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Direct enrichment of olive oil in oleuropein by ultrasound-assisted maceration at laboratory and pilot plant scale

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ABSTRACT

The possibility to improve the nutritional value of olive oil by enriching it in phenolic compounds from olive leaves (e.g., oleuropein) by ultrasonic maceration was studied. The experimental design used led to the following optimal extraction conditions: ultrasonic power of 60 W, temperature of 16 °C and sonication duration of 45 min. The high total phenolic content (414.3 ± 3.2 mg of oleuropein equivalent/kg of oil), oleuropein (111.0 ± 2.2 mg/kg of oil) and α-tocopherol (55.0 ± 2.1 g/kg of oil) concentrations obtained by optimized ultrasound-assisted extraction (UAE) proved the efficiency of this process when compared with the conventional solid–liquid extraction. Histochemical analyses showed that this efficiency is due to specific alteration of the phenol-containing leaf structures. Furthermore, the radical-scavenging activity of the processed oil (DPPH test) and its stability toward lipid autoxidation (heating test) confirmed its enrichment in antioxidants. Sensory evaluation of the enriched olive oil showed a slight increase in bitterness but an overall acceptability. Finally, the enriched olive oil was characterized by clear green color (L^*, a^*, b^* parameters).

1. Introduction

The inhabitants of Mediterranean countries have a longer life expectancy and lower risk of chronic diseases [1]. Epidemiological studies indeed suggest that the diet and lifestyle of these populations lead to decreased rates of cancer, diabetes and cardiovascular diseases [1,2]. Olive oil is typically the main lipid source in this region, being used for cooking, cosmetics and medical purposes. The nutritional and health effects of olive oil has been mainly assigned to an adequate fatty acid profile (98% of total oil weight) and to its content in secondary metabolites (2% of total oil weight) namely phenolic compounds (simple phenols and their acylglucosides, flavonoids) [3]. There is a great interest in natural antioxidants for oil supplementation aimed at inhibiting oxidation and keeping nutritional and sensorial quality [4]. Olive leaves, which have been widely used in folk medicine in the Mediterranean regions (e.g., to fight hypertension) [5], provide a rich source of phenolic compounds (namely, oleuropein, verbascoside, apigenin-7-glucoside, luteolin-7-glucoside, etc.) [6], which may act by different mechanisms (antioxidant, signaling) to protect against free radical attacks [4]. Moreover, olive leaves are considered one of the most abundant by-products of the olive oil industry (10% of total weight of the olives) [4,5] and can be used as a cheap source of high added-value phenolic compounds. Oleuropein is present in high amounts in olive leaves (60–90 mg/g of dry matter) [7], but only in traces in olive oil (of the order of parts per billion) [6]. *In planta* they have been shown to accumulate mainly in leaf hairs and cuticle where they ensure resistance to abiotic (radiation damage) and biotic (pathogens) stresses [32,33]. This secoiridoid is generally removed from olive leaves by conventional extraction (CE), which carried-out by maceration using organic solvents such us methanol or hexane [8].

The enrichment of edible oils with polyphenols of olive leaves can be performed in three different ways: the first one is to do a solvent extraction before to add the extract in oils (liquid–liquid extraction) [10]. The other method in which the purified and dried extract is partially dissolved into the oil (solid–liquid extraction) [6]. The third one is the combination of these procedures.

The first direct enrichment of olive oil, using olive leaves by continuous extraction assisted by ultrasound, was developed in 2008 by Luque de Castro and collaborators [6]. Ultrasound-assisted extraction (UAE) is now a well established technique commonly used in laboratories. Ultrasound have been applied to improve the extraction of natural products from plant material, mainly through the phenomenon of cavitation. The mechanical effect of

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ultrasounds is believed to accelerate the release of bioactive plant components due to cell wall disruption and mass transfer intensification [9].

The present work proposes an application of Luque de Castro method [6] as a procedure easily applied in laboratories and industrial scale. The process was then changed to static or batch system which is the most used with traditional small enterprises in Mediterranean area. We apply this method of direct enrichment of olive oil with olive leaves with static system (3 L) allowing an easy scale-up in industrial implementation (30 L); second, on its possible use as a novel food rich in antioxidant compounds. For this last purpose, several tests have been performed, as the radical-scavenging activity of the processed oil (DPPH test), the stability toward lipid autoxidation (heating test), sensory evaluation of the enriched olive oil and finally color assessment. This study was also completed with histochemical analyses to show ultrasound's efficiency in alteration of the phenol-containing leaf structures.

2. Materials and methods

2.1. Samples and chemicals

Olive leaves (*Olea europaea* L.), belonging to *Oleaceae* family and Bouchouk variety, were collected from the arboretum of Institut Technique d'Arboriculture Fruitière de Takrietz, Bejaia (Algeria), in March 2010. The plant was identified on the basis of its morphological characteristics, and a specimen has been deposited in the 3bs Laboratory (University of Bejaia). The sample was cleaned with distilled water, dried in the drying oven at 40 °C until constant weight. The dried olive leaf was ground using a grinder (IKA A11 BASIC, Germany) and sieved to <1 mm particle size.

Virgin olive oil was supplied by IeS LABO, Oraison (France). Solvents and reagents were all of analytical grade. *n*-Hexane, methanol, acetonitrile and formic acid were supplied by VWR International (Darmstadt, Germany). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma–Aldrich (Steinhaus, Germany). Standards of oleuropein, tyrosol, hydroxytyrosol and α-tocopherol were from Extrasynthese (Genay, France).

2.2. Instrumentation

2.2.1. Sonication apparatus

Ultrasound-assisted extraction (UAE) experiments were performed in a PEX3 ultrasonic bath (R.E.U.S., Contes, France) composed of an inox jug having 23 × 13.7 cm internal dimensions with a maximal capacity of 3 L (25 kHz, 150 W) and equipped with a transductor having 25 cm^2 surface shown on Fig. 1. The double layered mantle allowed us to control the temperature of the medium by cooling/heating systems. The output power of the generator was 150 W while the power dissipated in the medium was about 60 W, as measured by calorimetry [11].

