Concentrated Oleuropein Powder from Olive Leaves using Alcoholic Extraction and Supercritical CO_2 Assisted Extraction

Lucia Baldino[1], Giovanna Della Porta[2], Libero Sesti Osseo[1], Ernesto Reverchon[1,*], Renata Adami[1]

[1] Department of Industrial Engineering, University of Salerno, Via Giovanni Paolo II, 132, 84084, Fisciano (SA), Italy

[2] Department of Medicine, Surgery and Dentistry "Scuola Medica Salernitana", University of Salerno, Via Salvatore Allende, 1, 84081, Baronissi (SA), Italy

J Supercritical Fluids, revised form

** Corresponding author: Ernesto Reverchon; ereverchon@unisa.it; +39086964116;*

www.supercriticalfluidgroup.unisa.it

Abstract

In this work, a simple approach at oleuropein (OLE) extraction/concentration from olive leaves was proposed. OLE extraction yield was 5.4% w/w of dry leaves and a concentration of OLE of 20% w/w in the ethanol extract was obtained. Then, supercritical antisolvent extraction (SAE) was performed on the ethanol OLE solution, operating at different pressures between 100 and 200 bar and temperatures between 35 and 60 °C. SAE produced a powder rich in OLE up to 36% w/w at 35 °C, 150 bar. Precipitated powder showed different mean diameters dependent on SAE operating conditions: at 60 °C, 130 bar it consisted of micrometric and coalescing particles; at 35 °C, 150 bar it consisted of nanometric non coalescing particles. Therefore, OLE concentration was successful and its bioavailability should be improved by nanometric size of precipitates.

Keywords: Oleuropein; Olive leaves; Ethanol extraction; Supercritical Antisolvent Extraction; Supercritical CO_2.

1. Introduction

Olive leaves are byproducts of olive farming; they are attracting scientific and industrial interest due to their chemical composition. In particular, the presence of phenolic compounds, including flavones (luteolin-7-glucoside, apigenin-7-glucoside, diosmetin-7-glucoside, luteolin and diosmetin), flavonols (rutin), flavan-3-ols (catechin), substituted phenols (tyrosol, hydroxytyrosol, vanillin, vanillic acid and caffeic acid) and secoiridoids (oleuropein), makes olive leaves appealing for pharmaceutical and nutraceutical industry [1]. Oleuropein (OLE) is the most abundant among the different phenolic compounds and is also considered the most active; indeed, it exhibits anti-ischemic, antioxidative, hypolipidemic, antiviral, antimicrobial, antiatherogenic, cardioprotective, antihypertensive and anti-inflammatory properties [2-4].

OLE is generally extracted from olive leaves by organic solvents [5-7] or water [8]_ENREF_6. However, the traditional extraction techniques suffer of many disadvantages, such as: the use of large quantity of toxic organic solvents and low selectivity; moreover, these techniques pollute both the environment and the final product and the extracts generally contain low concentrations of the active compounds or they can be partly degraded. Malik and Bradford [9] showed that even temperatures around 60 °C can produce degradation of the active compounds contained in olive leaves. Water extraction is interesting since it does not use a polluting agent; nevertheless, this kind of process is not selective with respect to the components of interest (especially oleuropein) and high operating costs are connected to the vacuum, required to work at lower temperatures.

On the contrary, supercritical CO_2 (SC-CO_2) extraction (SFE) has been demonstrated to be particularly effective to concentrate active principles present in vegetable matter [10]. Due to the SC-CO_2 extraction selectivity, some authors attempted this process to obtain OLE and, generally speaking, phenolic compounds present in olive leaves. Le Floch et al. [11] tried the extraction of phenols from ground olive leaves using SC-CO_2 modified with 10% v/v methanol, operating at 334 bar, 100 °C. However, the extraction yield was only 45% with respect the one obtained using liquid methanol. Sahin et al. [12] also processed by SFE dried and comminuted olive leaves in the range

