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Optimization of ultrasound-assisted extraction of biomass from olive trees using response surface methodology

José Carlos Martínez-Patiño[a], Beatriz Gullón[b], Inmaculada Romero[a], Encarnación Ruiz[a,*], Mladen Brnčić[c], Jana Šic Žlabur[d], Eulogio Castro[a]

[a] Department of Chemical, Environmental and Materials Engineering, Universidad de Jaén, Campus Las Lagunillas, 23071 Jaén, Spain
[b] Department of Chemical Engineering, Institute of Technology, Universidade de Santiago de Compostela, 15782 Santiago de Compostela, Spain
[c] Department of Process Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, HR-10000 Zagreb, Croatia
[d] Faculty of Agriculture, University of Zagreb, Svetošimunska cesta 25, Zagreb, Croatia

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ABSTRACT

Olive tree pruning biomass (OTP) and olive mill leaves (OML) are the main residual lignocellulosic biomasses that are generated from olive trees. They have been proposed as a source of value-added compounds and biofuels within the biorefinery concept. In this work, the optimization of an ultrasound-assisted extraction (UAE) process was performed to extract antioxidant compounds present in OTP and OML. The effect of the three parameters, ethanol/water ratio (20, 50, 80% of ethanol concentration), amplitude percentage (30, 50, 70%) and ultrasonication time (5, 10, 15 min), on the responses of total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activities (DPPH, ABTS and FRAP) were evaluated following a Box–Behnken experimental design. The optimal conditions obtained from the model, taking into account simultaneously the five responses, were quite similar for OTP and OML, with 70% amplitude and 15 min for both biomasses and a slight difference in the optimum concentration of ethanol. (54.5% versus 51.3% for OTP and OML, respectively). When comparing the antioxidant activities obtained with OTP and OML, higher values were obtained for OML (around 40% more than for OTP). The antioxidant activities reached experimentally under the optimized conditions were 31.6 mg of TE/g of OTP and 42.5 mg of TE/g of OML with the DPPH method, 66.5 mg of TE/g of OTP and 95.9 mg of TE/g of OML with the ABTS method, and 36.4 mg of TE/g of OTP and 49.7 mg of TE/g of OML with the FRAP method. Both OTP and OML could be a potential source of natural antioxidants.

1. Introduction

Olive trees are cultivated mainly in Mediterranean countries, but nowadays their cultivation is spread around the world. Different wastes and by-products are generated in olive oil production, with olive tree pruning biomass (OTP) being the most abundant [1]. OTP is an agricultural residue generated in the pruning operation that is usually carried out every two years after fruit harvesting to remove the old branches and prepare the tree for the next crop. Normally, this biomass is eliminated by burning or grinding and spreading across the field for soil enrichment [2]. Although the proportions depend on different factors, a typical lot of OTP is composed of leaves (25% by weight), thin branches (50% by weight) and thick branches or wood (25% by weight) [3]. Another biomass generated in the early steps of the production of olive oil is the leaves and small branches that are generated during olive harvesting and that must be separated from the fruits before the extraction of the oil. They are usually removed using a blower during olive cleaning performed in olive mills, representing approximately 6% of the total olive weight [4]. This biomass has no current industrial application, but is partially used as an animal feed or discarded. As it consists mainly of leaves, this residue is called olive mill leaves (OML) in this work.

The main structural components of both OTP and OML are cellulose, hemicellulose and lignin. However, their extractive content is remarkably higher than other lignocellulosic biomasses, around 45% in the case of OML [4] and between 14.1 and 31.4% for OTP [1]. This extractive fraction contains, among others, sugars (mainly glucose in monomeric and oligomeric form), and mannitol and phenolic compounds [5]. In the context of biorefineries, OTP and OML could have a significant impact as raw materials for the production of fermentable sugars, antioxidant compounds, oligosaccharides, etc [6–9]. In the case of OTP, previous studies have shown that the removal of extracts in a first step can be positive to improve the effectiveness of the pretreatment and the yield of fermentation into bioethanol [5,10].

* Corresponding author.
E-mail address: eruiz@ujaen.es (E. Ruiz).

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Furthermore, from the perspective of the multipurpose cascading biorefinery, the extraction of these bioactive compounds from these cheap sources can improve the economic viability of the global process [11].

Olive leaves have been widely studied as a source of bioactive compounds [12]. Some research has also been carried out on antioxidant components of olive wood [13]. Therefore, biomass from olive trees can be of great interest to obtain high added-value compounds with applications in the pharmaceutical, food and cosmetic industries [14,15]. It is widely recognized that antioxidant compounds are associated with health maintenance and are also used as additives in food preservation [11,16]. In general, several bioactive compounds such as hydroxytyrosol, tyrosol, cycloolivil, 7-deoxyloganic acid, oleuropein, ligustroside and flavonoids have been identified in olive-derived biomass [13,15]. In the last few years, the demand for natural antioxidants has grown quickly as an alternative to less safe synthetic antioxidants.

For the recovery of bioactive compounds from residual biomass, Soxhlet extraction is traditionally used. However, this method requires large quantities of organic solvents, prolonged extraction times and can also cause thermal degradation of the targeted compounds [17,18]. Recently, ultrasound-assisted extraction (UAE) has been used successfully for the extraction of active compounds from different types of samples [19]. The UAE mechanism rely on collapse of the cavitation bubbles near or on the surface of the plant cell walls. This bubble collapse causes disruption of the cell walls on account of the primary mechanical and secondary thermal, and chemical effects helping the solvent to penetrate within the cell and has a consequence of increased mass transfer resulting in better diffusion of the cell material [20,21]. This is followed by structural modification of plant tissue, thus more cell material is released in shorter time of treatment and under lower temperatures [22,23]. Some authors [21,24,25] noticed interesting physical mechanisms of UAE like erosion, collapse pressure, turbulences, diffusion effects, detexturation, cell fragmentation and shear stresses.

