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

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Abstract

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

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

1. Introduction

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

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

as supercritical extraction with CO_2 (Bensebia et al., 2009), ultrasound assisted (Knorr et al., 2004; Zhang et al., 2009), microwave-assisted (Hayat et al., 2009) or superheated liquid extraction (Japón-Luján & Luque de Castro, 2006).

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

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

2. Materials and methods

2.1. Raw material

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

121

122 *2.2. Drying experiments*

123 The olive leaves, with an initial moisture content of 39.2 ± 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 ± 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^3.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

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

2.3.3. Ultrasound assisted extraction (USAE)

2.3.3.1 Experimental set-up and characterization of ultrasonic field

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

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

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

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

2.3.3.2 Parametric study

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

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

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

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

2.3.4. Conventional extraction

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

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

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

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 µL of sample were mixed with 200 µL of Folin-Ciocalteu's
224 phenol reagent (Sigma-Aldrich, Madrid, Spain) and 2 mL of distilled water. After
225 3 min at 25 ºC, 1 mL of Na_2CO_3 (Panreac, Barcelona, Spain) solution (Na_2CO_3-
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 µL of FRAP
243 reagent were used; this had been freshly prepared and heated to 37 °C and
244 mixed with 30 µL of distilled water and 30 µL of test sample or ethanol-water

(80:20, v/v) used as an appropriate reagent blank. The FRAP reagent contained 2.5 mL of a 10 mM TPTZ (Fluka, Steinheim, Germany) solution in 40 mM HCl (Panreac, Barcelona, Spain) plus 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ (Panreac, Barcelona, Spain) and 2.5 mL of 0.3 M acetate buffer (Panreac, Barcelona, Spain), pH 3.6 (Pulido et al., 2000). Readings at the maximum absorption level (595 nm) were taken using a spectrophotometer (Helios Gamma, Thermo Spectronic, Cambridge, UK). At least 4 replicates were made for each measurement. The AC was evaluated through a calibration curve that had been previously determined using the extracting solvent (ethanol-water 80:20, v/v) of a known Trolox (Sigma-Aldrich, Madrid, Spain) concentration and expressed as mg Trolox/g dry matter.

2.4.2.2. Trolox equivalent antioxidant capacity (TEAC)

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

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

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

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

Separation was carried out through a linear gradient method using 2.5 % acetic acid (A) and acetonitrile (B), starting the sequence with 10 % B and programming gradient to obtain 20 % B at 10 min, 40 % B at 35 min, 100 % B at 40 min, 100 % B at 45 min, 10 % B at 46 min and 10 % B at 50 min. In order to ensure the LC-MS pump performed accurately, 10% of organic solvent was premixed in the water phase. The flow-rate was 1 mL/min and the chromatograms were monitored at 240, 280 and 330 nm. The mass spectrometry operating conditions were optimized in order to achieve maximum sensitivity values. The ESI source was operated in negative mode to generate $[M–H]^-$ ions under the following conditions: a desolvation temperature of 365 °C and a vaporizer temperature of 400 °C; dry gas (nitrogen) and nebulizer were 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 $Y = (Y_\infty \cdot t)/(B+t)$ (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_∞ the
318 extraction yield at equilibrium and B (min) the extraction time needed to reach
319 half of Y_∞. The Excel™ Solver tool (Microsoft Corporation, Seattle, WA, USA)

was used to identify the model parameters (Y_∞ and B) that minimized the sum of the squared differences between the experimental and calculated Y. The explained variance (*VAR*) was used to determine the goodness of the model fit to the experimental data:

$$VAR = 1-(S^2_{xy}/S^2_y) \quad (3)$$

where S^2_{xy} is the variance of the estimation and S^2_y the variance of the sample. Moreover, the mean relative error (*MRE*) was calculated to establish the difference between the experimental (Y_{EXPi}) and calculated (Y_{CALi}) data:

$$MRE = (100/N)\sum_{i=1}^{N}(|Y_{EXPi} - Y_{CALi}|/Y_{EXPi}) \quad (4)$$

where *N* is the number of experimental data.

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

3. Results and discussion

3.1. Ultrasonic assisted extraction (USAE)

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

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^2 emitter
358 (intermediate surface) was nearly double that reached when using other
359 emitters (12.6 and 1.5 cm^2). 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^2). 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*

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

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

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

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_∞)
405 increased as the level of ultrasonic power applied rose, until reaching the
406 maximum level for the highest ultrasonic power tested (28.4 ± 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

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

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

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

3.1.2.3 Extraction temperature

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

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

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

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

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 ± 1.13 W (100% of electric power), 3.8 cm^2 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.

