New_possibilities_for_the_valorization_of_olive_oil_by-products

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ABSTRACT

In this contribution, the capabilities of pressurized liquid extraction (PLE) using food-grade solvents, such as water and ethanol, to obtain antioxidant extracts rich on polyphenolic compounds from olive leaves are studied. Different extraction conditions were tested, and the PLE obtained extracts were characterized *in-vitro* according to their antioxidant capacity (using the DPPH radical scavenging and the TEAC assays) and total phenols amounts. The most active extracts were obtained with hot pressurized water at 200ºC (EC_{50} 18.6 μg/ml) and liquid ethanol at 150ºC (EC_{50} 27.4 μg/ml), attaining at these conditions high extraction yields, around 40 and 30%, respectively. The particular phenolic composition of the obtained extracts was characterized by LC-ESI-MS. Using this method, 25 different phenolic compounds could be tentatively identified, including phenolic acids, secoiridoids, hydroxycinnamic acid derivatives, flavonols and flavones. Among them, hydroxytyrosol, oleuropein and luteolin-glucoside were the main phenolic antioxidants and were quantified on the extracts together with other minor constituents, by means of a UPLC-MS/MS method. Results showed that using water as extracting agent, the amount of phenolic compounds increased with the extraction temperature, being hydroxytyrosol the main phenolic component on the water PLE olive leaves extracts, reaching up to 8.542 mg/g dried extract. On the other hand, oleuropein was the main component on the extracts obtained with ethanol (6.156 – 2.819 mg/g extract). Results described in this work demonstrate the good possibilities of using PLE as a useful technique for the valorization of by-products from the olive oil industry, such as olive leaves.

1. INTRODUCTION.

Functional foods are increasingly gaining interest and attention within the food industry. This type of food is able to provide with additional benefits compared to a traditional food. At present, it is possible to find in the market a broad range of these products, including foods claiming antihypertensive, antihypercholesterolemic or antioxidant properties. Nevertheless, a lot of research is nowadays focused on the possible beneficial effects that some natural components might offer if consumed in the diet, such as anti-cancer activities [1,2] or neurodegenerative prevention [3,4], among others. These natural additives are clearly preferred by consumers over their synthetic counterparts. Ideally, in order to develop a new functional food, one or more natural ingredients with demonstrated activity are added to a traditional food in a way in which can exert a substantial beneficial action in the organism [5]. A possibility of obtaining these interesting components is their extraction from natural matrices, such as plants or algae [6,7]. However, another interesting approach is the extraction of such compounds from the food industry by-products, which usually are discarded or employed to produce animal feed. Different food-related by-products have been already studied, and different interesting compounds have been identified in some of them, such as lycopene in tomato by-products [8], isoflavones in soybean by-products [9], polyphenols in pomegranate peels [10], antioxidants in different plants [11], among many others. In this regard, leaves from olive tree (*Olea oleuropaea*) are produced in great amounts as a waste from the olive oil industry which is one of the main food products in the Mediterranean basin. Although the presence of interesting phenolic antioxidants in the olive leaf [12-14] is well known, this by-product is still underemployed. The polyphenols present in the olive leaves have been shown to possess important

antioxidant [15,16], anti-inflammatory [17,18], anti-atherogenic [19] and antimicrobial activities [20], and even possible anti-cancer effect [14,21,22].

On the other hand, pressurized liquid extraction (PLE) is a widely considered advanced extraction technique which is able to efficiently extract interesting compounds from natural matrices using low volumes of organic solvents, if any, as well as producing high extraction yields in short extraction processes. These good capabilities are a result of the particular extraction conditions used in which the extracting solvents are heated at high temperatures but maintained at high pressures in order to keep their liquid state during the whole extraction procedure. This technique has been already successfully applied to the extraction of phenolic antioxidants from different natural matrices [6]. Of particular interest is the application of PLE using water as solvent. In this case, this completely environmentally friendly technique is also called subcritical water extraction (SWE) or pressurized hot water extraction (PHWE). Here, the main variable is the dielectric constant of water (ε), as a measure of its polarity. When water is heated under pressure and its liquid state is kept, the dielectric constant decreases as temperature increased. This decrease on the water polarity may effectively modify its solvent properties, decreasing this parameter to values similar to those presented by some organic solvents, such as ethanol or methanol. Thus, the application of this green technique to the extraction of bioactive compounds from olive leaves could be of great interest, not only for the attaining of these natural active compounds but also for the possibility of re-using an important by-product from the industry. Although this technique has been also briefly explored for the extraction of target compounds from olive leaves [23,24], up to now, there is no published report systematically studying the influence of the different extraction conditions on the extraction of phenolic antioxidants from this material by using only food-grade solvents. Thus, the aim of the

present study was to test the PLE extraction conditions, using ethanol and water as solvents, to produce phenolic-rich antioxidant extracts from olive leaves and to study the phenolic composition of the PLE extracts, using advanced characterization techniques, and their relationship with the tested antioxidant activity.

