Food Chemistry 293 (2019) 161–168

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Extraction of oleuropein and luteolin-7-O-glucoside from olive leaves: Optimization of technique and operating conditions

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ARTICLE INFO

Chemical compounds studied in this article:
Oleuropein (PubChem CID: 5281544)
Luteolin-7-O-glucoside (PubChem CID: 5280637)

Keywords:
Bioactive compounds
Phenolics
Flavonoids
Dynamic maceration
Pressurized liquid extraction
Response surface methodology

ABSTRACT

Olive leaves have become a promising source of phenolic compounds and flavonoids with high added value. Phenolic compounds and flavonoids are important sources of antioxidants and bioactives, and one of the processes used to effectively produce them is extraction via solvents, using aqueous ethanol solutions. To obtain the highest extraction yield per kg of biomass, olive leaves were extracted using a conventional technique (dynamic maceration) and an emerging technology, such as pressurized liquid extraction. Studies of the factors that influence these processes were performed: temperature, leaf moisture content, solvent/solid, and aqueous ethanol concentration were optimized using the central composite and Box-Behnken experiment designs. Pressurized liquid extraction resulted in more efficient oleuropein and luteolin-7-O-glucoside extraction than dynamic maceration. The operational conditions for maximizing the recovery of phenolic compounds and flavonoids and antioxidant capacity were determined to be 190 °C, leaf moisture content of 5%, and aqueous ethanol concentration of 80%.

1. Introduction

Olive leaves, an agricultural waste obtained during harvesting or processing of olive fruits, are found in large quantities in the olive oil and olive table industries, where they are separated from olives using pneumatic separation systems and create a residue without industrial interest. However, from an economic point of view, as industrial residue from vegetable materials, olive leaves are an excellent source of phytochemicals. Olive leaves contain significant amounts of valuable compounds such as phenolic and flavonoid compounds, which have attracted considerable interest due to their potential use as food additives and/or nutraceuticals in both the food and pharmaceutical industries (Şahin et al., 2018). The amount of olive leaves that accumulate annually from these industries may exceed 1 million tons. Therefore, this olive industry residue can present interest in a biorefinery context. Moreover, it is worth recovering high added-value compounds from this material, as these compounds can present great interest for the pharmaceutical, food, and cosmetic sectors, due to the trend of using natural products instead of synthetic ones.

Since olive leaf extracts are rich in phenolic and flavonoid compounds, they present great potential as natural antioxidants for food preservation, bioactive food, etc. Oleuropein is the most abundant phenolic compound in olive leaves and the main compound responsible for the antioxidant and bioactive properties of hydroalcoholic extracts (Fakhraei et al., 2014). There has been increasing evidence that helped establish a scientific basis for the use of oleuropein-rich extracts as functional foods. The antioxidant and anti-inflammatory effects of oleuropein and the ability to treat pro-oxidant and inflammatory-related diseases (i.e., cancer, cardiovascular disease, hepatic disorders, obesity, diabetes, etc.) are its main biological activities, and these have been confirmed by previously published papers (Hassen, Casabianca, & Hosni, 2015). Qualitative and quantitative compositional analysis using high-performance liquid chromatography (HPLC) also revealed that luteolin-7-O-glucoside, a flavonoid, is another major polyphenolic compound present in olive leaf extracts (Guinda et al., 2015; Kiritsakis, Goula, Adamopoulos, & Gerasopoulos, 2018). This phytocompound can be a potent drug against colon carcinogenesis (Baskar, Ignacimuthu, Michael, & Al Numair, 2011) that also demonstrated gastroprotective effects when studied on different ulcer models using rats (Antonisamy et al., 2016).

Several methods have been used for the extraction of phenolic and flavonoid compounds from olive leaves and other by-products which originate from the olive oil industry (Romero-García et al., 2016). A traditional solvent extraction via maceration is a favorable process

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https://doi.org/10.1016/j.foodchem.2019.04.075
Received 4 January 2019; Received in revised form 11 April 2019; Accepted 21 April 2019
Available online 27 April 2019
0308-8146/

since heat sensitive compounds can be recovered at low temperatures. However, the extraction rate is limited by the dissolution, mass transfer to the solution by diffusion, and osmotic pressure of soluble compounds. Since slow extractions require large equipment to increase the yield, and long exposure to solvent may cause degradation, researchers have been seeking to provide alternative physical extraction methods using emerging technologies to replace the conventional processes (Cruz, Brito, Smirniotis, Nikolaidou, & Vieira, 2017).

Pressurized liquid extraction (PLE) uses solvents to carry out extraction at high pressure and temperature, always below their critical points, thus maintaining the liquid state of the solvents during the entire extraction process. When applying such conditions, extraction processes can occur faster, and typically, higher extraction yields are obtained using low volumes of organic solvents (e.g., 20 min and 10–50 mL solvent for PLE can be compared with a traditional extraction step where 10–48 h and up to 200 mL solvent are required), therefore decreasing the dilution of the samples. These characteristics are mainly due to the improvement in mass-transfer kinetics achieved at high temperature and pressure. Using high temperatures increases the solubility of the analytes in the solvent and decreases the viscosity and surface tension of the solvent, thus allowing for a better penetration of the solvent into the matrix (Herrero, Castro-Puyana, Mendiola, & Ibañez, 2013). PLE is broadly recognized as a green, eco-friendly, efficient, and cost effective extraction approach. As previously mentioned, it is mainly due to its low solvent consumption. Although the majority of PLE applications developed so far are aimed at the extraction of contaminants, herbicides and pesticides from different natural, food and environmental samples (Martínez, Gonzalo, Cruz, Álvarez, & Méndez, 2007), this technique has also demonstrated its usefulness for the extraction of bioactive compounds from natural matrices thus considering as a promising innovative extraction technology for recovering polyphenols from olive leaves. PLE has been used successfully in polyphenols extraction from various plant matrices and it can be implemented at industrial-scale (Putnik et al., 2017).