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

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

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

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

3.2.2. Influence on extract composition and antioxidant potential

In order to complete the study into the feasibility of ultrasound assisted extraction, it was necessary to evaluate not only the extraction rate but also the quality of the obtained extracts. For that purpose, a different batch of olive 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**

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

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

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644

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786

Figure captions

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

Fig. 2. Evolution of the total phenolic content (A) and antioxidant capacity (B; FRAP) of olive leaf extracts obtained by applying ultrasound at different electric powers supplied to the transducer (emitter surface 12.6 cm^2 and 25 °C extraction temperature).

Fig. 3. Influence of transducer emitter surface on the evolution of the total phenolic content of olive leaf extracts obtained by ultrasound assisted extraction (100% of the electric power supplied to the transducer and 25 °C extraction temperature).

Fig. 4. Evolution of antioxidant capacity (FRAP) at different temperatures of ultrasound assisted extraction (100% of the electric power supplied to the transducer, emitter surface 3.8 cm^2 and effective power 51.47 ± 1.13 W)**.**

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

(with agitation); USAE: ultrasound assisted extraction (100 % of the electric power supplied to the transducer; emitter surface 3.8 cm^2, effective power 51.47 ± 1.13 W and extraction temperature 25 °C).

Fig. 6. HPLC chromatograms at 280 nm of olive leaf extracts obtained at 25 °C by CVE (A; extraction time 24 h) and USAE (B; 100 % of the electric power supplied to the transducer, emitter surface 3.8 cm^2, effective power 51.47 ± 1.13 W and extraction time 15 min).

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^2)	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_∞ (mg GAE/g d. m.)[a]	B (min)[b]	R_0 [c]	VAR (%)[d]	MRE (%)[e]
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^2)	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_∞ (mg trolox/g d. m.)[a]	B (min)[b]	R_0 [c]	VAR (%)[d]	MRE (%)[e]
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^2)	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

[a] Y_∞ represents the extraction yield at equilibrium as mg of gallic acid (GAE) or mg of trolox per g of dry mass of olive leaves.

[b] B determines the extraction time needed to reach half of Y_∞.

[c] R_0 shows the relation Y_∞/B.

[d] VAR is the explained variance.

[e] 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).

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

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 Nº	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

