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Preparation of polyphenol fine particles potent antioxidants by a supercritical antisolvent process using different extracts of *Olea europaea* leaves

Chandrasekar Chinnarasu*,, Antonio Montes*,†, Clara Pereyra*, Lourdes Casas*, María Teresa Fernández-Ponce*, Casimiro Mantell*, Sangma Pattabhi**, and Enrique Martínez de la Ossa***

*Department of Chemical Engineering and Food Technology, Faculty of Sciences,
International Excellence Agrifood Campus (CeiA3), University of Cadiz, 11510 Puerto Real (Cadiz), Spain
**Department of Environmental Science, PSG College of Arts and Science, Coimbatore, Tamil Nadu 641 014, India
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Abstract−Various extracts from olive leaves have been precipitated by a supercritical antisolvent (SAS) process to evaluate the possibility of producing polyphenol fine particles with controlled size and size distribution. Olive leaves were initially extracted with subcritical fluids using mixtures of CO_2+ethanol at 10% and 50%, by pressurized liquid extraction (PLE) with water, ethanol and a hydroalcoholic mixture (50 : 50) (v/v), and also by conventional ethanol extraction (CE). PLE gave the extract with the highest yield and the best antioxidant activity. SAS precipitation was unsuccessful for the extracts obtained with pressurized water and with the hydroalcoholic mixture (50 : 50) (v/v). The SAS precipitates with the smallest particle sizes were produced from extracts obtained with subcritical fluids. The SAS precipitates obtained after the conventional ethanol extraction of olive leaves showed the best antioxidant activity.

Keywords: Olive Leaves, Supercritical CO_2, Ethanol, Polyphenol Particles, Antioxidant Activity

INTRODUCTION

Complex bioactive compounds from natural raw materials have recently received a great deal of attention for their beneficial effects on human health, low cost and environmentally benign extraction processes used to obtain them. Olive leaves are rich in polyphenolic compounds and these are of great interest due to their wide spectrum of biological activity [1]. Olive leaves contain important potentially bioactive compounds such as oleuropein, hydroxytyrosol, verbacoside, rutin, flavan-3-ols, vanillin, vanillic acid, tyrosol and caffeic acid. Bioactive compounds from olives have been associated with a range of functional roles. For example, such compounds have been reported as antioxidant, anti-ischemic and hypoglycemic agents, and they have also shown anti-HIV activity, anti-tumor activity in breast cancer cells, neuroprotective effects and antinociceptive activity [2-7].

The precipitation of natural antioxidant fine particles from plant leaf extracts is one of the critical steps in natural products research. Supercritical fluids (SCFs) have an impressive record due to their involvement in a variety of chemical phenomena that are not observed in conventional phases [8-10]. Supercritical fluids have diffusivities that are similar to those of gases, and this property results in higher mass-transfer rates. The properties of SCFs (solvent power and selectivity) can be adjusted continuously by altering the temperature and pressure, meaning that the system can be changed between gas-like (lower solvent power and higher selectivity) and liquid-like (higher solvent power and lower selectivity).

Moreover, SCFs can be removed from the experimental process by changing from supercritical to ambient conditions, a possibility that avoids difficult post-process treatments of liquid waste streams. More specifically, the supercritical antisolvent process (SAS) has been widely applied to the precipitation of drugs [11], polymers [12] and natural products [13-16] to give materials with a small particle size (PS) and narrow particle size distribution (PSD). In the SAS process a solution containing the solute of interest is sprayed through a nozzle into a vessel in which CO_2 is continuously flowing. Atomization of the solution into droplets favors diffusion of the supercritical fluid into the droplets, solubilizes the material and expands the solvent, thus leading to a high supersaturation of the solute solution and precipitation of a powder in the form of micro- or nanoparticles.

Supercritical CO_2 (sc-CO_2) is used as an antisolvent because of the relatively mild operating conditions required (T_C 31.1 °C and P_C 73.8 bar). Furthermore, the solvating power of CO_2 is comparatively close to that of organic solvents, and CO_2 is also nontoxic, nonflammable, inexpensive, chemically inert and environmentally safe.

The use of mild extraction techniques is essential prior to the application of the SAS process. In this way Ilbay et al. used ultrasound technology to improve the extraction of olives leaves according to environmentally friendly method [17]. For this reason, we applied two selective and green extraction techniques, namely subcritical fluid extraction (SFE) and pressurized liquid extraction (PLE), in conjunction with the SAS precipitation process. Moreover, conventional ethanol extraction (CE) was also employed prior to the SAS process for the sake of comparison.

In a previous study the authors optimized the precipitation process for olive leaf extracts and obtained spherical microparticles of olive leaf supercritical extract that had potent antioxidant activity

†To whom correspondence should be addressed.
E-mail: antonio.montes@uca.es

[13]. Nevertheless, to the best of our knowledge, a study of the SAS precipitation of olive leaf extracts obtained with different solvent systems has not been reported to date. It is feasible that modification of the extraction method may lead to changes in the properties of the precipitates, e.g., morphology, particle size and antioxidant activity, produced in the subsequent SAS process.