2. EXPERIMENTAL.

2.1. Samples and chemicals.

Olive tree leaves (variety Hojiblanca) generated as by-products from the olive oil industry were dried and provided by Oleoestepa (Sevilla, Spain). After extraction, cryogenic grinding of the sample was performed under liquid nitrogen. The samples were stored protected from light at 4ºC until their use. 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH, 95% purity), ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), potassium persulfate and caffeic acid were obtained from Sigma–Aldrich (Madrid, Spain), ethanol from VWR BDH Prolabo (Madrid, Spain) and methanol from Panreac Quimica (Barcelona, Spain). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) was provided by Fluka Chemie AG (Buchs, Switzerland). Folin-Ciocalteau phenol reagent and sodium carbonate (Na_2CO_3) were acquired from Merck (Darmstadt, Germany) whereas antioxidant standards, i.e., hydroxytyrosol, luteolin-7-glucoside, apigenin-7-glucoside, oleuropein, quercetin, apigenin and diosmetin were supplied by Extrasynthese (Genay, France). The water used was Milli-Q Water (Millipore, Billerica, MA, USA). For the LC-MS and UPLC-MS/MS analyses, MS grade ACN and water from LabScan (Dublin, Ireland) were employed.

2.2. Pressurized Liquid Extraction (PLE).

Extractions of olive leaves were performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA), equipped with a solvent controller. Two different solvents (i.e., ethanol and water) were used to obtain extracts with different compositions. In order to avoid any possible oxidation effect and to remove the dissolved oxygen, solvents were sonicated for 15 min prior to use. Extractions were performed at four different extraction temperatures (50, 100, 150, and 200 ºC) whereas the static extraction time was maintained for 20 min. An extraction cell heat-up step was carried out for a given time prior to any extraction. The warming up time changed depending on the extraction temperature (i.e., 5 min when the extraction temperature was 50 and 100 ºC, 7 min if the extraction temperature was 150 ºC, and 9 min if the extraction temperature was 200 ºC). All extractions were done using 11mL extraction cells, containing 2 g of sample. When water was used for the extraction, the extraction cell was filled with sand mixture on the top of the sample (3.0 g of sand) to prevent the clogging of the system.

The extracts obtained were protected from light and stored under refrigeration until dried. For solvent evaporation, a Rotavapor R-210 (from Büchi Labortechnik AG, Flawil, Switzerland) was used for the extracts obtained using organic solvents. For water extracts, a freeze-dryer (Virtis Unitop 400 SL, Gardiner, NY, USA) was employed.

2.3. Functional characterization of the PLE extracts.

2.3.1. Trolox equivalent antioxidant capacity (TEAC) assay.

Two *in-vitro* methods were employed to determine the antioxidant capacity of the olive leaves PLE extract. Trolox equivalent antioxidant capacity (TEAC) assay was performed as described by Re et. al. [25], with some modifications. ABTS radical cation

(ABTS$^{\cdot+}$) was produced by reacting 7 mM ABTS with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The aqueous ABTS$^{\cdot+}$ solution was diluted with ethanol for the ethanol extracts and with 5 mM phosphate buffer (pH= 7.4) for the water and water-ethanol extracts, to an absorbance of 0.70 (± 0.02) at 734 nm. Ten microliters of sample (different concentrations) were added to 1 mL of diluted ABTS$^{\cdot+}$ radical solution. After 50 min at 30 °C, 300 μL of the mixture were transferred into a well of the microplate, and the absorbance was measured at 734 nm in a microplate spectrophotometer reader (BioTek Instruments, Winooski, VT, USA). Trolox was used as reference standard and results were expressed as TEAC values (mmol Trolox/g extract). These values were obtained from at least four different concentrations of each extract tested in the assay giving a linear response between 20-80 % of the blank absorbance. All analyses were done at least in triplicate.

2.3.2. DPPH radical scavenging assay.

The other method employed to measure the antioxidant capacity of the obtained extracts was the DPPH radical scavenging method, based on a procedure described by Brand-Williams et al. [26]. Briefly, a solution was prepared dissolving 23.5 mg of DPPH in 100 mL of methanol. This stock solution was further diluted 1:10 with methanol. Both solutions were stored at 4 °C until use. Different concentrations of extracts were tested. Twenty five microliters of these extracts solutions were added to 975 μL of DPPH diluted solution to complete the final reaction medium (1 mL). After 4 h at room temperature, 300 μL of the mixture were transferred into a well of the microplate, and the absorbance was measured at 516 nm in a microplate spectrophotometer reader (BioTek). DPPH-methanol solution was used as a reference sample. The DPPH

concentration remaining in the reaction medium was calculated from a calibration curve. The percentage of remaining DPPH against the extract concentration was then plotted to obtain the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% or EC_{50}. Therefore, the lower the EC_{50}, the higher the antioxidant capacity. Measurements were done, at least, by triplicate.

2.3.3. Determination of total phenols.

Total phenols were estimated in the obtained PLE extracts as gallic acid equivalents (GAE), expressed as mg gallic acid/g d.m. (dry matter) according to the Folin-Ciocalteau assay [27]. The total volume of reaction mixture was miniaturized to 1 mL. Six hundred microliters water and 10 μL of sample were mixed, to which 50 μL undiluted Folin-Ciocalteu reagent was subsequently added. After 1 min, 150 μL of 2% (w/v) Na_2CO_3 were added and the volume was made up to 1.0 mL with water. After 2 h of incubation at 25 °C, 300 μL of the mixture were transferred into a well of the microplate. The absorbance was measured at 760 nm in a microplate spectrophotometer reader (BioTek) and compared to the gallic acid calibration curve (0.025 – 2 mg/mL) elaborated in the same manner. Data were presented as the average of duplicate analyses.