Ultrasound-assisted extraction compared with classical techniques has some advantages like higher extraction yields and reduction in time, solvent and energy consumption [26]. In a concept of green processing, UAE shows promising benefits. From the mechanism point of view, it is based on completely non-toxic waves propagation which does not pollute environment. In addition, regarding energy consumption, ultrasound has great potential, since it can decrease energy inputs significantly in comparison with traditional technologies [27]. Furthermore, as means of solvent usage, under same conditions, ultrasound could act as a processing tool with less solvent consumption. In some cases, it could give better results with water as a solvent than with ethanol or some other organic solvents. For waste emerged in food treatments and agro processing, ultrasound represents an excellent approach from both material and energy point of view. Namely, many components such as carotenoids, pigments, antioxidants and others could be extracted from wastes and by-products with UAE [28].

Generally, UAE is affected by several factors such as ultrasonic power and frequency, temperature, ultrasonication time, solvent properties and composition, particle size or solid to solvent ratio [29]. Therefore, the optimization of the extraction process is crucial to obtain antioxidant compounds with high bioactivity. The response surface methodology (RSM) is an effective mathematical and statistical tool to evaluate the effect of the independent variables and their interactions on the responses studied and their optimization [30]. Many researchers have used RSM to optimize bioactive compounds extraction from a number of biomass sources [11,18,31], including leaves collected from olive trees [32,33]. However, to the best of our knowledge, there is no literature on optimizing phenolic compounds extraction from OTP and OML by UAE.

The present study is an attempt to optimize the extraction of bioactive compounds with antioxidant activity from OTP and OML by UAE. The influence of some extraction parameters (ethanol/water ratio, amplitude percentage and ultrasonication time) was evaluated using RSM. Additional experiments based on the simultaneous maximization of all the evaluated responses (total phenolic content (TPC), total flavonoid content (TFC), and antioxidant properties (measured by DPPH, ABTS and FRAP) were performed in order to obtain extracts with high bioactivity.

2. Materials and methods

2.1. Raw material

Olive tree pruning biomass (OTP) was collected after fruit-harvesting in olive groves of the variety *Picual* in Jaén (Spain). Olive mill leaves (OML) were collected from the olive cleaning line when they were separated from the fruits in an olive mill also located in Jaén (SCA Unión Oleícola Cambil). These two categories of biomass were air-dried to the equilibrium moisture content and ground with an Ultra Centrifugal Mill (Retsch ZM200, Haan, Germany) with 1 mm sieve size.

2.2. Ultrasound assisted extraction (UAE)

OTP and OML extraction was performed using an ultrasound device (UP400S, Hielscher, Germany) with a power of 400 W and a frequency of 24 kHz. The liquid to solid ratio of extraction (v/w) was set at 20 mL/g. The biomass (15 g/300 mL of ethanol/water solution) was placed in a 400 mL beaker. A sonotrode of 22 mm in diameter was used. The sonotrode was submerged to 1.5 cm depth in the samples. Sonication was conducted in continuous mode with a full applied cycle (C = 1) which means that the ultrasound was propagated within the samples all the time. The samples were not cooled. After extraction, the solid and liquid fractions were separated by vacuum filtration and extracts were stored at −18 °C until further use.

2.3. Experimental design

A Box–Behnken experimental design was performed with a total of 17 experiments, with 5 replicates at the central point. The three variables studied were ethanol concentration (% v/v), ultrasonication time (min) and amplitude percentage (%). Amplitude percentage refers to the percentage of maximum power used. The natural and coded values for the independent variables are summarized in Table 1. During the extraction, the temperature of the samples increased, limiting the range of amplitude percentage and the ultrasonication time used, to avoid solvent evaporation. The temperature reached at the end of the experiments is shown in Table 2 (for OTP) and Table 3 (for OML), together with the experimental data. The experimental data were fitted using the following second-order polynomial equation:

$$y_j = \beta_0 + \sum_{i=1}^{3} \beta_i \cdot x_i + \sum_{i=1}^{3} \beta_{ii} \cdot x_i^2 + \sum \sum_{i<j=1}^{3} \beta_{ij} \cdot x_i \cdot x_j \quad (1)$$

where y_j is the different response (j = 1–5), β_0, β_i, β_{ij} and β_{ii} are the regression coefficients for the mean, linear, interaction and quadratic terms respectively calculated from the experimental results by the least squares method, and x_i and x_j are independent variables in coded values

Table 1
Independent variables involved in the study.

Independent variable	Nomenclature	Units	Value(s)		
			(−1)	0	(+1)
Ethanol concentration	[EtOH] or x_1	% v/v	20	50	80
Amplitude percentage*	Amp or x_2	%	30	50	70
Ultrasonication time	t or x_3	min	5	10	15

* Percentage of maximum power (400 W).

Table 2
Box-Behnken experimental design and experimental results obtained for the measured responses with olive tree pruning biomass (OTP).