2.2.2. HPLC analysis

HPLC analyses were performed at 35 °C on a Waters (Milford, MA) HPLC system equipped with a model 600 pump and a model 600 gradient controller, to which were connected a model 717 autosampler and a model 996 photodiode-array detector. Samples were analyzed using 20 μL of each solution (methanolic extract). The spectroscopic detection was performed in the range 200–800 nm with a resolution of 1.2 nm. All runs were acquired and processed using the Empower 2 software (Waters, Milford, MA, USA). Analyses were performed at least three times and only mean values were reported. Quantification was carried out by using the external standard method and the final concentrations were calculated in mg/kg of oil.

Separation of phenolic compounds from enriched olive oil was carried out on a 5 μm. The analytical column was a Purospher Star C18e (250 × 4.6 mm i.d.; 5 μm) from Merck (Darmstadt, Germany). The solvent flow rate was 1 mL/min. The binary solvent system used was composed of solvent A (13.3×10^{-3} mol/L formic acid in water) and solvent B (acetonitrile). The best separation was obtained with the following. The gradient was as follows: 0–25 min, 95–65% A and 5–35% B; 30–35 min, 65–0% A and 35–100% B. The wavelength used for the quantification of the phenolic compounds with the diode detector was 280 nm. The chromatograms were collected at 280 nm. HPLC-DAD quantitation (external standard) was expressed in mg/kg of oil for all analytes.

2.2.3. UV–vis spectroscopy

UV–vis spectra were recorded on a Hewlett–Packard 8453 diode-array spectrometer equipped with a magnetically stirred quartz cell (optical length = 1 cm) thermostated by a water bath.

2.3. Extraction procedures

2.3.1. Preliminary study

A preliminary study was performed in order to determine optimal solid–liquid ratio for the rest of investigation. Amount of olive leaves subjected to enrichment of VOO varied from 5 to 30 g for 100 mL of oil under stirring during 30 min. Experiments were followed using Folin–Ciocalteu's reagent after extraction of total

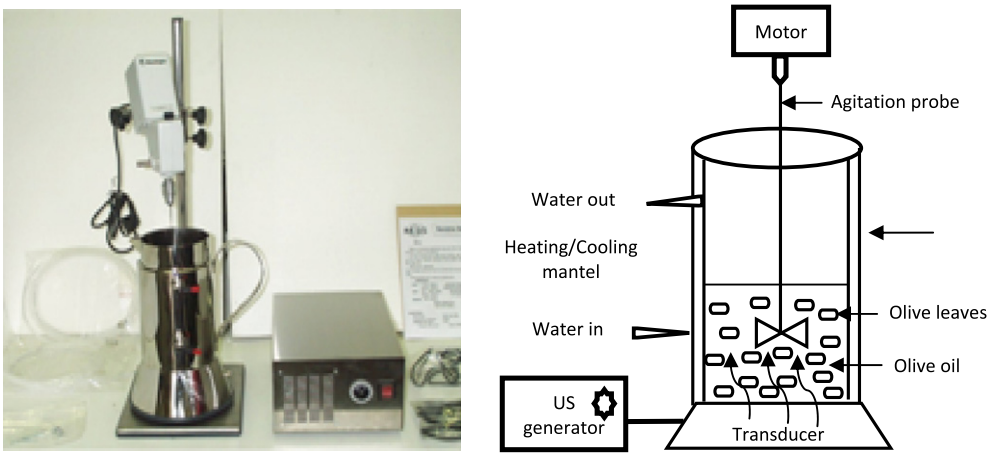

Fig. 1. Ultrasonic device (www.etsreus.com).

phenolic compounds. Results were reported as mg oleuropein equivalent per kg of oil.

2.3.2. Ultrasound assisted maceration

One hundred and fifty grams of dried and milled olive leaves were added to 1 L of olive oil as solvent into the ultrasonic device during 45 min. A rotating pale (50 mm diameter) allowed homogenization of the mixture at 225 rpm during all the experiments. Mixture was then filtered through a coffee filter in order to remove traces of leaves before analyses. Various extraction experiments were made in triplicate (except for experimental design).

2.3.3. Conventional maceration

Conventional maceration, made for comparison [6,12], was carried out in the exactly same conditions without ultrasound; 150 g of dried and milled olive leaves were added to 1 L of olive oil into the ultrasonic reactor during 45 min under stirring.

2.4. Extraction of polyphenols from olive oil

Phenolic extracts of olive oil were obtained following the procedure described by Tsimidou et al. [13]. Thus, 20 ml of olive oils were mixed with hexane (1:1, V/V) and extracted with methanol (60%, 1:1, v/v) three times. The extracts were combined, washed with hexane and then filtered through 0.45 μm pore size filter. The methanolic extracts were stored at −18 °C for subsequent analyses.

2.5. Folin–Ciocalteu assay

The Total phenolic content (TPC) was determined using Folin–Ciocalteu [14] reagent and following the procedure described by the seller of the kit [15]. In a typical procedure, 20 μL of filtered extract were mixed with 2.0 mL of chromogen reactant (bromine 0.1–0.2%, ACl 2.5–10%, H_3PO_4 2.5–10%) and 1.0 mL of buffer solution. The mixtures were allowed to stand for 30 min in the dark at room temperature. Then, their absorbance was determined using a spectrophotometer at 750 nm against a blank prepared using the same conditions, replacing extract by methanol/distilled water. TPC was then given in mg of oleuropein equivalent per 1 kg of oil sample (mg OE/kg oil).