2.4. Chemical characterization of the PLE extracts.

2.4.1. LC-MS characterization of the PLE extracts.

To chemically characterize the PLE extracts obtained, a LC-MS method was used. The instrument employed was an Agilent 1200 liquid chromatograph (Agilent, Santa Clara, CA, USA) equipped with a DAD and autosampler, directly coupled to an ion trap mass spectrometer (Agilent ion trap 6320) via an electrospray interface. To carry out the

analyses, a Hypersil C_{18}-AR column (150 mm×4.6 mm, d.p. 3 μm) (Thermo Scientific, San Jose, CA) was employed using as mobile phases ACN (A) and water (0.1% formic acid, B) eluted according to the following gradient: 0 min, 95% B; 5 min, 95% B; 50 min, 40% B; 53 min, 5% B; 57 min, 5% B; 60 min, 95% B. The optimum flow rate was 0.4 mL/min while the injection volume was 10 μL. The diode array detector recorded the spectra from 200 to 550 nm. On the other hand, the MS was operated under ESI negative ionization mode using the following parameters: dry temperature, 350 ºC; dry gas flow, 9 L/min; nebulizer gas pressure, 40 psi; capillary voltage, 3500 V. The instrument acquired data in the range m/z 90-1200.

2.4.2. Quantification of phenolic antioxidants by UPLC-MS/MS.

The UPLC-MS/MS analyses were carried out using an Accela (Thermo Scientific, San Jose, CA) liquid chromatograph equipped with a DAD and an autosampler. The chromatograph was coupled to a TSQ Quantum (Thermo Scientific) triple quadrupole analyzer via an electrospray interface. The analytical conditions employed consisted of the use of a Hypersil Gold column (50mm×2.1mm, d.p. 1.9μm) (Thermo Scientific) using as mobile phases ACN (0.1% formic acid, A) and water (0.1% formic acid, B) eluted according to the following gradient: 0min, 95% B; 0.35 min, 95% B; 6.5 min, 40% B; 7 min, 5% B; 7.5 min; 5% B; 8 min, 95% B. The optimum flow rate was 0.4 mL/min while the injection volume was 5 μL. The diode array detector recorded the spectra from 200 to 500 nm.

To quantify the antioxidants, the mass spectrometer was operated in the negative ESI multiple reaction monitoring (MRM) with a Q1 and Q3 resolution of 0.7 Da FWHM using scan width 0.010 Da and scan time of 0.040 s. The values corresponding to the

tube lens voltage and collision energy as well of the ion transitions employed for the quantification were optimized for each compound as indicated below.

2.5. Statistical analysis.

Microsoft Excel 2003 Program was employed for statistical analysis of the data with the level of significance set at 95%. One-way analysis of variance (ANOVA) was used to assess statistical differences between extractions. Differences were considered as significantly different at a value of $p < 0.05$.

3. RESULTS AND DISCUSSION.

3.1. Extraction of olive leaves and functional characterization.

To study the capabilities of PLE using food-grade solvents (i.e., water and ethanol) to extract bioactive compounds from olive leaves, different extraction conditions were tested. Four different temperatures were tested, namely 50, 100, 150 and 200 ºC, in order to cover the entire instrument's working range. However, according to previous reports conducted in our lab [28,29], both the extraction time and pressure were always maintained constant at 20 min and 1500 psi, respectively. These works demonstrated that both parameters did not have a statistically significant influence on the result from the extraction. However, the temperature might have a critical influence on the chemical composition and characteristics of the obtained extracts. In order to cover the entire instrument's working range, four different extraction temperatures were tested, i.e., 50, 100, 150 and 200 ºC for the two solvents employed. The obtained extracts were subsequently characterized in terms of extraction yield, antioxidant activity and total phenols content. Data collected from these assays is summarized in Table 1. As it can

be observed, the total extraction yield obtained after the PLE extraction increased as a result of the increment on the extraction temperature. Interestingly, both solvents (ethanol and water) provided with similar extraction yields at the same temperature, although water extracts had a slightly higher extraction yield. In this case, the influence of the temperature is very important, considering that increasing this value from 50 to 200 ºC it is possible to produce ca. 2.5 times more yield. This behaviour was expected and it has been previously reported in the PLE extraction of other natural matrices. An increase on the extraction temperature, while keeping the solvent in the liquid state, produces an enhancement on the solubility of the analytes, an improvement of water diffusivity and a decrease in water viscosity, which allows a better penetration into the matrix. These facts are translated during the PLE process into an increase of the mass transfer rate, and therefore, in an improved extraction yield (C. C. Teo, S. N. Tan, J. W. H. Yong, C. S. Hew, E. S. Ong, J. Chromatogr. A, 1217 (2010) 2484).

As mentioned, two different *in-vitro* assays were used in order to gain insight on the possible antioxidant mechanisms present; the DPPH radical scavenging and the TEAC assay. As it is shown in Table 1, both methods provided with comparable results; in fact, for water extracts, an increment of the antioxidant activity (lower EC_{50} and higher TEAC value) was observed according to an increase in the extraction temperature, being the antioxidant activity at 200°C significantly different ($p < 0.05$) than those obtained at 100 and 150 ºC. However, ethanol extracts presented a maximum of activity at 150 ºC, showing a decrease when the extraction temperature was raised to 200 ºC. Although results for both, water and ethanol, considering the DPPH radical scavenging assay were quite similar, the antioxidant activity of water extracts was much higher against the ABTS radical employed in the TEAC assay; for instance, comparing the

antioxidant activity of extracts produced at 150 ºC using both solvents, it could be seen that water was, by far, more active (more than 2-fold).