In addition, the extraction of bioactive compounds from plant materials requires several preliminary steps, such as drying, size reduction, or carrying out studies on their solvent retention capacities and extraction kinetics, to obtain higher extraction yields. These require labor-intensive and time-consuming preparation procedures. The recovery of phenols from olive leaves is clearly influenced by a number of factors, and the effect of drying plays a significant role in the amount of phenols recovered and their antioxidant capacities. Drying is the most common commercial technique employed before the extraction of high added-value compounds from plant materials. Using this technique helps to reduce the water content of the plants and prevents their microbial spoilage as enzymatic degradation (Ahmad-Qasem et al., 2016). Investigation results demonstrated that the oleuropein content (OC) extracted from fresh olive leaves was low, thus indicating that drying the leaves would be required for high oleuropein recovery (Afaneh, Yateem, & Al-Rimawi, 2015; Kamran, Hamlin, Scott, & Obied, 2015; Şahin et al., 2018). However, no research has been conducted on the influence of the moisture content (MC) of leaves on the extraction process. To optimize the recovery of bioactive compounds, this parameter should be investigated.

In this study, we used the response surface methodology (RSM) to optimize the extraction of phenolic compounds and flavonoids from olive leaves, with focus on oleuropein and luteolin-7-O-glucoside. The application of the RSM allows studying the influence of several process factors on one or more responses of the design. Experimental designs are able to determine interactions between parameters and obtain and predict in a quick and reliable way optimum extraction conditions using a minimum number of experimental runs (Arabi, Ghaedi, & Ostovan, 2016; Ostovan et al., 2018). Experimental design methodology has recently used in the optimized extraction of biologically active constituents and several biological indicators in different samples (Arabi, Ghaedi, & Ostovan, 2017; Ostovan, Ghaedi, & Arabi, 2018; Bagheri et al., 2019). We used dynamic maceration (DM) and PLE as extraction methods, which allowed us to compare our results with those obtained using conventional extraction methods. The aim of this work was to obtain antioxidant rich-oleuropein and luteolin-7-O-glucoside extracts featuring high antioxidant capacities. The effects of treatment temperature, MC of leaves, solvent/solid ratio, and ethanol concentration on the contents of oleuropein and luteolin-7-O-glucoside biophenols in the extracts and on their antioxidant capacities were studied using both techniques, and corresponding mathematical models were developed for determining the optimal operational conditions which would favor a better valorization of this plant by-product.

2. Materials and methods

2.1. Samples and reagents

Olive leaves from *Olea europaea* L. var. Picual were manually harvested from several specimens located on the campus of the University of Jaén. After picking, olive leaves were dried in a stove at 105 °C for different times, until the required MC was reached, and then they were stored in the dark until extraction. The initial MC of the olive leaves was 48.56%. Before extraction the leaves were ground using a Retsch™ SM 100 model cutting mill (Fisher Scientific S. L., Madrid, Spain) featuring a 5 mm sieve. The average particle diameter by mass was further calculated (D_{50} = 1.21 mm). This required sieving the ground olive leaves using a Retsch™ AS 200 sieve shaker and five test sieves, their aperture sizes being 2 mm, 1 mm, 850 µm, 500 µm, and 150 µm. The solvent retention capacities of olive leaves at different aqueous ethanol concentrations (20, 60, and 80%) were also determined. Excess solvent was added to 20 g dry olive leaves. The mixture was stirred using magnetic stirring for 24 h to achieve complete wetting. The wet sample was weighted after centrifugation at 4000 g for 10 min and filtered using a Kitasato flask for 5 min and then reweighted after oven drying at 105 °C for 24 h. The olive leaves presented a solvent retention capacity of 104 ± 6%.

Ethanol, methanol, and acetonitrile (HPLC grade) were purchased from PanReac AppliChem (Barcelona, Spain). Standards of oleuropein and luteolin-7-O-glucoside were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Extraction procedures

Two extraction methods were assayed for the recovery of phenolic compounds and flavonoids from olive leaves.

2.2.1. DM method

This conventional extraction method was performed using leaves of different MCs (10, 20, 30, and 40%). We macerated 20 g biomass in aqueous solutions of various concentrations of ethanol (20, 40, 60, 80, and 96%) using different solvent/solid ratios (6:1, 9:1, 12:1, and 15:1 v/w), and at different temperatures (25, 40, 55, 70, and 85 °C) for 6 h. We determined that the minimum quantity of solvent necessary to achieve a uniform stirring of the mixture corresponded to a solvent/solid ratio of approximately 6:1. Previous studies allowed to delimit the leaf drying and extraction times. Hydroalcoholic extracts were obtained using a MultiMix D magnetic stirrer (OVAN, Barcelona, Spain) (at a constant speed of 350 rpm) using 500 mL Erlenmeyer flasks submerged in a thermostatic water bath, according to the extraction conditions defined by the experimental design. At the end of the experiments, the extracts were filtered using grade 1 Whatman® qualitative filter paper.

2.2.2. PLE method

We performed PLE using a Dionex™ ASE™ 350 accelerated solvent extractor (Thermo Fisher Scientific Inc., Waltham, USA) equipped with 22 mL stainless steel extraction cells and 60 mL collection vials. Specifically, 3.0 g ground olive leaves were thoroughly mixed with 2 g

Ottawa sand and loaded into the cells. The extraction procedure was performed in static mode (5 min), without using the solvent saver mode of the extractor. Again, a binary solvent such as ethanol–water was used, as it is a non-harmful, low-toxicity, and easily removable solvent that presents minimum environmental impact. This mixture is commonly used for extracting phenolic compounds from agri-food by-products and was a good choice for the PLE process. Preliminary studies were carried out to determine the static extraction time and consecutive number of cycles of the experiments, since these parameters may play a predominant role in the PLE. The effects of these PLE parameters were assessed on the OC and luteolin-7-O-glucoside content (LC) from olive leaves extracts using aqueous ethanol (60%, v/v).

