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Set-up of Sonotrode Based Extraction to Recover Phenolic Compounds from Olive Leaves †

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Abstract: Olive leaves are a waste by-product obtained during the olive oil production and pruning. They contain phenolic compounds that possess antioxidative, antimicrobial, anti-atherogenic and anti-inflammatory properties, among others. For that reason, a procedure based on ultrasound-assisted extraction via sonotrode was developed to evaluate the recovery of these phenolic compounds from olive leaves. To establish the sonotrode extraction, a Box-Behnken design based on response surface methodology (RSM) was used to optimize the effects of factors such as solvent composition (30–100% EtOH), extraction time (1–10 min) and amplitude (20–100%). Qualitative and quantitative analyses of phenolic compounds were performed using HPLC coupled to DAD and mass spectrometer detectors. The highest content of phenolic compounds was 40.9 ± 0.2 mg/g d.w. and it was obtained using 55:45 ethanol/water (*v*/*v*) for 8 min and 100% of the amplitude. The optimal conditions selected for the sonotrode were compared with the result obtained by a conventional ultrasonic bath achieving similar concentrations. Therefore, sonotrode could be considered as an efficient extraction technique that allows a good recovery of phenolic substances from olive leaf that could be easily scale-up at industrial level.

Keywords: olive leaves; phenolic compounds; sonotrode; Box-Behnken; HPLC-MS

1. Introduction

Olive leaves represents around 10% of the weight of olives collected for the oil production (25 kg per olive tree) and during the tree pruning [1]. Part of this by-product is used in the animal food or energetic biomass, whereas a great quantity of olive leaves are discarded generating a great cost and a high environment impact [2]. Nevertheless, olive leaves are a potential source of phenolic compounds that possess numerous beneficial properties attributed to their antioxidant activity [3]. Therefore, its reutilization can be profitable for the Food Industry in order to obtain nutraceuticals or functional foods. The phenolic composition of olive leaves varies according to many factors such as the date of collection [2,4], cultivation zone [5] and cultivar [2,6,7]. Phenolic compounds in olive leaves can be classified in phenolic acids, phenolic alcohols, flavonoids and secoiridoids [7]. The main

phenolic compounds in olive leaves are hydroxytyrosol, rutin, verbascoside, luteolin-7-glucoside, luteolin-4-o-glucoside, oleuropein, oleuropein aglycone, and ligstroside aglycone [3,7,8].

The extraction process is the most important step in the phenolic recovery. Conventional techniques such as maceration have been used for a long time. However, they require high volume of solvents, long extraction times and possess a low selectivity, low reproducibility and low efficiency [9,10]. Nowadays, in order to reduce extraction times, new techniques such as microwave extraction, supercritical fluid extraction and pressurized liquid extraction have been applied in the phenolic recovery from olive leaves [2,11–13]. Nevertheless, most of these techniques generate high energy costs because they operate at high pressures/temperatures. For that reason, ultrasound assisted extraction (UAE) can be the best choice due to it is an effective and low-cost extraction technique [14]. Ultrasound assisted extraction can be carried out by using two types of devices, ultrasonic bath or ultrasonic probe (sonotrode) (US) equipment [15]. The ultrasonic bath is the most used for the phenolic extraction at lab level because it is cheap and allows the extraction of various samples simultaneously. However, by comparison with probe systems, they possess a low reproducibility and low power of ultrasound delivered directly to the sample [15]. Nevertheless, the sonotrode system is more powerful because of an ultrasonic intensity delivered through a smaller surface (the tip of the probe), in comparison with the ultrasonic bath [16].

In view of the above, the purpose of this work was to evaluate the recovery of phenolic compounds from olive leaves by optimization of a sonotrode ultrasonic-assisted extraction method. For that purpose, response surface methodology (RSM) was performed to evaluate extraction parameters % EtOH/H_2O (*v*/*v*), amplitude and extraction time with an experimental Box–Behnken design. In addition, it was carried out a conventional ultrasonic bath extraction in order to compare with the optimized by sonotrode technique.