2.6. Response surface methodology

The investigation of the performance of the UAE was obtained by the response surface methodology (RSM), using the software STATGRAPHICS PLUS (Rockville, USA, 2000). Box-Wilson design, also called central composite design (CCD), is used to evaluate the relevance and interaction of the three controlled factors namely ultrasonic power (w) (P), temperature (°C) (T), and sonication time (min) (t). The CCD application comprises a two-level full factorial design (coded ±1), superimposed by center points (coded 0) and "star points" (coded ±α) located on variables axes at a distance α from the center (Fig. 2). A CCD can be represented by a cube where each factor is corresponding to an axis. Thus, this study provides a complete exploration of the experimental domain to be studied with a number of experiments optimized. The coded levels: −α (=−1.68), −1, 0, +1, +α (=1.68) and their natural values were selected on the basis of previous experiments (preliminary study, data not shown). Variables are presented in Table 1 and involved a total of 20 experiments, including six replicates of center point, each designated by the coded value 0, were chosen in random order according to a CCD configuration for three factors, to prevent effects of extraneous variables. The selected optimization parameters were TPC (Y1), oleuropein concentration (Y2), hydroxytyrosol (Y3), tyrosol (Y4). The surface responses of data obtained were analyzed with ANOVA and presented by a Standardized Pareto Chart. The experimental and predicted values of oleuropein and TPC were compared in order to determine the validity of the model. To confirm the results, runs were carried out in triplicate under the selected optimized conditions.

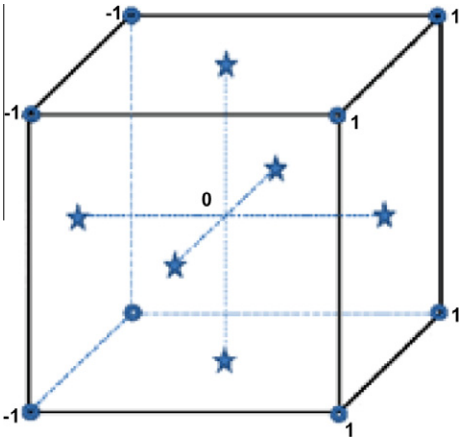

Fig. 2. Representation of CCD variables in a virtual cube.

Table 1
Sets of variables selected in central composite design.

Variables	Levels				
	−α	−1	0	1	+α
Ultrasonic power (W)	19	27	39	52	60
Temperature (°C)	16	25	38	51	60
Sonication time (min)	05	15	30	45	55

2.7. Kinetic studies

A comparative study was conducted between the conventional and ultrasound-assisted techniques after the optimization of conditions. For each enrichment of VOO, uptake of 10 mL from the mixture was performed each 10 min until 60 min, to determine the corresponding TPC values. The results obtained were analyzed with a mathematical model derived from Fick's second law [16]. The first order accumulation of TPC in solution can be expressed as follows:

$$C_t = C_\infty(1 - e^{-kt})$$

C_t, TPC at time t; C_∞, final TPC; k, apparent first-order rate constant of extraction, calculated from the linear plots of $\ln\left(\frac{C_\infty}{C_\infty - C_t}\right)$ against time.

2.8. Determination of α-tocopherol

α-Tocopherol content was evaluated according the Official method (AOCS, Ce 8-89). Two grams of each sample was dissolved in 25 mL n-hexane before injection. The separation was performed with a 5 μm nucleosil silica column (250 × 4.6 mm i.d.; 5 μm particle size from Merck), eluting with n-hexane:isopropanol 99/1 (V/V) at a flow rate 1.5 mL/min. The wavelength of 292 nm was used for determination of α-tocopherol.

2.9. Radical-scavenging test

The radical-scavenging activity of samples was evaluated by the DPPH assay. DPPH (2,2-diphenyl-1-picrylhydrazyl) is a stable

highly colored free radical that can abstract labile hydrogen atoms from phenolic antioxidants with concomitant formation of a colorless hydrazine (DPPH-H) [19,20]. The free radical-scavenging activity (RSA) of an extract can be expressed as the percentage of DPPH reduced by a given amount of extract. The free radical-scavenging activity (RSA) was measured, following the modified method of Goupy et al. [17]. Briefly, an aliquot of methanol extract from the studied oils was subjected to four serial dilutions. 1 ml of each solution was added to 2 ml of DPPH solution (2×10^{-4} mol/L in methanol) and the mixture was left in the dark at room temperature for 30 min. The absorbance was measured at 517 nm. The quantity (μg) of dry extract per mL of reaction medium necessary to decrease the initial DPPH radical concentration by 50% (EC_{50}) was determined using an exponential curve. The total RSA of each extract was expressed as the percentage of DPPH reduced and was calculated by the following equation:

$$\mathrm{RSA} = \left(\frac{A_0 - A}{A_0}\right) \times 100$$

A_0, absorbance of DPPH solution without any antioxidant; A, absorbance of DPPH solution after reaction with the extract. All experiments were performed in triplicate.

2.10. Heating conditions

Effect of heating conditions was studied by the determination of Total Polar Materails (TPM). Four hundred grams of oil (VOO, VOO-CV and VOO-US) was heated under domestic frying conditions, i.e. 180 ± 5 °C during more hours [18]. The temperature was monitored by a thermocouple (ATC-300) inserted directly into the domestic deep-fat electric fryers. All samples were evaluated before the first heating sessions and at 1 h of heating until oil discard. The end of heating assays was determined by the value of TPM, max 25% in accordance to the French law (Article3-3 of decree N°86-857 of 18/07/86). This maximal legal content of TPM in frying oils, including hydrolysis products (diglycerides, monoglycerides and free fatty acids) and a complex distribution of oxidation products encompassing polymers, all formed at temperature below 180 °C (French law N°86-857). The TPM value is usually assessed in restaurants and the agrofood industry by fast commercial tests (mostly based on colorimetric readings), which have proven to correlate well with values obtained by official standards [18].

2.11. Color assessment

The L^*, a^*, and b^* values (CIE 1976) were determined using a spectrocolorimeter (Minolta CR1000, color space CIELAB). The colorimetric coordinates of the colorant solutions (oils) were computed in the CIELAB scale. In this scale, each color is numerically specified by a unique set of three cylindrical coordinates ($L^*a^*b^*$): L^* indicates the luminance and changes from 0 for black to 100 for white, a^* changes from -60 for green to +60 for red, b^* changes from −60 for blue to +60 for yellow. The hue angle is defined as h_{ab} = arctan (b^*/a^*). h_{ab} is near 90° for yellow colors and near 180° (or −180°) for green colors [19].