2.3. Experimental design and statistical analysis

RSM was used to study the effects of temperature (T), leaves MC, solvent/solid ratio (S) and ethanol concentration (E) on the extraction of phenolic and flavonoid compounds from olive leaves. We selected six responses: the extraction yield or soluble solids content of the liquid extract (R), total phenolic and flavonoid contents (TPC and TFC, respectively), antioxidant capacity (2,2-diphenyl-1-picrylhydroazyl (DPPH) free radical scavenging capacity), OC, and LC of the extracts. To evaluate the relationships between the experimental factors and observed results and to optimize the operating conditions, two experimental designs were developed: a central composite design (CCD) involving four factors, consisting of thirty randomized trials, and including six repetitions of the central point, and a Box-Behnken design involving a total of seventeen randomized runs and including five central points. The coded and actual values for the experimental designs are listed in Tables 1 and 2. The factors were coded according to the transformation described by Vidal et al. (2018).

The experimental results were evaluated using the Design-Expert® v8.0.7.1 software (Stat-Ease, Inc., Minneapolis, USA) and RSM. Analysis of variance (ANOVA) was used to determine the significance of the results. To describe the behavior of the response data, a quadratic model for each response was used, according to Eqs. (1) and (2).

$$Y = \beta_0 + \beta_1 T + \beta_2 MC + \beta_3 S + \beta_4 E + \beta_{12} T\hat{A}\cdot MC + \beta_{13} T\hat{A}\cdot S + \beta_{14} T\hat{A}\cdot E + \beta_{23} MC\hat{A}\cdot S + \beta_{24} MC\hat{A}\cdot E + \beta_{34} S\hat{A}\cdot E + \beta_{11} T^2 + \beta_{22} MC^2 + \beta_{33} S^2 + \beta_{44} E^2 \pm \quad (1)$$

$$Y = \beta_0 + \beta_1 T + \beta_2 MC + \beta_3 E + \beta_{12} T\hat{A}\cdot MC + \beta_{13} T\hat{A}\cdot E + \beta_{23} MC\hat{A}\cdot E + \beta_{11} T^2 + \beta_{22} MC^2 + \beta_{33} E^2 \pm \quad (2)$$

Table 2
Box-Behnken design in terms of actual and coded factors used for the pressurized liquid extraction.

Run	Temperature (T)		Leaf moisture content (MC)		Ethanol concentration (E)	
	Actual (°C)	Coded	Actual (%)	Coded	Actual (%)	Coded
1	70	−1	5.3	−0.97	70	0
2	130	0	13.5	−0.15	70	0
3	190	1	4.7	−1.03	70	0
4	130	0	4.7	−1.03	60	−1
5	130	0	14.1	−0.09	70	0
6	190	1	13.5	−0.15	60	−1
7	130	0	22.6	0.76	80	1
8	130	0	5.3	−0.97	80	1
9	190	1	14.1	−0.09	80	1
10	190	1	22.6	0.76	70	0
11	70	−1	14.1	−0.09	60	−1
12	70	−1	14.1	−0.09	80	1
13	130	0	14.1	−0.09	70	0
14	130	0	22.6	0.76	60	−1
15	130	0	14.1	−0.09	70	0
16	70	−1	22.6	0.76	70	0
17	130	0	14.1	−0.09	70	0

Table 1
Central composite design in terms of actual and coded factors applied to the extraction by dynamic maceration.

Run	Temperature (T)		Leaf moisture content (MC)		Solvent/solid ratio (S)		Ethanol concentration (E)	
	Actual (°C)	Coded	Actual (%)	Coded	Actual (%)	Coded	Actual (%)	Coded
1	55	0	32.4	0.24	12	0	60	0
2	55	0	29.5	−0.06	18	2	60	0
3	70	1	18.8	−1.13	9	−1	40	−1
4	25	−2	29.3	−0.07	12	0	60	0
5	40	−1	18.7	−1.13	9	−1	80	1
6	55	0	32.4	0.24	12	0	60	0
7	55	0	29.5	−0.06	12	0	100	2
8	70	1	42.0	1.20	9	−1	80	1
9	40	−1	41.5	1.15	9	−1	80	1
10	70	1	42.0	1.20	15	1	80	1
11	40	−1	41.5	1.15	15	1	40	−1
12	55	0	48.7	1.87	12	0	60	0
13	55	0	29.5	−0.06	12	0	20	−2
14	70	1	42.0	1.20	15	1	40	−1
15	40	−1	18.7	−1.13	9	−1	40	−1
16	55	0	8.9	−2.11	12	0	60	0
17	40	−1	41.5	1.15	9	−1	40	−1
18	70	1	42.0	1.20	9	−1	40	−1
19	55	0	32.4	0.24	12	0	60	0
20	85	2	29.5	−0.05	12	0	60	0
21	70	1	18.8	−1.13	9	−1	80	1
22	70	1	18.8	−1.13	15	1	80	1
23	55	0	32.6	0.26	12	0	60	0
24	55	0	32.6	0.26	12	0	60	0
25	40	−1	18.7	−1.13	15	1	80	1
26	70	1	18.8	−1.13	15	1	40	−1
27	55	0	32.6	0.26	12	0	60	0
28	40	−1	18.7	−1.13	15	1	40	−1
29	40	−1	41.5	1.15	15	1	80	1
30	55	0	29.5	−0.06	6	−2	60	0

where T is the coded temperature, MC is the coded leaf moisture content, S is the coded solvent/solid ratio, E is the coded ethanol concentration and SD is the standard deviation. The predicted responses were correlated with the following set of coefficients (β): the intercept (β_0), linear (β_1, β_2, β_3, and β_4), interaction (β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34}) and quadratic (β_{11}, β_{22}, β_{33}, and β_{44}) coefficients. The statistical significance of the models was determined for a 0.1% probability level (p-value < 0.001) and that of the coefficients for a 5% probability level (p-value < 0.05), while the lack of fit was determined for a 10% probability level (p-value > 0.1).

