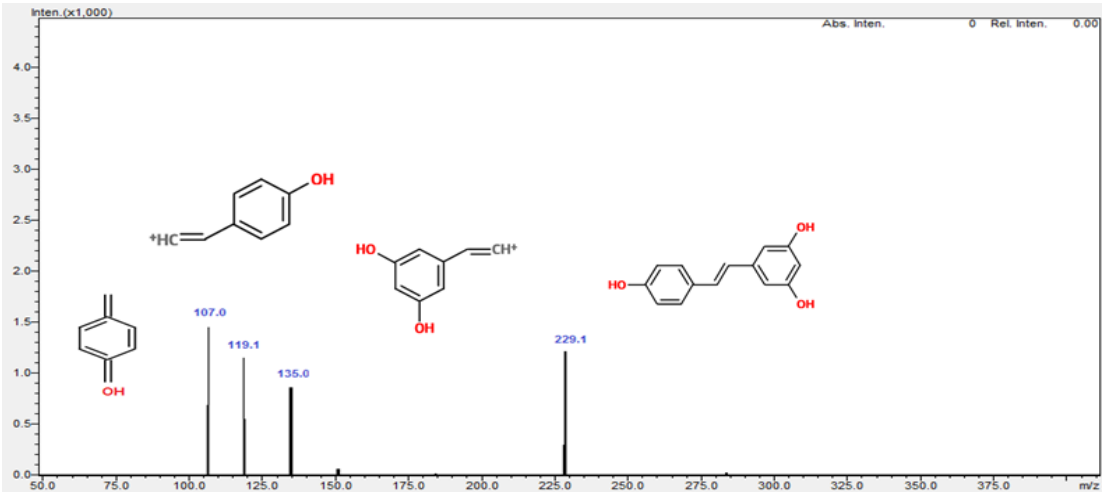

Figure 4. The main ion and fission ions of RES

3. CONCLUSIONS

The presently obtained results are comparatively evaluated under the light of the available literature records as of the blueberry species growing under different climatic and regional conditions. Considering the use of the extraction methods (PLE, PLE/SPE), the results showed that PLE 60°C conditions with methanol could be used to obtain high-value EA and RES for blueberry species. The obtained results of the levels of EA and RES in the blueberry species grown in Turkey are found comparable to the other blueberry species in the literature (Table 4). It is considered that the HPLC method developed in this study would be helpful in the determination of the polyphenolic compounds contained in the fruits.

Table 4. The amount of EA and RES in the literature and results of obtained from this study

Blueberry	*EA*	*RES*	*Extraction / Analysis method*	*Reference*
Berkeley Bluecrop	6.10 µg/g 19.10 µg/g	-	Fruit, Extraction with Methanol/Water/HCl, HPLC	[3]
Hybrids species (TH 161, TH 440, TH 442, Sharpblue)	7.5-66.5 µg/g	-	Fruit, Extraction with HCl /Methanol, HPLC	[16]
Northblue, Northcountry	≤ 100 µg/g	-	Lyophilized fruit, Extraction with HCl /Methanol, HPLC	[19]
Varieties grown in Slovenia	1 µg/g	4 µg/g	Fruit, SPE Extraction, LC/MS	[17]
Bluecrop	-	1.8-3.5 µg/g	Fruit, Acetone/Formic acid - SPE Extraction, HPLC	[25]
Bluecrop Bluejay Jersey	-	4.04 µg/g 7.40 µg/g 4.37 µg/g	Lyophilized fruit, Methanol/Water/Formic acid – Enzymatic hydrolysis, GC/MS	[30]
Duke Blueray	-	63 µg/g 78 µg/g	Fruit, Extraction with Methanol/HCl, HPLC	[18]
Bluecrop Brigitta Darrow Bluejay	1.65 µg/g 4.20 µg/g 9.16 µg/g 1.73 µg/g	3.45 µg/g 9.31 µg/g 2.95 µg/g -	Lyophilized fruit, Extraction with ASE/Metanol, HPLC	The resulst obtained from this study
Bluecrop Brigitta Darrow Bluejay	1.66 µg/g 4.29 µg/g 9.35 µg/g 1.83 µg/g	3.67 µg/g 10.09 µg/g 3.19 µg/g 1.08 µg/g	Lyophilized fruit, Extraction with ASE/Metanol, LC/MS/MS	The resulst obtained from this study

http://dx.doi.org/10.29228/jrp.313
J Res Pharm 2023; 27(1): 311-319

4. MATERIALS AND METHODS

4.1. Materials

Four cultivated highblush blueberry (Vaccinium corymbosum) fruits (Bluecrop, Brigitta, Darrow and Bluejay), grown in the Black Sea Region of Turkey were collected on the same location in Hayrat (Trabzon, Turkey). Samples are immediately frozen in liquid nitrogen, lyophilized and stored at -20°C until analysis time. All chemicals and solvents were reagent or HPLC grade. EA and RES were purchased from Sigma Chemical Co. (St. Louis, MO, USA), the other chemicals and organic solvents were purchased from Merck (Darmstadt, Germany). For the solid-phase extraction, a LiChrolut EN (40–120 m) of Merck was employed. Bidistilled deionized water was Milli-Q quality.

