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Additional Information

1 **Kinetic and compositional study of phenolic extraction from olive leaves**  
2 **(var. Serrana) by using power ultrasound**

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27 **Abstract**

28 Power ultrasound is being used as a novel technique for process  
29 intensification. In this study, the feasibility of using power ultrasound to improve  
30 the phenolic extraction from olive leaves was approached taking both  
31 compositional and kinetic issues into account and also determining the  
32 influence of the main process parameters (the electric power supplied, emitter  
33 surface and temperature). For this purpose, the extraction kinetics were  
34 monitored by measuring the total phenolic content and antioxidant capacity and  
35 mathematically described by Naik's model, and HPLC-DAD/MS-MS was used  
36 to identify and quantify the main polyphenols. The electric power supplied and  
37 the emitter surface greatly affected the effective ultrasonic power applied to the  
38 medium, and hence the extraction rate. However, the influence of temperature  
39 on ultrasound assisted extraction was not clear. Compared with conventional  
40 extraction, ultrasound assisted extraction reduced the extraction time from 24 h  
41 to 15 min and did not modify the extract composition.

42

43 *Key words:* Olive leaves, Byproducts; Antioxidant capacity; Polyphenols;  
44 Ultrasonics

45

## 46 **1. Introduction**

47 Olive (*Olea europaea* L.) is one of the most important crops in the  
48 Mediterranean countries, one which has traditionally played an important role in  
49 human diet because of the high nutritional value of olive oil (Ryan et al., 2001).  
50 Olive fruit is rich in phenolic compounds with bioactive properties providing,  
51 among other things, antiviral, antitumoral and antioxidant activity (Della Ragione  
52 et al., 2000; Liu et al., 2003). Nowadays, the harvesting of olive fruit and the  
53 pruning of olive trees generate an important number of byproducts, such as  
54 branches and leaves, both mainly used as animal feed or to be removed by  
55 burning. However, bioactive compounds have been found in these byproducts  
56 (Japón-Luján & Luque de Castro, 2007) which exhibit similar antioxidant  
57 potential to those found in olive fruit (Malik & Bradford, 2006). Therefore, the  
58 extraction of phenolic compounds could represent an interesting means of  
59 increasing the value of these byproducts (Guinda et al., 2004; Tabera et al.,  
60 2004).

61 The conventional extraction of bioactive compounds from plants or seeds  
62 has been carried out by maceration using liquid solvents, which is considered a  
63 slow process requiring long extraction times. The extraction rate may be  
64 improved by choosing the best combination of process variables, such as the  
65 type of solvent or level of agitation (Rodríguez-Bernaldo de Quirós et al., 2010).  
66 Using high temperatures does lead to a kinetic improvement, but it is limited by  
67 the fact that polyphenols are sensitive to high temperatures. Thus, although  
68 heat treatments can improve extraction kinetics, they reduce both the phenolic  
69 content and antioxidant capacity. Recent studies into future industrial  
70 applications have addressed some alternatives to conventional extraction, such

71 as supercritical extraction with CO<sub>2</sub> (Bensebia et al., 2009), ultrasound assisted  
72 (Knorr et al., 2004; Zhang et al., 2009), microwave-assisted (Hayat et al., 2009)  
73 or superheated liquid extraction (Japón-Luján & Luque de Castro, 2006).

74         Ultrasound assisted extraction is considered one of the most interesting  
75 techniques by which to intensify the extraction of valuable compounds from  
76 vegetal materials (Vilkhu et al., 2008). This is due to the fact that it is not only a  
77 simple, efficient and inexpensive alternative to conventional extraction  
78 procedures (Huang et al., 2009), but it also induces mechanical effects in the  
79 medium being applied. In liquids, ultrasound enhances mass transfer mainly by  
80 inducing cavitation. The implosion of gas bubbles in liquid generates high  
81 localized pressures and micro-streaming, causing plant tissue disruption and  
82 improving the release of intracellular substances into the solvent (Knorr et al.,  
83 2002). Ultrasound also produces other effects coupled to cavitation, like  
84 interfacial instabilities and successive compressions and expansions that can  
85 influence both external and internal mass transfer. Two common ultrasonic  
86 devices are employed in solid/liquid extraction, namely baths and probe-type  
87 systems. Although ultrasound baths are more widely used, probe-type systems  
88 offer the advantage of providing more intense and localized ultrasonic  
89 application, which heightens the effects in solid-liquid systems (Priego-Capote &  
90 Luque de Castro, 2004). In addition, probes allow a wider choice of process  
91 parameters than ultrasonic baths, which is highly interesting for research  
92 purposes. The effectiveness of ultrasound application is directly related to the  
93 ability of the ultrasonic probe to introduce energy into the solvent medium. This  
94 fact mainly depends on how well the emitter surface fits the solvent medium and  
95 product being treated, which is extremely complicated to predict and, therefore,

96 should be determined in each specific application. Other process parameters,  
97 such as electric amplitude supplied to the ultrasonic transducer, sonication time,  
98 temperature, solvent composition (Herrera & Luque de Castro, 2005) or number  
99 of extraction steps (Jerman et al., 2010) could also affect the ultrasound  
100 assisted extraction process. Ultrasound assisted extraction from olive leaves  
101 has previously been reported by Japón-Luján et al. (2006) and Sánchez-Ávila et  
102 al. (2007), who for analytical purposes studied, optimized and characterized the  
103 extract composition using different process parameters (Esclápez et al., 2011).  
104 However, the compositional study should be accompanied by a thorough  
105 analysis of the kinetics taking into account the effective power applied to the  
106 medium, a fact which is not included in previous research and which is highly  
107 relevant for industrial applications. Thereby, the aim of this work was to address  
108 the power ultrasound assisted extraction of olive leaf bioactive compounds by  
109 evaluating the influence of some process parameters (the electric amplitude,  
110 the emitter surface and temperature) on both the extraction kinetics and the  
111 extract composition.

112

## 113 **2. Materials and methods**

### 114 *2.1. Raw material*

115 Olive leaves (*Olea europaea*, var. Serrana) were collected on a farm  
116 located in Segorbe (Castellón, Spain) in February (approximately 2 months after  
117 the fruit harvest), packaged, stored at 4 °C and processed in less than 48 hours.  
118 The initial moisture content was determined by drying until constant weight in a  
119 vacuum chamber at 70 °C (AOAC, 1997).

120

121

## 122 *2.2. Drying experiments*

123 The olive leaves, with an initial moisture content of  $39.2 \pm 0.9$  % (kg  
124 water/kg total), were dried at 120 °C in a forced air laboratory drier (FD, Binder,  
125 Tuttlingen, Germany) according to Ahmad-Qasem et al. (2012). Samples were  
126 dried until constant weight, which corresponded to a loss of  $40 \pm 1$  % of the  
127 initial weight. After drying, the olive leaves were stored at 4 °C until subjected to  
128 extraction.

129

## 130 *2.3. Extraction experiments*

### 131 *2.3.1. Olive leaf sample preparation*

132 In order to perform the extraction experiments, dried olive leaves were  
133 milled (Blixer 2, Robot Coupe USA, Inc., Jackson, MS, USA). The obtained  
134 powder was sieved (Metallic mesh 0.05 mm, Filtra Vibración, Barcelona, Spain)  
135 to select particles with a diameter of less than 0.05 mm and a density of 426.2  
136 kg/m<sup>3</sup>. Thus, using this small particle diameter, it was possible to increase the  
137 active surface area of the olive leaf sample.

138

### 139 *2.3.2. Extraction solution and extract preparation*

140 The solvent (extracting medium) used was an 80:20 (v/v) ethanol-water  
141 solution. The extracts obtained were centrifuged for 10 min at 5000 rpm  
142 (Medifriger BL-S, J.P. Selecta, Barcelona, Spain), filtered (nylon filters of 0.45  
143 µm) and stored in opaque vials at 4 °C until analyzed. The extraction kinetic  
144 was monitored in both ultrasound assisted extraction experiments as well as in

145 conventional solid-liquid maceration. Both extraction methods are described in  
146 the following sections.