The aim here was to identify a suitable extraction method for use in conjunction with the SAS precipitation technique, with particular emphasis on morphology, PS and PSD. The antioxidant activities of the SAS precipitates were evaluated by a DPPH assay/UV-Visible spectrophotometry.

EXPERIMENTAL

1. Plant Materials

Olea europaea leaves were collected in 2013 in the region of Jaén (Spain). The leaves were desiccated to constant weight at ambient temperature and were stored in a freezer in the absence of light. Prior to extraction the leaves were ground in a Bosch 6,000 W grinder with a sieve of approximately 5 mm.

2. Solvents and Reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH) reagent was purchased from Sigma-Aldrich (Steinheim, Germany). CO_2 with a maximum purity of 99.8% was obtained from Linde (Spain). Ethanol (HPLC grade) was purchased from Panreac (Barcelona, Spain). Double-distilled Milli-Q grade water was used in all experiments.

3. High Pressure Extraction

High pressure extractions were performed in a high pressure apparatus purchased from Thar Technologies (Pittsburgh, PA, USA, model SF 1000). A schematic diagram of the equipment used in this work is shown in Fig. 1. The experimental set-up was described in detail in our previous publications [13,18]. The vessel was filled with approximately 150 g of olive leaves and the solvent mass/feed ratio (S/F) was around 24 for all extractions. The extracts were recovered in a cyclonic separator and then collected in brown glass bottles.

Two high pressure extraction methods were evaluated: subcritical fluid extraction (SFE) and pressurized liquid extraction (PLE). SFE was performed using CO_2 plus ethanol as co-solvent (ethanol is a GRAS solvent, i.e., 'generally recognized as safe') under the following experimental conditions: pressure of 100 bar and temperature of 55 °C for 3 h. The total flow rate was 20 g/min and two percentages of ethanol were evaluated (10% and 50%). The extraction conditions were selected bearing in mind the results of previous studies by the authors, which showed that a low pressure (100 bar) and relatively high temperature (55 °C) are most suitable to recover antioxidant compounds such as polyphenols and anthocyanins from several raw materials [18-20].

PLE was carried out using water, ethanol and a hydroalcoholic mixture (50 : 50 v/v ethanol/water). The operating conditions were a temperature of 100 °C, a pressure of 120 bar and a total flow rate of 20 g/min for 3 h. These extraction conditions were selected on the basis of previous results [20,21]. In PLE the pressure does not have a significant influence on the extraction yield, and the use of high temperatures (100-120 °C) increases the yield of the process. However, a temperature of 100 °C ensures that the extracts have a high antioxidant capacity [22].

4. Conventional Ethanol Extraction (CE)

The olive leaf extract was prepared by heating 100 g of olive leaves in 500 mL of ethanol at 30 °C for 12 h. The extract was filtered under vacuum and stored in brown glass bottles prior to assay.

5. Supercritical Antisolvent Precipitation (SAS)

A schematic diagram of the SAS equipment, which was purchased from Thar Technologies (model SAS200), is shown in Fig. 2 and the experimental procedure was described previously [11,20]. All experiments were carried out under the same operating conditions: pressure (P) of 150 bar, temperature (T) of 50 °C, CO_2 mass flow rate (Q_{CO_2}) of 20 g/min, sample solution flow rate (Q_L) of 2 mL/min with a nozzle diameter (θ_n) of 100 μm and a washing time (t_w) of 60 min.

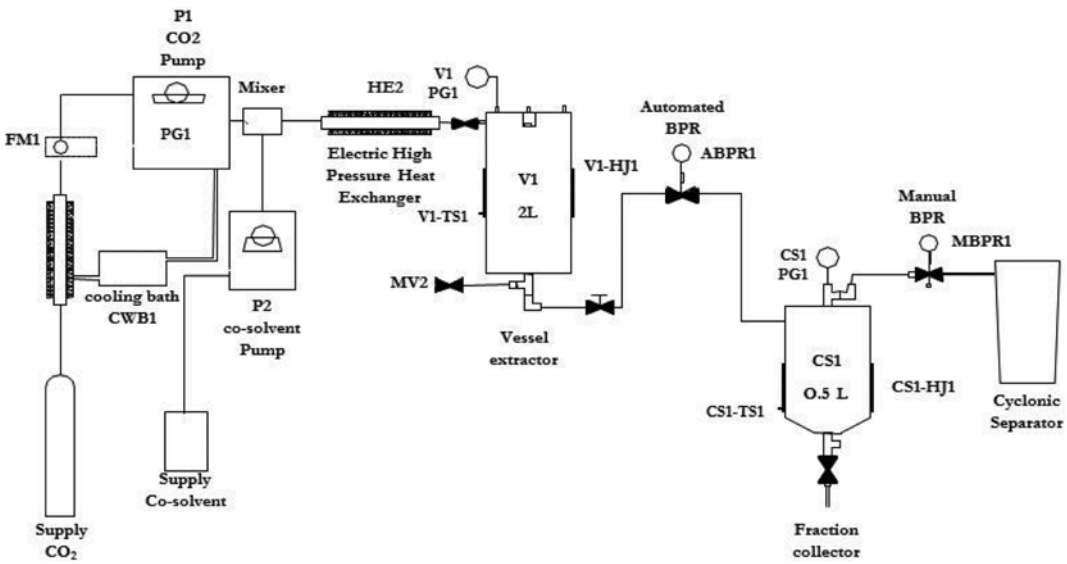

Fig. 1. Schematic diagram of the subcritical fluid extraction (SFE) process.