To carry out optimization, once analyzed each response to establish the appropriate model, the optimization tool in Design-Expert® allows searching for a combination of factor levels that simultaneously satisfy the requirements placed on each of the responses and factors. Optimization of one response or the simultaneous optimization of multiple responses were performed numerically. The desired goals for each factor and response were "in range" and "maximize", respectively. To optimize one or more responses, the models are combined into a global desirability function. The software seeks to maximize this function.

2.4. Extraction yield or residual dry weight

The extraction yields were calculated using the ratios between the residual weight of the dry extract after evaporating the extraction solvent and the weight of dry biomass fed into the extraction cell. To determine the weight of the dry extract, 2 mL of extract filtered through a 0.45 µm filter was dried using a BMO35 electronic moisture analyzer (BOECO, Hamburg, Germany) at 105 °C until constant weight. Since the initial volume of the extract was known, the total extract amount could be calculated. The extraction yields were expressed in g of extract per kg of dry olive leaf.

2.5. TPC and TFC of olive leaves extract

The TPC of the olive leaves extract was determined by adding the Folin–Ciocalteu reagent (1.5 mL, 0.2 M) and 0.7 M sodium carbonate solution (1.2 mL) to a suitably diluted aliquot (300 µL) of extracts. After maintaining the sample at 25 °C in the dark for 10 min, its absorbance at 655 nm was measured. The results were expressed as mmol gallic acid equivalent/kg dry olive leaf (mmol gallic acid/kg).

The TFC of the olive leaves extract was determined using the aluminum chloride colorimetric method (Chang, Yang, Wen, & Chern, 2002) with modifications. In brief, 1 mL extract was reconstituted to 1 mL using methanol and mixed with 4 mL distilled water. Then, 0.3 mL of 5% $NaNO_2$ and 0.3 mL of 10% $AlCl_3$ solutions were added after 5 min of incubation, and the mixture was allowed to stand for 6 min. Subsequently, 2 mL of 1 mol/L NaOH solution was added, and the final volume of the mixture was brought to 10 mL using double-distilled water. The mixture was allowed to stand for 15 min, and its absorbance was measured at 510 nm. The total flavonoid content was calculated from a calibration curve, and the results were expressed as mmol rutin equivalent per kg dry olive leaf (mmol rutin/kg).

2.6. Antioxidant capacities of olive leaves extract

The antioxidant capacity of the extracts was determined as the DPPH free radical scavenging capacity, according to the method described by Vázquez-Roncero, Janer del Valle, and Janer del Valle (1973). The percentage of inhibition of the DPPH radicals was calculated after 15 min at 515 nm according to the following equation:

$$\%DPPH_{inhibition} = \frac{([DPPH]_{control} - [DPPH]_{extract})}{[DPPH]_{control}} 100 \quad (3)$$

where $[DPPH]_{control}$ and $[DPPH]_{extract}$ are the DPPH concentrations of the control and extract, respectively. The DPPH concentrations were calculated by interpolating the calibration curve of absorbance versus DPPH concentration. The percentage of inhibition was converted into antioxidant capacity using Trolox as standard antioxidant. Another calibration curve of the percentage of inhibition against the Trolox concentration was used for this purpose. The antioxidant capacity of the extracts was obtained in terms of Trolox concentration and was expressed as mmol Trolox equivalent/kg dry olive leaf (mmol Trolox/kg).

2.7. HPLC analysis and quantification

All extracts were analyzed using a Shimadzu Prominence UFLC device equipped with a DGU-20A5 degasser, LC-20AD quaternary pump, SIL-20AC HT auto sampler, SPD-M20A diode array detector and CTO-10AS VP column oven. A BDS HYPERSIL C18 column (250 mm × 4.6 mm, 5 µm particle size, Thermo Fisher Scientific Inc., Waltham, USA) was used to analyze the phenolic compounds. The mobile phase was a mixture of Milli-Q® water/0.2% orthophosphoric acid (solvent A), methanol (solvent B), and acetonitrile (solvent C) with an initial composition of 96/2/2 (v/v/v). The gradient elution changed according to the following conditions: B from 2 to 25%, C over 40 min; a linear increase to 30% B, C over 5 min; another linear increase to 50% B, C over 15 min; and isocratic at 50% B, C for 8 min followed by a linear decrease to 2% B and C over 4 min. The column was equilibrated for 8 min at starting conditions before each injection. A flow rate of 1.0 mL/min at 30 °C and an injection volume of 20 µL were used. The detection of phenolic compounds was performed at 280 nm.

The quantitative evaluation of oleuropein and luteolin-7-O-glucoside was based on comparing their retention times with that of the reference compound and recording the UV spectra in the 190–350 nm range. The quantification was performed using five individual stock solutions of known concentration for each compound. The OC and LC levels were expressed as g polyphenol per kg of dry olive leaves (g polyphenol/kg). The calibration curves were constructed by plotting the average of peak areas versus the concentrations of compounds selected. For linearity determination, calibration curves were fitted to linear least squares regression with coefficients of determination (R^2) ≥ 0.99. The calibration functions ($y_i = ax_i + b$) were calculated from the measured values, where y_i is peak area at AU (absorbance units), x_i the compound's concentration in g/L, b is the intercept, and a is the slope. Oleuropein and luteolin-7-O-glucoside were quantified with the following calibration curves: $y = 5.59 \cdot x + 11.91$ ($R^2 = 0.999$) and $y = 30.40 \cdot x - 113.09$ ($R^2 = 0.999$), respectively.