147

### 148 *2.3.3. Ultrasound assisted extraction (USAE)*

#### 149 *2.3.3.1 Experimental set-up and characterization of ultrasonic field*

150 The experimental set-up used to carry out the ultrasonic assisted  
151 extraction experiments is shown in Fig. 1. During the experiments, the  
152 temperature was held constant and measured with a Pt100 sensor located in  
153 the centre of the extraction vessel and wired to a process controller (E5CK,  
154 Omron, Hoofddorp, Netherlands). A peristaltic pump (302 S, Watson-Marlow,  
155 Postfach, Germany), driven by the controller, recirculated a glycol solution (10  
156 % glycol) at -10 °C from the cooling reservoir, equipped with a chiller (Frigedor,  
157 J.P. Selecta, Barcelona, Spain), through a jacketed extraction vessel.  
158 Ultrasound was continuously applied (cycle 100 %) using a probe system  
159 (UP400S, Dr. Hielscher, Teltow, Germany), which allows the tip probe to be  
160 changed, thus being able to test different emitter surfaces. The ultrasonic  
161 emitter was immersed 1 cm into the solution. In order both to avoid the negative  
162 effect of light on phenolic compounds and to preserve the original composition  
163 of extracts, the extraction vessel was protected from light in every experiment.

164 A calorimetric procedure was used to determine the effective ultrasonic  
165 power transferred into the medium for every condition tested (Raso et al.,  
166 1999). For this purpose, the temperature of the solvent was logged every 3 s for  
167 the first 3 min of ultrasound application without controlling the temperature.  
168 Thus, using the temperature rise caused by cavitation, the ultrasonic power  
169 applied (P, W) was calculated as:



170  $P = (M \cdot C_p) \cdot (dT/dt)$  (1)

171 where  $M$  (kg) is the solvent mass,  $C_p$  (J/kg °C) the heat capacity and  $dT/dt$  the  
172 slope of the logged temperature-time curve. The ultrasonic power was  
173 measured, at least in triplicate, for every condition tested.

174

### 175 *2.3.3.2 Parametric study*

176 A parametric study was performed in order to identify the influence of  
177 process variables in the ultrasonic assisted extraction. The parameters taken  
178 into account were the electric power supplied to the ultrasonic transducer, the  
179 emitter surface and the extraction temperature. The first two parameters affect  
180 the ultrasonic intensity applied to the medium that could produce a different  
181 extension of ultrasound effects, while the extraction temperature could have an  
182 effect on both the extraction kinetic and final yield.

183 A first set of experiments was carried out supplying different levels of  
184 electric power to the transducer (40, 60, 80 and 100 % of the total power of the  
185 system, 400 W) using an emitter surface of 12.6 cm<sup>2</sup>. Afterwards, using the  
186 electric power which provided the extracts with the highest antioxidant capacity,  
187 the influence of the emitter surface (12.6, 3.8 and 1.5 cm<sup>2</sup>) on the extraction  
188 yield was evaluated in a second set of experiments. Both extraction tests were  
189 carried out at 25 °C for 15 min. Finally, a third set of experiments was carried  
190 out for 15 min at 6 different extraction temperatures (25, 30, 35, 40, 45 and 50  
191 °C). In this case, the electric power supplied and the emitter surface were fixed  
192 by the first two experiments.

193 Each extraction experiment was carried out using a ratio of olive leaf  
194 mass to solvent volume of 6.25 g/200 mL (0.031 g/mL). In order to determine

195 the extraction kinetics, the samples were taken (2 mL) at preset times (0, 3, 6,  
196 9, 12 and 15 min) replacing the extract volume with new solvent. At least 3  
197 replicates were made for each extraction condition tested.

198

#### 199 *2.3.4. Conventional extraction*

200 In order to determine conventional extraction kinetics, experiments were  
201 carried out without (static extraction, ST) and with agitation (CVE) at 170 rpm in  
202 a thermostatic shaking water bath (Stuart, Staffordshire, UK). From previous  
203 experiments, it was stated that this level of agitation was enough to maintain a  
204 high degree of turbulence in the medium. The same ratio between olive leaf  
205 mass and solvent volume (0.031 g/mL) was used as in section 2.3.3.2. In  
206 addition, kinetics were also monitored by taking samples (2 mL) at preset times  
207 (0, 3, 6, 9, 12 and 15 min) and replacing the extract volume with new solvent.

208 Moreover, additional conventional extraction experiments were carried  
209 out using the ratio of olive leaf mass to solvent volume (0.125 g/mL) proposed  
210 as optimum by other authors (Japón-Luján & Luque de Castro, 2006; Sánchez-  
211 Ávila et al., 2009). These experiments were prolonged until equilibrium was  
212 reached, which needed nearly 24 hours. During extraction, the samples were  
213 also stirred at 170 rpm using the thermostatic shaking water bath. In this case,  
214 the extraction kinetic was not evaluated and only the final extract (24 hours)  
215 was analyzed.

216 Every conventional extraction test was carried out at  $25 \pm 1$  °C in sealed  
217 containers protected from light. At least, 3 extraction replicates were made for  
218 each extraction condition.

219

## 220 2.4 Quality evaluation of olive leaf extracts

### 221 2.4.1 Total phenolic content (TPC)

222 The TPC was determined by the Folin-Ciocalteu method (Singleton et al.,  
223 1999). Briefly, 100  $\mu$ L of sample were mixed with 200  $\mu$ L of Folin-Ciocalteu's  
224 phenol reagent (Sigma-Aldrich, Madrid, Spain) and 2 mL of distilled water. After  
225 3 min at 25 °C, 1 mL of Na<sub>2</sub>CO<sub>3</sub> (Panreac, Barcelona, Spain) solution (Na<sub>2</sub>CO<sub>3</sub>-  
226 water 20:80, p/v) was added to the mixture. The reaction was kept in dark at  
227 room temperature for 1 h. Finally, absorbance was read at 765 nm using a  
228 spectrophotometer (Helios Gamma, Thermo Spectronic, Cambridge, UK).  
229 Measurements were taken at least in triplicate. A standard curve of gallic acid  
230 (Sigma-Aldrich, Madrid, Spain) was previously prepared using solutions of a  
231 known concentration in ethanol-water (80:20, v/v) solution. Results were  
232 expressed as mg gallic acid (GAE)/g of dry weight of olive leaves.

233

### 234 2.4.2. Antioxidant capacity (AC)

235 The AC was determined by the Ferric-reducing ability power method  
236 (FRAP) in order to monitor the extraction kinetics. Moreover, the Trolox  
237 equivalent antioxidant capacity (TEAC) method was also used to compare the  
238 quality of USAE and CVE extracts.

239

#### 240 2.4.2.1. Ferric-reducing ability power (FRAP)

241 The FRAP method was applied following the procedure described by  
242 Benzie & Strain (1996), with some modifications. Briefly, 900  $\mu$ L of FRAP  
243 reagent were used; this had been freshly prepared and heated to 37 °C and  
244 mixed with 30  $\mu$ L of distilled water and 30  $\mu$ L of test sample or ethanol-water

245 (80:20, v/v) used as an appropriate reagent blank. The FRAP reagent contained  
246 2.5 mL of a 10 mM TPTZ (Fluka, Steinheim, Germany) solution in 40 mM HCl  
247 (Panreac, Barcelona, Spain) plus 2.5 mL of 20 mM FeCl<sub>3</sub>•6H<sub>2</sub>O (Panreac,  
248 Barcelona, Spain) and 2.5 mL of 0.3 M acetate buffer (Panreac, Barcelona,  
249 Spain), pH 3.6 (Pulido et al., 2000). Readings at the maximum absorption level  
250 (595 nm) were taken using a spectrophotometer (Helios Gamma, Thermo  
251 Spectronic, Cambridge, UK). At least 4 replicates were made for each  
252 measurement. The AC was evaluated through a calibration curve that had been  
253 previously determined using the extracting solvent (ethanol-water 80:20, v/v) of  
254 a known Trolox (Sigma-Aldrich, Madrid, Spain) concentration and expressed as  
255 mg Trolox/g dry matter.