In order to find possible correlations between the chemical nature of the extracts and their antioxidant activity, the amount of total phenols was determined using the Folin assay. Data corresponding to these determinations is also shown in Table 1. As it can be observed, the amount of total phenols present on the extracts followed the same trend as the antioxidant activity, with maxima in the water and ethanol extracts at 200 and 150 ºC, respectively. Therefore, the antioxidant activity present on these extracts can be highly correlated to the presence of phenolic compounds. Ethanolic extracts from olive leaves obtained using conventional solvent extraction have also shown strong correlations between their total phenols content and their respective antioxidant activities (E. Altiok, D. Bayçin, O. Bayraktar, S. Ulku, Sep. Purif. Technol. 62 (2008) 342). However, the data obtained for the two sets of antioxidant activity measurements are not mathematically correlated to the total phenols; for instance, similar amount of phenols were determined in the extracts obtained with water and ethanol at 50 ºC (see Table 1), whereas their antioxidant activities were significantly different. It is well known that not only phenolic antioxidant may give a positive response to the Folin assay, but also other components containing phenols groups that might not contribute to the final antioxidant activity. Therefore, this determination provides with useful information regarding the nature of the extracts, but it is not enough to completely characterize them. For this reason, the next step consisted on the chemical characterization of the obtained extract paying special attention to those which presented the highest values of antioxidant activity and total phenols, that is, the extracts obtained with water at 200 ºC and with ethanol at 150 ºC.

298 **3.2. LC-MS characterization of the olive leaves PLE extracts.**

299 A new RP-LC method was optimized, using a slow gradient, and applied to the olive
300 leaves' PLE extracts to attain a good separation of the main components. Figure 1
301 shows the chromatograms (280 nm) corresponding to the most active extracts, that is,
302 ethanolic extract at 150 ºC and water extract at 200 ºC. Besides, Table 2 lists the
303 compounds that could be tentatively identified. The assignment of these components
304 was made according to the information provided for the two detectors connected in
305 series after the separation, the DAD and the MS detector. In fact, combining the
306 information provided by the UV-Vis spectra of the separated compounds as well as the
307 information of their MS spectra and MS/MS fragmentation patterns, it was possible to
308 significantly increase the certainty on the tentative assignments. These data are also
309 shown in Table 2.

310 As it can be observed in Figure 1, all the main peaks separated in these analyses could
311 be identified. Ethanol extract was characterized by the presence of hydroxytyrosol and
312 different secoiridoids, mainly oleuropein and its related compounds, as well as by
313 several flavonoids, whereas the extract produced with water was richer in
314 hydroxytyrosol and contained less flavonoids. Nevertheless, the negative ESI ionization
315 conditions employed for the detection of the phenolic compounds did not allow the
316 identification of the main peak present on the water extracts (peak 1). With the aim to
317 identify this component, positive ESI ionization analysis were carried out. Its analysis
318 under positive ESI conditions revealed the existence of a molecular ion ($[M+H]^+$) at
319 m/z 127.1 that produced a main fragment at m/z 109.1. The MS^3 analysis of this
320 fragment produced an ion at m/z 93.1. The combination of this information with its UV-
321 Vis spectrum, with a maximum of absorbance at 283 nm, provided the tentative
322 identification of this peak as 5-hydroxymethylfurfural. This compound was probably

not naturally present on the analysed samples. In fact, it is well known that 5-hydroxymethylfurfural can be produced as a result of Maillard reaction as well as from dehydration of sugars under strong temperature conditions [30]. Different studies conducted in our lab have previously demonstrated the occurrence of Maillard reaction and other chemical events during PLE extraction processes with water at very high temperatures [31,32]. Therefore, the existence of this compound in the water extract obtained at 200ºC could be a consequence of dehydration of sugars present on the sample at this high temperature (e.g., cellulose) and/or Maillard reaction processes. The close study of the formation of this compound from olive leaves during PLE processes with water at high temperatures as well as its associated activities will be the aim of a forthcoming research.

3.2.1. Secoiridoids.

Among the secoiridoids detected, oleuropein (peak 19) was the main compound in both extracts. This very well known compound present in olive has been described to possess interesting functional properties including antioxidant, anti-inflammatory, anti-atherogenic, anti-cancer and antimicrobial activities, among others [33]. This peak presented a maximum of absorbance at 280 nm and a clear molecular ion m/z 539 ([M-H]-). The fragmentation of this ion produced fragments with m/z 377, 307 and 275 (Figure 2A). Both the molecular ion as well as the fragments confirmed the presence of oleuropein. The fragmentation pattern of this compound is well described, presenting a fragment as a result of the loss of a hexose (m/z 377), and different ions derived from the further fragmentation of the oleuropein aglycon residue (m/z 307, 275). However, besides this main peak corresponding to oleuropein, two adjacent minor peaks could be also detected possessing exactly the same characteristics (see peaks 20 and 21). It has