2.12. Sensory evaluation

Evaluation of sensory properties of olive oils (VOO, VOO-CV and VOO-US) was performed according to the method of Lalas et al. [20] The panel was constituted by twelve tasters, selected from laboratory staff. Panelists evaluated the overall acceptability of each sample (taking into account any off-flavor and undesirable taste), using a numerical scale 1–5 (1 = not acceptable, 5 = extremely good), as well as bitterness (1 = no bitterness, 5 = extremely bitter).

2.13. Histochemistry

The efficiency of the tested extraction methods was assessed using 3 μm thick microtome sections stained with toluidine blue (TB) that is a phenol-revealing histochemical test [34]. Leaf fragments (approximately 5 mm^2) were excised from untreated leaves (control), and from leaves subjected to optimized UAE and to maceration and immediately fixed in FAA (formalin/acetic acid/alcohol, 1/1/8 : V/V/V). After 48 h fixation, samples were rinsed in distilled water, dehydrated in graded series of alcohol (70–100%), embedded in historesine and serially sectioned as previously described. [35] Sections were stained for 5 min in 0.05% TB mounted and analyzed using a light microscope equiped with a digital camera for image capture.

3. Results and discussion

3.1. Preliminary study

The effect of the variable of solid–liquid ratio on the rate of TPC was evaluated without ultrasonic assistance. The highest concentration of TPC (328 ± 2.4 mg OE/kg oil), was obtained for a 15% (w/v) of solid–liquid ratio. Thus it applied for the rest of investigation.

3.2. Optimal conditions

Twenty experimental points run randomly according to the UAE experiment planning, the triple coded values of independent variables and responses obtained in the multivariate study for each experiment are shown in Table 2. The predicted models can be described by the following second-order polynomial equations:

$$\begin{aligned} Y1 = {} & 149.043 + 1.39526P + 1.25675T + 0.545167t \\ & - 0.00607069P^2 - 0.0186007PT + 0.0122571Pt \\ & - 0.00220471T^2 - 0.00960256Tt - 0.00985282t^2 \end{aligned}$$

$$\begin{aligned} Y2 = {} & -70.4684 + 2.1032P + 2.34551T + 0.255201t \\ & - 0.00708299P^2 - 0.0297645PT + 0.0103793Pt \\ & - 0.0109315T^2 - 0.00576923Tt - 0.00552187t^2 \end{aligned}$$

$$\begin{aligned} Y3 = {} & 41.0034 + 0.131473P + 0.264437T - 0.0035804t \\ & - 0.0002238P^2 - 0.00236849PT + 0.000390968Pt \\ & - 0.00156028T^2 - 0.000641026Tt + 0.000395376t^2 \end{aligned}$$

$$\begin{aligned} Y4 = {} & 18.9167 + 0.121655P + 0.0260203T - 0.0156432t \\ & + 0.000161694P^2 - 0.00184968PT - 0.000830685Pt \\ & + 0.00045267T^2 + 0.0Tt + 0.00113739t^2 \end{aligned}$$

where $Y1$ is TPC (mg GAE/kg oil), $Y2$, $Y3$, and $Y4$ are oleuropein, tyrosol and hydroxytyrosol concentration (mg/kg oil), respectively. P is the applied power (W), t is the sonication time (min) and T (°C), the temperature in coded units.

The analysis of the variance (ANOVA) data (Table 3) for oleuropein represented on a Pareto chart (Fig.3) shows significant effects ($P < 0.05$) for all linear variables and for the PT factor. The length of the bars is proportional to the absolute magnitude of the estimated coefficients while the dashed line represents the minimum magnitude of statistically significant effects (95% of the confidence interval) with respect to the response. It can be seen

Table 2
Fully coded central composite design and responses obtained.

Run	Responses						
	Power (W)	Temperature (°C)	Time (min)	TPC (mg O/kg oil)	Oleuropein (mg O/kg oil)	Tyrosol (mg T/kg oil)	Hydroxytyrosol (mg HE/kg oil)
1	27	25	45	335.16	44.00	22	50
2	39	38	30	350.37	60.00	24	51
3	39	38	30	346.45	50.25	23	50
4	39	38	55	358.35	60.45	25	52
5	39	38	05	290.12	30.00	24	50
6	39	38	30	346.45	55.50	23	51
7	27	51	45	321.75	37.50	23	50
8	52	51	45	337.05	45.00	23	50
9	39	38	30	345.49	51.00	23	51
10	39	60	30	326.15	36.00	22	50
11	39	38	30	340.31	46.50	24	51
12	52	51	15	322.52	34.50	25	50
13	60	38	30	340.51	49.50	25	51
14	39	16	30	345.23	51.00	26	50
15	52	25	15	358.19	68.00	26	52
16	52	25	45	394.14	84.00	26	53
17	19	38	30	309.26	31.50	23	50
18	27	25	15	335.28	42.00	23	50
19	39	38	30	345.56	53.00	24	51
20	27	51	15	307.09	39.00	22	50

Table 3
ANOVA for oleuropein obtained in the CDD. The bold values show significant effects ($p<0.05$) for all linear variables (P,T,t) and for the PT factor.

Source	Sum of squares	*Df*	Mean square	*F*-ratio	*P*-value
P: power	733.446	1	733.446	33.78	**0.0021**
T: temperature	868.391	1	868.391	40.00	**0.0015**
t: time	453.816	1	453.816	20.90	**0.0060**
P^2	126.311	1	126.311	5.82	0.0607
PT	508.096	1	508.096	23.40	**0.0047**
Pt	79.609	1	79.609	3.67	0.1137
T^2	49.0309	1	49.0309	2.26	0.1932
Tt	10.125	1	10.125	0.47	0.5250
t^2	20.8163	1	20.8163	0.96	0.3725
Lack-of-fit	390.971	5	78.1942	3.60	0.0930
Pure error	108.552	5	21.7104		
Total (corr.)	3274.78	19			

$R^2 = 84.75\%$, R^2 (adjusted for *Df*) = 71.02%.