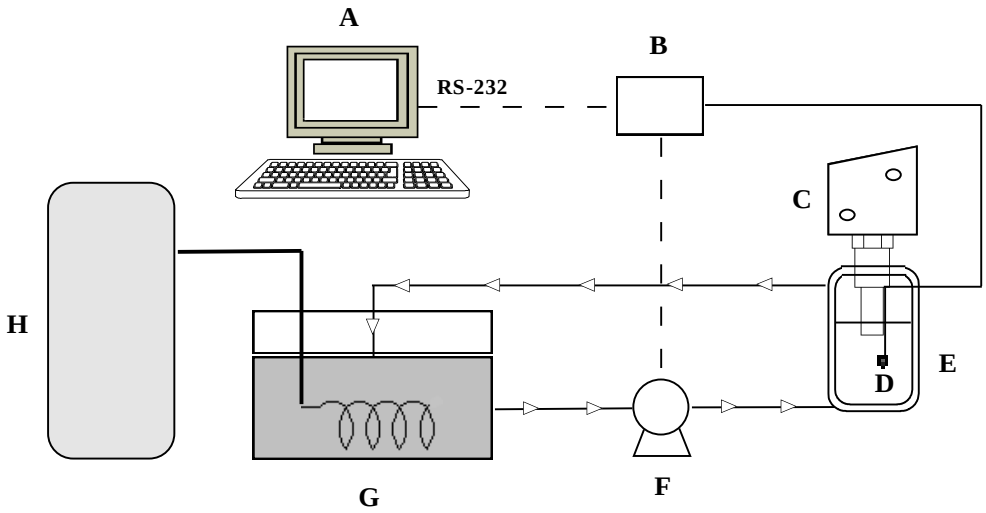


Figure 2

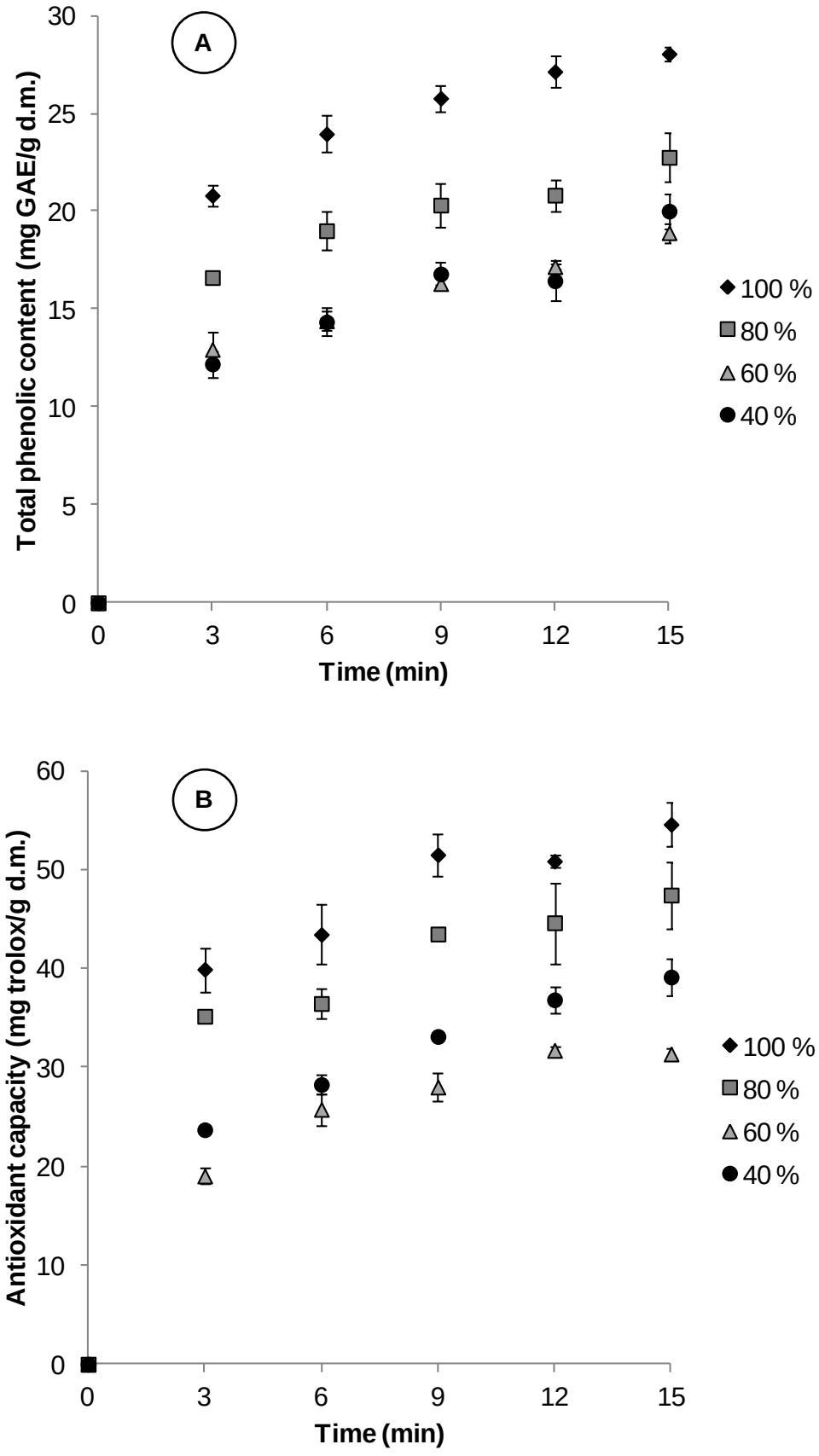

A
Total phenolic content (mg GAE/g d.m.)
30
25
20
15
10
5
0
0
3
6
9
12
15
Time (min)
100 %
80 %
60 %
40 %
B
Antioxidant capacity (mg trolox/g d.m.)
60
50
40
30
20
10
0
0
3
6
9
12
15
Time (min)
100 %
80 %
60 %
40 %