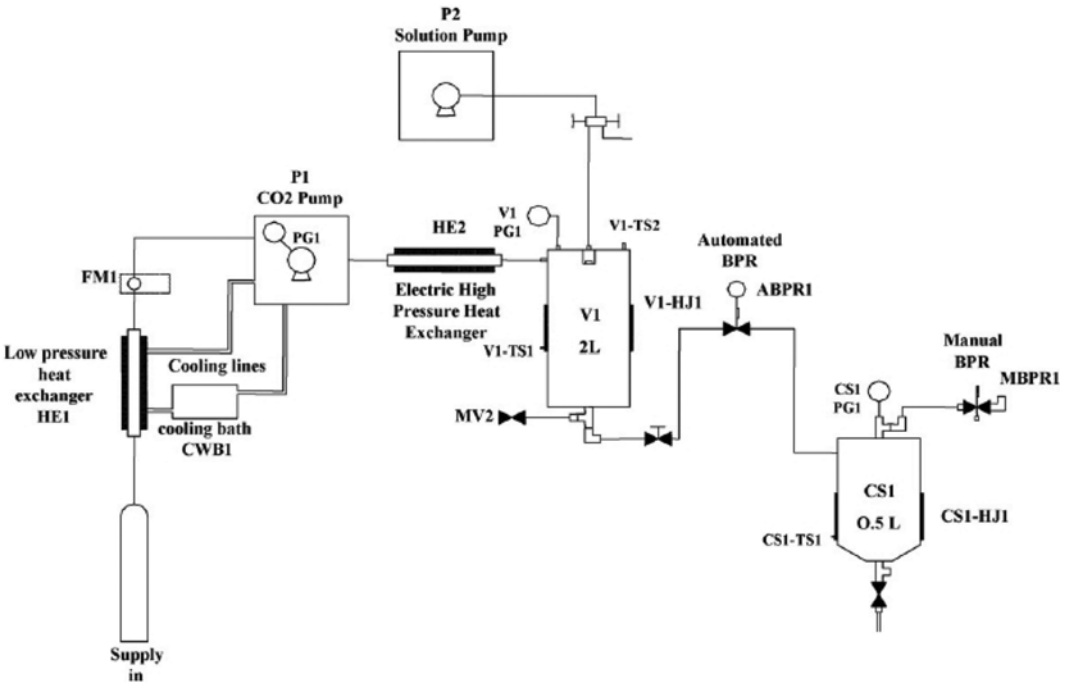

Fig. 2. Schematic diagram of the supercritical antisolvent (SAS) precipitation process.

For the SAS process the extracts were used as produced, except for the water and hydroalcoholic extracts, which were evaporated to dryness and redissolved in ethanol because the use of water is not recommended in the SAS process.

Experiments were carried out according to the following procedure: CO_2 was pumped into the vessel and when supercritical conditions had been achieved, the sample solution was also pumped into the vessel and sprayed through the nozzle. The small drops of solvent were dissolved by the supercritical CO_2, causing supersaturation of the liquid solution and consequent precipitation of the olive leaf antioxidants (OLA) in the form of a powder. The precipitate was recovered from both the inner wall and the frit of the precipitator vessel.

6. Sample Characterization

A small portion of the extract was evaporated to dryness on a rotary evaporator (IKA® RV 10) at 40 °C and subsequently studied by scanning electron microscopy to analyze the morphology of the extract prior to the SAS process.

Scanning electron microscopy (SEM) images of the dry olive leaf extract (OLE) and the SAS-processed olive leaf fine particles were obtained on a QUANTA 200 SEM system. Prior to analysis, the dry OLE and OLA fine particles were placed onto tape and then covered with a coating of gold using a sputter coater. The SEM images were processed using Scion image analysis software (Scion Corp.) to obtain the particle sizes. The mean particle size and particle size distribution, as a measure of the width distribution, were calculated using Statgraphics plus 5.1 software. About 300 particles were counted in each experiment.

High pressure liquid chromatography (HPLC) was carried out on an Agilent HPLC series 1100 system (Agilent, Germany) equipped with a degasser, a quaternary pump, an autosampler and a UV/vis detector. ChemStation® HP software was used for data analysis. A Lichrospher 100 RP-18 column (5 μm) (Agilent Technologies, Germany) was employed. Gradient elution was carried out with solvent A (water/2% acetic acid) and solvent B (methanol/2% v/v acetic acid) at a constant flow rate of 0.45 mL/min. A linear gradient profile with the following proportions (v/v) of solvent B was used [t (min), % B]: (10, 10), (18, 20), (20, 20), (30, 40), (40, 50), (50, 100). Finally, washing and reconditioning steps for the column were included as follows [t (min), % B]: (95, 100), (97, 100). The injection volume for all samples was 20 μL. Compounds were detected at 280 nm according to the retention time.