Exp.	Ethanol (%v/v)	Amp (%)	Time (min)	T (°C)*	TPC (mg GAE/g OTP)	TFC (mg RE/g OTP)	DPPH (mg TE/g OTP)	ABTS (mg TE/g OTP)	FRAP (mg TE/g OTP)
1	50	50	10	56	29.1	64.4	29.1	54.6	31.2
2	20	30	10	48	19.3	38.6	17.4	39.2	20.6
3	50	70	15	75	30.7	69.9	30.0	57.6	33.4
4	50	50	10	57	28.2	62.2	26.4	53.9	28.3
5	20	50	5	44	19.9	37.2	16.6	40.4	20.2
6	50	50	10	57	28.1	62.5	26.5	56.6	27.9
7	50	30	5	40	25.5	57.7	24.7	49.1	26.0
8	50	30	15	56	27.5	63.8	29.0	53.9	28.5
9	50	70	5	54	27.3	63.3	25.7	53.5	29.2
10	50	50	10	57	27.0	61.6	26.2	52.1	29.3
11	80	50	15	63	26.7	62.6	25.3	52.5	26.8
12	80	70	10	70	27.8	68.7	26.2	55.5	29.3
13	80	50	5	43	21.9	52.7	21.4	44.7	22.1
14	80	30	10	44	23.0	55.3	21.2	44.2	24.1
15	50	50	10	58	28.3	65.2	26.8	54.0	30.5
16	20	70	10	67	25.8	59.5	24.6	47.1	25.8
17	20	50	15	65	24.2	58.6	23.5	45.7	25.2

GAE: Gallic acid equivalents.
RE: Rutin equivalents.
TE: Trolox equivalents.
* *Higher temperature reached, measured at the end of the experiment.

ranging from −1 to 1. Commercial software (Design Expert 7.0.0, Stat-Ease Inc., Minneapolis, USA) was used to analyze the results and optimize the conditions of all the responses. The optimal UAE conditions were tested experimentally in triplicate to check the validity of the model.

2.4. Total phenolic content (TPC) and total flavonoid content (TFC) determination

Total phenolic compounds were measured by spectrophotometry using the Folin–Ciocalteu method [34]. Gallic acid was used as standard and the results were expressed as mg of gallic acid equivalents (GAE)/g of dry biomass. Total flavonoid content (TFC) was determined following the colorimetric assay described by Zhishen et al. [35]. Rutin was the reference standard and the results were expressed as mg of rutin equivalents (RE)/g of dry biomass. All samples were analyzed in triplicate.

2.5. Antioxidant capacity

Three different methods were used to determine the antioxidant capacity of the extracts obtained from OTP and OML. In all assays, trolox (6-hydroxy-2,5,7,8-tetramethylchromen-2-carboxylic acid) was used as standard and the results were expressed in mg of trolox equivalents (TE)/g of biomass. The determinations were carried out in triplicate and the mean was calculated.

2.5.1. DPPH radical scavenging

A DPPH assay was performed according to the procedure described by Brand-Williams et al. [36] with some modifications. The reduction of absorbance at 517 nm after 15 min was measured when 0.2 mL of the samples were added to 2 mL of $6{\cdot}10^{-5}$ M DPPH (2,2-diphenyl-1-picrylhydrazyl) methanol solution.

2.5.2. ABTS cation radical scavenging

This assay was carried out following the method described by Cano et al. [37]. ABTS radical cation ($ABTS^{+}$) was generated by reacting

Table 3
Box-Behnken experimental design and experimental results obtained for the measured responses with olive mill leaves (OML).

Exp.	Ethanol (%v/v)	Amp (%)	Time (min)	T (°C)*	TPC (mg GAE/g OML)	TFC (mg RE/g OML)	DPPH (mg TE/g OML)	ABTS (mg TE/g OML)	FRAP (mg TE/g OML)
1	50	50	10	59	39.1	87.5	44.4	82.9	43.5
2	20	30	10	51	32.9	72.3	38.4	74.0	37.2
3	50	70	15	74	42.9	98.8	49.2	98.8	50.5
4	50	50	10	58	37.2	81.8	45.2	86.2	42.8
5	20	50	5	44	31.2	66.7	37.7	67.9	35.3
6	50	50	10	58	38.0	84.8	48.0	88.9	43.3
7	50	30	5	40	35.0	77.7	43.8	78.6	37.9
8	50	30	15	55	38.8	85.0	46.7	83.6	40.8
9	50	70	5	54	37.0	84.9	48.8	84.0	40.2
10	50	50	10	58	37.8	84.8	46.4	87.9	42.5
11	80	50	15	63	36.2	80.8	43.0	83.9	39.6
12	80	70	10	70	35.7	84.8	44.9	86.0	42.7
13	80	50	5	43	30.0	71.0	39.1	75.0	33.9
14	80	30	10	44	32.0	71.0	39.5	76.2	34.3
15	50	50	10	59	36.3	84.9	46.8	82.8	42.1
16	20	70	10	68	36.0	85.9	46.0	85.1	41.3
17	20	50	15	66	35.7	81.2	43.4	76.9	39.2

GAE: Gallic acid equivalents.
RE: Rutin equivalents.
TE: Trolox equivalents.
* Higher temperature reached, measured at the end of the experiment.

7 mM ABTS (2,2′-azino-di(3-ethylbenzothiazoline-6-sulfonic acid) stock solution with 2.45 mM potassium persulfate (final concentration). This solution was incubated for 12–16 h at room temperature and protected from light. Then, the ABTS reagent was diluted with phosphate buffer (PBS, pH 7.4) until reaching an absorbance of 0.7 at 734 nm. The assay consisted in the addition of 20 μL of extracts to 2 mL of diluted ABTS reagent and measuring the decrease in absorbance after 6 min.