256

#### 257 *2.4.2.2. Trolox equivalent antioxidant capacity (TEAC)*

258 The TEAC method was performed as previously described by Laporta et  
259 al. (2007). Briefly, an ABTS radical cation (ABTS<sup>•+</sup>) was produced by reacting  
260 ABTS (Sigma-Aldrich, Europe) stock solution with 2.45 mM potassium  
261 persulfate (final concentration) and keeping the mixture in the dark at room  
262 temperature for 12-24 h before use. The ABTS<sup>•+</sup> solution was diluted with  
263 distilled water until an absorbance value of 0.714 ± 0.02 at 734 nm was  
264 reached. For the photometric assay, an absorbance of 200 µL of the ABTS<sup>•+</sup>  
265 solution, or blank, was measured in a spectrophotometer (Spectrostar Omega,  
266 BMG Labtech, Offenburg, Germany). Then 20 µL of antioxidant extract, or  
267 blank, were added and, after 29 min, the final absorbance was measured at 734  
268 nm (Spectrostar Omega, BMG Labtech, Offenburg, Germany). The AC was  
269 determined from the difference between the initial and final absorbance and the

270 calibration curve of Trolox (Sigma-Aldrich, Madrid, Spain). At least 3 replicates  
271 were made for each extract. The AC results were expressed as mg Trolox/g dry  
272 matter.

273

#### 274 *2.4.3 Identification and quantification of polyphenols by HPLC-DAD/MS-MS*

275 In order to identify and quantify the main polyphenols present in the  
276 USAE and CVE extracts, these were analyzed using a HPLC instrument  
277 (Agilent LC 1100 series; Agilent Technologies, Inc., Palo Alto, CA, USA)  
278 controlled by the Chemstation software. The HPLC instrument was coupled to  
279 an Esquire 3000+ (Bruker Daltonics, GmbH, Bremen, Germany) mass  
280 spectrometer equipped with an ESI source and ion-trap mass analyzer, and  
281 controlled by Esquire control and data analysis software. A Merck Lichrospher  
282 100RP-18 (5  $\mu$ m, 250 x 4 mm) column was used for analytical purposes.

283 Separation was carried out through a linear gradient method using 2.5 %  
284 acetic acid (A) and acetonitrile (B), starting the sequence with 10 % B and  
285 programming gradient to obtain 20 % B at 10 min, 40 % B at 35 min, 100 % B at  
286 40 min, 100 % B at 45 min, 10 % B at 46 min and 10 % B at 50 min. In order to  
287 ensure the LC-MS pump performed accurately, 10% of organic solvent was  
288 premixed in the water phase. The flow-rate was 1 mL/min and the  
289 chromatograms were monitored at 240, 280 and 330 nm. The mass  
290 spectrometry operating conditions were optimized in order to achieve maximum  
291 sensitivity values. The ESI source was operated in negative mode to generate  
292  $[M-H]^-$  ions under the following conditions: a desolvation temperature of 365 °C  
293 and a vaporizer temperature of 400 °C; dry gas (nitrogen) and nebulizer were  
294 set at 12 L/min and 70 psi, respectively. The MS data were acquired as full scan

295 mass spectra at 50–1100 m/z by using 200 ms for the collection of the ions in  
296 the trap.

297 The main compounds were identified by means of a HPLC-DAD analysis,  
298 comparing the retention time, UV spectra and MS/MS data of the peaks in the  
299 samples with those of authentic standards or data reported in literature.

300 Only the main olive leaf polyphenols were quantified using commercial  
301 standards: oleuropein (Extrasynthese, Genay Cedex, France) and luteolin-7-O-  
302 glucosyde (Phytolab, Vestenbergsgreuth, Germany). A purified verbascoside  
303 standard (96.85 %), obtained from Universidad Miguel Hernández (Elche,  
304 Spain), was used for quantification. The quantitative evaluation of compounds  
305 was performed with a calibration curve for each polyphenol, using ethanolic  
306 (oleuropein) or methanolic (verbascoside and luteolin) solutions of known  
307 concentrations. USAE and CVE extracts were analyzed at least in triplicate and  
308 results were expressed as mg polyphenol/g dry matter.

309

## 310 *2.6. Modeling of extraction kinetics and statistical analysis*

311 The monitoring of the total phenolic content (TPC) and antioxidant  
312 capacity (AC) of extracts during extraction allowed the extraction kinetics to be  
313 evaluated. The Naik model was used to mathematically describe the extraction  
314 kinetics (Naik et al., 1989):

$$315 \quad Y = (Y_{\infty} \cdot t) / (B + t) \quad (2)$$

316 where  $Y$  represents the extraction yield (TPC or AC) (mg gallic acid (GAE) or  
317 mg Trolox/g dry matter of olive leaves),  $t$  (min) the extraction time,  $Y_{\infty}$  the  
318 extraction yield at equilibrium and  $B$  (min) the extraction time needed to reach  
319 half of  $Y_{\infty}$ . The Excel™ Solver tool (Microsoft Corporation, Seattle, WA, USA)

320 was used to identify the model parameters ( $Y_{\infty}$  and B) that minimized the sum  
321 of the squared differences between the experimental and calculated Y. The  
322 explained variance (VAR) was used to determine the goodness of the model fit  
323 to the experimental data:

$$324 \quad VAR = 1 - (S_{xy}^2 / S_y^2) \quad (3)$$

325 where  $S_{xy}^2$  is the variance of the estimation and  $S_y^2$  the variance of the sample.  
326 Moreover, the mean relative error (MRE) was calculated to establish the  
327 difference between the experimental ( $Y_{EXPi}$ ) and calculated ( $Y_{CALi}$ ) data:

$$328 \quad MRE = (100/N) \sum_{i=1}^N \quad (4)$$

329 where N is the number of experimental data.

330 Analysis of Variance (ANOVA) was performed using Statgraphics®  
331 Centurion XV (Statpoint Technologies Inc., Warrenton, VA, USA) in order to  
332 identify significant ( $p < 0.05$ ) differences among the extracts, while the Fisher's  
333 Least Significant Difference (LSD) intervals were used for comparison of  
334 means.

335

### 336 **3. Results and discussion**

#### 337 *3.1. Ultrasonic assisted extraction (USAE)*

338 USAE was addressed in depth in order to estimate how the process  
339 parameters affect the ultrasonic field intensity and to identify an adequate  
340 combination of parameters with which to improve antioxidant extraction from  
341 olive leaves. First of all, the ultrasonic field was characterized as a means of  
342 establishing the energy applied to the medium by different emitters and electric  
343 powers. Moreover, a parametric study was carried out into the main process  
344 parameters that affect the ultrasound application.

$$\sum (|Y_{EXPi} - Y_{CALi}| / Y_{EXPi})$$

345

### 346 *3.1.1 Ultrasonic field characterization*

347         The intensity reached in the ultrasonic field during the different tests was  
348 measured by means of calorimetry, as was explained in section 2.3.3.1. Thus, it  
349 was possible to assess the effective power transferred by the transducer into  
350 the medium (ethanol-water 80:20, v/v) and choose the proper combination of  
351 electric power supplied to the transducer and emitter surface. From  
352 experimental results, it was observed that the greater the supply of electric  
353 power to the transducer, the more the ultrasonic power applied to the medium  
354 (Table 1). This relationship was linear for all the emitters tested.

355         The emitter surface also had a significant ( $p<0.05$ ) influence on the  
356 ultrasonic power applied to the medium. For every level of electric power  
357 supplied to the transducer, the ultrasonic power achieved by the 3.8 cm<sup>2</sup> emitter  
358 (intermediate surface) was nearly double that reached when using other  
359 emitters (12.6 and 1.5 cm<sup>2</sup>). Therefore, this emitter achieved the best coupling  
360 between the ultrasonic probe and the medium and led to the maximum figure of  
361 the effective ultrasonic power 51.47 W (100 % of the electric power and emitter  
362 surface of 3.8 cm<sup>2</sup>). In this case, it should be remarked that the yield  
363 electric/ultrasonic was only of approximately 13 % (51 W/400 W), which  
364 indicates that the energy conversion degree was low and there exists a wide  
365 range for the improvement of the ultrasonic devices.

366

### 367 *3.1.2 Parametric study*

#### 368 *3.1.2.1 Electric power supplied*



369 First of all, the effect of the electric power supplied to the transducer was  
370 monitored in olive leaf extraction kinetics by taking TPC and AC measurements.  
371 Different percentages of electric power, from 40 to 100 % of the total, were  
372 tested using an ultrasonic probe with a 12.6 cm<sup>2</sup> emitter. Thus, as is shown in  
373 Table 1, the effective ultrasonic power applied ranged from 12.6 to 28.4 W.