X-ray diffraction (XRD) analysis was performed on a Bruker D8 Advance diffractometer to determine the amorphous or crystalline nature of the precipitates obtained by the SAS process. All diffraction patterns were scanned from 2° to 50° in 2θ angles with a step size of 0.02° and 1 s as the step time.

7. Antioxidant Activity Assay with DPPH

The antioxidant activity of the extracts and the corresponding SAS-precipitated microparticles was determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical assay. The DPPH assay is based on the methods described by Brand-Williams [24] and Scherer and Godoy [25]. In this procedure 0.1 mL aliquots of ethanolic solutions of samples or standard at various concentrations were added to 3.9 mL of 6×10^{-5} mol/L ethanolic DPPH solution. The absorbance was monitored spectrophotometrically at 515 nm at 0 min and every 2 min until the reaction reached the steady state. All tests were performed in triplicate. The DPPH concentration (C_{DPPH}) in the reaction medium was calculated from a calibration curve determined by linear regression with Eq. (1):

$$Abs=12.709.\ C_{DPPH}+0.002 \quad (1)$$

The percentage of DPPH remaining was calculated by using Eq. (2):

$$\% \text{ DPPH remaining} = C_{DPPHt}/C_{DPPHo} \times 100 \quad (2)$$

The EC_{50} (effective concentration that provides 50% inhibition) was calculated graphically using a non-linear curve fitting by plotting the sample concentration vs. % DPPH remaining at the steady state. The antioxidant activity is expressed as the Antioxidant Activity Index (AAI), which was calculated by considering the final concentration of DPPH and the EC_{50} of the compound tested in the reaction, as shown in the following equation:

$$\text{AAI} = \text{Final concentration of DPPH } (\mu g/mL)/EC_{50}\ (\mu g/mL) \quad (3)$$

The final concentration of DPPH was calculated with respect to the concentration of DPPH in the reaction medium. The antioxidant activity is considered to be poor when AAI≤0.5, moderate when AAI is between 0.5 and 1.0, strong when AAI is between 1.0 and 2.0, and very strong when AAI≥2.0 [19].

RESULTS AND DISCUSSION

1. Extraction of Olive Leaves

In this study high pressure extraction techniques (SFE and PLE) and conventional extraction were compared. SFE is an efficient technique that is widely applied to the separation of active compounds from natural products [8]. This technique is widely used due to the very high solvent power and the distinctive physicochemical properties of supercritical fluids (SCFs). The relatively low viscosity (near to the value for the gas) and the high diffusivity of SCFs help these solvents to penetrate porous solid materials more efficiently than liquid solvents, thus resulting in faster and more efficient extractions [20]. PLE involves the use of hot pressurized liquid solvents, including water or ethanol, below their critical point. The use of solvents at high temperature and high pressure enhances the extraction performance in comparison to processes carried out at room temperature and atmospheric pressure [21].

The extraction yields and antioxidant capacities of the olive leaf extracts are listed in Table 1. In SFE, ethanol was used as a CO_2 modifier to increase the solubility of the polar compounds with antioxidant capacity. The extraction yield was found to increase on increasing the amount of ethanol from 10% to 50% (see Table 1).

Table 1. Extraction yields and antioxidant capacities of olive leaf extracts obtained by different extraction methods

Extraction methods	Extraction yield (%)	Antioxidant capacity (μg DPPH/μg extract)
SFE		
CO_2+10% ethanol	6.43	0.44
CO_2+50% ethanol	13.02	0.65
PLE		
Water	26.54	1.22
Ethanol	34.86	0.66
Ethanol-water 50 : 50	36.51	1.37
CE	21.75	0.63

SFE: 100 bar, 55 °C at 20 g/min for 3 h
PLE: 120 bar, 100 °C at 20 g/min for 3 h
CE: atmospheric pressure, 30 °C during 12 h

In addition, the antioxidant capacity of the extracts increased from 0.44 to 0.65 μg DPPH/μg extract. The addition of co-solvent (ethanol) increased the solvent power of CO_2 and this led to high extraction yields and improved the quality of the extracts. Co-solvents such as alcohols induce dipole/dipole interactions, phenol-alcohol interactions and hydrogen bonding with polar groups in the solute-matrix, thus increasing the solubility of polar compounds [26,27].

The use of PLE was evaluated in an effort to increase the yield and the antioxidant activity of olive leaf extracts. Different solvent compositions, namely pure ethanol, ethanol/water (50 : 50) and water, were studied in order to select the most suitable solvent system. It can be seen from the results in Table 1 that there was a significant increase in the yield (26-36%) on changing from ethanol to ethanol/water mixtures. Furthermore, extracts obtained with water and the hydroalcoholic mixture showed strong antioxidant properties (1.22 and 1.37 μg DPPH/μg extract, respectively).