4.2. Sample extraction

4.2.1. Pressurized liquid extraction (PLE)

An ASE 100 Model (Dionex, Germany), an automated extraction system for pressurized liquid extraction, was used to extract phenolic compounds from blueberry fruits. Two grams of lyophilized samples were mixed with diatomaceous earth (purified sand) and placed in the extraction cartridge. Diatomaceous earth was added thoroughly in order to pack the extraction cartridge. Extraction was carried out with the following parameters: heat, 5 min; static, 5 min; flush volume, 60% of the extraction cell volume; purge, 60 s; pressure, 1500 psi; temperature, 60°C and 90°C; extraction solvent was methanol in three cycles. After extraction, the solvent was evaporated using a rotary evaporator with vacuum control (Heidolph Instruments) and thermostatic bath held at 60°C. The extract was transferred to a 10 mL volumetric flask which was made up to its volume with methanol/water (1:1) and was stored in glass vials at -20°C in darkness until chromatographic analysis. The extract filtered through a 0.45 µm nylon filter (Biocrom MN 718020, Phonex nylon fitler 25 mm) prior to injection into the HPLC system.

4.2.2. Pressurized liquid extraction (PLE) combined to solid phase extraction (SPE)

In the first step 2.5 g of the adsorbent of LiChrolut EN was placed in the cartridge. In the second step the adsorbent, a cellulose paper (Dionex) was inserted to separate it from the sample to be extracted. In the third step the remaining space was filled with diatomaceous earth. Extraction was carried out with the following parameters: heat, 5 min; static, 5 min; flush volume, 60% of the extraction cell volume; purge, 60 s; pressure, 1500 psi; the extraction was carried out 40°C with water. The sequential extraction was carried out with methanol in three cycles at 60°C and 90°C and the same parameters were used for the methanolic extract.

4.3. HPLC analysis

All extracts were analyzed on the HPLC-DAD system, Agilent Technologies, High Performance Liquid Chromatography was a combination of a Model G1311A quaternary pump, a Model G1322A vacuum degasser and a Model G1315A diode array detector, 1200 series auto sampler. The separation was performed on Phenomenex, Luna, 5 µm, C18 ODS-RP 250 x 4.6mm column. The column temperature was 30°C. The mobile phase consisted of A (5mM potassium dihydrogen phosphate solution, pH 2.6 (adjusted by o-phosphoric acid)) and B (acetonitrile). Gradient elution conditions were: 0-20 min 18% B, 20-30 min from18% B to 35% B, 30-35 min from 35% B to 40% B, 35-40 min from 40% B to 18% B, 40-50 min 18% B. Flow rate was 1.0 mL /min and injected volume 20 µL. The EA and RES were detected at 260 nm, 313 nm, respectively. EA and RES stock solution were prepared in methanol/water (1:1) and stored at 4°C in darkness. Calibration was performed by injecting the standards three times at five different concentrations. Identification of ellagic acid and resveratrol were carried out by comparing retention times and spectral data with those of authentic standards. Quantitative determinations were carried out using calibration curves of the standards. Samples were injected in duplicate. Results were expressed as mg/kg sample lyophilized weight.

4.4. LC-MS / MS analysis

A Shimadzu LCMS-8030 series HPLC system (Shimadzu, Japan) via an electrospray ionization (ESI) source was used for quantitative analysis of EA and RES. The column used for the chromatographic separation was a Restek Aqueous C18, 100A^0 (3 µm, 100 mm x 2.1 mm). The mobile phases used were water with 0.1 % formic acid (eluent A) and acetonitrile with 0.1 % formic acid (eluent B) and was delivered at a flow rate of 0.3 mL / min. The gradient programme started with 5% B, followed by 0.0-3.0 min from 5% B to

95% B, 3.0-5.0 min 95% B, 5.0-5.5 min from 95% B to 5% B, 5.5-8.0 min 5% B. The mass spectrometer was operated in ESI mode with multiple reaction monitoring (MRM) at unit resolution. Nitrogen was used as the nebulizer, heater and curtain gas as well as the collision activated dissociation (CAD) gas. Mass spectrometer instrumental parameters were tuned to maximize the generation of precursor and fragment ions by infusion of a solution of EA and RES into the ESI source at 20 µL. The optimum ESI conditions included a vaporizer temperature of 400°C, nitrogen drying gas temperature of 250°C at 15.0 L/min, ion spray needle voltage 4500 V. Selected ion monitoring (SIM) was used to record the abundance of the protonated molecule of EA at m/z 301, RES at m/z 229. Quantitative determinations were carried out using calibration curves of the standards.

4.5. Statistical analysis

Results are presented graphically as means with calculated standard deviations (SD) represented by vertical bars. Statistical significance was applied using Microsoft office Excel (2016). Difference in values of recoveries were expressed tested by Student's t-test ($P<0.05$). All results are given as the mean ± STD. Statistical significance was defined as $p<0.05$.

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