Figure 3

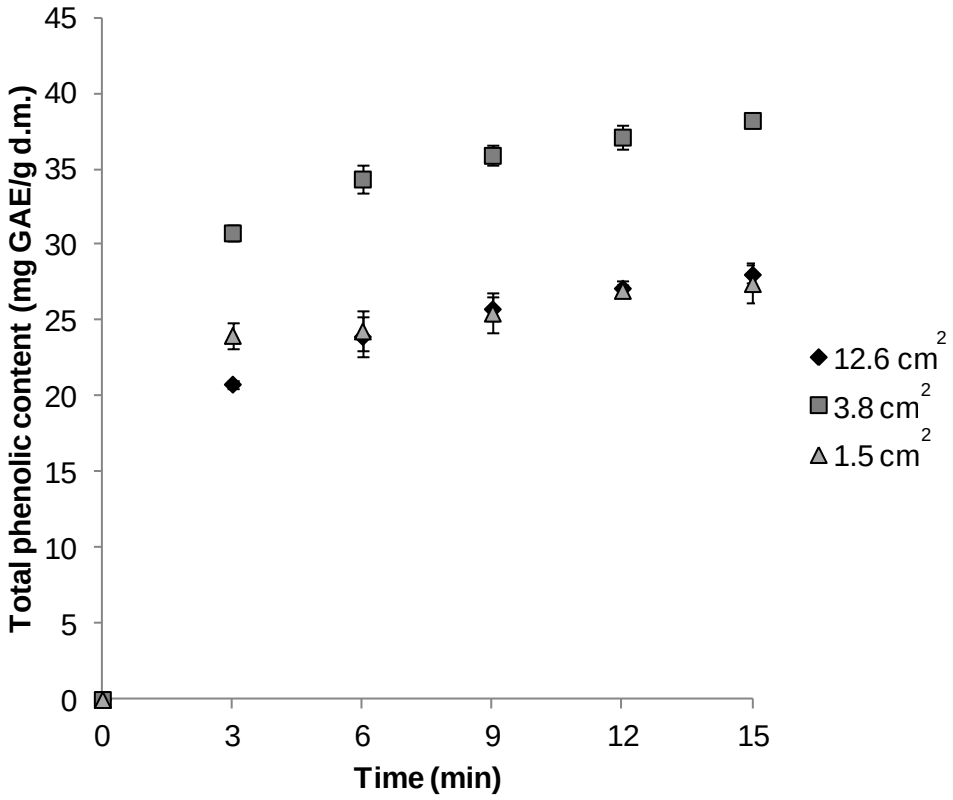

Total phenolic content (mg GAE/g d.m.)
45
40
35
30
25
20
15
10
5
0
0
3
6
9
12
15
Time (min)
12.6 cm2
3.8 cm2
1.5 cm2