The extraction with the ethanol/water mixture gave the highest yield (36.86%) and also the extract with the highest antioxidant activity (1.37 μg DPPH/μg extract). This behavior has been reported by several authors [20,21] and can be explained as being due to the use of a binary mixture, particularly a mixture of an organic solvent and water, which improves the extraction efficiency because the organic solvent enhances the solubility of the analyte and water increases the analyte desorption.

The yield obtained by CE and the antioxidant capacity of the extract were lower than those obtained on using PLE. Moreover, CE requires long extraction times and this causes degradation of the active compounds.

2. SAS Precipitation

The SAS precipitation results are shown in Table 2. Four of the six experiments (1, 2, 5 and 6) led to the successful precipitation of olive leaf antioxidants. The extracts obtained with pressurized water and the hydroalcoholic mixture (50 : 50) (v/v) were not suitable for the SAS process because supercritical CO_2 is not a very effective solvent for aqueous solutions, which in turn means that the anti-solvent effect of CO_2 would be weak [28,29]. For this reason, these extracts were dried and subsequently dissolved in ethanol before the SAS process was employed. In any case, runs 3 and 4 were unsuccessful in the SAS precipitation - possibly because the extracted compounds have a non-negligible solubility in the CO_2/solvent mixture and the difference between the solubility of the compounds

Table 2. SAS precipitation of olive leaf antioxidants (experimental conditions: (P) 150 bar; (T) 50 °C; (QCO_2) 20 g/min; (Q_L) 20 mL/min; (θ_n) 100 μm and (t_w) 60 min)

Extraction methods	Runs	Success	PS (μm)	Antioxidant capacity (μg DPPH/μg extract)
SFE				
CO_2+10% ethanol	1	+	0.34±0.07	0.84
CO_2+50% ethanol	2	+	0.32±0.08	1.14
PLE				
Water	3	–	-	-
Ethanol-water 50 : 50	4	–	-	-
Ethanol	5	+	1.97±1.08	1.11
CE	6	+	0.55±0.32	1.56

and this mixture is very small, which leads to failure of the precipitation [30].

Images of the precipitates recovered from a single phase on the inner wall of the precipitator and nozzle are shown in Fig. 3. The morphologies of the unprocessed dried extracts and their corresponding SAS-precipitated spherical microparticles, which have mean particles sizes in the range 0.30 to 1.97 μm, are shown in Figs. 4(A)-(D). The PSD of each SAS precipitate is shown in the corner

Fig. 3. Photographs of (a) wall precipitator and (b) nozzle with OLA fine particles.