2.5.3. Ferric reducing antioxidant power (FRAP)

A FRAP assay was done according to Benzie et al. [38] with some modifications. Briefly, 0.1 mL of the diluted extracts was added to 3 mL of FRAP reagent. The FRAP reagent was prepared by mixing 100 mL of 300 mM acetate buffer (pH 3.6); 10 mL of 10 mM of TPTZ (2,4,6-Tri(2-pyridyl)-1,3,5-triazine) in 40 mM HCl solution and 10 mL of 20 mM $FeCl_3{\cdot}6H_2O$ solution. The absorbance was measured at 593 nm after 6 min.

3. Results and discussion

3.1. Model adequacy

A Box–Behnken design was used to evaluate the influence of three variables (ethanol concentration, amplitude percentage and ultrasonication time) in an ultrasound-assisted extraction of OTP and OML. Tables 2 and 3 show the operational conditions assayed and experimental results for the five responses analyzed in this work (TPC, TFC, DPPH, ABTS and FRAP). The selected independent variables and their variation ranges were selected based on other related investigations [18,27,39]. In this work, ethanol was selected as the extraction solvent due to its low cost, non-toxicity and its use in food applications [11,18]. A preliminary screening was performed on general parameters such as particle size and solids concentration (data not shown), and 1 mm and 5%, respectively, were selected based on these results. With respect to amplitude and time, as mentioned in Section 2.3, the experimental domain was not extended to avoid the evaporation of the solvent, due to the increase in the temperature produced. Thus, the temperatures ranged from 40 °C in the case of the experiments performed at the lowest level of amplitude and time (experiment 7 in Tables 2 and 3) to 74–75 °C at the highest levels (experiment 3 in Tables 2 and 3).

The regression coefficients in terms of coded values determined by analysis of variance (ANOVA) for each model, and the statistical parameters F-values, coefficient of determination (R^2), adjusted R^2, coefficient of variation (CV) and lack of fit (p-value) are summarized in Tables 4 and 5. The high F value for all responses evaluated (24.08–69.74) indicated that the model obtained was statistically significant. The coefficient of determination (R^2) for all of the responses was higher than 0.932, which indicates the good accuracy of the model. The adjusted determination coefficients (R^2_{adj}) were also satisfactory, suggesting a high degree of correlation between the experimental and predicted values. Furthermore, in all cases the coefficient of variation (CV) was less than 5%, which confirms the good precision and reproducibility of the model. Moreover, the p-value for lack of fit was insignificant in all cases ($p > 0.1$), meaning the dispersion of experimental data was model-independent measure of the pure error.

In general, all these statistical parameters indicated that the model used represents adequately the relationship between the independent variables and the different responses.

3.2. Total phenolic content (TPC)

Phenolic compounds had an aromatic and a benzene ring with one or more hydroxide groups and had the ability to donate hydrogen and form stable radical intermediates which give them their antioxidant capacity. The experimental values of TPC ranged between 19.3 and 30.7 mg GAE/g in the case of OTP (Table 2) and between 30.0 and 42.9 for OML (Table 3). The differences between both residual biomasses could be attributed to the higher proportion of leaves present in OML, since the content of phenolic compounds in olive wood and small branches is lower than in olive leaves [4,40,41].

Table 4
Model equation coefficients and statistical parameters for olive tree pruning biomass (OTP).

Coefficient	TPC	TFC	DPPH	ABTS	FRAP
b_0	28.07[a]	62.86[a]	27.15[a]	54.36[a]	29.14[a]
b_1	1.27[a]	5.68[a]	1.97[a]	3.04[a]	1.30[a]
b_2	2.41[a]	8.15[a]	1.31[b]	3.89[a]	2.31[a]
b_3	2.11[a]	7.89[a]	2.44[a]	3.26[a]	2.07[a]
b_{12}	NS	−1.88[c]	NS	NS	NS
b_{13}	NS	−2.89[b]	NS	NS	NS
b_{23}	NS	−4.62[b]	NS	NS	NS
b_{11}	−4.01[a]	−6.93[a]	−5.60[a]	−8.20[a]	−4.87[a]
b_{22}	NS	NS	NS	NS	NS
b_{33}	−0.98[b]	−3.55[b]	NS	NS	NS
F-value	60.70	60.70	37.42	46.86	40.49
R^2	0.9681	0.9858	0.9315	0.9446	0.9364
Adj R^2	0.9521	0.9695	0.9067	0.9244	0.9133
CV	2.81	2.76	4.80	3.06	4.01
Lack of fit (p-value)	0.5629	0.3939	0.5383	0.6123	0.6844

NS: equation term not significant ($p > 0.1$).
[a] Highly significant ($p < 0.01$).
[b] Moderately significant ($0.01 < p < 0.05$).
[c] Significant ($0.05 < p < 0.1$).

Table 5
Model equation coefficients and statistical parameters for olive mill leaves (OML).