100-300 bar, 50-100 °C. When SC-CO_2 alone was used, only small OLE amounts were extracted. Therefore, the authors added to SC-CO_2 co-solvents like ethanol, methanol and water at 20% v/v. The largest OLE yield was obtained operating at 300 bar, 100 °C and using methanol 20% v/v. Soxhlet method was also used for comparison purposes: the efficiency was excellent; but, the extracts had an unpleasant smell, due to the long extraction time and high temperature, that probably produced the decomposition of some compounds. Xynos et al. [13] used SC-CO_2 plus 20% w/w ethanol, selected as the most useful combination of SC-solvent and co-solvent for OLE extraction, operating at 300 bar and 50 °C. After drying and lyophilization, they obtained an extract at 30% w/w OLE. Instead, using 5% w/w ethanol, no OLE was detected in the extracts. However, the overall process was not successful, considering the procedure suggested by the authors at the end of their work; they proposed to use SC-CO_2 extraction, to remove lipids and chlorophylls from the vegetable matter, followed by pressurized liquid extraction (PLE) using subcritical water to continue to the extraction of the polar compounds, achieving an OLE recovery of 4.6%.

The use of co-solvents to improve CO_2 performance in SFE, especially when high percentages of co-solvent are used (typically 20% v/v in the previous works), does not resolve the problems related to the use of organic solvents. Indeed, even if smaller quantities of these solvents are used to improve the solvent power of SC-CO_2, they drastically reduce the process selectivity, producing the co-extraction of undesired compounds. Moreover, the extracts still require complex post-processing to separate the liquid solvent from the solid extraction products [13]. Finally, the use of high process temperatures (up to 150 °C) induces the decomposition of thermolable compounds contained in the extracts.

Chinnarasu et al. [14] followed a different approach: first extracted olive leaves using SC-CO_2 plus ethanol 20% w/w at 100 bar, 55 °C; then, they used the extract diluted in ethanol to perform SC-CO_2 antisolvent extraction. This last process is similar to the well known supercritical antisolvent micronization (SAS) [15] and has been frequently named supercritical antisolvent extraction (SAE) [16]. Only the scope of the process is different: SAS is generally aimed at

producing micrometric or nanometric powders of single compounds; whereas, SAE is mainly used to eliminate the solvent in a single process step from complex mixtures, after traditional extraction. These authors, during SAE, used ethanol extracts with concentrations of 16 and 32 mg/mL and operated at pressures from 100 to 200 bar and temperatures between 35 and 60 °C. Different CO_2 and liquid solution flow rates were also used. The experiments performed at 16 mg/mL did not produce a precipitate; i.e., the solutes were extracted by the mixture ethanol+CO_2. The tests performed at 32 mg/mL, produced small sub-microparticles that resulted to be more concentrated in antioxidant compounds than the starting material, since some compounds, that not participate to the antioxidant activity, were co-extracted during SAE experiments. The limits of this work are that the authors performed two complex steps of SC-CO_2 processing and only measured the overall increase of the antioxidant activity of precipitates; but, did not quantify the OLE content before and after processing.

Considering the analysis of the previous literature, in this work, a simpler and different approach at oleuropein extraction/concentration from olive leaves is proposed. The first part of the process is a traditional liquid-solid extraction of ground olive leaves. Ethanol will be used as selected solvent, since it is not harmful and can be efficiently eliminated from the final product. Then, the liquid ethanol extracts is fed to SAE apparatus, where ethanol-SC-CO_2 solution is formed and OLE is concentrated in a precipitated powder. This overall process scheme, eliminates the expensive SC-CO_2 extraction step proposed by Xynos et al. [13], in which a very large mass of the starting vegetable matter is treated using SC-CO_2. The new process arrangement should be very efficient and less expensive when finalized to industrial scale.

2. Materials and methods

Olive leaves were supplied by Planta Medica s.r.l. (Pistrino di Citerna (PG) - Italy), with a water content of 8% w/w. They were accurately ground using a mill (Waring Commercial Blender) at room temperature, obtaining a final mean diameter of about 1 mm. Ethanol (purity >99.8%) was

purchased from Sigma-Aldrich; oleuropein standard 99.9% was given by Extrasynthese (Lyon - France). CO_2 (purity 99.9%) was supplied by Morlando Group s.r.l. (Sant'Antimo (NA) - Italy).

Liquid extraction using ethanol

37.5 g of ground olive leaves were contacted with 400 mL ethanol in an agitated vessel at room temperature (20 °C). Then, samples of the solution were drawn at fixed time intervals (30 min each, for 40 h) to measure OLE content. In particular, they were filtered and evaporated in a rotary evaporator (model R-210, Buchi, Assago (MI) - Italy) at 30 °C under vacuum. The calibration curve was obtained using the oleuropein standard and was used for quantitative measurement of the OLE content in the extracts and precipitates. OLE percentage in the extracts was expressed as (mass of OLE in the extract)/(mass of initial dried sample used)x100.