3. Results and discussion

Due to the development of modern analytical techniques, many novel extraction procedures, such as PLE, gained popularity, but certain aspects (more complex equipment and higher costs than those for traditional methods) made them difficult to use in industrial-scale applications. Therefore, it is essential and desirable to optimize these extraction methods and determine the economical and high-efficiency conditions required for the selective extraction of phenolic compounds from olive leaves. Very few research articles specifically address the PLE procedure for the recovery of polyphenols from olive leaves (Taamalli et al., 2012) while also using the RSM (Xynos et al., 2014; Putnik et al., 2017); therefore, it is necessary to continue studying other factors that may affect the extraction process.

3.1. Previous drying studies

Some researchers have reported that hot air oven-drying of olive leaves at high temperatures (105 or 120 °C) is an excellent pre-treatment, preferable to other drying options, and can be used prior to obtaining olive leaf extracts rich in bioactive compounds (Ahmad-Qasem, Barrajón-Catalán, Micol, Mulet, & García-Pérez, 2013; Kamran et al., 2015). In our study, olive leaves were dried using a forced air stove at

105 °C for different times. The evolution of moisture loss during stove-drying was used to determine the times necessary to reach the required MCs to perform the desired assays, while paying attention to the objective of investigating the effect of various degrees of drying on the extraction of olive leaves. The drying kinetics of olive leaves was adjusted to a first-order model (Eq. (4)):

$$MC = 58.78e^{-0.0752t} \quad (4)$$

with $R^2 = 0.978$, SD of 2.14 and a kinetic constant of 0.0752 min^{-1}. *MC* is the moisture content (% referred to fresh leaf) that remains at a time *t* (min) of drying. Eq. (4) predicts that the kinetic process of drying starts at 2.54 min after introducing the sample in the stove, for the moisture indicated in Section 2.1.

3.2. DM method

The extraction time is a factor that must be considered for equilibrium extractive methods because of the minimum time required for the solvent to solubilize the compounds (Ameer, Shahbaz, & Kwon, 2017). Therefore, to reduce the processing times of the samples and improve the extraction of phenolic compounds and flavonoids, prior to applying the technique, the variation of the extraction yield was determined versus time. Extraction kinetics was performed using 20 g dry olive leaves (at 105 °C for 24 h) by maintaining the temperature (25 °C), stirring speed (350 rpm), solvent/solid ratio (6:1, v/w), and aqueous ethanol concentration (80% (v/v)) constant. The extraction yield for each time interval was determined using thermogravimetric analysis by measuring the mass of the residue after removing the solvent. It was determined that the maximum extraction yield expressed as percentage of dry residue was obtained after 6 h (27.55 ± 0.03%), when equilibrium was reached. Afterward, this time value was further used for the optimization process using the DM method. The experimental data of the extraction kinetics was adjusted to a first-order model (Eq. (5)):

$$C = 15.56e^{-0.0303t} \quad (5)$$

with $R^2 = 0.970$ and SD of 0.70, where *C* represents remaining extractive content in the olive leaves as a function of time *t* and a kinetic constant of 0.0303 min^{-1}. For $t = 0$, the Eq. (5) predicts an instantaneous solubilization of 11.99 g of extractives/100 g of dry olive leaves.

The CCD was applied to determine the influence of four factors on the DM of olive leaves (Table 1). For this design, five levels ($-\alpha$, -1, 0, $+1$, $+\alpha$) of the factors were tested. The axial points (α) were calculated based on the number of factors to consider; for four factors $\alpha = 2.11$. However, the coded levels for MC did not coincide with these values since, in practice, the actual levels of the initial design were replaced by those obtained using the drying kinetic equation (Eq. (3)), which allowed to calculate the times required by the olive leaves to achieve specific moisture levels. The experimental results determined for the DM extracts obtained from olive leaves using the conditions described in Table 1 can be found in the Supplementary material S1.

As can be observed in Table 3, for each response, the effects of each factor and their interactions in terms of coded factors were obtained. All models were statistically significant, they did not exhibit lack of fit, and the terms not significant of the models were not included according to the p-values indicated in the Section 2.3. Combining the coefficients of determination and variation (relative error) confirmed that the models adjusted very well to the experimental results. The models indicated that the MC significantly influenced all responses, including larger than temperature mainly for TFC, DPPH, OC, and LC. The effects were negative for all cases. This was different than the response obtained for the temperature and solvent/solid ratio, thus, the highest response values were obtained when olive leaves with the lowest MC were used.

Fig. 1a illustrates the response surface predicted using the model obtained for the antioxidant capacity of the extracts obtained utilizing DM with respect to the temperature and leaf MC. The effect of the ethanol concentration on the antioxidant capacity of the DM extracts or the MC of the PLE extracts was negligible, and vice versa. Although the negative quadratic term which was greater, in absolute value, than the main level, caused the response to exhibit a maximum at approximately 60 °C. For the DM of olive leaves, the improvement in OC and LC was observed when the decrease in MC was significant (Fig. 2a and c).

Using the models for the studied responses, we determined the conditions that maximized the antioxidant capacity of the extracts, OC, and LC to be as follows: temperature of 70 °C, MC of 20%, solvent/solid ratio of 13 v/w, and ethanol concentration of 60.8%. Under these conditions, an optimized hydroalcoholic extract with R (287.0 g/kg), TPC (210.40 mmol gallic acid/kg), TFC (111.10 mmol rutin/kg), OC (18.60 g/kg), LC (1.0 g/kg) and DPPH (149.90 mmol Trolox/kg) would be obtained.