Coefficient	TPC	TFC	DPPH	ABTS	FRAP
b_0	37.66[a]	84.40[a]	46.32[a]	86.46[a]	42.83[a]
b_1	NS	NS	NS	2.13[c]	NS
b_2	1.61[a]	6.04[a]	3.38[a]	5.19[a]	3.06[a]
b_3	2.55[a]	5.69[a]	2.44[a]	4.71[a]	2.85[a]
b_{12}	NS	NS	NS	NS	1.08[b]
b_{13}	NS	NS	NS	NS	NS
b_{23}	NS	1.65[c]	−2.30[b]	2.45[c]	1.82[a]
b_{11}	−4.34[a]	−9.01[a]	−4.30[a]	−8.23[a]	−4.65[a]
b_{22}	0.82[b]	2.65[b]	NS	2.09[c]	0.70[c]
b_{33}	NS	NS	−1.04[c]	−2.31[c]	−1.18[a]
F-value	69.74	55.65	37.16	24.08	77.54
R^2	0.9588	0.9620	0.9489	0.9547	0.9837
Adj R^2	0.9450	0.9447	0.9234	0.9150	0.9710
CVply	2.06	2.24	2.34	2.59	1.71
Lack of fit (p-value)	0.9236	0.6595	0.9273	0.7824	0.2560

NS: equation term not significant ($p > 0.1$).
[a] Highly significant ($p < 0.01$).
[b] Moderately significant ($0.01 < p < 0.05$).
[c] Significant ($0.05 < p < 0.1$).

The significant terms in the model equation obtained with OTP (Table 4) were the linear terms of the three independent variables and the quadratic terms of the ethanol concentration and ultrasonication time. Fig. 1a shows the surface response of TPC as a function of ethanol concentration and amplitude percentage (ultrasonication time constant at 10 min, $x_3 = 0$) while Fig. 1b shows the TPC response as a function of amplitude percentage and ultrasonication time for an ethanol concentration of 50% v/v ($x_1 = 0$). As can be seen in Fig. 1a, the influence of ethanol concentration was positive until reaching an optimum level (ethanol concentration of 55% v/v) and then the TPC decreased. This behaviour is due to the high influence of the quadratic term of temperature on the extraction yield equation. In this context, it has been reported that the use of a mixture of ethanol with water is more effective for the phenolic compounds extraction than the corresponding single solvent [11,42]. Similar trends were observed with other biomass such as the flower of *Limonium sinuatum* [18] or grapefruit solid wastes [43]. On the other hand, the amplitude percentage and ultrasonication

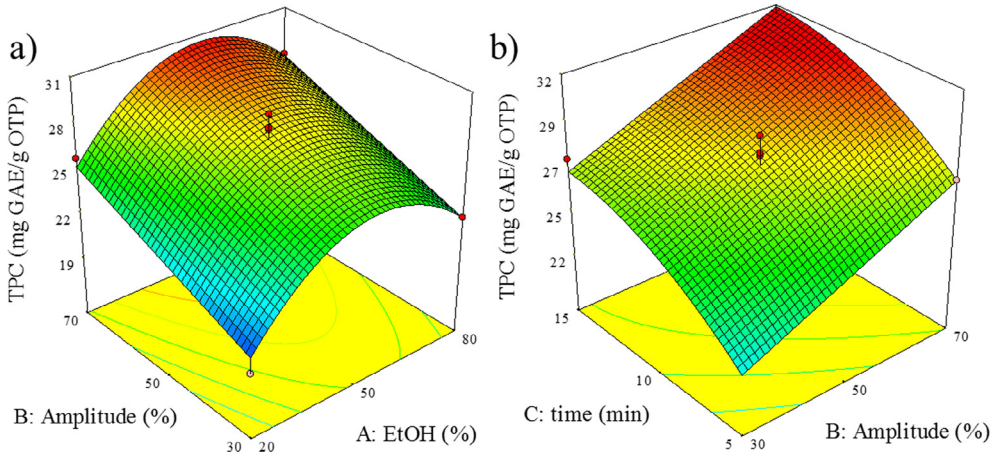

Fig. 1. Response surface of total phenolic content (TPC) for olive tree pruning biomass (OTP) as a function of a) ethanol concentration and amplitude percentage and b) amplitude percentage and ultrasonication time.

time showed a positive linear effect on the TPC in all the range of variation studied. Therefore, the maximum TPC predicted by the model was 31.8 mg GAE/g OTP at 70% amplitude, 15 min and 54.7% ethanol concentration.

Regarding the results of the model obtained for OML (Table 5), the significant terms of the model were the lineal terms of amplitude percentage and ultrasonication time and the quadratic terms of ethanol concentration and amplitude percentage. The behaviour of the TPC response was similar to the one observed for OTP. All the surface response plots for OML are shown in Figs. S1–S3. Accordingly with the values of the model coefficients presented in Table 5, the quadratic term of ethanol concentration showed a negative effect on TPC. This implied that an increase in ethanol concentration above a certain point (50%) caused a decrease in this response. The linear coefficients of the ultrasonication time and amplitude percentage showed a positive influence on TPC (maximum predicted TPC of 42.6 mg GAE/g OML, attained at 70% amplitude, 15 min and 50% ethanol concentration). Several factors can affect the extraction of phenolic compounds from olive leaves, such as leaf age, geographical origin or olive tree cultivar, among others [32]. The results found in the literature when olive leaves were submitted to an ultrasound-assisted extraction showed important variations. For example, Şahin and Şamli [32] obtained 25.1 mg GAE/g with olive leaves of the variety *Tavsan yuregi*, extracted under optimal conditions (50 mg/L of solid to solvent ratio, 60 min and 50% of ethanol), while Shrizad et al. [33] reached 183.4 mg of GAE/g with olive leaves of the variety *Koroneiki* under optimized conditions (51% ethanol, 15 min and 65 °C).