2. Materials and Methods

2.1. Chemicals and Reagents

Ethanol and methanol were purchased from Fisher Scientific (Leicestershire, UK), and water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). For HPLC analysis, LC-MS grade acetronitrile was purchased from Fisher (Fisher Scientific UK, Leicestershire, UK) and ultrapure water was obtained with the Milli-Q system described above. The acetic acid used was purchased from Fluka (Buchs, Switzerland). The standard compounds used for the quantification were hydroxytyrosol, and apigenin, which were purchased from Sigma-Aldrich (Saint Louis, MO, USA), and oleuropein was from Extrasynthèse (Lyon, France).

2.2. Samples

Olive leaves 'Koroneiki' were collected from at "IFAPA, Centro Alameda del Obispo" in Córdoba, Spain (37°51′36.5″ N 4°47′53.7″ W). Samples were collected at mid-December (fruit-ripening) in 2020. Olive leaves were air dried under controlled temperature. Subsequently, leaves were ground using IKA A 10 Basic Mill (Retsch GmbH, Haan, Germany) and the resulting powder was stored at −20 °C until the extraction.

2.3. Extraction of Phenolic Compounds from Olive Leaves by US Sonotrode and Ultrasonic Bath Extraction

The extraction was achieved with an US sonotrode UP400St (Hielscher Ultrasonics GmbH, Teltow, Germany). 0.25 g of powdered olive leaves were extracted using 100 mL of EtOH/H_2O. The percentage of ethanol/water, extraction time and the US amplitude were varied according to the experimental design.

The ultrasonic bath extraction of phenolic compounds was performed as described previously by Talhaoui et al. 2015 with certain modifications [2]. Briefly, powdered leaves (0.1 g) were extracted using 10 mL of EtOH/H2O (80:20, *v*/*v*) by using an ultrasonic bath (Bandelin, Sonorex, RK52, Berlin,

Germany) operating at a frequency of 35 kHz during 20 min. Two replicates of each sample were processed.

After the extraction, the olive leaf extracts were centrifugated at 1000 g for 10 min, the supernatant was collected, evaporated, and reconstituted in 5 mL of methanol/water (1:1, *v*/*v*). The final extracts were filtered through 0.2 μm polytetrafluoroethylene (PTFE) syringe filters and stored at −18 °C until the analyses.

2.3. Experimental Design

A Box-Behnken design with 3 variables was carried out to optimize the extraction parameters to obtain the highest phenolic content from olive leaves. In this study, three independent variables were %EtOH/H_2O (X_1), Amplitude (X_2) and time (X_3), with 3 levels for each variable and the response variable (Y) was the sum of the phenolic compounds and elenolic acid (total compounds). The parameters range established were percentage of ethanol/water (30, 65 and 100 %), amplitude (20, 60, and 100%) and extractions times (1, 5.5 and 10 min), which were similar to a previous study that reported UAE factors of 20–80% EtOH, 20–70% of amplitude and 5–15 min in olive mill leaves [17]. Amplitude percentage refers to the percentage of maximum power used. The extraction time was limited to 10 min due to during the extraction the temperature increased. In addition, the range of extraction time was chosen from 1 min according to a previous study, which employed short sonication times from 1 to 5 min in olive leaves [18]. The design consisted of 15 combinations including 3 center points.

The response variables were fitted to a second-order polynomial model equation obtained by the response surface methodology (RSM) (Equation (1)).

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} {X_{ii}}^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ii} X_i X_j \quad (1)$$

Were Y is the response variable, which was the total compounds in olive leaves obtained by HPLC-MS. Xi and Xj are the independent factors, whereas β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients of the model for the mean, linear, quadratic and interaction term calculated from the experimental results by the least of squares method. Model building, experimental results and designs were processed using STATISTICA 7.0 (2002, StatSoft, Tulsa,OK).