3.3. PLE method

From the results of the preliminary studies (data not shown), we concluded that the extraction time exhibited no significant influence on OC and LC at any temperature. However, only at 70 °C at least two consecutive cycles were necessary for the recovery of oleuropein and luteolin-7-O-glucoside; hence, taking into account the extraction time and energy consumption savings, 5 min and 1 cycle were considered sufficient for the extraction procedures of the experimental design. These results were consistent with the findings of other authors (Xynos et al., 2014; Putnik et al., 2017), and were chosen as the maximum range of static extraction time and number of cycles in subsequent experimental designs.

Extractions were performed according to the Box-Behnken design illustrated in Table 2. The experimental results for the PLE extracts obtained from olive leaves under the conditions described in Table 2 can be found in the Supplementary material S2. At first glance, the extraction yield predicted for PLE was much higher than that for DM. Thus, at the center point of the model, the first and second indicated yields of 380.99 and 224.18 g/kg of dry leaf. On the other hand, the PLE process required shorter extraction times.

These results indicated that temperature and MC represented the

Table 3
Models, in coded factors, of the responses in the Supplementary material S1 and main statistical parameters.

Responses	Models	R^{2a}	CV (%)[b]
R	$224.18 + 0.56\,T - 11.37\,MC + 19.54\,S - 10.97\,E - 11.92\,T{\cdot}MC - 12.05\,MC{\cdot}E + 7.11\,T^2 + 25.08\,MC^2 - 9.80\,E^2 \pm 13.20$	0.912	5.41
$(TPC)^{2.5}$	$434259 + 57106\,T - 62{,}901\,MC + 82190\,S - 5942\,E - 1718\,T{\cdot}MC - 21{,}214\,MC{\cdot}E - 30226\,T^2 + 76635\,MC^2 - 39{,}088\,S^2 - 80{,}260\,E^2 \pm 22241$	0.990	5.90
TFC	$78.97 + 7.83\,T - 23.84\,MC + 4.68\,S + 11.11\,E - 7.09\,T^2 + 6.26\,MC^2 - 6.75\,S^2 + 9.81\,E^2 \pm 4.56$	0.985	5.84
log(DPPH)	$1.972 + 0.037\,T - 0.127\,MC + 0.038\,S - 0.018\,E + 0.029\,T{\cdot}MC - 0.017\,T{\cdot}S + 0.018\,T{\cdot}E + 0.029\,T^2 + 0.041\,MC^2 - 0.037\,S^2 - 0.087\,E^2 \pm 0.027$	0.983	1.40
$(OC)^{0.5}$	$2.036 + 0.082\,T - 1.470\,MC + 0.072\,S - 0.001\,E - 0.047\,T{\cdot}MC - 0.057\,MC{\cdot}E - 0.102\,T^2 + 0.772\,MC^2 - 0.158\,S^2 - 0.276\,E^2 \pm 0.111$	0.996	5.25
$(LC)^2$	$0.677 + 0.044\,T - 0.211\,MC + 0.075\,S + 0.014\,E - 0.036\,MC{\cdot}S - 0.066\,T^2 + 0.037\,MC^2 - 0.081\,S^2 - 0.162\,E^2 \pm 0.052$	0.980	11.67

[a] Coefficient of determination.
[b] Coefficient of variation.

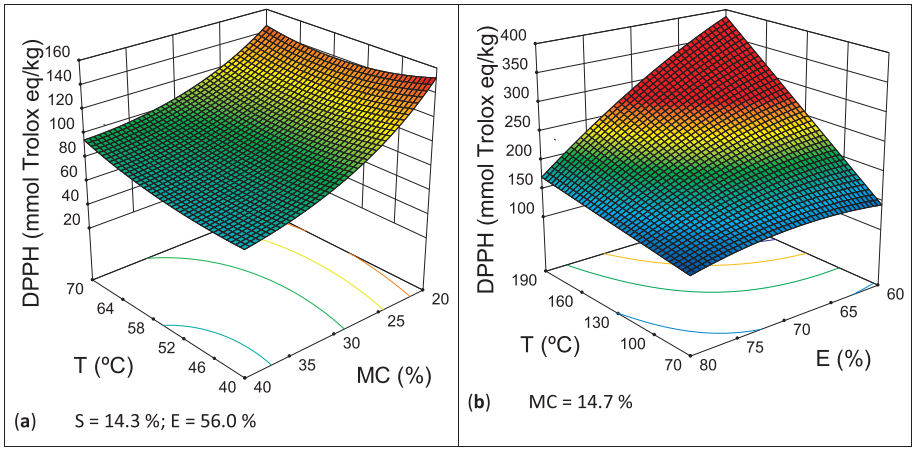

Fig. 1. Antioxidant capacity of the extracts (DPPH) with respect to temperature (T), leaf moisture content (MC) and ethanol concentration (E): (a) dynamic maceration, (b) pressurized liquid extraction. The fixed factors, solvent/solid ratio (S), E and MC, correspond to the maximum response.