3.3. Total flavonoid content (TFC)

Flavonoids represent an important group of polyphenolic compounds present in natural sources and are of special interest due to their potential antioxidant activity as well as their possible beneficial effects on human health [11]. The maximum experimental value of TFC for OTP (69.9 mg RE/g of dry raw material) was reached in experiment 3 (50% v/v of ethanol concentration, 70% of amplitude and 15 min); under these same conditions the value obtained for OML was considerably higher (98.8 mg RE/g of dry raw material) (Tables 2 and 3).

Attending to the coefficients of the model of OTP (Table 4), all terms were significant except the amplitude percentage quadratic term. Fig. 2 shows the response surface for TFC a) as a function of ethanol concentration and ultrasonication time for a power of 50% ($x_2 = 0$), and b) as a function of amplitude percentage and ultrasonication time for an ethanol concentration of 50% v/v ($x_1 = 0$). Similar to the trend observed for TPC, the ethanol concentration has a positive influence on TFC until reaching a maximum value at 56% of ethanol concentration. However, above this optimum level, TFC is negatively affected by the increase of ethanol concentration. Several authors have also reported that mixtures of ethanol and water are more effective in extracting flavonoids [11,33,44]. In the case of amplitude percentage, this variable showed a positive influence on the recovery of flavonoids throughout the experimental domain. The ultrasonication time also affected the flavonoid content positively. However, at the highest amplitude percentage values, the value of the TFC response decreased from time values higher than 12 min, as can be observed in Fig. 2b. This behaviour is due to the negative sign of the term of the interaction between the amplitude percentage and ultrasonication time. Thus, the highest content of flavonoids (72 mg RE/g OTP) was predicted by the model at 56% of ethanol concentration, 70% of amplitude and 12 min of ultrasonication time. This result might be due to the fact that when high amplitude percentages and extended ultrasonication times are used, high temperatures (> 75 °C) are reached (Table 2), which may lead to degradation of these compounds. In this context, it has been reported that flavonoids were thermo-sensitive compounds [11,45].

In the case of OML, the lineal terms of amplitude percentage and ultrasonication time, the quadratic terms of ethanol concentration and amplitude percentage and the interaction between amplitude percentage and ultrasonication time had significant impacts on the TFC (Table 5). An increase in the ultrasonication time and amplitude percentage provoked an increase in the TFC response in all of the range studied. The ethanol concentration had a similar influence to that observed for OTP. This same trend was also reported by Shirzad et al. [33] when they studied the extraction of antioxidants from olive leaves using ultrasound.

3.4. Antioxidant capacity

Three different assays were performed to evaluate the impact of ultrasound treatment conditions on the antioxidant capacity of OTP and OML extracts. The DPPH or ABTS assays are related with the neutralization of free radicals generated in both assays systems by the compounds present in the extracts with antioxidant capacity. The FRAP assay measures the antioxidant activity for the reduction of Fe^{3+} (ferric iron) to Fe^{+2} (ferrous iron).

The experimental antioxidant activity for OTP extracts was between 16.6 and 30.0 mg of TE/g of OTP in the DPPH assay; 39.2 and 57.6 mg of TE/g of OTP in the ABTS assay and 20.2 and 33.4 g of TE/g of OTP in the FRAP assay. The model coefficients (Table 4) showed that the three linear terms and the quadratic term of ethanol concentration significantly affect the antioxidant activity for all assays. Fig. 3(a–c) shows the effect of the ethanol concentration and amplitude percentage on the DPPH, ABTS and FRAP assays responses, while Fig. 3(d–f) depicts the

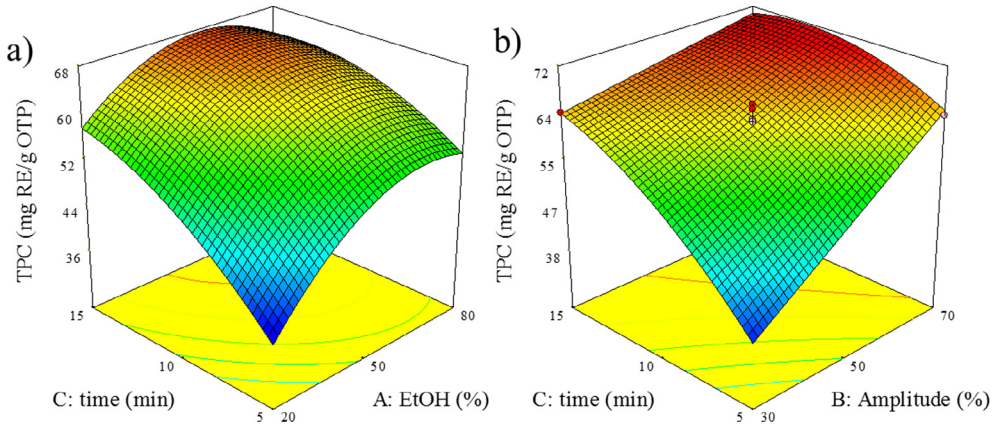

Fig. 2. Response surface of total flavonoid content (TFC) for olive tree pruning biomass (OTP) as a function of a) ethanol concentration and ultrasonication time and b) amplitude percentage and ultrasonication time.

influence of the ultrasonication time and amplitude percentage on the same responses. The ultrasonication time and amplitude percentage had a positive influence in all of the operational range studied. When considering the concentration of ethanol, two different behaviours were observed: a positive effect until the first half of its variation range (approximately 55%) and above this value a negative influence, as in the other responses evaluated in this work. Sharmila et al. [39] obtained the highest DPPH (90.5%) and FRAP (96.2 mM Fe^{2+}/g) activities in extracts of *Cassia auriculata* leaves with solvent concentrations of 60%, short times (5 min) and the highest power (50 W).