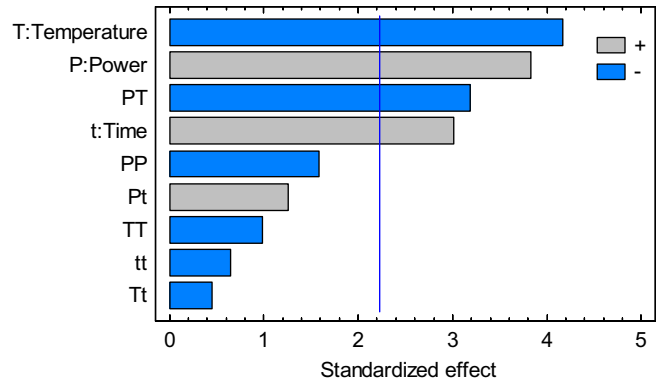

Fig. 3. Standardized Pareto chart for oleuropein (mg O/kg oil).

that temperature has the most important influence on oleuropein contents, followed by ultrasound power, sonication time and interaction of power and temperature, whereas the cross product terms (P^2, Pt, T^2, Tt, t^2) show no significant effects. The lack of significance of these terms suggests the absence of interactions between variables in the studied zone.

The lack of fit test (Table 3) was designed to determine whether the selected model is adequate to describe the observed data. Since the *P*-value for lack-of-fit in the ANOVA table is greater than 0.05, the model appears to be adequate for our experimental results at 95% confidence level.

The optimal conditions were obtained from the first derivatives of the second-order polynomial equations. The second derivatives were then equaled to 0 and solved in an equation system. The coded values obtained from these equations were thus decoded and rounded in order to be applied to the device. The obtained values corresponding to optimal conditions were as follows: $Y1$ = 60 W, 16 °C, 45 min; $Y2$ = 60 W, 16 °C, 45 min; $Y3$ = 60 W, 16 °C, 17 min; $Y4$ = 60 W, 18 °C, 55 min. As expected and according to the response surfaces (Fig.4), the extraction efficiency in terms of TPC, oleuropein, hydroxytyrosol and tyrosol concentrations increases by increasing P and t and by decreasing T and PT. Thus, the values finally selected correspond to the maximal values chosen to define the experimental domain. [21] On the basis of our principle responses ($Y1$ and $Y2$), the optimal settings were checked as follows: ultrasonic power of 60 W, temperature of 16 °C and sonication duration of 45 min. A repeatability study was conducted by using these optimal conditions to assess the predictive ability of the models. Under the optimized conditions, the experimental values for oleuropein (111.0 ± 2.2 mg/kg oil) and TPC (414.3 ± 3.2 mg OE/kg oil) were very close to the predicted values (409.5 mg OE/kg oil; 104.4 mg/kg oil) respectively. This implied that there was a high degree of correlation between observed and predicted data from the regression model ($R^2 > 84.75$, $R^2_{adj} > 71.02$). Hence, the response surface modeling could be applied effectively to predict enrichment of olive oil with oleuropein from olive leaves.

3.3. Comparison of UAE and CE methods

A comparison was carried out between ultrasound-assisted and conventional enrichments of virgin olive oil with phenolic compounds from olive leaves. TPC values of VOO-US (60 W at 16 °C for 45 min under stirring) and of VOO-CV (idem except sonication) is shown on Fig. 5. UAE showed a very significant increase in TPC from 342.5 ± 1.5 to 414.2 ± 3.2 mg/kg oil for conventional and UAE, respectively. With reference to the original TPC value in VOO (*ca.* 282.3 ± 1.7 mg/kg oil), the gain in TPC is more than twice higher by UAE than by CE. The kinetics of VOO

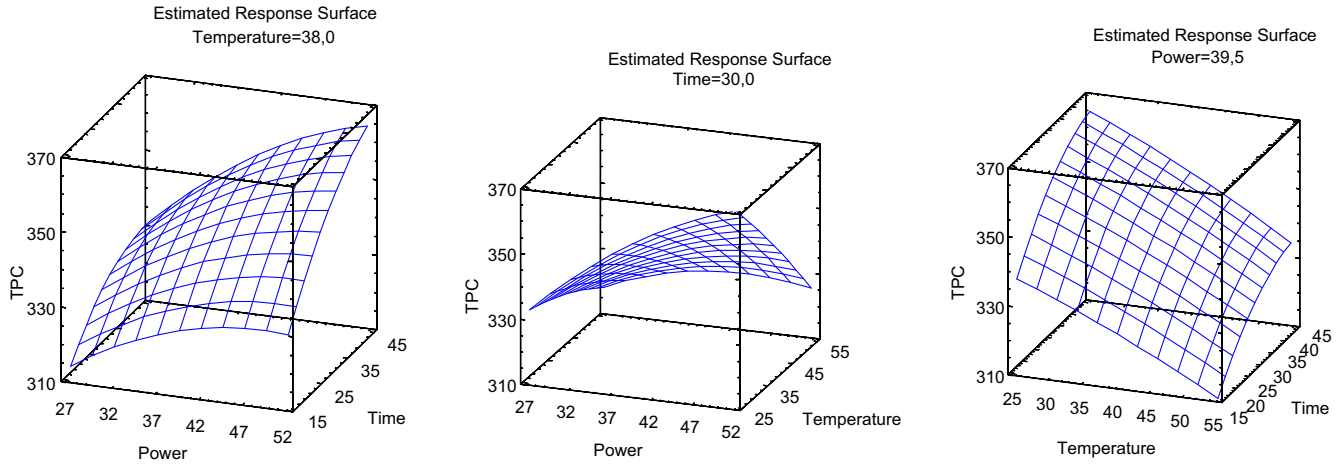

Fig. 4. Optimization parameters by response methodology: (a) estimated oleuropein, power, temperature response surface, (b) estimated oleuropein, temperature, extraction time response surface and (c) estimated oleuropein, power, extraction time response.