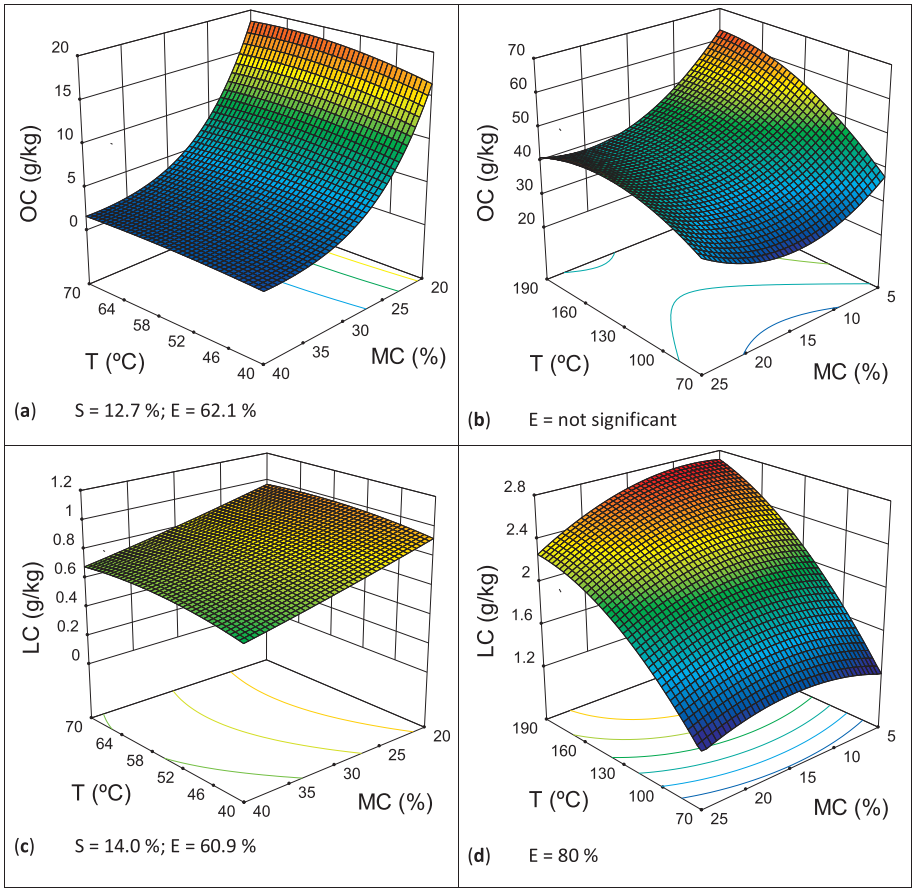

Fig. 2. Oleuropein and luteolin-7-O-glucoside contents (OC and LC, respectively) of the extracts with respect to temperature (T) and leaf moisture content (MC): (a, c) dynamic maceration, (b, d) pressurized liquid extraction. The fixed factors, solvent/solid ratio (S) and ethanol concentration (E), correspond to the maximum response.

Table 4
Models, in coded factors, of the responses in the Supplementary material S2 and main statistical parameters.

Responses	Models	R^2[a]	CV (%)[b]
R	$380.99 + 146.75\,T + 12.98\,MC - 3.52\,E + 11.33\,T{\cdot}E - 17.38\,MC{\cdot}E + 25.85\,T^2 \pm 10.66$	0.993	2.80
TPC	$281.12 + 115.68\,T - 18.76\,MC - 10.21\,E - 11.14\,T{\cdot}MC + 9.81\,T{\cdot}E - 16.12\,MC{\cdot}E + 21.03\,T^2 \pm 7.25$	0.996	2.45
TFC	$190.20 + 46.6\,T - 5.01\,MC + 2.74\,E - 17.53\,T^2 + 7.00\,MC^2 \pm 2.92$	0.995	1.61
DPPH	$224.34 + 71.04\,T - 1.68\,MC - 57.26\,E - 50.76\,T{\cdot}E - 26.42\,MC^2 - 16.12\,E^2 \pm 6.20$	0.992	3.25
OC	$40.75 + 7.59\,T - 5.79\,MC - 5.22\,T{\cdot}MC - 6.14\,T^2 + 10.13\,MC^2 \pm 1.47$	0.984	3.46
LC	$2.191 + 0.542\,T - 0.114\,MC + 0.011\,E - 0.109\,T{\cdot}MC + 0.108\,T{\cdot}E - 0.228\,T^2 - 0.179\,MC^2 + 0.043\,E^2 \pm 0.026$	0.998	1.28

[a] Coefficient of determination.
[b] Coefficient of variation.

key recovery factors (Table 4). The analysis of variance (ANOVA) of the responses indicated that all models were significant and there was no lack of fit, thus, implying that the models could be used to predict the responses. The terms not significant of the models were not included according to the p-values indicated in the Section 2.3. A positive correlation was observed between all responses and the temperature, which was the most influential factor. Our findings on the effect of temperature on the extraction process were in agreement with those reported in the literature. Fig. 2b and d illustrate the response surfaces of the effects of changing the temperature and MC on the OC and LC of olive leaves, while maintaining the other factor (ethanol concentration) at the value that yielded the maximum response. The models in Table 4 and response surfaces depicted in Fig. 2b and d indicate that temperature exhibited the greatest positive influence on the extraction of oleuropein and luteolin-7-O-glucoside. Higher temperatures generally improve the solubility and diffusivity of compounds, thus increasing the mass transfer between the plant matrix and bulk solvent (Carciochi, Sologubik, Fernández, Manrique, & D'Alessandro, 2018). This effect was slightly enhanced for R, TPC, and LC by increasing the ethanol concentration due to a positive interaction with temperature. The results obtained for TPC (maximum of 76.4 mg gallic acid/g dry leaf) were comparable to those reported by other authors (Putnik et al., 2017).

It was observed that levels of the extraction yield were slightly enhanced when the MC increased (positive coefficient), but the increase was counteracted by a significant interaction with the concentration of ethanol, while the rest of the responses decreased as the MC increased. A completely different behavior of the extraction yield could be observed during the DM process. Therefore, the MC of olive leaves played a significant role in the amounts of oleuropein and luteolin-7-O-glucoside recovered. From this study, it was deduced that it would be convenient to reduce the MC of olive leaves to improve the recovery of biophenols using PLE.