374 The extraction kinetics are shown in Figure 2 for the different  
375 experimental conditions. As can be observed, the more the electric power  
376 supplied, the higher the TPC or AC of the extract. Thereby, the best results  
377 were obtained supplying 100 % of the total electric power to the ultrasound  
378 transducer, which corresponded with the highest ultrasonic power applied (28.4  
379 ± 0.6 W) to the medium (Table 1). Since the acoustic energy transmitted into  
380 the medium is directly related to the extension of the ultrasonic effects, the more  
381 the ultrasonic power applied, the greater the cavitation intensity. Cavitation  
382 makes it easier for the solvent to penetrate into the matrix and eases interface  
383 transport (Luque de Castro & Priego-Capote, 2006), increasing the extraction  
384 efficiency of antioxidant compounds present in the sample (Dash et al., 2005).

385 The statistical analysis confirmed that the electric power applied only had  
386 a significant influence ( $p < 0.05$ ) on the final extracts, those obtained after 15 min  
387 of extraction, when it was above a certain threshold, which was  $18.5 \pm 0.5$  W  
388 (60 % electric power) for TPC and  $23.7 \pm 0.3$  W (80 % electric power) for AC.  
389 No influence of the ultrasound application was observed when less power was  
390 applied. These results agree with the ones reported by Cárcel et al. (2007a and  
391 2007b), who also found that the ultrasound effect on mass transfer during the  
392 osmotic treatment of apple was only significant ( $p < 0.05$ ) when the ultrasonic  
393 power applied was above 10.8 W/cm<sup>2</sup> (Cárcel et al., 2007a) and 50 W/cm<sup>2</sup>

394 during meat brining (Cárcel et al., 2007b). However, another study into the  
395 ultrasound assisted extraction of the triterpenic fraction of olive leaves  
396 concluded that irradiation power was not a significant ( $p < 0.05$ ) factor within the  
397 range under study (10-50 % electric power, 450 W) (Sánchez-Ávila et al.,  
398 2007). It is likely that in this case, the ultrasonic power range applied was too  
399 low, which prevented any significant differences from being observed.

400 Naik's model was used to quantify the influence of the ultrasonic power  
401 applied on the evolution of TFC and AC of olive leaf extracts during extraction  
402 process (Table 2). The model provided a close fit of experimental kinetics: the  
403 percentage of explained variance (VAR) was over 92 % and the mean relative  
404 error (MRE) lower than 9 %. The TPC and AC of extracts at equilibrium ( $Y_{\infty}$ )  
405 increased as the level of ultrasonic power applied rose, until reaching the  
406 maximum level for the highest ultrasonic power tested ( $28.4 \pm 0.6$  W, 100 %  
407 electric power). As far as the initial extraction rate is concerned ( $R_0$ ), it also  
408 increased as the level of power applied went up in both the TPC and AC.  
409 Therefore, ultrasound quickened the extraction process, which allowed the final  
410 TPC and AC of the extracts to increase, the effect being dependent on the  
411 electric power applied. Thereby, the highest electric power (100 %) was chosen  
412 to evaluate the influence of other process variables, such as the emitter surface  
413 of the ultrasonic probe and the temperature.

414

#### 415 3.1.2.2 *Emitter surface*

416 Experiments were carried out using 100 % of the total electric power  
417 supplied to the ultrasonic transducer and varying the ultrasonic emitter surface

418 (1.5, 3.8 and 12.6 cm<sup>2</sup>). This variable was evaluated since the ultrasonic probe  
419 used in this work allowed the use of different emitters by changing the probe tip.

420 Experimental results showed that the intermediate emitter surface tested  
421 (3.8 cm<sup>2</sup>) provided higher TPC and AC in the extracts than the smaller (1.5 cm<sup>2</sup>)  
422 or larger (12.6 cm<sup>2</sup>) emitter surfaces (Fig. 3). This fact could be explained from  
423 the measurement of the effective acoustic power applied (Table 1). While  
424 probes of 1.5 and 12.6 cm<sup>2</sup> provided a power applied of 33.3 ± 0.5 and 28.4 ±  
425 0.6 W, respectively, the emitter of 3.8 cm<sup>2</sup> increased the ultrasonic power  
426 transferred into the medium up to 51.47 ± 1.13 W (Table 1). The smallest  
427 emitter surface (1.5 cm<sup>2</sup>) greatly concentrates the ultrasound energy, producing  
428 an intense cavitation but only in a very limited zone located around the tip,  
429 resulting in a non-homogeneous application in the medium. On the other hand,  
430 using the largest surface tip (12.6 cm<sup>2</sup>) led to a more homogenous treatment  
431 but decreased the intensity of the ultrasonic power. Therefore, the best coupling  
432 between the application system (probe) and the volume treated of the extraction  
433 medium was achieved with the intermediate emitter surface (3.8 cm<sup>2</sup>), which  
434 was able to introduce the highest energy level per volume treated.

435 Modeling supported the previous results regarding the adequacy of the  
436 intermediate emitter surface, which provided the highest equilibrium of TPC and  
437 AC. Moreover, in the experiments carried out with the smallest emitter (1.5  
438 cm<sup>2</sup>), a high value of the initial extraction rate ( $R_0$ ) was found. This fact could be  
439 linked to the snapshot cavitation generated by the intense cavitation of this  
440 emitter in a very limited volume.

441

442 *3.1.2.3 Extraction temperature*

443 Temperature could have an influence on ultrasound application since  
444 high temperatures can decrease surface tension, increase the vapor pressure  
445 and produce less cavitation energy conversion. In addition, it could also affect  
446 extraction composition since some bioactive compounds may be sensitive to  
447 heat exposure. Thereby, the extraction temperature is an important variable to  
448 be considered. In this work, the influence of temperature was studied in the  
449 range of 25 to 50 °C, by carrying out a set of experiments applying 100 % of the  
450 electric power and using a 3.8 cm<sup>2</sup> emitter surface, which allowed 51.47 ± 1.13  
451 W to be applied to the medium.

452 The influence of the temperature on experimental kinetics was not very  
453 clear, as is observed in the evolution of both TPC and AC (Fig. 4). A statistical  
454 analysis showed that the influence of temperature was significant (p<0.05) on  
455 TPC, the content of which was significantly (p<0.05) higher at 45 °C. These  
456 results agreed with those previously found in the literature, since it is widely  
457 recognized that temperature enhances mass transfer by the improvement of the  
458 extraction rate. This fact can be explained by the effect temperature has on the  
459 vapor pressure, surface tension and viscosity of the liquid medium  
460 (Muthukumaran et al., 2006), which facilitates mass transfer. Moreover, the  
461 increase observed in the extraction yield may be linked to the increased ease  
462 with which solvent diffuses into cells and the enhancement of desorption and  
463 solubility at high temperatures (Esclápez et al., 2011). However, temperature  
464 had no significant (p<0.05) influence on the AC of extracts; the experimental  
465 error and/or the natural variability of raw matter could contribute to mask the  
466 slight differences produced by the extraction temperature. In addition, the  
467 introduction of a given amount of ultrasound energy into the medium could also

468 contribute to mask the effect of temperature. This fact has already been  
469 reported in literature, where there is controversy surrounding the influence of  
470 temperature in antioxidant extraction processes. Thus, Jerman et al. (2010)  
471 reported an increase in extraction efficiency at temperatures of up to 45 °C in  
472 olive fruit phenolic compounds. The same fact was observed by Zhang et al.  
473 (2009) in the range of 15 - 45 °C, where high temperatures reduced the  
474 extraction yield. However, Zhang et al. (2011) found that extraction yields rose  
475 as the temperature increased from 60 to 80 °C, while Rostagno et al. (2007)  
476 found that phenolics underwent an important degradation at temperatures of  
477 over 60 °C. Therefore, it seems that the temperature influence may be product-  
478 dependent, it being necessary to determine the proper extraction temperature  
479 for a specific commodity. The use of high temperatures, over the optimum,  
480 should be avoided due to the fact that they lead to solvent loss by volatilization,  
481 higher energy costs and more extraction impurities (Esclápez et al., 2011).