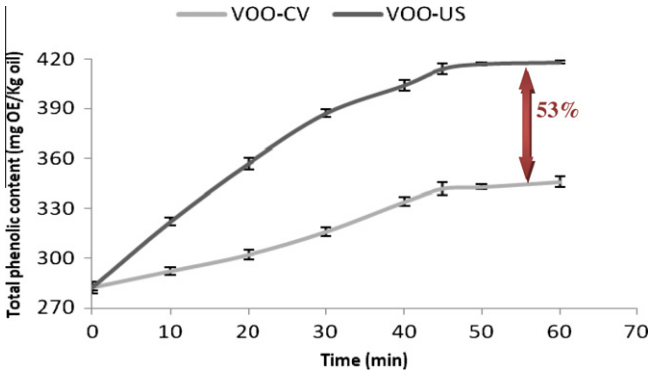

Fig. 5. Comparison of TPC from virgin olive oil ultrasound assisted enrichment (VOO-US) and conventional method (VOO-CV).

enrichment was clearly improved, as the extraction of total phenols was found three times as fast under ultrasounds (k = 0.26 min^{-1}) as in the conventional method (k = 0.08 min^{-1}), which could be attributed to ultrasonic cavitation. Similar kinetic effects were evidenced by Chemat et al. [22] for UAE of carvone and limonene from caraway seeds in hexane and by Virot et al. [23] and Khan et al. [12] for UAE of polyphenols from apple pomace and orange peel, respectively.

Characterization of oleuropein, hydroxytyrosol and tyrosol was achieved by comparing their HPLC retention times and UV spectra with standards. Whereas oleuropein is undetectable in VOO, this secoiridoid was present in VOO-US where its concentration was twice higher than in VOO-CV (Table 4). Representative HPLC chromatograms from olive oil before and after its supplementation are displayed in Fig. 6.

The phenols originally present in virgin olive oil, hydroxytyrosol and tyrosol [24], are not degraded under sonication. Thus, their concentrations were not decreased (Table 4, Fig. 6). Indeed, the

Table 4
Characterization of VOO, VOO-CV and VOO-US.

	VOO	VOO-CV	VOO-US
TPC (mg O/kg oil)	282.0 ± 1.7	342.5 ± 1.5	414.3 ± 3.2
Phenols (mg/kg oil)			
Oleuropein	ND	50.7 ± 1.7	111.0 ± 2.2
Tyrosol	31.7 ± 0.3	29.1 ± 0.2	25.5 ± 0.1
Hydroxytyrosol	58.7 ± 0.2	55.7 ± 0.7	52.4 ± 0.5
Antioxidant capacity			
RSA (%)	52.9 ± 0.9	65.0 ± 0.8	86.2 ± 0.2
EC_{50} (μg/mL)	109.4 ± 0.09	97.43 ± 0.04	81.34 ± 0.01
ARP ($1/EC_{50}$)	09.1 ± 0.4	10.3 ± 0.5	12.3 ± 0.3
α-Tocopherol content (g/kg of oil)	43.0 ± 0.2	46.5 ± 1.2	55.0 ± 2.1
TPM (%)			
Before frying	4.25 ± 0.28	4.16 ± 0.20	4.08 ± 0.08
Frying time for 25% TPM (h)	6.9 ± 0.1	10.1 ± 0.1	12.6 ± 0.1
Sensory analysis			
Overall acceptability	4	3	3
Bitterness	1	2	4
Lab test			
L^*	58.8 ± 0.2	51.6 ± 0.1	37.1 ± 0.1
h_{ab}	89.9°	137.0°	148.4°

Values are the mean ± standard deviation (n = 3).
VOO, virgin olive oil; VOO-CV, virgin olive oil enriched with conventional method; VOO-US, virgin olive oil ultrasound assisted enrichment.