2.4. Analysis of the Phenolic Compunds by High-Performance Liquid Chromatography Coupled to Mass Spectrometry (HPLC- MS)

Analyses of the phenolic compounds of olive leaves were carried out following the previously validated method of Talhaoui et al. [7] using Agilent 1200 Series Rapid Resolution liquid chromatography system (Agilent Technologies, CA, USA), which is comprised of a binary pump, degasser, and auto sampler and coupled to DAD and ion-trap-MS. Phenolic compounds were separated using a Poroshell 120 EC-C18 (4.6 × 100 mm, 2.7 mm) from Agilent Technologies, at 25 °C and a flow rate of 0.8 mL min^{-1}. The mobile phases were 1% of acetic acid as mobile phase A and acetonitrile as mobile phase B. The conditions of the solvent gradient were as follows: 0 min, 5% B; 4 min, 9% B; 7 min, 12% B; 8 min, 15% B; 9 min, 16% B; 14 min, 20% B; 15 min, 22% B; 18 min, 28% B; 19 min, 30% B; 20 min, 31% B; 21.50 min, 32% B; 23 min, 34% B; 24 min, 35% B; 25.5 min, 40% B; 27 min, 50% B; 30 min, 100% B; 34 min, 100% B; 36 min, 5% B.

Hydroxytyrosol, tyrosol, oleuropein, rutin, luteolin-7-glucoside, apigenin-7-glucoside and luteolin were the standard used for the quantification of compounds in the olive leaf extracts. The calibration curves were prepared at seven concentration levels from the limit of quantification (LOQ) to 100 mg/L.

3. Results and Discussion

3.1. Characterization of Phenolic and Other Compounds from Olive Leaves US Sonotrode Extracts by HPLC-MS

Phenolic compounds were identified by rendering their mass spectra according to the previous experience of the group and using the data reported in in previous studies [7,19–21]. A total of 36 compounds were identified and quantified in olive leaf obtained by US sonotrode. The quantification of individual compounds in each experiment was carried out by the calibration curve of standards. In addition, all calibration curves showed a good linearity ($r^2 > 0.9910$).

Regarding the results, the minimum content of total compounds was 24.92 ± 0.06 in SON 10 (100% EtOH, 60% of amplitude and 10 min), whereas the maximum total content was 33.0–34.15 in SON 2 (30% EtOH, 100% amplitude and 5.5 min), SON 7 (65% EtOH, 20% amplitude and 10 min), SON 8 (65% EtOH, 100% amplitude and 10 min) and in the central points in SON 13, 14 and 15 at 60% EtOH, 60% amplitude and 5.5 min.

3.2. Fitting the Model

The evaluation of the model was carried out according to the significance of the regression coefficients. According with other works, the level of significance was $\alpha < 0.1$ in order to increase the number of significant variables.

In addition, the p-value of lack-of-fit was used to verify the adequacy of the model, which was non-significant ($p > 0.05$), so this means that the model fits well.

3.3. Analysis of Response Surfaces (RSM) and Comparison between Sonotrode and Ultrasound Bath Extraction

In order to determine the optimal levels of independent variables for the extraction of the total content of phenolic compounds from olive leaves, responses surfaces were plotted. Each pair of variables was depicted in three-dimensional surface plots, while the other one variable was kept constant at central level. The final step of the RSM after selecting the optimal conditions was to predict the accuracy of the mathematical model. The highest phenolic content was obtained at optimum conditions: 100% Amplitude, 55% EtOH and water to obtain a predictable value of total compounds of 35.54 mg g^{-1} d.w. To verify the suitability of the model for total compounds, the predictable value of total compounds was compared with experimental values obtained at optimal conditions. Analysis of the results revealed an acceptable variance (CV = 9.05%) between the theoretical and experimental data, therefore, the model was considered suitable

A similar total phenolic content was obtained by UAE sonotrode and UAE bath.

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Conflicts of Interest: The authors declare no conflict of interest.

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