482 Naik's model parameters (Table 2) confirmed the scarce effect of  
483 temperature on extraction kinetics. As can be observed, the differences among  
484 the values identified at the temperatures tested were small. For example, the  $Y_{\infty}$   
485 ranged from 40.4 at 25 °C to 45.8 at 45 °C. The highest initial extraction ( $R_0$ )  
486 rate was achieved at 25 and 35 °C for AC and TPC, respectively, the identified  
487 values being very close to those found at 45 °C. Thus, taking into account both  
488 energy consumption and the slight improvement gained due to the increase in  
489 extraction temperature, the temperature of 25 °C was chosen as the most  
490 suitable for the ultrasound assisted extraction of polyphenols from olive leaves.

491

492 *3.2. Ultrasound assisted extraction (USAE) versus conventional extraction*

493           Once the best choice of process parameters for ultrasound application  
494 was identified:  $51.47 \pm 1.13$  W (100% of electric power), 3.8 cm<sup>2</sup> emitter and 25  
495 °C; the feasibility of USAE was addressed. An overall study was conducted  
496 comparing USAE with conventional extraction processes, considering not only  
497 kinetic but also compositional issues.

498

### 499 *3.2.1. Effect on extraction kinetics*

500           The kinetic of the ultrasound assisted extraction (USAE) was compared  
501 with conventional extraction with agitation (CVE; 170 rpm) and conventional  
502 static extraction (STE).

503           Experimental results highlighted that solvent agitation significantly  
504 affected ( $p < 0.05$ ) extraction kinetics. As is shown in Fig. 5, the kinetic of TPC  
505 extraction was faster in CVE than in STE experiments. Obviously, the  
506 turbulence created by agitating the extracting medium reduced the external  
507 resistance to mass transfer, thereby, improving phenolic extraction.  
508 Nevertheless, CVE was significantly ( $p < 0.05$ ) slower than USAE. By applying  
509 ultrasound both TPC and AC were improved in extracts, causing phenolic  
510 compounds to migrate into the solvent faster. For example, after 3 min the AC  
511 in USAE was 119 and 332 % higher than in CVE and STE, respectively.  
512 Moreover, the TPC in USAE after 3 min was almost double that obtained after  
513 15 min in CVE. Previous works have also reported an improvement in bioactive  
514 compounds extraction brought about by the application of power ultrasound.  
515 Thus, Jiang-Bing et al. (2006) and Zhang et al. (2009) reported increases in the  
516 amount of extracted bioactive compounds of 16.5 and 60 %, respectively.

517 In this study, the ultrasound application led to an immediate leaching of  
518 polyphenols into the solvent; thus, 84 % of TPC was extracted during the first 5  
519 min of US treatment. Therefore, ultrasound effects accelerated the solubilization  
520 of accessible antioxidant compounds (washing effect) and contributed to the  
521 extraction of the non-accessible compounds. A review of the literature also  
522 brings opposite results to light, thus, Jerman et al. (2010) determined that the  
523 extraction efficiency of polyphenols from olive fruit was low for the first 4 min of  
524 ultrasound application, indicating that longer times were needed for wall  
525 disruption. This mild effect could be linked to the level of ultrasonic power  
526 applied, since these authors carried out the experiments in an ultrasonic bath,  
527 which actually supplies lower ultrasonic intensities than probe systems like the  
528 one used in the current study.

529 On the other hand, in USAE experiments, the increase in the TPC and  
530 AC of the extracts was almost negligible after 15 min of extraction. This fact  
531 suggests that long sonication times were not effective. During extraction times  
532 of over 15 min, the TPC and AC were kept constant, which also indicates that  
533 continuous ultrasound application seems to have no effect on bioactive  
534 compounds. These results agreed with Rodrigues et al. (2008), who indicated  
535 that 15 min of sonication time were enough to extract phenols from coconut.  
536 The effect of ultrasound could be mainly linked to the phenomenon of cavitation  
537 and the generation of microstreaming, alternative pressures or interfacial  
538 instabilities. The implosion of cavitation bubbles generates macro-turbulence,  
539 high-velocity inter-particle collision and perturbation in the micro-porous  
540 particles of the biomass accelerating the eddy diffusion and internal diffusion,  
541 thereby, increasing mass transfer (Jian-Bing et al., 2006). Moreover, the

542 asymmetric implosion of bubbles near vegetable particles generates micro-jets  
543 (Mason & Lorimer, 2002) that hit cellular surfaces disrupting them and allowing  
544 their contents to be extracted.

545 Naik's model fitted the extraction kinetics for both CVE and USAE  
546 experiments well, such as is observed in Fig. 5. The initial extraction rate  
547 identified for USAE experiments,  $R_0$ , was three times higher than the one  
548 identified for CVE ones (37.3 and 11.6 mg GAE/min-g d.m., respectively)  
549 indicating the significant effect of ultrasound on the extraction rate. As far as  
550 equilibrium is concerned, the identified value of  $Y_\infty$  was  $41 \pm 2$  mg GAE/g d.m.  
551 for USAE and  $22 \pm 1$  mg GAE/g d.m. for CVE. The  $Y_\infty$  value identified for CVE  
552 experiments should be considered a modeling artifact since the experimental  
553 conditions are not a valid means of identifying the equilibrium point. This is due  
554 to the fact that, at the longest time tested (15 min), the system is a long way  
555 from equilibrium, which under these conditions was reached after approximately  
556 24 hours. Therefore, the results obtained showed just how effective ultrasound  
557 application is at extracting antioxidants from olive leaves, thus reducing  
558 extraction times. This fact could be very interesting for industrial purposes,  
559 since ultrasound assisted extraction would make it possible to improve process  
560 rates and, consequently, reduce processing times and costs.

561

### 562 *3.2.2. Influence on extract composition and antioxidant potential*

563 In order to complete the study into the feasibility of ultrasound assisted  
564 extraction, it was necessary to evaluate not only the extraction rate but also the  
565 quality of the obtained extracts. For that purpose, a different batch of olive  
566 leaves was collected and processed as already explained in section 2.1. The



567 extracts were obtained by USAE after 15 min and CVE after 24 h and  
568 characterized (Table 3). The TPC of extracts obtained by CVE and USAE was  
569 similar (66 mg GAE/g d. m.). As for AC, FRAP and TEAC methods gave slightly  
570 different results. While no significant ( $p < 0.05$ ) differences were observed  
571 between USAE and CVE extracts when using TEAC, the use of FRAP implied a  
572 significant ( $p < 0.05$ ) increase (10 %) in AC when USAE was applied. This fact  
573 could be explained by the fact that these methods are based on different  
574 chemical principles, which involves a different sensitivity towards evaluating  
575 changes in extract composition linked to antioxidant capacity.

576 The extracts obtained from USAE and CVE extraction were also  
577 analyzed by chromatography, which allowed the main phenolic compounds  
578 present in olive leaf extracts to be identified (Table 4). Chromatograms from  
579 USAE and CVE extracts were very similar, as is observed in Fig.6. Thus,  
580 ultrasound application did not promote the formation of new phenolic  
581 compounds or induce phenolic degradation. The main polyphenols identified in  
582 this study: oleuropein, verbascoside and luteolin-7-O-glucoside have been  
583 already reported in previous studies of olive leaf extracts (Benavente-García et  
584 al., 2000; Japón-Luján & Luque de Castro, 2006). However, other known  
585 phenols, such as tyrosol and hydroxytyrosol, which are characteristic of olive  
586 fruit and leaf, were not found in either CVE or USAE extracts. It is likely that  
587 these differences could be explained by the olive cultivar and collecting season.

588 In this study, only the main polyphenols were quantified (oleuropein,  
589 verbascoside and luteolin-7-O-glucoside) using standard compounds. No  
590 significant ( $p < 0.05$ ) difference was found between the verbascoside and  
591 luteolin-7-O-glucoside content of USAE and CVE extracts. In the case of

592 oleuropein, however, USAE extracts exhibited a 12 % significantly ( $p < 0.05$ )  
593 lower content than CVE ones. Jerman et al. (2010), who studied ultrasound  
594 assisted extraction of olive fruit phenolic compounds, found that the extraction  
595 method had a significant ( $p < 0.05$ ) influence on the content of all the compounds  
596 quantified in this study. In all likelihood, these authors did not compare extracts  
597 obtained at equilibrium, as the result is masked by a kinetic effect linked to  
598 ultrasound application.