Supercritical fluid antisolvent extraction

A membrane high-pressure pump (mod. LDB1 M210S, LEWA, Mazzo di Rho, Italy) was used to deliver liquid CO_2 and an HPLC pump (mod. 305, Gilson, Middleton, USA) was used to feed the liquid solution. The precipitator was a stainless steel vessel (V= 490 mL, i.d.= 50 mm) and a 180 μm nozzle diameter on the top of the precipitator allowed the injection of the liquid solution. A stainless steel filter with an overall porosity of 0.1 μm, located at the bottom of the precipitator, allowed the collection of the powder. A pressure reduction valve (mod. 60VRMM4882, Autoclave engineering, Pennsylvania, USA), operating at 25 bar and a separator, located downstream the precipitator, were used to recover the liquid solvent and non precipitated materials; then, ethanol was recovered by evaporation. A simplified process scheme is reported in Figure 1.

To perform SAE experiments, first, CO_2 was pumped to the precipitator at fixed temperature until the desired condition of pressure was reached; then, the pressure was regulated by a micrometering valve located between the precipitator and the separator. When a constant flow rate of CO_2 was established, pure solvent was sent through the nozzle to the precipitator until steady

state conditions for solvent and antisolvent system were reached. At this point, the delivery of the ethanol solution started. The fast extraction of the solvent by SC-CO_2 produced the precipitation of the solute. At the end of the ethanol solution delivering, the precipitator was purged for 30-40 min with pure SC-CO_2 at the process conditions, to wash away the solvent solubilized in the supercritical antisolvent. Finally, the precipitation vessel was depressurized and the precipitated compounds were collected.

Identification of phenolic compounds

The separation and identification of phenolic compounds were performed by HPLC-UV/Vis method with an Agilent HPLC series 1100 instrument, equipped with ChemStation software. A C_{18} LiChrospher column (150 mm x 3.0 mm i.d., 5 μm, Phenomenex) was operated at room temperature. The mobile phase consisted of two solutions: 2.5% acetic acid in water (solution A) and 10% acetonitrile (solution B); the gradient used for separation was: 95% (A) and 5% (B), 75% (A) and 25% (B) for 20 min; 20 min at 50% (A) and (B); 10 min at 20% (A) and 80% (B); 10 min (total time 60 min) to return at the starting composition. The injection volume was 20 μL with a solvent flow of 1 mL/min; the elution time was 60 min at isobaric conditions. The OLE detection was performed at 280 nm; the calibration curve to determine OLE amount was y= 4.21*x.

OLE powder was sputter coated with Gold (Agar Auto Sputter Coater mod. 108 A, Stansted, UK) at 30 mA for 120 s and analyzed by a FESEM (Field Emission Scanning Electron Microscope, mod. LEO 1525, Carl Zeiss SMT AG, Oberkochen, Germany) to study particle morphology.

Particle size distributions (PSDs) of the OLE powder were measured from FESEM photomicrographs using the Sigma Scan Pro image analysis software (release 5.0, Aspire Software International Ashburn, VA); approximately 1000 particles were analyzed for each sample.

Ethanol residues were measured using a gas chromatograph equipped with a flame ionization detector (GC-FID, mod. 6890 GC-SYSTEM, HP, Agilent Technologies Mfg. Gmbh & Co. KG, USA). GC conditions were: oven temperature of 60 °C for 5 min and increase to 200 at 20 °C/min.

The injector was maintained at 250 °C, the detector was maintained at 280 °C and nitrogen was used as the carrier gas (15 mL/min). Samples were prepared in 10 mL vials loaded with 950-1050 mg of OLE particles plus 5 mL of internal standard and diluted to 10 mL with dimethylformamide (DMF). The internal standard was prepared by dissolving 40 mg of absolute ethanol in DMF to obtain 100 mL; 2.5 mL were, then, diluted to 25 mL with DMF. Analyses were performed in triplicate.