been previously described the presence of different isomers of oleuropein both in olive fruits and leaves. According to these previous reports [16], peak 21 would correspond to oleuroside, an isomer of oleuropein that differ from it in the position of the olefinic double bond in the elenolic acid moiety. Peak 20, more closely eluting to oleuropein, would correspond to another oleuropein isomer already described [14,34]. Moreover, different detected peaks showed ions at m/z 701.4 (peaks 14 and 18). Their fragmentation patterns were identical. An example of one of them (peak 14) is shown in Figure 2B. These ions showed fragments at m/z 539 that could most probably be derived from the loss of a hexose moiety (162 Da) together with other fragments at m/z 377, 307 and 175 corresponding to those typical from oleuropein and its aglycone. Thus, these two peaks were tentatively identified as oleuropein diglucosides. A similar fragmentation pattern was found for a small peak detected in the olive leaves water extract (peak 22). In this case an ion at m/z 601.6 was detected, presenting the same fragments as the oleuropein diglicosides. Therefore, considering the MS information together with its UV-Vis spectra and retention time, this compound was assigned to an oleuropein derivative. Besides, another oleuropein derivative was also detected and tentatively assigned in the water extracts (peak 11). This peak presented a molecular ion at m/z 555.4. The main fragments produced from this ion presented m/z 537, 403 and 393. The loss of 18 Da could indicate the presence of an OH, whereas the fragment m/z 393 corresponded to the loss of a hexose, therefore being the aglycone. This fragment has been already described in olive derived products as 10-hydroxy oleuropein aglycon. Consequently, peak 11 was assigned to 10-hydroxy oleuropein.

Different compounds closely related to elenolic acid were also detected. Among them, peak 23 was identified as ligstroside. This peak possessed a molecular ion ($[M-H]^-$) m/z 523.4 and presented different characteristic fragments, such as the loss of a hexoside

(m/z 361) or the loss of a C_4H_6O (m/z 291). This latter fragmentation was also produced in the case of oleuropein, as it has been shown above. Moreover, peak 7 could be identified as elenolic acid glucoside, thanks to the detection of an ion at m/z 403.1 and MS/MS fragments at m/z 371, 222 and 179. These compounds had been already determined in olive leaves [14]. The UV-Vis spectra of these two components with maxima at ca. 290 and 321 nm provided further proof of their identity. Lastly, among the different detected secoiridoids, oleoside was also detected (peak 4). This compound, which presented a m/z 389.1 was previously detected in olive pomace [14]; further fragmentation of this ion by MS/MS provided ions at m/z 227, 183, 165 and 121 that confirmed the tentative identification as oleoside.

3.2.2. Tyrosols.

Another important group of compounds found in olive leaves' PLE extracts was formed by tyrosol-related compounds. Among them, hydroxytyrosol was the main component (peak 3). In fact, this was the main identified phenolic compound in water extracts. The identification of this compound was possible thanks to its UV-Vis maximum at 280 nm and m/z 153.9, presenting a typical fragment at m/z 123.8. This fragment corresponded to a loss of the CH_2OH group. Hydroxytyrosol was found in high amounts in both extracts; different important functional properties have been associated to its presence such as antioxidant, anti-inflammatory, antiproliferative or antifungal activities, among others [35-37]. In fact, it has been already shown how olive leaves extracts enriched on hydroxytyrosol were able to exhibit a cell cycle blocking in the G1 phase within human breast cancer cells (Z. Bouallagui, J. Han, H. Isoda, S. Sayadi, Food Chem Toxicol 49 (2011) 179. Besides, in water extracts two related compounds could be also determined; a small peak eluting before hydroxytyrosol was identified as its glucoside (peak 2),

presenting an ion at m/z 315.9 that gave fragments at m/z 153 (loss of hexose) and 123, typical of hydroxytyrosol. On the other hand, another peak presenting the same UV-Vis spectra as hydroxytyrosol was detected possessing a m/z 137.5. Accordingly, this peak was assigned to tyrosol. Finally, another tyrosol-related compound could be detected in both extracts (peak 16). Considering that this peak presented a maximum of absorbance at 280 nm as well as its molecular ion at m/z 195.6 and its relative retention time with respect to hydroxytyrosol, this compound was tentatively identified as hydroxytyrosol acetate. This component has been previously described as a phenolic component of olive oil [38].

3.2.3. Hydroxycinnamic acid derivatives.

Two different hydroxycinnamic acid derivatives could be indentified in the olive leaves extracts. Firstly, compound 6 was tentatively identified as a coumaroyl derivative, considering its particular UV-Vis spectra, matching those from these compounds, and the presence of an important fragment at m/z 163 coming from the molecular ion m/z 491.2 as well as another at m/z 325. This fragment might, therefore, correspond to a coumaric acid glucoside. Besides, other hydroxycinnamic acid derivative, verbascoside, commonly found in olive leaves was also identified on the PLE extracts. This peak presented a molecular ion at m/z 623.5. The MS/MS fragmentation of this ion produced ions at m/z 461 and 325 corresponding to the loss of a hexose moiety and of a rhamnose residue, respectively. This information, together with its UV-Vis spectra allowed the identification of this compound (see Figure 3). Although being a minor component, verbascoside has been demonstrated to significantly contribute to the overall antioxidant capacity of several olive leaves extracts [39].