599 As regards the extraction yields reached in this study, the polyphenol  
600 content was higher than that published by other authors using other extraction  
601 methods. As an example, the oleuropein content was 222 % and 347 % higher  
602 than that determined by Japón-Luján & Luque de Castro (2006) in olive leaves  
603 and Jerman et al. (2010) in olive fruits, respectively. Thus, extracts with a higher  
604 content of oleuropein (65-74 mg/g d. m.), verbascoside (18.5-18.7 mg/g d. m.)  
605 and luteolin-7-O-glucoside (9.7-11 mg/g d. m.) were obtained. Although there  
606 are many factors which can affect the extract composition, such as the cultivar  
607 or sampling season, both extraction methods used in this study can be  
608 considered adequate and efficient procedures. Moreover, it is necessary to  
609 highlight that ultrasound application reduced the extraction time from the 24 h  
610 needed in the conventional method to 15 min, maintaining the phenolic  
611 composition and antioxidant potential of the extracts. In this sense, the  
612 application of ultrasound would be an interesting alternative method to  
613 conventional procedures, since it greatly increased the extraction rate and was  
614 able to generate extracts rich in bioactive compounds.

615

#### 616 **4. Conclusions**

617           The application of ultrasound energy could be considered an interesting  
618 alternative as a means of intensifying the extraction process of phenolic  
619 compounds from olive leaves. The ultrasound effect was mostly dependent on  
620 the effective ultrasonic power applied to the medium, and was influenced not  
621 only by the amount of electric power supplied but also by how well the emitter  
622 surface and extracting medium coupled. Thereby, it was highlighted that the  
623 greatest improvement of polyphenolic extraction was achieved by supplying 100  
624 % of the total electric power to the ultrasonic device and using the intermediate  
625 emitter surface tested (3.8 cm<sup>2</sup>) for an extracting medium of 200 mL. Moreover,  
626 temperature was found to have no clear effect on extraction kinetics. Therefore,  
627 compared with conventional techniques, ultrasound assisted extraction can be  
628 considered a more efficient procedure, being able to provide olive leaf extracts  
629 with a similar content of bioactive compounds, such as oleuropein,  
630 verbascoside and luteolin-7-O-glucoside, but markedly shortening the extraction  
631 time, from 24 hours to 15 min.

632           The ultrasonic assisted extraction is still a challenge on an industrial  
633 scale. Therefore, further research is necessary in order to develop efficient  
634 ultrasonic transducers and thus improve the extraction processes. These facts  
635 would allow the processing costs to be minimized, giving rise to a new more  
636 competitive market in which the bioactive properties would remain intact.

637

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644

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786

787 **Figure captions**

788

789 **Fig. 1.** Experimental set-up for ultrasonic assisted extraction of olive leaf  
790 phenolic compounds. A: Computer; B: Process controller; C: Ultrasonic probe  
791 system; D: Temperature sensor (Pt100); E: Jacketed extraction vessel; F:  
792 Peristaltic pump; G: Glycol reservoir; H: Chiller.

793

794 **Fig. 2.** Evolution of the total phenolic content (A) and antioxidant capacity (B;  
795 FRAP) of olive leaf extracts obtained by applying ultrasound at different electric  
796 powers supplied to the transducer (emitter surface  $12.6 \text{ cm}^2$  and  $25 \text{ }^\circ\text{C}$   
797 extraction temperature).

798

799 **Fig. 3.** Influence of transducer emitter surface on the evolution of the total  
800 phenolic content of olive leaf extracts obtained by ultrasound assisted extraction  
801 (100% of the electric power supplied to the transducer and  $25 \text{ }^\circ\text{C}$  extraction  
802 temperature).

803

804 **Fig. 4.** Evolution of antioxidant capacity (FRAP) at different temperatures of  
805 ultrasound assisted extraction (100% of the electric power supplied to the  
806 transducer, emitter surface  $3.8 \text{ cm}^2$  and effective power  $51.47 \pm 1.13 \text{ W}$ ).

807

808 **Fig. 5.** Influence of extraction method on the total phenolic content. STE: static  
809 extraction (no agitation of extracting medium); CVE: conventional extraction

810 (with agitation); USAE: ultrasound assisted extraction (100 % of the electric  
811 power supplied to the transducer; emitter surface  $3.8 \text{ cm}^2$ , effective power  $51.47$   
812  $\pm 1.13 \text{ W}$  and extraction temperature  $25 \text{ }^\circ\text{C}$ ).

813

814 **Fig. 6.** HPLC chromatograms at 280 nm of olive leaf extracts obtained at  $25 \text{ }^\circ\text{C}$   
815 by CVE (A; extraction time 24 h) and USAE (B; 100 % of the electric power  
816 supplied to the transducer, emitter surface  $3.8 \text{ cm}^2$ , effective power  $51.47 \pm$   
817  $1.13 \text{ W}$  and extraction time 15 min).

818

**Table 1.** Ultrasonic power (W) applied to the medium as a function of the percentage of the total electric power (400 W) supplied to the ultrasonic transducer and the emitter surface of the probe tip.

Tip diameter (cm)	Emitter surface (cm <sup>2</sup> )	Electric power supplied to transducer			
		40%	60%	80%	100%
4.0	12.6	12.6 ± 0.3	18.5 ± 0.5	23.7 ± 0.3	28.4 ± 0.6
2.2	3.8	24 ± 2	32.4 ± 0.2	41.75 ± 1.13	51.47 ± 1.13
1.4	1.5	11.85 ± 0.17	16.9 ± 0.6	27.6 ± 1.5	33.3 ± 0.5

**Table 2.** Identified parameters of Naik's model. Influence of process parameters on the total phenolic content and antioxidant capacity (FRAP) of olive leaf extracts.

Extraction variables		Total phenolic content				
		$Y_{\infty}$ (mg GAE/g d. m.) <sup>a</sup>	B (min) <sup>b</sup>	$R_0$ <sup>c</sup>	VAR (%) <sup>d</sup>	MRE (%) <sup>e</sup>
Electric Power (%)	40	21.6	2.6	8.2	95.3	6.3
	60	21.9	2.3	9.5	95.4	6.3
	80	23.0	1.2	19.6	97.2	4.6
	100	29.1	1.2	24.1	98.1	3.4
Emitter surface (cm <sup>2</sup> )	1.5	27.0	0.4	64.5	97.9	3.9
	3.8	40.4	1.1	36.8	99.0	2.7
	12.6	29.1	1.2	24.1	98.1	3.4
Temperature (°C)	25	40.4	1.1	36.8	99.0	2.7
	30	40.5	1.3	30	99.4	2.2
	35	39.1	0.8	46.6	95.6	4.9
	40	42.2	1.0	41.6	99.2	2.5
	45	45.8	1.1	43.2	99.1	2.6
	50	43.4	1.6	26.5	96.0	5.9

Extraction variables		Antioxidant capacity (FRAP)				
		$Y_{\infty}$ (mg trolox/g d. m.) <sup>a</sup>	B (min) <sup>b</sup>	$R_0$ <sup>c</sup>	VAR (%) <sup>d</sup>	MRE (%) <sup>e</sup>
Electric Power (%)	40	43.4	2.7	15.8	96.9	4.8
	60	41.1	3.0	13.8	92.8	8.7
	80	50.7	1.7	30.0	96.9	5.3
	100	57.2	1.7	33.7	96.2	5.7
Emitter surface (cm <sup>2</sup> )	1.5	49.9	0.2	318.0	99.5	1.8
	3.8	73.2	0.8	95.8	95.8	6.2
	12.6	57.2	1.7	33.7	96.2	5.7
Temperature (°C)	25	73.2	0.8	95.8	95.8	6.2
	30	77.0	1.6	48.9	97.6	4.2
	35	83.2	1.2	68.3	97.3	4.2
	40	84.2	1.2	67.8	98.4	3.3
	45	89.2	1.4	63.1	95.9	5.7
	50	81.7	1.2	66.0	94.6	6.0

<sup>a</sup>  $Y_{\infty}$  represents the extraction yield at equilibrium as mg of gallic acid (GAE) or mg of trolox per g of dry mass of olive leaves.

<sup>b</sup> B determines the extraction time needed to reach half of  $Y_{\infty}$ .

<sup>c</sup>  $R_0$  shows the relation  $Y_{\infty}/B$ .

<sup>d</sup> VAR is the explained variance.

<sup>e</sup> MRE is the mean relative error.