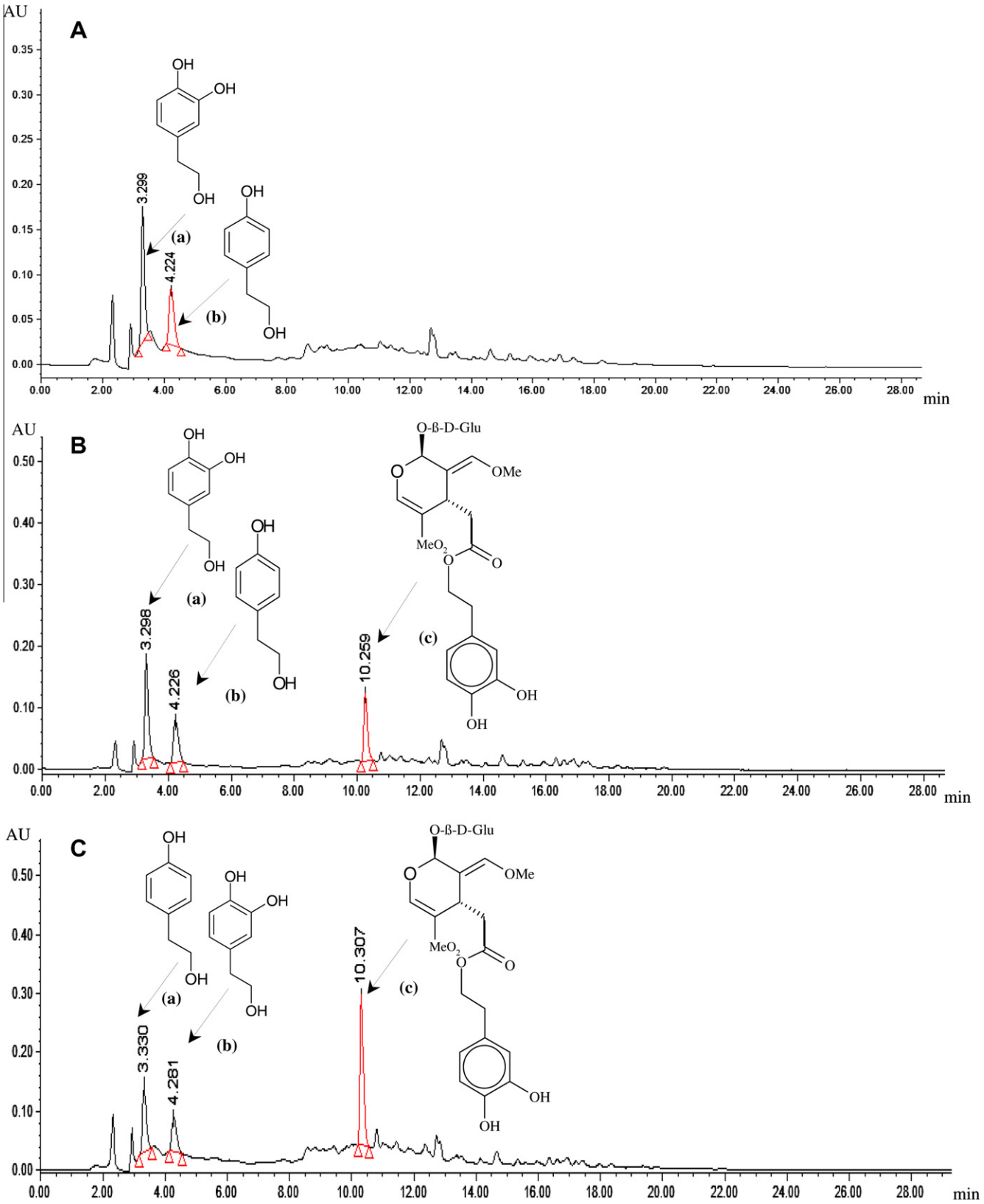

Fig. 6. HPLC-DAD chromatograms at 280 nm of methanolic extracts of VOO (A), VOO-CV (B) and VOO-US (C). Peak identification: (a) hydroxytyrosol, (b) tyrosol, (c) oleuropein.

ultrasonic degradation of phenols is typically slow in comparison with more volatile aromatics that diffuse more readily into the cavitation bubble for pyrolysis [25]. In addition, phenol degradation is favored at higher frequencies (required for the generation of the hydroxyl radical by water homolysis) than the one selected in this work (25 kHz).

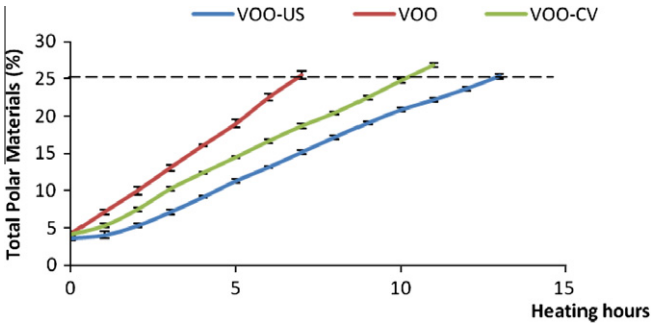

Fig. 7. The total polar materials during the heating sessions (180 °C) of different virgin olive oils.

The results obtained by the proposed method were different with the information reported by the study of Luque de Castro and collaborators [6], that have demonstrated the enrichment of olive oil with biophenols of olive leaves (oleuropein, verbascoside, apigenin-7-glucoside and luteolin-7-glucoside), using a dynamic ultrasound approach. Several reasons can explain this fact: technique of enrichment, solid–liquid ratio, temperature, sonication time and variety of olive leaves. These parameters supposed have an important effect on the enrichment of olive oil.

3.4. Histochemistry

Microscopic observation of cross sections from control samples showed that even dried the olive leaf conserved its well-known drought-adapted anatomy with the presence of a thick adaxial cuticle and multicelled, umbrella-like hairs (Fig. 9a). When treated with TB these two structures stained blue-green indicating the presence of phenolic substances [34]. Compared to control the hairs of leaf fragments that have been subjected to conventional method exhibited reduced TB-positive material particularly in inner cell walls (Fig. 9b). However, with this treatment, the cuticle appeared to retain a blue-green staining similar to control. Sections from UAE extracted samples displayed a noticeable decrease in the TB-positive substances both in hairs and cuticle (Fig. 9c) suggesting an efficient extraction of phenolic substances *via* this procedure. This was presumably due to the well known, ultrasound-mediated phenomenon of cavitation believed to improve the extraction of plant metabolites using UAE technologies.

3.5. Antioxidant capacity

Several authors have determined the RSA of oils by measuring the consumption of the DPPH radical at 517 nm [5,26]. In this work, all oils were shown to exhibit RSA to a greater or lesser extent. However, the methanolic extract of VOO-US displayed the highest scavenging efficiency. EC_{50} is a parameter widely used to evaluate the antioxidant or radical-scavenging capacity. The lower EC_{50}, the higher the antioxidant capacity. The antiradical power can also be defined as $ARP = 1/EC_{50}$. The higher the ARP, the higher the antioxidant activity. Based on the data in Table 3, VOO-US appeared to be more potent at scavenging the DPPH radical than VOO-CV and VOO in agreement with its higher concentration of phenolic antioxidants (mainly oleuropein), which confirmed the

Fig. 8. Pilot plant ultrasonic device (30 L) used for ultrasound-assisted enrichment.