Figure 4

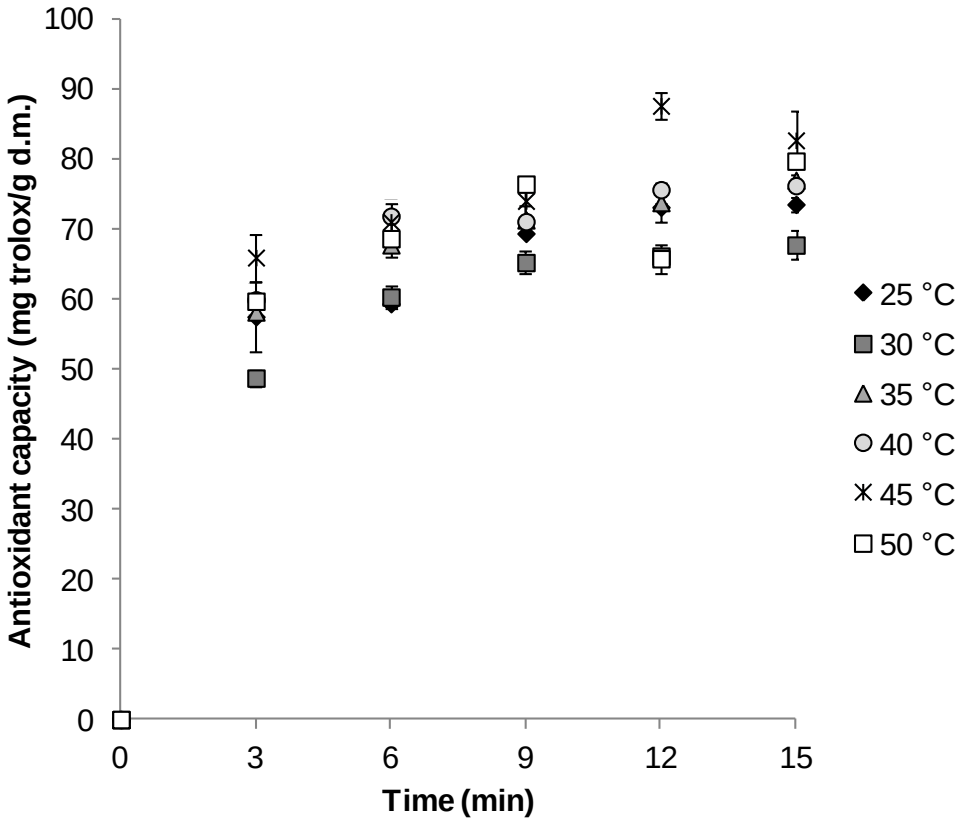

Antioxidant capacity (mg trolox/g d.m.)
100
90
80
70
60
50
40
30
20
10
0
0
3
6
9
12
15
Time (min)
25 °C
30 °C
35 °C
40 °C
45 °C
50 °C