**Table 3.** Characterization of olive leaf extracts obtained by conventional (CVE, 24 h, 170 rpm) and ultrasound assisted extraction (USAE, 15 min, 51.47 W).

		<b>CVE</b>	<b>USAE</b>
Oleuropein (mg/g d. m.)		74 ± 2 <sup>a</sup>	65 ± 2 <sup>b</sup>
Verbascoside (mg/g d. m.)		18.7 ± 0.3 <sup>a</sup>	18.5 ± 0.6 <sup>a</sup>
Luteolin -7-O-glucoside (mg/g d. m.)		9.7 ± 0.4 <sup>a</sup>	11 ± 4 <sup>a</sup>
Total phenolic content (mg GAE/g d. m.)		66 ± 3 <sup>a</sup>	66 ± 8 <sup>a</sup>
Antioxidant capacity	FRAP	102 ± 3 <sup>a</sup>	112 ± 6 <sup>b</sup>
(mg trolox/g d. m.)	TEAC	6.2 ± 0.3 <sup>a</sup>	7.2 ± 1.2 <sup>a</sup>

Note: The subscripts a and b show homogeneous groups established from LSD (Least Significance Difference) intervals ( $p < 0.05$ ).

**Table 4.** Identification of the main phenolic compounds present in olive leaf extracts.

Peak N°	Phenolic compound	Molecular mass (g/mol)	Retention time (min)
1	Cafeoil	354.31	4.70
2	Apigenin-6,8-diglucoside	594.52	9.41
3	Verbascoside	624.6	13.85
4	Luteolin-7-O-rutinoside	578.52	14.57
5	Luteolin-7-O-glucoside	448.38	15.27
	Luteolin-7-O-glucoside(isomer)	448.38	18.50
6	Oleuropein glucoside	702	16.45
7	Apigenin rutinoside	578.53	17.11
8	Apigenin-7-O-glucoside	432.37	18.24
9	Oleuropein	540.52	19.02
10	Luteolin	286.24	25.50

Figure 1

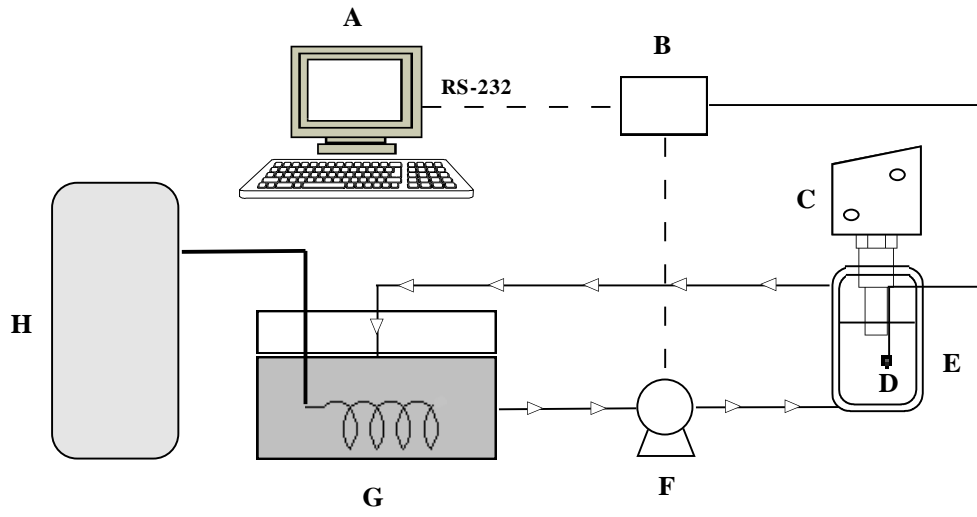




Figure 2

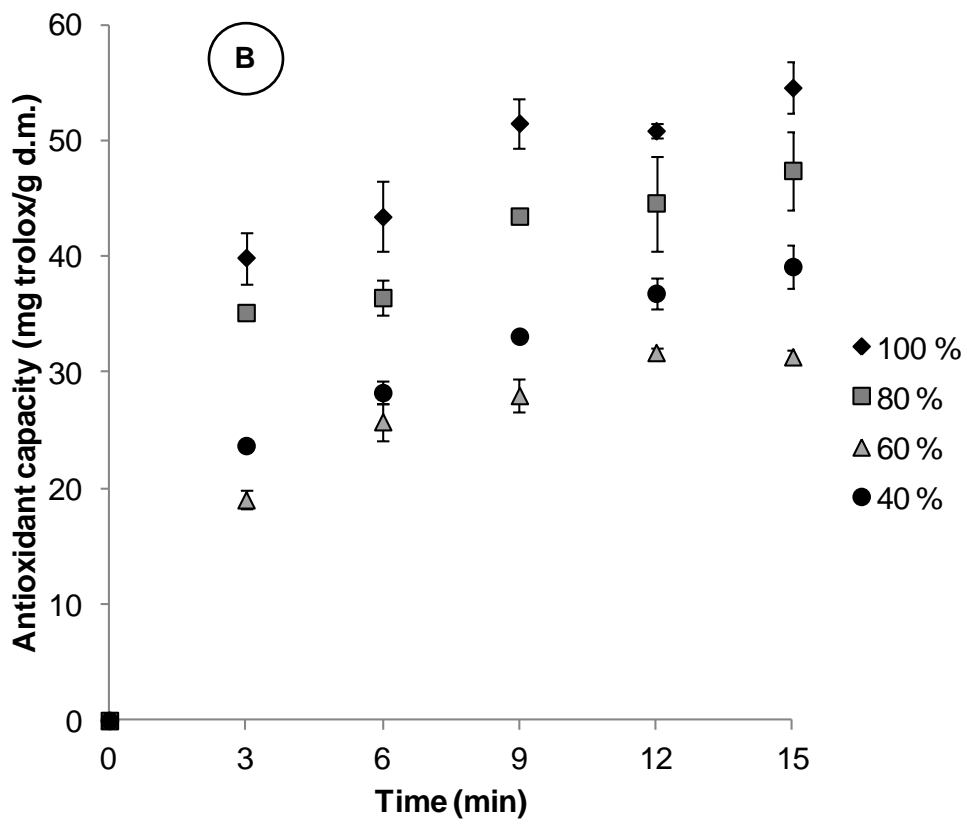
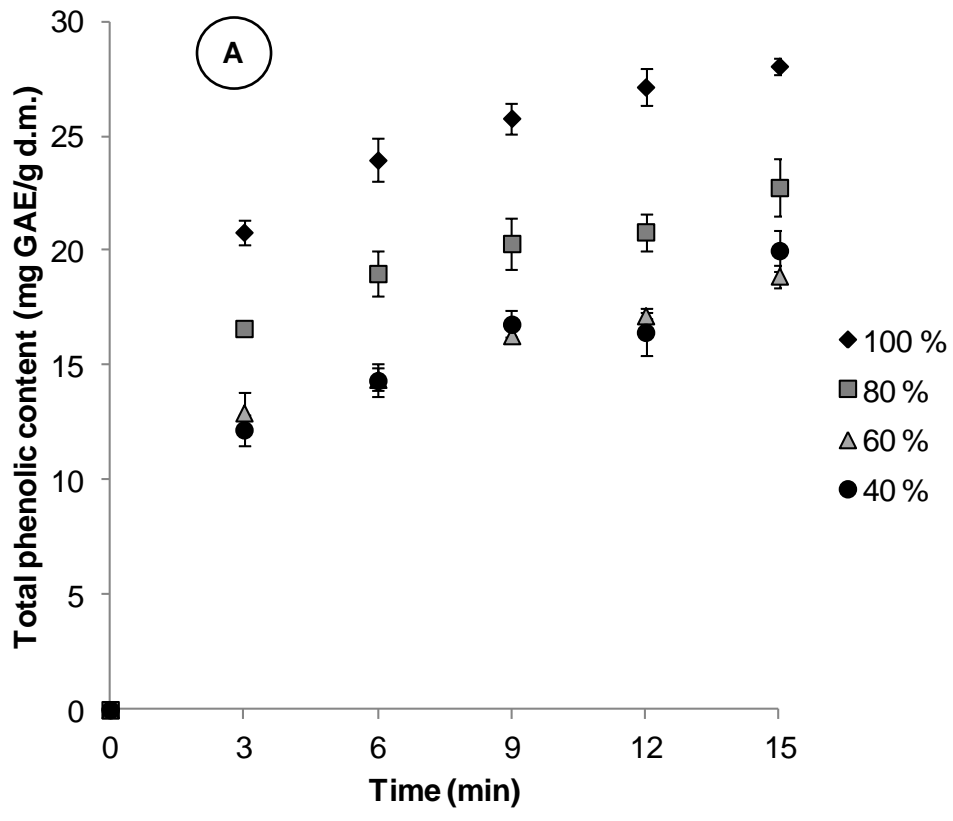