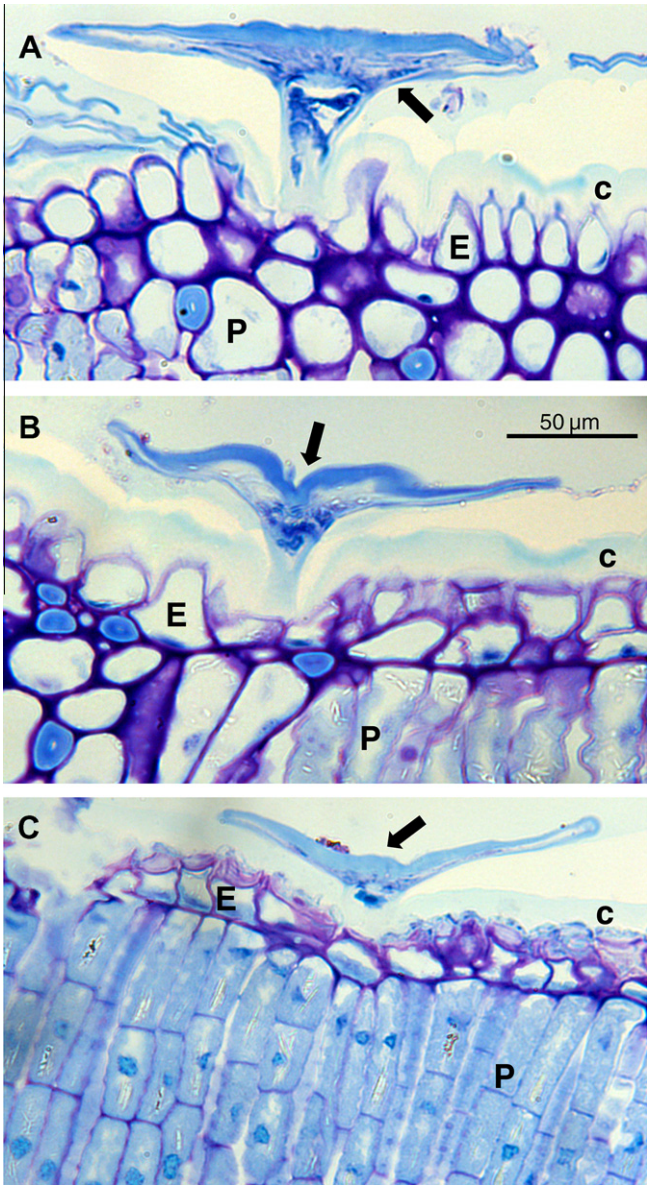

Fig. 9. Structure of olive leaves after different treatments. (A) Control, (B) maceration and (C) sonication.

usual correlation between antioxidant activity and TPC [27]. Indeed, phenolic antioxidants are typically able to quickly reduce reactive oxygen species (ROS) including free radicals, thereby protecting biomolecules (e.g. polyunsaturated fatty acids) against oxidation [28]. Oleuropein was already shown to exert a potent antioxidant activity [29], which could contribute to the nutritional properties of olive oil.

3.6. α-Tocopherol content

In VOO, α-tocopherol dominates with its concentration reaching 95% of the total tocopherol content [30]. Tocopherols exert both vitaminic and antioxidant properties [31]. Their presence in oil is also crucial for the stability of the autoxidation-sensitive polyunsaturated lipids [18]. The α-tocopherol content in VOO is 43.0 ± 0.2 g/kg and the oil supplementation with olive leaves by UAE led to a higher α-tocopherol concentration (55.0 ± 2.1 g/kg) than by CE (46.5 ± 1.2 g/kg). Indeed, olive leaves are rich in tocopherols [2] and oil may be regarded as a suitable green solvent for their extraction.

3.7. Oil enrichment and heating test

All olive oils displayed close TPM values before heating (Table 4, Fig. 7) but total *heating* times required to reach the maximal TPM value of 25% were quite different: 12.6 ± 0.1 h for VOO-US, 10.1 ± 0.1 h for VOO-CV and 6.9 ± 0.1 h for VOO. Hence, the efficient ultrasound-assisted supplementation of VOO with TPC must significantly prolong the shelf-life of olive oil. The increased concentrations of oleuropein (with its H-donating hydroxytyrosol moiety) and, to a lesser extent, α-tocopherol (the potent chain-breaking antioxidant) must confer a higher antioxidant protection to olive oil primarily responsible for this effect.

3.8. Color assessment

The color of oils was determined according the $L^*a^*b^*$ space, which models the human eye in its appreciation of luminance and chrominance. [23] As shown in Table 3, the luminance (L^*) of samples increased in the series VOO > VOO-CV > VOO-US whereas the reverse order was observed for the hue angle (h_{ab}): VOO-US > VOO-CV > VOO. Thus, the colors expressed by VOO, VOO-CV and VOO-US were yellow, yellow-green and clear green, respectively. This difference can be attributed to enrichment of VOO with chlorophyll.

3.9. Sensory evaluation

Sensory characteristics of oils indicated that the overall acceptability was equally good for all samples, although the bitterness was increased in VOO-CV and VOO-US. This bitter taste is related to oleuropein [3], the concentration of which increased during CE and UAE (Table 3). However, further evaluation of these oils by trained panels is needed in the perspective of product development.

3.10. Extraction pilot study, industrial trials

To scale up laboratory experiments, a larger reactor of 30-l extraction tank has been also used in this study. This reactor consist of a quadriple output of ultrasound at 25 kHz and 4 × 200 W. Pump systems are coupled to the ultrasonic bath in order to fill it, to stir the mixture and to empty the system at the end of the experiment (Fig. 8). From the previous lab study, the selected conditions for the ultrasound extraction pilot study were at the optimum conditions. Although, the yield of polyphenols from the ultrasound extraction pilot scale was equal to laboratory scale UAE and 53% higher than the conventional maceration. The result showed that the potential use of ultrasound extraction was promising for extraction on an industrial scale. UAE could shorten the extraction time, enhancing final yield, and lower the operating temperature, which resulted in considerably lower operating costs. UAE could also be used to produce larger quantities of natural antioxidants by using existing big scale ultrasound extraction reactors suitable for enrichment of edible oils with volumes of 100 to 1000 liters. These ultrasound reactors are suitable for the extraction of 15 or 150 kg of dry plant material per time.

4. Conclusion

The ultrasound-assisted process greatly facilitates the enrichment of VOO in phenolic compounds compared to conventional maceration. Tyrosol and hydroxytyrosol were not significantly degraded by sonication. Olive oil enriched in phenolic compounds

by UAE displayed a higher radical-scavenging capacity and an improved stability to thermal degradation (DPPH and heating tests) that may be attributed to higher oleuropein and α-tocopherol concentrations. In conclusion, this work suggests that ultrasound-assisted extraction of phenolic compounds from olive leaves may be a viable process to enhance the stability and nutritional quality of olive oil with little impact on the sensorial qualities.

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