Figure 5

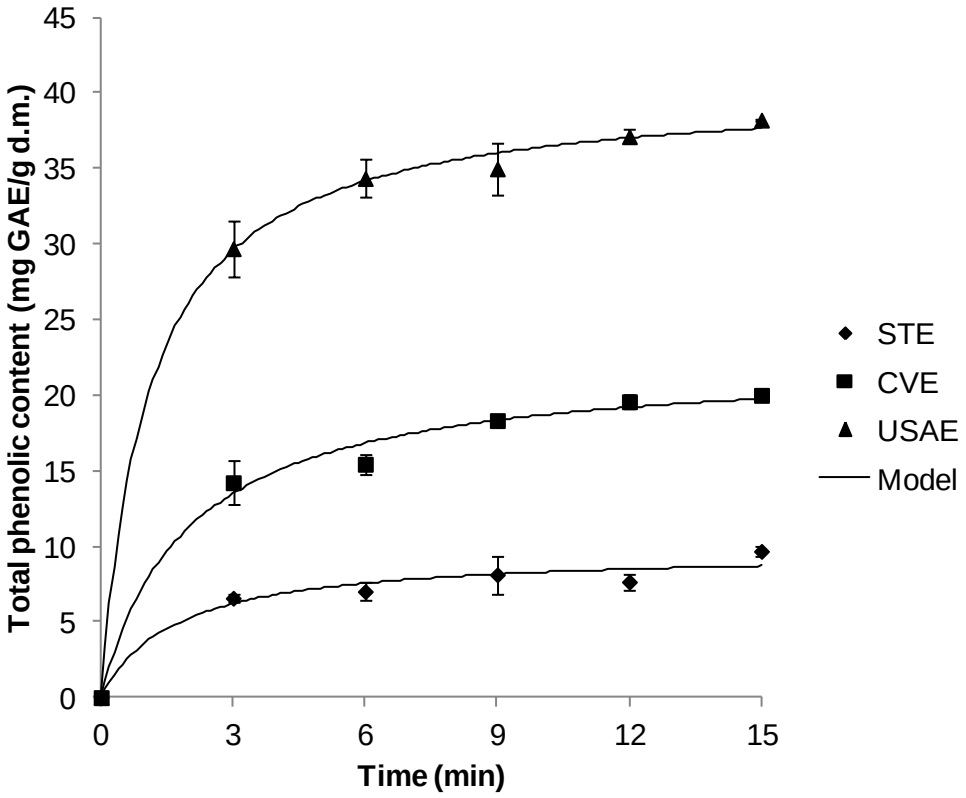
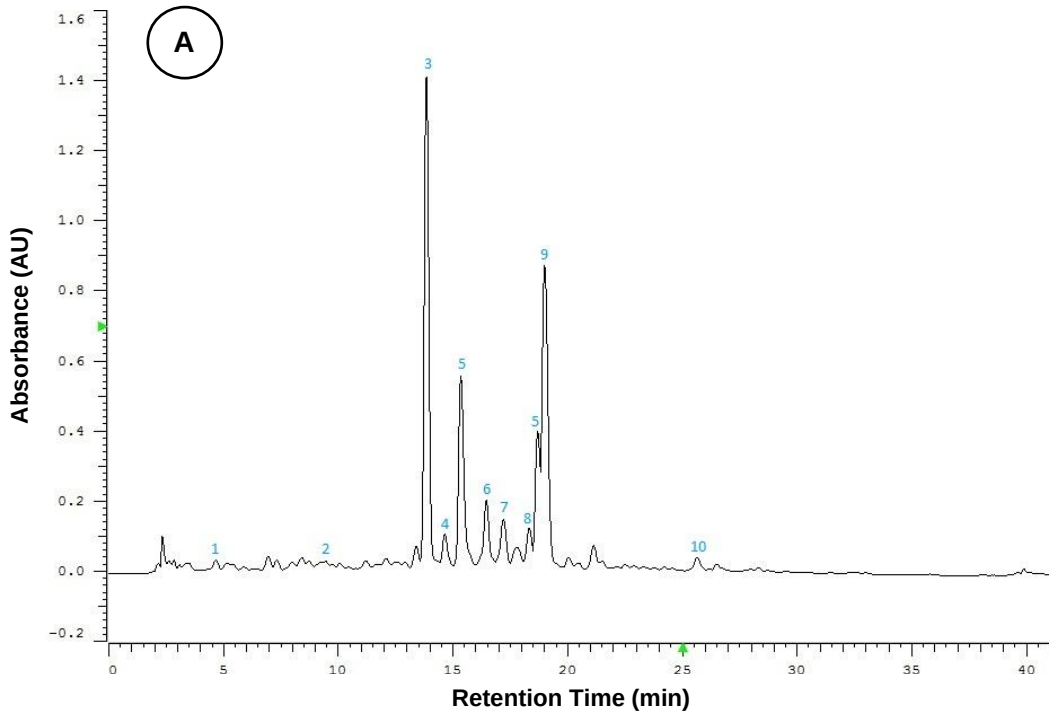
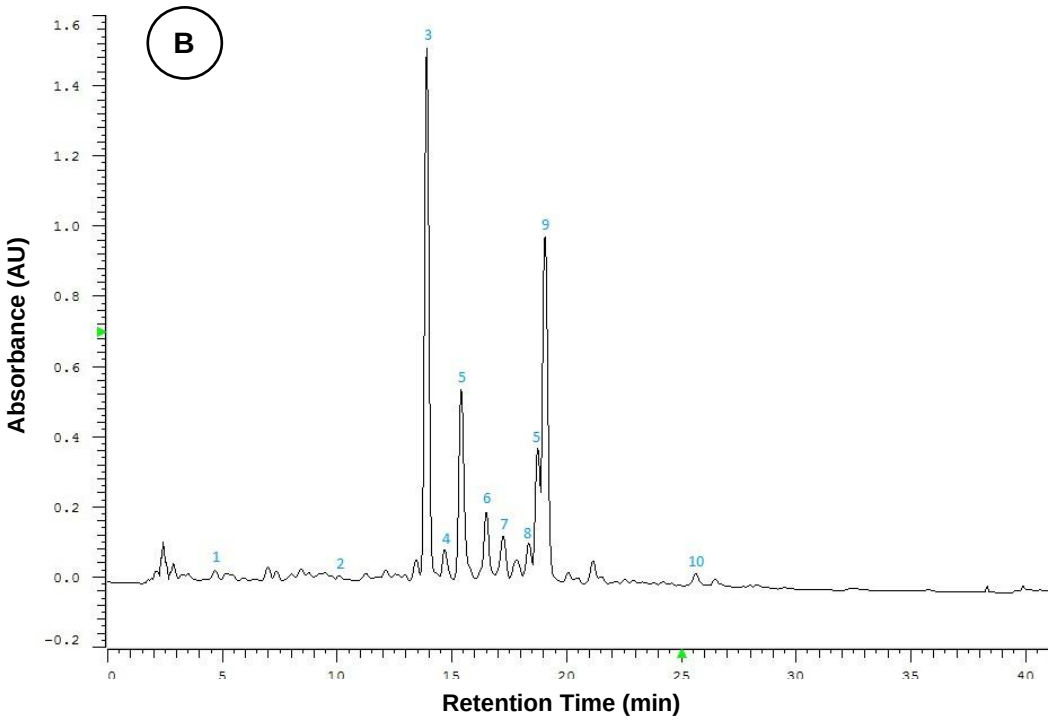

Total phenolic content (mg GAE/g d.m.)
45
40
35
30
25
20
15
10
5
0
0
3
6
9
12
15
Time (min)
STE
CVE
USAE
Model

Figure 6


A
Absorbance (AU)
Retention Time (min)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0
5
10
15
20
25
30
35
40
1
2
3
4
5
6
7
8
9
5
10


B
Absorbance (AU)
Retention Time (min)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0
5
10
15
20
25
30
35
40
1
2
3
4
5
6
7
8
9
5
10