Figure 3

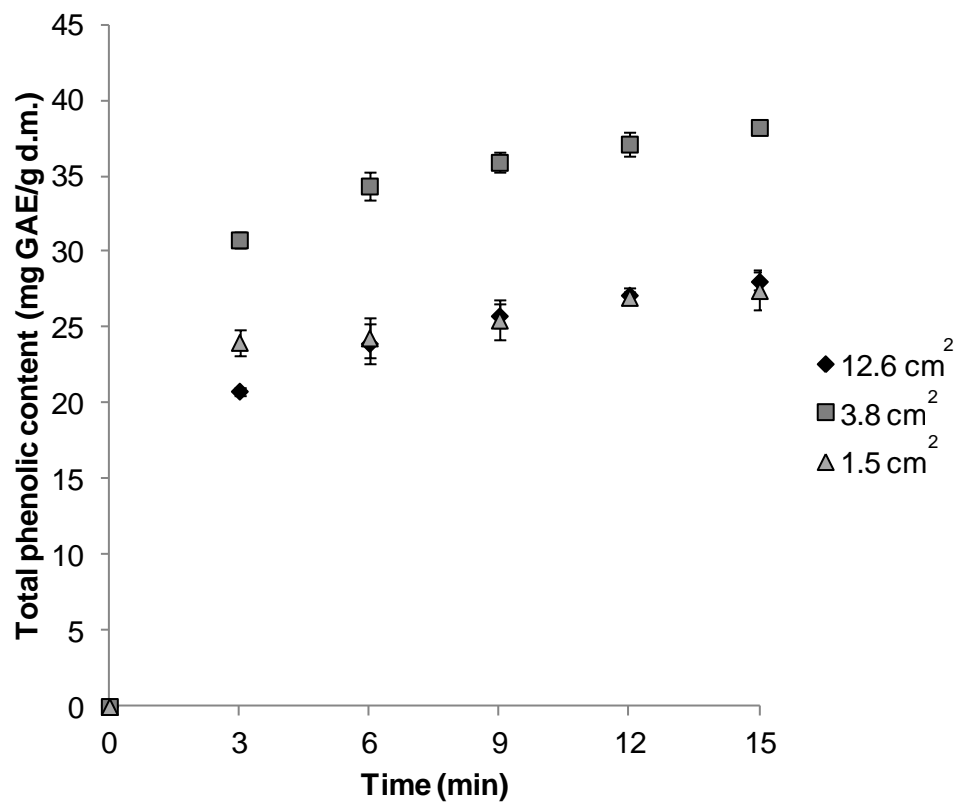


Figure 4

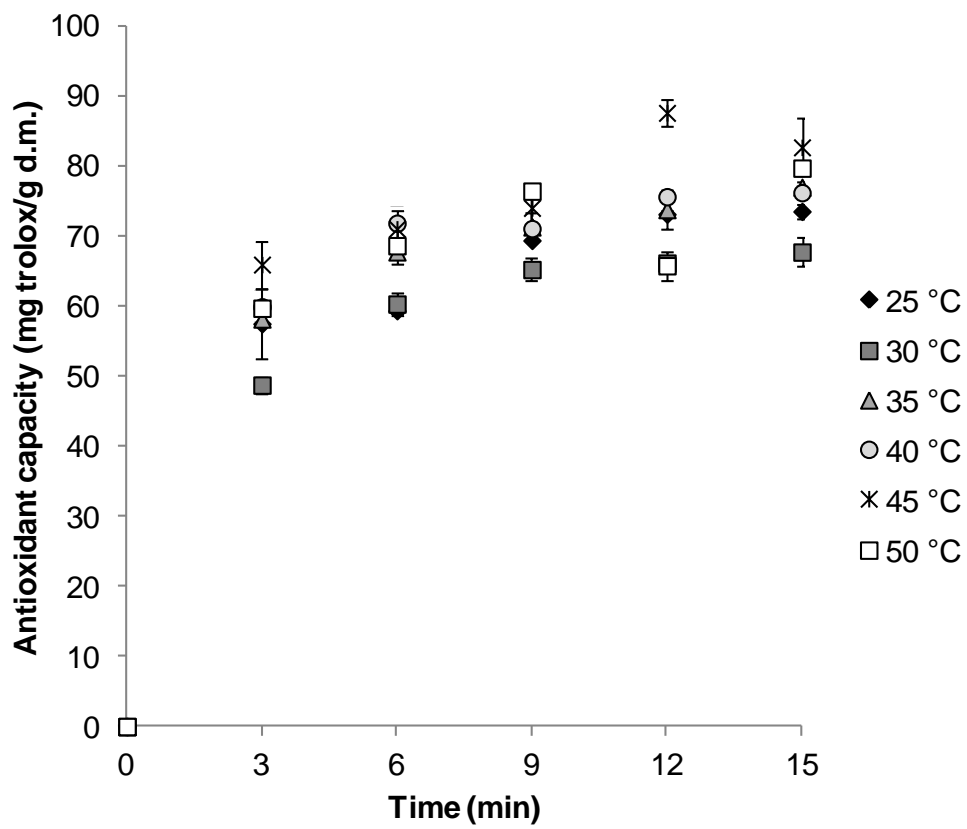


Figure 5

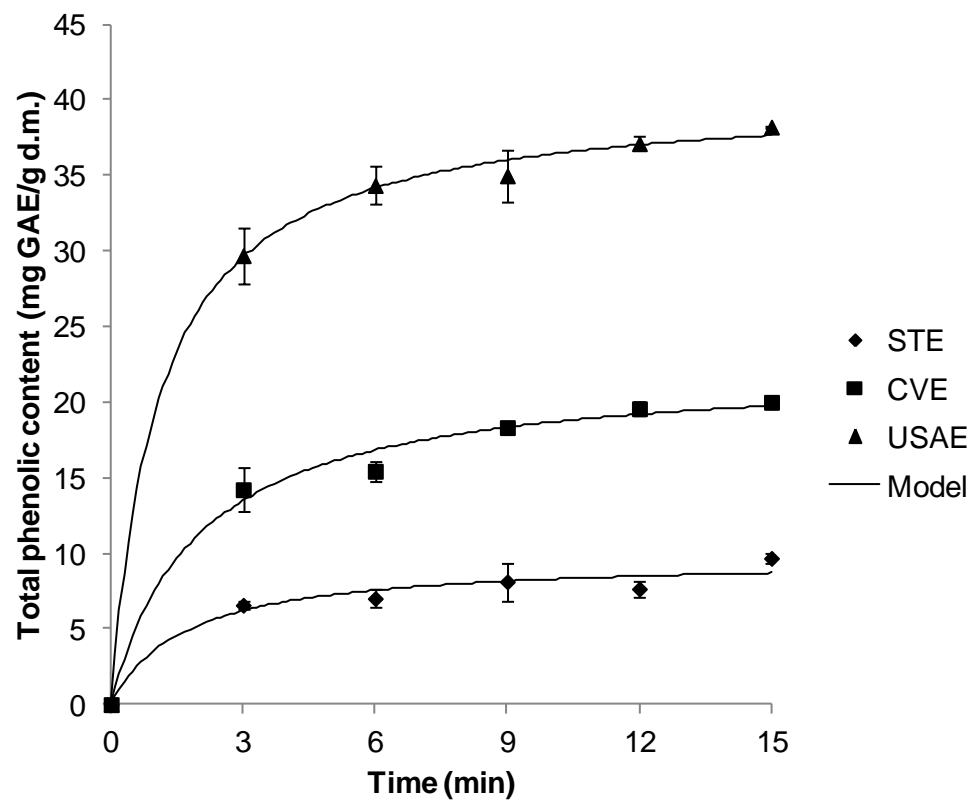


Figure 6