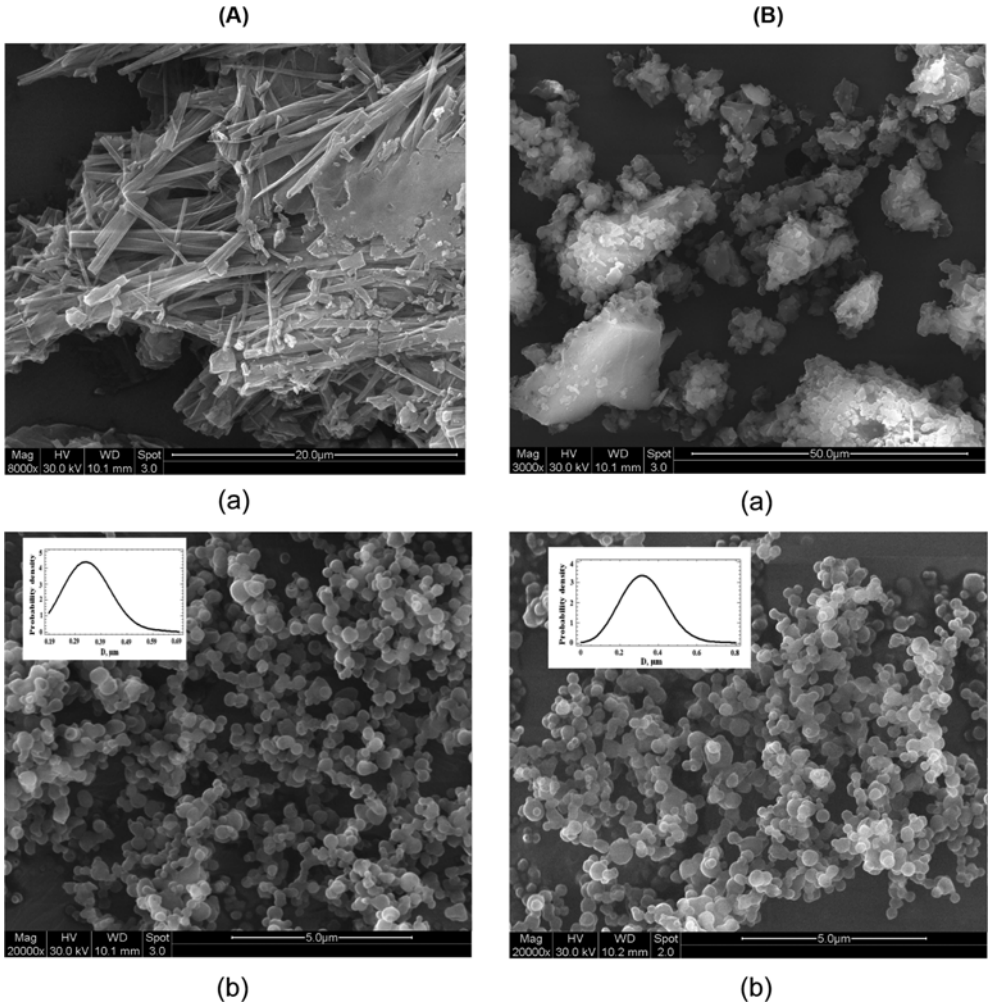

Fig. 4. (A) SEM images of (a) SFE-10% ethanol dry extract (b) SAS processed OLA fine particles. (B) SEM images of (a) SFE- 50% ethanol dry extract (b) SAS processed OLA fine particles. (C) SEM images of (a) Pressurized ethanol extraction dry extract (b) SAS processed OLA fine particles. (D) SEM images of (a) CE dry extract (b) SAS process OLA fine particles.

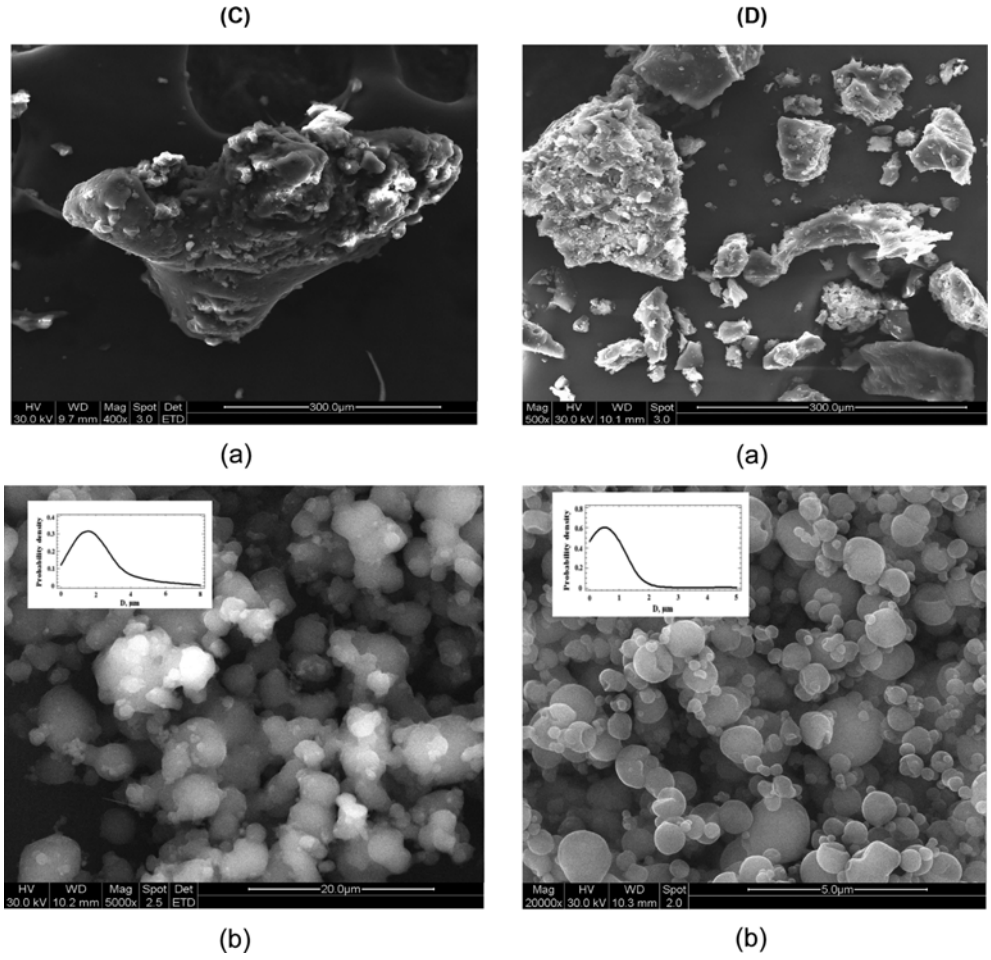

Fig. 4. Continued.

of the SEM image (Figs. 4(A)-(D)). Olive leaf extracts obtained by SFE led to SAS-precipitated particles with the smallest PS (0.32-0.34 μm) and the narrowest distribution (runs 1, 2 and 6). By contrast, the precipitated particles obtained from the pressurized ethanol extract presented the highest PS (1.97 μm) and the broadest size distribution (run 5).