DSC (DSC 30 Mettler, Toledo) was performed on the oleuropein standard, in the temperature range between 25 and 150 °C, with a heating rate of 10 °C/min; the inert gas was nitrogen, feed at a flow rate of 50 L/min.

3. Results and discussion

Liquid extraction results

Liquid extraction of ground olive leaves was performed as described in the previous part of the work and extraction kinetics were obtained. Extraction time was defined looking at the extraction yield kinetics. After 40 h contact between ethanol and olive leaves, no significant increase of the extraction yield was found; i.e., yield asymptotized to the value reported in this work. The overall quantity of the extract was 2.0 g, corresponding to 5.4% w/w of the starting vegetable matrix. The extracted OLE was 0.4 g, namely 20% w/w of the ethanol extract.

SAE results

SAE can be used to process the alcoholic extract; ethyl alcohol can be selectively extracted in a single step operation by SC-CO_2, leaving a dry powder. Operating in this way, SC-CO_2 is used as the antisolvent, instead of as the solvent in supercritical processing. Considering this process from another point of view, it is the supercritical version of the conventional liquid solvent-liquid antisolvent crystallization/precipitation with all the advantages connected to the formation of a supercritical solution between ethanol and CO_2, obtaining a solute practically solvent free. Given

this explanation, the phenolic extract obtained using ethanol was used to perform the subsequent SAE precipitation, looking at the recovery of a dry micronized precipitate, possibly enriched in OLE.

Preliminarily, a DSC analysis was performed on the OLE standard: the result is that OLE starts to degrade at temperatures larger than about 55 °C, as shown in Figure 2. For this reason, in this work, all the experiments were performed at low temperatures and only one experiment was performed at 60 °C. In the following part of the work, lower processing temperatures will be considered as preferable when similar process performances will be obtained.

A series of SAE experiments was performed on the ethanol OLE solution, changing the operative pressure between 100 and 200 bar, temperature between 35 and 60 °C. SC-CO_2 flow rate was fixed at 40 g/min and liquid solution flow rate at 1 mL/min, corresponding to a CO_2 molar fraction of 0.98. These process conditions allow to operate SAE over the mixture critical point (MCP) of the solution ethanol-CO_2; therefore, complete solubilization of ethanol in SC-CO_2 is assured. A selection of the most significant SAE experiments performed, is reported in Table 1.

T (°C)	**P (bar)**	**CO_2 density (g/cm^3)**	**OLE (% w/w) ± 0.8%**
35	150	0.82	36
40	100	0.62	27
	150	0.78	29
	200	0.84	36
60	130	0.51	27

Table 1. Operative conditions adopted for SAE experiments, the corresponding CO_2 density and OLE concentration (% w/w) in the final product.

SAE precipitate had a pale green color, due to the presence of trace of chlorophyll. Some SAE experiments were also performed at 150 bar, 35 °C, changing the molar fraction of CO_2 (x_{CO2}) from 0.98 to 0.85; but, they were unsuccessful since less uniform precipitates were obtained at the lower

x_{CO2} values and the product was partly extracted. This result could be expected since, at these CO_2 molar fractions, the operation was performed within the miscibility hole for the system CO_2-ethanol under pressure.

Precipitate composition, with respect to phenolic compounds, was analyzed by HPLC, as described in Materials and Methods. The major compounds identified in the extract and in the SAE precipitate were OLE, hydroxytyrosol, -7-glucosides of luteolin, apigenin and verbascoside. These results are in general agreement with the previous literature [17]; however, OLE was largely the most abundant phenolic compound in these products and it was, therefore, assumed as the reference during all the analyses performed in this work.

Precipitates morphology was observed by SEM. Two examples of particles morphology are reported in Figure 3 and are referred to the experiments performed at 60 °C, 130 bar and at 35 °C, 150 bar. The particles related to the experiment performed at 60 °C, 130 bar (lower CO_2 density) were micrometric with an irregular morphology and showed coalescence phenomena; the mean diameter of the single microparticles was approximately estimated as 1.6 μm (Figure 3a). On the contrary, concerning experiments performed at 35 °C, 150 bar (higher CO_2 density), particles collected were clearly nanometric, with a mean diameter of about 360 nm (Figure 3b). This result is in agreement with the general knowledge of supercritical antisolvent precipitation: the higher is CO_2 density, the smaller are the particles collected. Moreover, coalescence of the particles, observed in the test performed at 60 °C, 130 bar, could also depend on the instability of the precipitated material, since, as previously shown, OLE degradation starts at about 55 °C.