3.2.4. Flavonoids.

Different compounds included in the group of flavonoids could be detected in the olive leaves' PLE extracts. Among them, the most frequently found was luteolin and its related compounds. In fact, luteolin-diglucoside (peak 8), luteolin-rutinoside (peak 10), and two isomers of luteolin-glucoside (peaks 13 and 17) were found in these extracts. These compounds could be identified thanks to their typical flavone UV-Vis spectra as well as for the detection of molecular ions corresponding to the different glycosides and their related fragments. In Figure 4, an example of the identification and differentiation among these compounds is shown. Different luteolin-glucoside isomers have been described to occur simultaneously [13]. The first eluting peak was identified as luteolin-7-glucoside comparing its retention time to that of the commercial standard. The other luteolin-glucoside isomer could not be unequivocally assigned, although according to previous reports, peak 17 may most probably be lutelin-4-glucoside [13]. On the other hand, the differential fragmentation pattern allowed the identification of peak 8 and 9 as luteolin-diglucoside and rutin (quercetin 3-O-rutinoside), respectively. Although the two peaks presented molecular ions at m/z 609 and eluted closely, the fragmentation of peak 8 produced clear fragments corresponding to luteolin-glucoside (m/z 447) and luteolin aglycone (m/z 285) whereas the MS/MS analysis of m/z 609 at peak 9 provided with fragments with m/z 301 and 179, typical from quercetin. Apigenin-rutinoside (peak 15) could be identified following the same reasoning than the rest of flavonoid glucosides. Finally, the aglycones of luteolin, apigenin and diosmetin could also be detected in the ethanolic extracts. In this case, retention times as well as typical molecular ions and UV-Vis spectra were used in order to conclude their assignment. In general, it has been already demonstrated that the flavonoids present on the composition of olive leaves might have an important contribution to the overall antioxidant capacity

of the extracts [39]. Consequently, although hydroxytyrosol and oleuropein have been pointed out as the main bioactive compounds in this matrix, the importance and influence of the flavonoids present should not be underestimated.

3.3. Quantification of phenolic antioxidants by UPLC-MS/MS.

Once the PLE extracts from olive leaves were chemically characterized, the quantification of some of the main phenolic antioxidants, for which commercial standards were available, was carried out. To do that, the separation method was transferred to a UPLC instrument coupled to a MS equipped with a triple quadrupole analyzer. The use of this detector allowed the attaining of very low LODs thanks to its high selectivity. The selected antioxidants included the main phenolic compounds detected in both, ethanol and water extracts, that is, oleuropein and hydroxytyrosol. Besides, other important phenolic antioxidants detected in the extracts were quantified, namely, luteolin-glucoside, apigenin and diosmetin. On the other hand, other compounds described in olive tree related products, such as caffeic acid, apigenin-glucoside and quercetin, were also quantified although its presence could not be confirmed using the above described method. The UPLC method was adapted to a new gradient using an analytical column with sub 2 μm particles, being possible to have the complete separation of the analyzed antioxidants in less than 8 min. Table 3 lists the quantified compounds together with the detection parameters optimized for their quantification using multiple reaction monitoring (MRM). As it can be observed, the transition between the [M-H]- ion and the corresponding most intense fragment ion was optimized individually, along with the optimum collision energy and tube lens values. Once the transitions were optimized, calibration curves for each compound were constructed, using different concentration ranges, but including, in any case, at least 5

different concentration points. The calibration curves obtained, together with the concentration ranges employed and the LODs and LOQs obtained for each compound are shown in Table 4. As it can be seen, R^2 values higher than 0.993 were obtained for all the quantified antioxidants. The detection limits ranged typically from 0.010 μg/ml for diosmetin to 0.065 μg/ml for caffeic acid. The only compound outside this sensitive range was hydroxytyrosol, for which a LOD of 0.443 μg/ml was obtained due to the background noise obtained for the detection of this peak. The reproducibility of the UPLC method was also very good, with RSD (%) values for retention times always lower than 3.2 %. Figure 5 shows the MRM chromatograms corresponding to the quantified phenolic antioxidants.

Next, the obtained olive leaves extracts were analyzed under the optimum UPLC-MS/MS conditions; results are shown in Table 5. As it can be observed, there is a strong influence of the solvent polarity in the type of compounds extracted by PLE. In general, significantly higher amounts of the more polar phenolic antioxidants, such as hydroxytyrosol and caffeic acid were obtained using water as solvent, whereas ethanol was more selective towards the extraction of less polar flavonoids. Nevertheless, not all the studied compounds had a similar behaviour as a result of the change in the extraction conditions. For instance, it can be seen how the extraction of hydroxytyrosol improved with the increasing temperature for the two solvents tested, although the use of water was, by far, more favourable for the extraction of this potent antioxidant. However, in the case of flavonoids the highest amount recovered with water was found at medium extraction temperatures. At the highest tested temperatures, the amount of these compounds was lower, most probably due to thermal degradations. In fact, observing the recoveries of these compounds when using ethanol, that was the most appropriate solvent, it could be observed how the highest amounts were obtained at the

lowest temperatures tested, thus confirming a degradation at higher temperatures. In general, as it can be observed in Table 5, the highest amounts of the quantified phenolic antioxidants extracted were obtained using water. Although using ethanol high proportions of the less polar phenolic compounds could be obtained, the total recovered amount was always lower as compared with water, at the same extraction temperature, except at 50ºC. Therefore, it could be concluded that the use of water in PLE processes might provide extracts with higher proportions of phenolic antioxidants, mainly hydroxytyrosol and oleuropein. By using ethanol, less amount of the quantified compounds could be obtained but the composition of the extracts was more complex, including significantly less amounts of hydroxytyrosol but higher proportions of flavonoids. As can be seen comparing data obtained for the total phenols quantified (Table 5) with the total phenols measured using the Folin assay (Table 1), trends are in quite good agreement, mainly for water extracts, less complex than ethanol; discrepancies are due to the inability to quantify all phenolic compounds and on the basis of Folin reaction that, as mentioned previously, allows a positive response for many compounds and therefore, it is expected an overestimation of the final results [40].