The influence of MC on the antioxidant capacity was small compared to those of the rest of the factors, but it presented a negative and slightly significant quadratic term that marked a maximum capacity around the central point (15%). In this case, as mentioned above, the temperature was the main factor responsible for the increase in the antioxidant capacity. This dependence has been observed by other authors and for other by-products (Machado, Pasquel-Reátegui, Barbero, & Martínez, 2015). The other parameter exhibiting significant and negative influence on the response was the ethanol concentration of the solvent mixture. Due to its strong negative interaction with the temperature, its negative influence doubled in the upper end of the temperature range, whereas in the lower end of the temperature range its influence was canceled out. A similar trend was observed for the temperature, due to its interaction with the concentration of ethanol. However, because the coefficient of the main level was higher, the effect of this interaction was less significant than that of the concentration of ethanol, as can be seen in Fig. 1b. In general, the concentration of ethanol is considered an important parameter for the extraction process because it could affect the solubility of the compounds. However, the results indicated that, over the variation range of the factor, it would not be convenient to increase the concentration of ethanol to optimize the responses. The maximum antioxidant capacity coincided with the maximum TPC, OC, and LC values.

We obtained optimal results for the OC of the extracts at 190 °C and after one extraction cycle. Regarding the OC of the extracts, similar results were reported by other authors using a mixture of water/ethanol (43:57) at 190 °C for 1 extraction cycle (Xynos et al., 2014). The response surface for OC (Fig. 2b) is curved and twisted due to the important quadratic terms and interactions between factors. The absolute value coefficients were similar to those of the main level. The OC and LC extracted from olive leaves ranged from 27.01 to 63.35 g/kg and from 1.18 to 2.74 g/kg, respectively. Oleuropein and luteolin-7-O-glucoside were the major components in the olive leaves extracts (see chromatograms in the supplementary material S3). The response surface for luteolin-7-O-glucoside (Fig. 2d) also indicates that the highest values were obtained for the highest temperature and lowest MC. The aim of this study was to obtain an extract of olive leaves which would be rich in oleuropein and luteolin-7-O-glucoside and would exhibit high antioxidant capacity. The temperature, MC, and ethanol concentration values that maximized the individual responses for the PLE process, were as follows: 190 °C, 5% and 63.1% for OC (63.35 g/kg); 190 °C, 8.74% and 80% for LC (2.74 g/kg) and 190 °C, 14.68% and 60% for DPPH (387.3 mmol Trolox/kg), respectively. If the three responses were optimized simultaneously, the optimal conditions would be: 190 °C, 5% and 60% (63.35 g/kg, 2.47 g/kg and 362.55 mmol Trolox/kg) for OC, LC, and DPPH, respectively.

Again, although the ethanolic composition of the mixture of solvents (percentage of ethanol) was considered by other authors (Xynos et al., 2014) to be an important parameter for the OC, in our work, this factor presented no statistical significance and did not affect the recovery of oleuropein or luteolin-7-O-glucoside in the selected range.

Pressurized liquid extraction is quite more effective than the conventional process of dynamic maceration on all of the studied responses. PLE has proven to be effective in the extraction of phenolics from several sources (Santos, Veggi, & Meireles, 2012) and it outperformed other traditional methods that have been used for this task. The results show as additional advantages a lower energy consumption than dynamic maceration (an extraction time of 5 min is required to achieve higher yields versus 6 h needed for dynamic maceration). Besides, pressurized liquid extraction also consumes lower quantities of solvent compared to dynamic maceration if we pay attention to the solvent/solid ratio used (7:1 versus 13:1, respectively), which would reduce the operational costs at industrial scale (Veggi, Cavalcanti, & Meireles, 2014).

PLE can also be compared with other emerging technologies such as microwave-assisted extraction (MAE) and ultrasound assisted extraction (UAE) (Rahmanian, Jafari, & Wani, 2015; Cruz et al., 2017). MAE has also been used for the extraction of biophenols from dried olive leaves. After microwave irradiation at 800 W, a complete extraction of oleuropein was reached at 10 min (Procopio et al., 2009). For PLE, oleuropein optimal extraction conditions were achieved in half the time. Ahmad-Qasem et al. (2013) used UAE to intensify the extraction process of phenolic compounds from olive leaves such as oleuropein and luteolin-7-O-glucoside. Again, compared with UAE, PLE reduced the extraction time from 15 to 5 min yielding higher TPC values (66 vs 76 mg GAE/g dry leaf, respectively) with only a single cycle of extraction.

4. Conclusions

The results of this work could contribute to the optimization and simulation of the PLE process used for the capitalization of olive leaves. It can be concluded that PLE is an efficient extraction technique featuring superior oleuropein and luteolin-7-O-glucoside recovery yields from olive leaves using the following optimized parameters: temperature of 190 °C, leaf MC of 5%, static extraction time of 5 min, and aqueous ethanol concentration of 80%. Under these conditions 63.35 g oleuropein and 2.71 g luteolin-7-O-glucoside per kg dry olive leaf were obtained, but the active antioxidant capacity of the extract was 146.5 mmol Trolox/kg. To obtain an extract with high antioxidant capacity, an aqueous ethanol concentration of 60% should be used (2.47 g luteolin-7-O-glucoside/kg and DPPH = 362.55 mmol Trolox/kg).

Future research studies in this area should being focused on overcoming those essential aspects for developing this PLE technique on an industrial scale so that the significant benefits which are obtained by improved extraction of bioactive compounds from olive leaves using this process can be exploited by biomass biorefinery plants. Although the investment to implement a PLE plant may be high, it is generally compensated by a significant reduction of operational costs, mainly

because the solvent consumption and energy expense of the involved unit operations are much lower.

Acknowledgments

This work was supported by the Universidad de Jaén through Acción 6 (EI_TEP1_2017) and the Ministerio de Economía, Industria y Competitividad (ENE2017-85819-C2-1-R). The technical and human support provided by CICT of the Universidad de Jaén (UJA, MINECO, Junta de Andalucía, FEDER) is gratefully acknowledged. Dr. Lama Muñoz would like to specially thank Mr. Óscar del Pico Hualde for his kind help and support during HPLC analyses.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.04.075.

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