Particle size of SAE precipitates can be relevant in view of a possible application, since, the smaller is particle size the larger is the bioavailability of the corresponding nutraceutical/pharmaceutical formulation, due to the faster dissolution in water based systems of difficult to solubilize compounds. More specifically, nanometric precipitates show a very large surface area that should maximize their solubilization rate.

Another very interesting result reported in Table 1 is that the percentage of OLE in the precipitates largely increases with CO_2 density used during SAE, whereas the green color of the powder becomes lighter. The explanation of this result is relatively simple: when CO_2 density is increased in SAE experiments, its solvent power towards small molecular weight, non-polar or slightly polar compounds increases; therefore, larger quantities of undesired compounds are extracted from the starting solution and, correspondingly, the concentration of phenolic compounds in the precipitates increases; operating in this way, not only the extract is precipitated in a simple one step process in submicro/nanoparticles, but also a partial fractionation of the extract is performed. Indeed, the liquid collected downstream the precipitator (ethanol plus extracted compounds) showed a bright green color related to the presence of chlorophyll, but also showed very small concentration of phenolic compounds. These last observations are even more interesting considering that OLE percentage in the ethanol extract is about 20% w/w; whereas, its concentration in the final product is, at the highest densities tested in this work, 36% w/w; i.e., about 1.8 the value in the starting solution. It is also useful to remember that 20% w/w also represents the maximum OLE content in the commercial products.

Solvent (EtOH) residue in the precipitate was also measured and was always smaller than 500 ppm, that is largely lower than the limit residue of EtOH allowed in pharmaceutical compounds [18]. This result confirms the efficiency of SAE in the elimination of the organic solvents from vegetable extracts and pharmaceutical compounds, that was observed yet in other scientific papers [16, 19].

4. Conclusions

The adoption of the hybrid processing scheme formed by traditional ethanol extraction from olive leaves, followed by SAE precipitation, confirmed to be effective in concentrating powders, containing up to 36% w/w OLE; moreover, the powder was formed by nanometric quasi-spherical particles.

A best operating temperature of 35 °C can be indicated for OLE concentration in the final product, due to the necessity of operating at temperature largely lower than 60 °C, identified as the temperature at which OLE degrades.

Very low ethanol residues were measured in the final product, confirming the effectiveness of SAE.

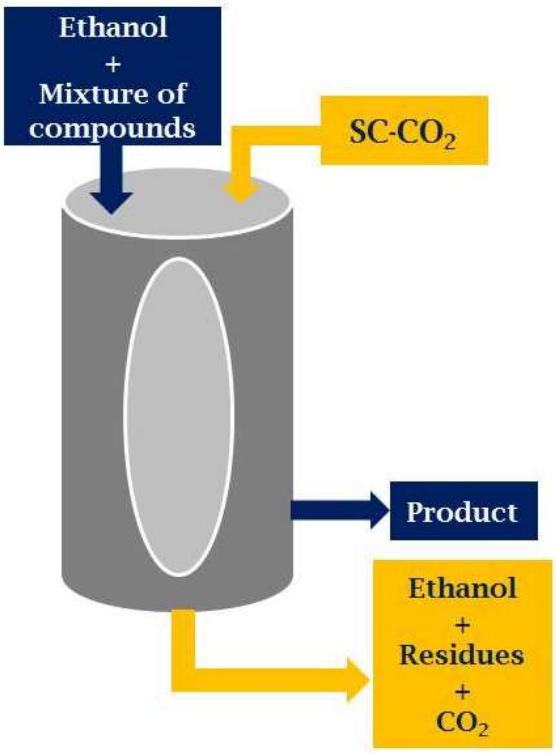


Figure 1. Simplified process scheme.