4. CONCLUSIONS.

In this work it has been demonstrated the capabilities of PLE using food-grade solvents, such as water and ethanol, to obtain antioxidant extracts from olive leaves rich on polyphenolic compounds. The extraction conditions that provide with the best results in terms of antioxidant capacity included the use of liquid water at 200 ºC (ca. 40% extraction yield) and liquid ethanol at 150 ºC (ca. 30% extraction yield). Around 25 different phenolic compounds could be tentatively identified on these extracts by LC-

MS, including phenolic acids, secoiridoids, hydroxycinnamic acid derivatives, flavonols and flavones. Among them, the most important phenolics described on this plant, such as hydroxytyrosol, oleuropein or luteolin-glucoside were found. The quantification of these components by UPLC-MS/MS showed that the amount of the quantified compounds in the water extracts was increased along with the extraction temperature, being hydroxytyrosol the main phenolic compound on the water PLE olive leaves extracts. On the other hand, oleuropein was the main component of ethanolic extracts; in this case, the amount of phenolic compounds recovered decreased with the increasing temperature except in the case of hydroxytyrosol. In conclusion, this work shows the good possibilities of coupling advanced environmentally clean extraction mechanisms to powerful analytical techniques in order to produce and characterize natural antioxidant extracts from by-products from the olive oil industry, such as olive leaves.

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FIGURE LEGENDS.

Figure 1. Chromatograms (280 nm) obtained from the LC-MS analysis of the olive leaves PLE extracts produced with ethanol at 150 ºC (A) and water at 200 ºC (B). For peak identification, see Table 2.

Figure 2. UV-Vis and MS spectra, MS/MS fragmentation pattern and proposed chemical structures for A) oleuropein (peak 19) and B) oleuropein-diglucoside (peak 14).

Figure 3. UV-Vis and MS spectra, MS/MS fragmentation pattern and proposed chemical structures for verbascoside (peak 12).

Figure 4. MS spectra and MS/MS fragmentation pattern of the luteolin-related compounds found in the olive leaves PLE extracts. A) luteolin diglucoside (peak 8), B) luteolin-rutinoside (peak 10), C) luteolin-7-glucoside (peak 13), and D) luteolin (peak 24).

Figure 5. MRM extracted UPLC-MS/MS chromatograms corresponding to a mixture of 0.39 μg/ml of each of the quantified phenolic antioxidants present in the olive leaves PLE extracts.

628 **Table 1.** Values of extraction yield (% dry weight), antioxidant activity (measured by
629 means of DPPH radical scavenging assay, EC_{50} (μg/ml) and TEAC assay (mmol/g)) and
630 total phenols (as mg gallic acid/mg extract) obtained for the different PLE extractions
631 performed at the indicated conditions. Superscripts indicate pairs of values not
632 significantly different ($p > 0.05$).

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Solvent	Extraction Temperature (ºC)	Time (min)	Extraction yield (%)	Antioxidant activity		Total phenols (mg gallic acid/g extract)
				EC_{50} (μg/ml)	TEAC (mmol/g)	
Water	50	20	15.5	39.7 ± 1.7	1.129 ± 0.038	28.3 ± 1.7
	100	20	21.3	27.2 ± 1.3[a]	1.699 ± 0.113[a]	42.8 ± 2.1[a]
	150	20	33.6	29.9 ± 2.7[a]	1.609 ± 0.074[a]	43.4 ± 1.9[a]
	200	20	37.8	18.6 ± 0.4	2.661 ± 0.188	58.7 ± 0.9
Ethanol	50	20	13.5	52.7 ± 5.3	0.273 ± 0.016	26.2 ± 1.1
	100	20	23.0	35.3 ± 2.2	0.536 ± 0.014[c]	33.6 ± 0.2
	150	20	29.0	27.4 ± 0.7	0.677 ± 0.025	45.8 ± 0.6[b]
	200	20	37.5	31.1 ± 0.6	0.573 ± 0.019[c]	43.2 ± 0.9[b]

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Table 2. Identification of antioxidant compounds in the olive leaves PLE extracts. UV-Vis and MS characteristics.

ID	Retention time (min)	Identification	UV-Vis maxima (nm)	[M-H]-	Main fragments detected
1	12.1	5-Hydroxymethylfurfural	283	127.1[b]	109.1, 93.1[b]
2	16.4	Hydroxytyrosol glucoside	280	315.9	153.5, 123.7
3	16.7	Hydroxytyrosol [a]	278	153.9	123.8
4	17.4	Oleoside	176	389.1	226.8, 182.9, 164.9, 121.1
5	21.2	Tyrosol	280	137.5	
6	22.5	Coumaroyl derivative	295s, 310	491.2	325.1, 162.9
7	24.0	Elenolic acid glucoside	296, 321	403.1	371.1, 222.9, 179.0
8	25.1	Luteolin diglucoside	331	609.5	447.2, 285.3
9	27.5	Rutin	340	609.4	300.9, 179.1
10	28.1	Luteolin-rutinoside	340	593.2	285.0
11	28.2	10-hydroxy-oleuropein	280	555.4	403.2, 393.4, 323.3, 291.0
12	28.5	Verbascoside	290, 325	623.5	461.2, 315.1
13	29.3	Luteolin-7-glucoside [a]	346	447.6	284.9
14	29.8	Oleuropein-diglucoside	280	701.4	539.4, 377.4, 307.2, 275.3
15	30.3	Apigenin-rutinoside	335	577.7	269.0
16	31.4	Hydroxytyrosol acetate	280	195.6	151.0, 110.9
17	32.1	Luteolin-glucoside	336	447.8	284.9
18	32.7	Oleuropein-diglucoside	280	701.4	539.2, 377.1, 307.1, 275.0
19	33.3	Oleuropein [a]	280	539.3	377.2, 307.4, 275.7
20	33.9	Oleuropein isomer	280	539.3	377.1, 307.2, 275.6
21	34.1	Oleuroside	280	539.4	377.2, 307.2, 275.4
22	35.1	Oleuropein derivative	280	601.6	539.2, 377.1, 307.1, 275.2
23	36.1	Ligstroside	280, 320s	523.4	361.1, 291.1, 259.4
24	38.0	Luteolin	344	285.7	
25	41.9	Apigenin [a]	332	269.7	
26	42.4	Diosmetin [a]	347	299.9	