The antioxidant activity of OML extracts ranged from 37.7 to 49.2 mg of TE/g of OML in the DPPH assay; from 67.9 to 98.8 mg of TE/g of OML in the ABTS assay and from 33.9 to 50.5 mg of TE/g of OML in the FRAP assay (Table 3). In the case of OML, other terms of the model equation were significant (Table 5). For example, in the three model equations of antioxidant activity for OML, the quadratic term for ultrasonication time and the interaction between the amplitude and time were significant. In addition, the quadratic term of amplitude percentage was significant in the ABTS and FRAP equations, while the interaction term between the ethanol concentration and amplitude percentage was only significant in the FRAP equation. However, the influence of the factors was quite similar to that in the case of OTP, as it was positive in all of the range assayed for amplitude percentage and ultrasonication time, and the ethanol concentration also achieved a maximum around 52%. Shirzad et al. [33] observed the same influence of ethanol concentration and time extraction on the FRAP activity of olive leave extracts. Nevertheless, the DPPH activity was increased with the ethanol concentration (75%v/v). Also, Şahin and Şamli [32] obtained higher DPPH activities in ethanol pure solvent.

Several authors have investigated the degradation of phenolic compound by UAE [46,47]. Different results were reported depending on the chemical nature of the phenolics, although the mechanism involved is unclear in some cases. Styaningsih et al. [47], in a stability study of 40 phenolic compounds during UAE, reported a slight degradation of some of the studied compounds starting at 60 or 70 °C, while all the 40 phenolics remained stable when UAE between 10 and 50 °C was applied. Liazid et al. [46] studied the stability of several compounds of the flavonoid family using different extraction

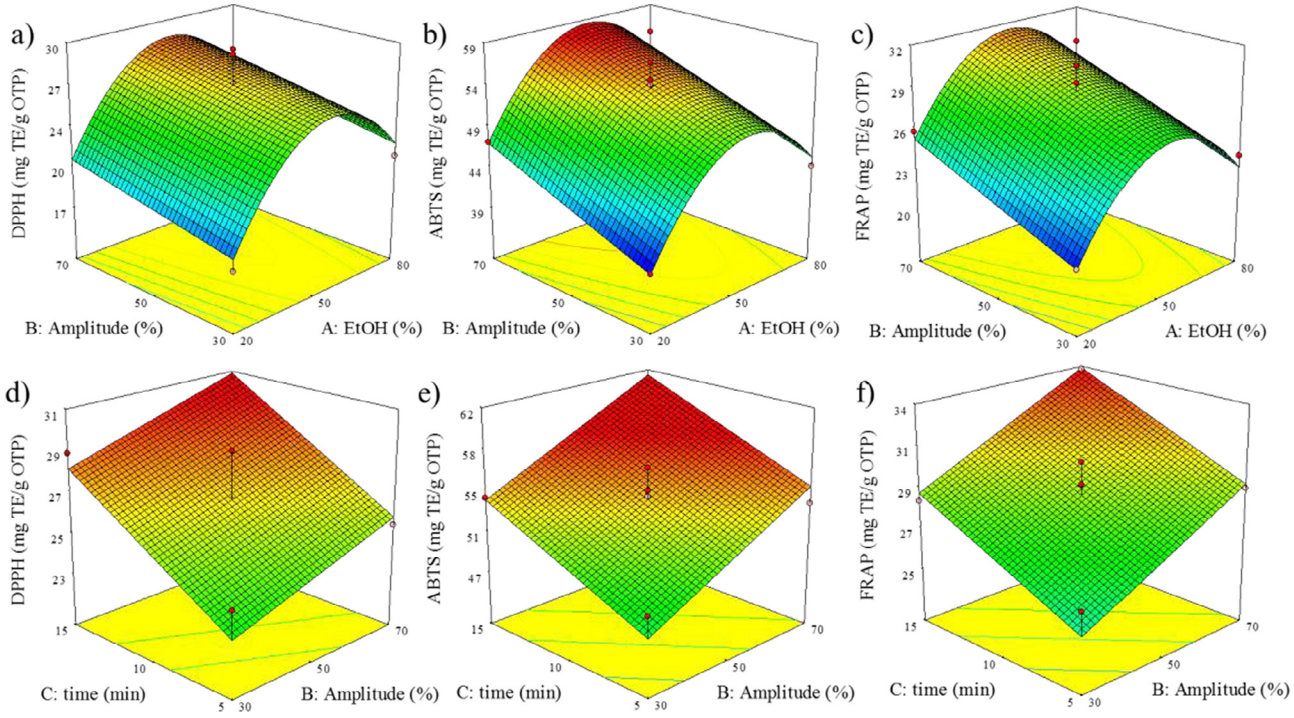

Fig. 3. Response surface plots for olive tree pruning biomass (OTP) of a, d) DPPH assay; b, e) ABTS assay and c, f) FRAP assay.

techniques, showing that all the studied compounds remained stable after UAE extraction (performed below 75 °C). These results are in agreement with those obtained in the present work, since no decrease in antioxidant activity was evidenced when amplitude ant time (and consequently temperature) were increased in the range studied. Nevertheless, a detailed analysis of the extracted compounds would be of great interest and will constitute the focus for further research.