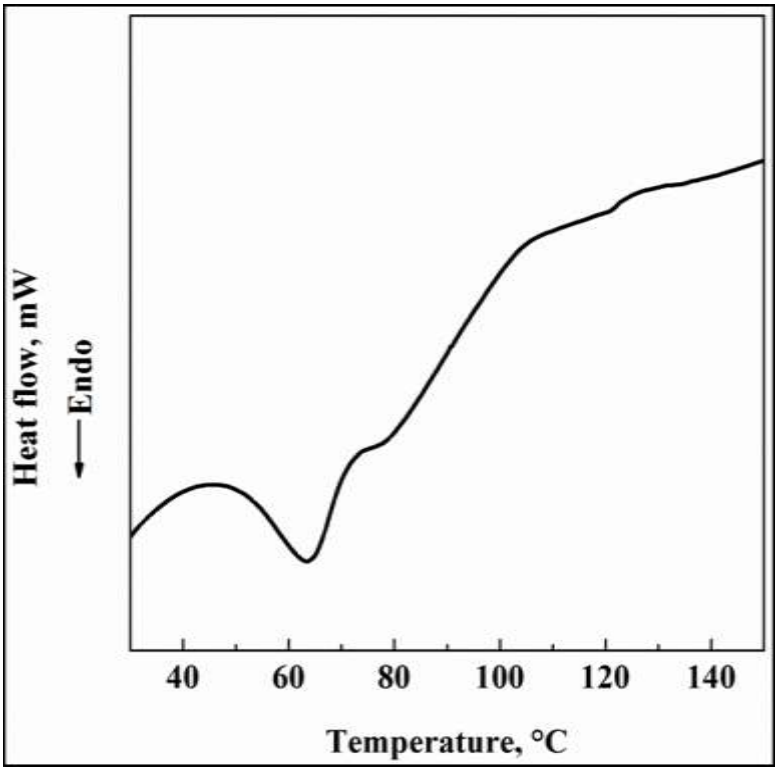
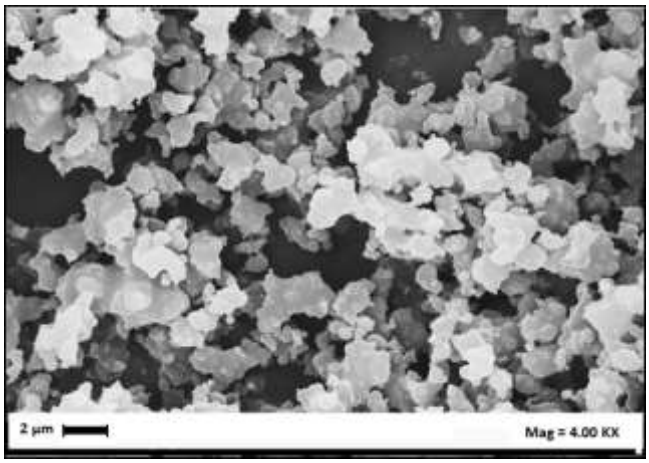
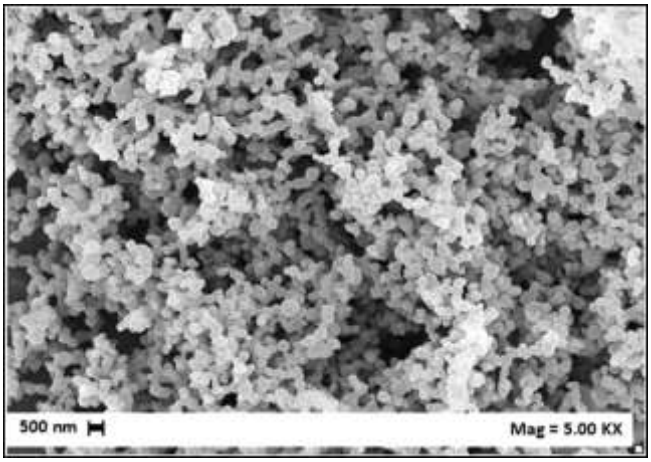


Figure 2. OLE standard thermogram.



(a)



(b)

Figure 3. FESEM images of powder obtained in the experiments performed at: a) 60 °C and 130 bar; b) 35 °C and 150 bar.