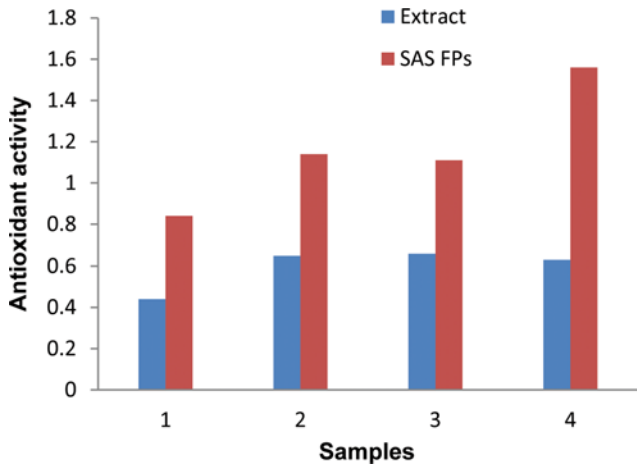

Fig. 5. Antioxidant activities of the extracts and OLA fine particles: (1) SFE-10% ethanol, (2) SFE- 50% ethanol, (3) pressurized ethanol extraction and (4) CE methods.

3. Antioxidant Activity of OLE and OLA Fine Particles

Olive leaf extracts and SAS precipitates were analyzed for their radical scavenging activity by the DPPH assay. The antioxidant activity of the extracts and precipitates is represented in Fig. 5. The OLA fine particles showed greater antioxidant activity than the respective extracts. However, numerous differences were found depending on the extract used. For example, the highest antioxidant activity was observed for the SAS precipitate formed from the sample that was obtained by CE, followed by those obtained by SFE using 50% ethanol, pressurized ethanol and SFE using 10% ethanol.

Chromatographic analysis of the extracts indicated that the majority of the compounds extracted are the same regardless of the extraction process employed. Oleuropein, with a retention time of around 30 min, was identified as a major component in all of the samples. A family of compounds with a retention time of around 38 min, possibly flavone glycosides, was also detected in all extracts. This family of compounds generally had a lower antioxidant activity than oleuropein. It can be seen from Fig. 6(a) that oleuropein, which has a high antioxidant activity, is more concentrated in the material obtained by SFE using 50% ethanol than in the corresponding sample obtained using 10% ethanol because the peak at 38 min,