3.5. Process optimization and validation of the model

An optimization of the variables studied was performed with the aim of maximizing the five responses simultaneously (TPC, TFC and antioxidant activities by DPPH, ABTS and FRAP methods) due to the relationship between the content of antioxidant compounds and their bioactive properties. The optimal experimental conditions predicted by the model for OTP were: ethanol concentration of 54.6% v/v, amplitude of 70%, and ultrasonication time of 15 min. In the case of OML the optimal conditions were quite similar: 51.9% v/v of ethanol concentration, 70% of amplitude and 15 min. From these results it can be deduced, on the one hand, that the different proportions of leaves and wood present in OML and OTP do not noticeably affect the optimum conditions of operation of UAE. This behaviour is a positive factor for the potential use of this biomass in biorefineries, since different mixtures of OTP and OML with different content of leaves, small branches and wood could be used together as raw material. On the other hand, the optimal conditions obtained were quite similar to the optimization performed for each of the five responses separately (Sections 3.1–3.3). This fact suggests a positive correlation between the extraction of TFP and TFC and its antioxidant activity. Zekovic et al. [26] also found a good correlation between the TPC content and antioxidant activity of UAE of sage by-products.

Experiments under optimal conditions were carried out in triplicate in the ultrasonic device to validate the adequacy of the model. The predicted and the experimental values for the different responses are shown in Table 6. As can be observed, the experimental values were close to the predicted values, confirming the validity of the model to obtain the optimal UAE conditions of antioxidants from OTP and OML. The ultrasonic energy introduced in the system was evaluated according to the literature [48], leading to 180 W/L for the optimal conditions, for an amplitude percentage of 70% of the maximum ultrasonic power (280 W).

Comparing the experimental results obtained for both biomasses, the TPC and the TFC for OML were 35% and 29% higher, respectively, than the ones obtained for OTP. Regarding the antioxidant activity, it was between 35% and 44% higher for OML than for OTP, depending on the different assays. The same fact has also been reported for different extracts of leaves and bark of *Solidago Canadensis* L., which showed higher TPC, TFC and antioxidant activities in the case of foliar extracts [49]. These authors, comparing different extraction methods, found the best results for TPC (3.8 mg GAE/g) and DPPH activity (0.547 mg acid ascorbic equivalent/g) in the case of UAE of leaf extracts from *Solidago Canadensis* L. Better results are obtained in this work with UAE of OML and OTP (Table 6).

There is a huge potential of ultrasound use as a novel and non-thermal approach for extraction. Many authors reported benefits of the UAE with various raw materials [25,50,51]. In addition to laboratory-level research, different studies have also been performed at pilot plant level, as well as optimization of scale-up to semi-industrial and industrial level. For example, in a comprehensive review of UAE [50] the approaches for the extraction of olive oil in semi-industrial scale were reported. A batch reactor for treatment of 4.25 L of the sample coupled with 150 W of power and 35 kHz of frequency was employed and results were compared with existing traditional technologies. Significant reduction of processing time and yield increase was observed. In another research conducted by Achat and collaborators [52], the olive oil with oleouropein was manufactured in ultrasonic bath with 30 L of volume. The olive oil sample was treated under 25 kHz of sonication and maximal 200 W. The results obtained showed that the time of treatment was threefold shortened in comparison with traditional extraction, and also the olive oil produced with UAE showed larger radical scavenging capacity.

4. Conclusion

In this paper, ultrasound-assisted extraction was used to extract phenolic compounds from olive tree pruning biomass (OTP) and olive mill leaves (OML). The mathematical models obtained by RSM describe appropriately the relationship between the parameters studied and the different responses (TPC, TFC, DPPH, ABTS and FRAP). The results showed a positive influence of ultrasonication time and amplitude percentage for both OTP and OML in the range studied. Ethanol concentration had the greatest impact on all variables studied for both OTP and OML, followed by amplitude and ultrasonication time. The five responses optimized separately led to an optimum ethanol concentration between 54.0 and 55.8% in the case of OTP and between 50.0 and 53.9% for OML. These results agree with those obtained when all the responses were maximized simultaneously. Therefore, similar operating conditions could be used for both residual biomasses in potential industrial applications. The higher values of TPC, TFP and antioxidants activities of the extracts found in OML with respect to OTP could be attributed to the higher proportion of leaves present in OML. UAE can be used to obtain natural antioxidants from OTP and OML as a first step of the process in a biorefinery context.

Table 6
Predicted and experimental values obtained under the optimum conditions resulting from the simultaneous optimization of the five responses considered: total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activities (DPPH, ABTS and FRAP).

	TPC (mg GAE/g OTP)	TFC (mg RE/g OTP)	DPPH (mg TE/g OTP)	ABTS (mg TE/g OTP)	FRAP (mg TE/g OTP)
Olive tree pruning biomass (OTP)[a]					
Predicted value	31.8	70.7	31.1	61.8	33.6
Experimental value	31.0 ± 0.4	74.2 ± 1.5	31.6 ± 1.4	66.5 ± 2.7	36.4 ± 1.2
Error	2.6%	4.7%	1.5%	6.8%	7.7%
Olive mill leaves (OML)[b]					
Predicted value	42.6	100.4	48.8	98.7	50.1
Experimental value	42.0 ± 0.3	96.0 ± 0.6	42.5 ± 0.4	95.9 ± 1.4	49.7 ± 2.0
Error	1.4%	4.5%	14.8%	2.9%	0.8%

GAE: Gallic acid equivalents.
RE: Rutin equivalents.
TE: Trolox equivalents.
[a] [EtOH]:54.6%; Amp: 70%; t: 15 min.
[b] [EtOH]:51.9%; Amp: 70%; t: 15 min.

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