References

[1] N. El Sedef, S. Karakaya, Olive tree (Olea europaea) leaves: potential beneficial effects on human health, Nutr. Rev. 67 (2009) 632-638.
[2] I. Singh, M. Mok, A.-M. Christensen, A.H. Turner, J.A. Hawley, The effects of polyphenols in olive leaves on platelet function, Nutr., Metab. Cardiovasc. Dis. 18 (2008) 127-132.
[3] H. Jemai, A. El Feki, S. Sayadi, Antidiabetic and Antioxidant Effects of Hydroxytyrosol and Oleuropein from Olive Leaves in Alloxan-Diabetic Rats, J. Agric. Food Chem. 57 (2009) 8798-8804.
[4] I. Andreadou, E.K. Iliodromitis, E. Mikros, M. Constantinou, A. Agalias, P. Magiatis, A.L. Skaltsounis, E. Kamber, A. Tsantili-Kakoulidou, D.T. Kremastinos, The olive constituent oleuropein exhibits anti-ischemic, antioxidative, and hypolipidemic effects in anesthetized rabbits, J. Nutr. 136 (2006) 2213-2219.
[5] C. Savournin, B. Baghdikian, R. Elias, F. Dargouth-Kesraoui, K. Boukef, G. Balansard, Rapid high-performance liquid chromatography analysis for the quantitative determination of oleuropein in Olea europaea leaves, J. Agric. Food Chem. 49 (2001) 618-621.
[6] E. De Marco, M. Savarese, A. Paduano, R. Sacchi, Characterization and fractionation of phenolic compounds extracted from olive oil mill wastewaters, Food Chem. 104 (2007) 858-867.
[7] A. Guinda, A. Lanzón, J. Rios, T. Albi, The isolation and quantification of the components from olive leaf: hexane extract, Grasas y Aceites 53 (2002) 419-422.
[8] R. Japón-Luján, M.D. Luque de Castro, Superheated liquid extraction of oleuropein and related biophenols from olive leaves, J. Chromatogr. A 1136 (2006) 185-191.
[9] N.S. Malik, J.M. Bradford, Recovery and stability of oleuropein and other phenolic compounds during extraction and processing of olive (Olea europaea L.) leaves, J. Food, Agric. Environ. 6 (2008) 8.
[10] E. Reverchon, I. De Marco, Supercritical fluid extraction and fractionation of natural matter, J. Supercrit. Fluids 38 (2006) 146-166.
[11] F. Le Floch, M. Tena, A. Rıos, M. Valcarcel, Supercritical fluid extraction of phenol compounds from olive leaves, Talanta, 46 (1998) 1123-1130.
[12] S. Şahin, M. Bilgin, M.U. Dramur, Investigation of oleuropein content in olive leaf extract obtained by supercritical fluid extraction and soxhlet methods, Sep. Sci. Technol. 46 (2011) 1829-1837.
[13] N. Xynos, G. Papaefstathiou, M. Psychis, A. Argyropoulou, N. Aligiannis, A.-L. Skaltsounis, Development of a green extraction procedure with super/subcritical fluids to produce extracts enriched in oleuropein from olive leaves, J. Supercrit. Fluids 67 (2012) 89-93.
[14] C. Chinnarasu, A. Montes, M. Fernández-Ponce, L. Casas, C. Mantell, C. Pereyra, E.M. de la Ossa, Precipitation of antioxidant fine particles from Olea europaea leaves using supercritical antisolvent process, J. Supercrit. Fluids 97 (2015) 125-132.
[15] E. Reverchon, I. De Marco, R. Adami, G. Caputo, Expanded micro-particles by supercritical antisolvent precipitation: interpretation of results, J. Supercrit. Fluids 44 (2008) 98-108.
[16] M.A. Meneses, G. Caputo, M. Scognamiglio, E. Reverchon, R. Adami, Antioxidant phenolic compounds recovery from Mangifera indica L. by-products by supercritical antisolvent extraction, J. Food Eng. 163 (2015) 45-53.
[17] O. Benavente-Garcıa, J. Castillo, J. Lorente, A. Ortuno, J. Del Rio, Antioxidant activity of phenolics extracted from Olea europaea L. leaves, Food Chem. 68 (2000) 457-462.

[18] C. Witschi, E. Doelker, Residual solvents in pharmaceutical products: acceptable limits, influences on physicochemical properties, analytical methods and documented values, Eur. J. Pharm. Biopharm. 43 (1997) 215-242.
[19] L. Martín, A. González-Coloma, R. Adami, M. Scognamiglio, E. Reverchon, G. Della Porta, J. Urieta, A. Mainar, Supercritical antisolvent fractionation of ryanodol from Persea indica, J. Supercrit. Fluids 60 (2011) 16-20.