[a] Identification confirmed using commercial standards

[b] Parent and fragment ions detected as $[M+H]^+$

s, shoulder

642 **Table 3.** Main optimized parameters for the MS/MS detection of the phenolic
643 antioxidants quantified and the optimum fragmentation values and ion transitions
644 employed for each one.

Compound	Parent ion [M-H]-	Product ion	Collision energy (V)	Tube lens offset (V)
Hydroxytyrosol	153.1	123.151	15	72
Caffeic acid	179.2	135.119	17	65
Luteolin-7-glucoside	447.2	284.95	28	79
Apigenin-7-glucoside	431.2	268.963	25	101
Oleuropein	539.3	377.051	18	113
Quercetin	301.1	151.012	22	79
Apigenin	269.1	117.13	39	75
Diosmetin	299.1	284.028	22	72

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Table 4. Calibration curves and concentration ranges employed for the quantification of the phenolic antioxidants, and limits of detection (LOD) and limits of quantification (LOQ) reached using the optimized UPLC-MS/MS method.

Compound	Tr (min) ± RSD (%)	Concentration range (μg/ml)	Calibration curve	R^2	LOD (μg/ml)	LOQ (μg/ml)
Hydroxytyrosol	1.14 ± 3.2	0.098 – 100	y = 38364x + 84999	0.9952	0.443	1.477
Caffeic acid	2.17 ± 1.2	0.098 – 6.25	y = 431678x + 19330	0.9994	0.065	0.217
Luteolin-7-glucoside	3.14 ± 0.4	0.098 – 100	y = 251541x + 414600	0.9971	0.037	0123
Apigenin-7-glucoside	3.49 ± 0.3	0.098 – 6.25	y = 275193x + 60995	0.9963	0.012	0.041
Oleuropein	3.88 ± 0.4	0.098 – 100	y = 189185x + 440844	0.9980	0.021	0.070
Quercetin	4.22 ± 0.3	0.098 – 6.25	y = 359042x + 50847	0.9970	0.024	0.081
Apigenin	4.73 ± 0.3	0.098 – 6.25	y = 241311x + 67250	0.9956	0.014	0.045
Diosmetin	4.85 ± 0.2	0.098 – 6.25	y = 2658922x + 784558	0.9931	0.010	0.035

Table 5. Quantification of the phenolic antioxidants found in the olive leaves extracts. Concentrations indicated as mg/g extract ± sd. Values are the mean of, at least, three replicates.

Solvent	**Temp. (ºC)**	**Hydroxytyrosol** (mg/g extract)	**Caffeic acid** (mg/g extract)	**Luteolin-7-glucoside** (mg/g extract)	**Apigenin-7-glucoside** (mg/g extract)	**Oleuropein** (mg/g extract)	**Quercetin** (mg/g extract)	**Apigenin** (mg/g extract)	**Diosmetin** (mg/g extract)	**Total** (mg/g extract)
Water	50	3.326 ± 0.224	0.060 ± 0.004	0.824 ± 0.030	0.680 ± 0.013	3.116 ± 0.095	n.d.	0.009 ± 0.000	0.008 ± 0.000	8.023
	100	3.418 ± 0.140	0.015 ± 0.000	1.386 ± 0.039	0.491 ± 0.008	7.993 ± 0.091	0.003 ± 0.000	0.001 ± 0.000	0.001 ± 0.000	13.308
	150	5.930 ± 0.278	0.013 ± 0.000	1.449 ± 0.031	0.071 ± 0.012	5.295 ± 0.123	0.001 ± 0.000	n.d.	< LOQ	12.759
	200	8.542 ± 0.150	0.018 ± 0.002	0.507 ± 0.021	0.012 ± 0.000	4.341 ± 0.090	< LOQ	n.d.	< LOQ	13.420
Ethanol	50	0.351 ± 0.046	0.001 ± 0.000	2.630 ± 0.068	0.475 ± 0.028	6.156 ± 0.083	0.028 ± 0.003	0.040 ± 0.002	0.028 ± 0.001	9.613
	100	0.678 ± 0.075	0.004 ± 0.000	2.778 ± 0.062	0.469 ± 0.044	4.661 ± 0.095	0.077 ± 0.005	0.015 ± 0.002	0.022 ± 0.000	8.704
	150	2.235 ± 0.037	0.008 ± 0.001	2.213 ± 0.107	0.492 ± 0.043	4.509 ± 0.121	0.129 ± 0.002	0.009 ± 0.001	0.016 ± 0.000	9.611
	200	2.519 ± 0.186	0.013 ± 0.001	1.927 ± 0.061	0.398 ± 0.027	2.819 ± 0.053	0.086 ± 0.002	0.032 ± 0.001	0.037 ± 0.001	7.831