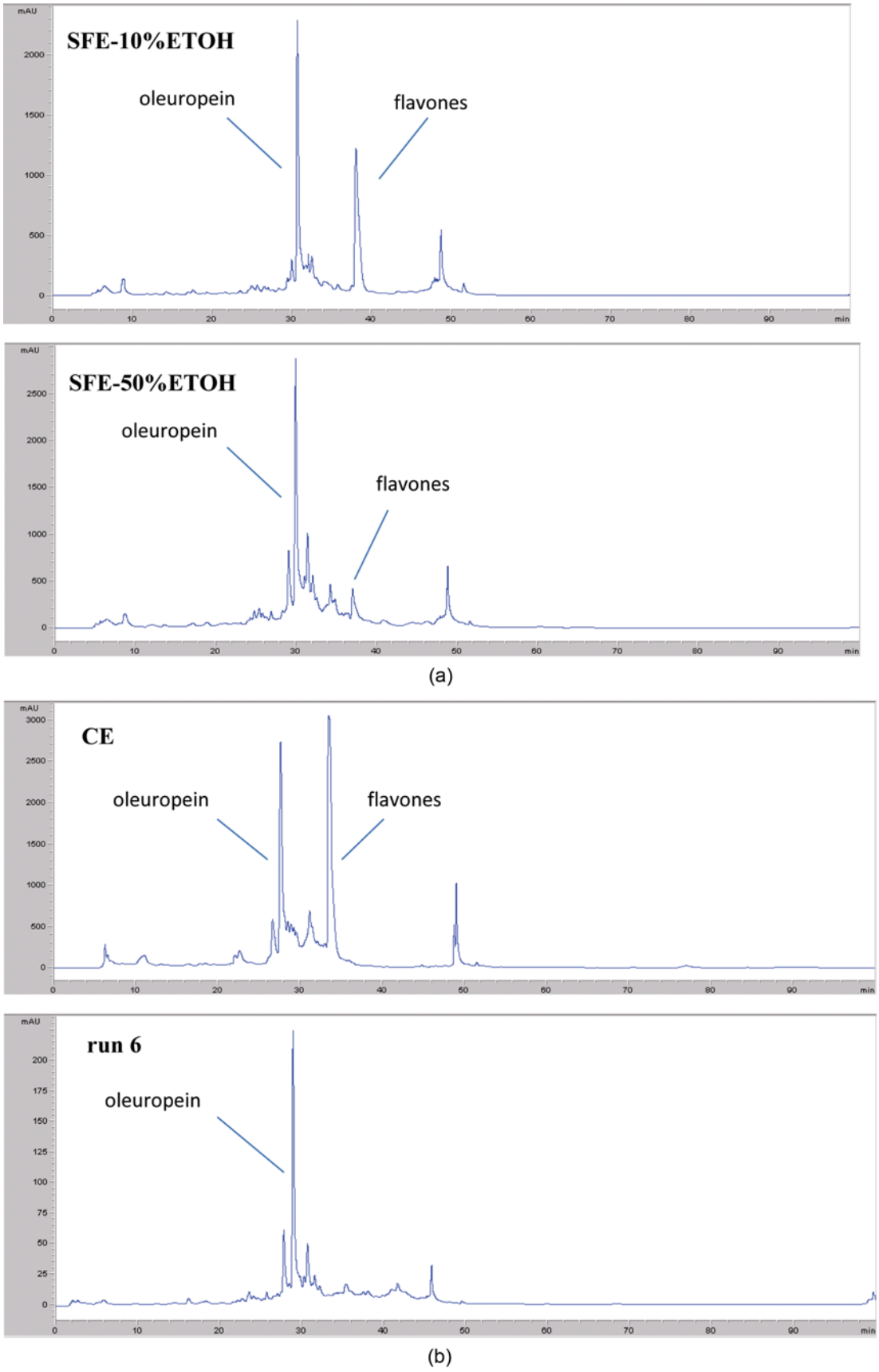

Fig. 6. (a) HPLC chromatograms at 280 nm of SFE with different percentages of co-solvent. (b) HPLC chromatograms at 280 nm of CE and OLA fine particles (run 6).

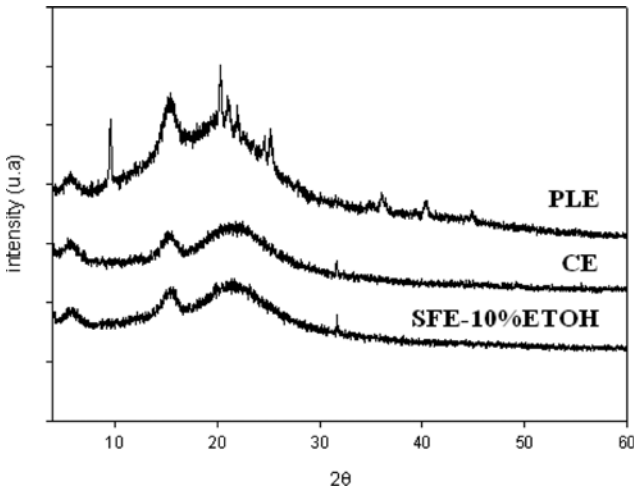

Fig. 7. XRD patterns of SAS processed fine particles.

corresponding to flavone glycosides, was markedly smaller. This finding explains the higher antioxidant activity of the extract obtained by SFE using 50% ethanol than that obtained using 10% ethanol [26,31]. The addition of greater quantities of ethanol with CO_2 is recommended in SFE to concentrate compounds with strong antioxidant activity, e.g., oleuropein.

Extracts obtained with pressurized ethanol and by CE, in which ethanol is also used, had strong antioxidant activity because the contents of flavone glycosides were lower. A comparison between the chromatograms of SAS precipitates and their respective original extracts shows that the flavones with a retention time of around 38 min are not present in the precipitates, which become more concentrated in oleuropein (Fig. 6(b)). Taking into account the conditions under which the chromatograms were obtained, the less polar compounds would have longer retention times. These less polar compounds would not be expected to precipitate in the SAS process as they would be removed by the CO_2 and solvent. This situation was also described in a previous publication concerning a preliminary study on the SAS precipitation of olive leaf extracts [13].

X-ray diffractograms of SAS-processed extracts obtained by PLE, SFE and CE are shown in Fig. 7. It can be seen that microparticles resulting from PLE have more crystalline regions than the others. The amorphous or crystalline nature of a sample can be related to the dissolution rates so samples with a more amorphous nature (SFE and CE microparticles) would have faster dissolution rates than the samples with a more crystalline nature (PLE microparticles) [32]. This fact can be exploited in cases where low solubility limits absorption in that an amorphous character may improve the dissolution.

CONCLUSIONS

The study reported here focused on the use of green solvents (CO_2, C_2H_5OH and H_2O) for the extraction of olive leaves and the subsequent supercritical antisolvent (SAS) precipitation process. In general, pressurized liquid extraction gave the highest extraction yields followed by conventional extraction and subcritical extraction. The SAS process was successful on using extracts obtained with CO_2 and ethanol. Nevertheless, olive leaf extracts obtained by extraction with water and hydro-ethanol extraction were not suitable for the SAS process. In comparison to SAS precipitates obtained after the subcritical extraction of olive leaves, the SAS precipitates obtained from conventional extracts of olive leaves gave higher antioxidant activity. This finding is probably related to the high concentration of oleuropein (higher antioxidant activity) and lower levels of flavone glycosides (lower antioxidant activity) in the precipitates. The addition of greater quantities of ethanol with CO_2 is recommended in subcritical extraction in order to concentrate compounds such as oleuropein, which have strong antioxidant activity. SAS precipitates obtained from subcritical fluid extracts of olive leaves led to the smallest particle size and narrowest particle size distribution, and these had moderate and strong antioxidant activities. Microparticles obtained from samples prepared by CE and SFE have fewer crystalline regions than PLE microparticles and this enables faster dissolution rates to be achieved.

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LIST OF SYMBOLS

Nd : nozzle diameter [m]
QCO_2 : molar flow rate of carbon dioxide [mol/s]
QL : liquid solution flow rate [m^3/s]
P : pressure [Pa]
PS : particle size [m]
T : temperature [K]

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