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# Phenolic extract obtained from steam-treated olive oil waste: Characterization and antioxidant activity

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ABSTRACT

Alperujo or olive oil waste was hydrothermally treated at 160 °C for 60 min to increase the phenols in the liquid phase. The extract obtained from the liquid using ethyl acetate extraction was divided into 27 phenolic fractions using adsorbent and polyamide resins. Phenolic alcohols and acids, secoiridoid molecules, elenolic acid derivatives, flavonoids, verbascoside, degradation products of sugar and a polymeric phenolic fraction (PPF) were characterized using HPLC-ESI-MS. The antiradical activity, ferric reducing power and the inhibition of primary and secondary oxidation were examined for each fraction. Hydroxytyrosol was the most abundant phenol in the ethyl acetate extract and possibly the main component responsible for the in vitro antioxidant activity of the entire phenol extract. However, other phenolic and secoiridoid molecules with interesting biological properties were also identified, and the crude extract could be considered a potential source of natural antioxidants.



## 1. Introduction

Many epidemiological studies show that the Mediterranean diet provides protection against a wide array of common chronic pathological conditions, including coronary heart disease, cancer and neurodegenerative disorders (Pérez-Jiménez, 2005). This effect may be attributed to many components of the diet, including the phenols that exhibit free radical scavenging activity and protect organisms against oxidative damage (Covas et al., 2006). Thus, the daily consumption of olive oil, rich in phenolic compounds improves health by reducing oxidative damage to the human body. However, after olive oil extraction, only a small percentage (1–2%) of the total phenols present in the olive fruit remain in the oil, with the majority remaining in the olive mill wastes like alperujo (two phase extraction system) that may be used as a promising source of these phenolic compounds.

To increase the concentration of these phenols and to extract them in high yield from alperujo, an environmentally friendly process based on hydrothermal treatment in which an autohydrolytic process occurs has been developed (PCT/ES2011/070583). In a previous study, phenols were selectively extracted from the autohydrolysis liquid using ethyl acetate, yielding extracts with antioxidant activity levels comparable to vitamin E (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2012).

The aim of this study was to determine the phenolic composition of the ethyl acetate extract obtained after hydrothermal treatment of the alperujo at 160 °C for 60 min. Fractionation of the extract is used to facilitate the characterization and identification of the main phenolic and secoiridoid components. A second purpose of this study was to assess the antioxidant activity of each fraction to determine the relative contribution of the various compounds to the above activities for the entire phenolic extract. This study will help to evaluate the use of this extract as a potential source of natural antioxidants.

## 2. Materials and methods

### 2.1. Materials

The sample of alperujo was obtained in March 2009 from Picual olives processed at a Spanish oil mill (Instituto de la Grasa, Seville).

### 2.2. Standard compounds

Hydroxymethylfurfural, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, p-coumaric acid, caffeic acid and 3,4-dihydroxyphenylglycol were obtained from Sigma–Aldrich (Deisenhofer, Germany). Tyrosol and 3,4-dihydroxyphenylacetic acid

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were obtained from Fluka (Buchs, Switzerland). Oleuropein, verbascoside and luteolin-7-O-glucoside were obtained from Extrasynthese (Lyon Nord, Geney, France). (±)-α-Tocopherol ≥95% purchased from Sigma–Aldrich was used as reference compound. Hydroxytyrosol was obtained using the method described by Fernández-Bolaños et al. (2002).

### 2.3. Thermal treatment

In total, 20 kg of alperujo was treated for 60 min at a fixed temperature of 160 °C in the patented reactor (100 L). Then, the wet material was centrifuged at 4700 g (Comteifa, S.L., Barcelona, Spain), obtaining 51 L of liquid, and an aliquot of 10 L was collected for the experiment and was concentrated to 1 L.

### 2.4. Determination of the total phenolic content

The phenolic content was measured according to the Folin–Ciocalteu method and expressed as grams of gallic acid equivalents per kilogram of fresh alperujo (Singleton, & Rossi, 1965).

### 2.5. Phenol extraction

The liquid portions (1 L) obtained after treatment were washed with hexane (500 mL) to remove the lipid fraction. The mixture was shaken vigorously, and the phases were then separated by decantation and washed twice (De Marco, Savarese, Paduano, & Sacchi, 2007).

Phenolic compounds were extracted with ethyl acetate (500 mL per 200 mL of sample) using a continuous extraction at 77 °C for 8 h. The organic phase was rotary-evaporated under vacuum at 30 °C.

### 2.6. Chromatographic fractionation of the ethyl acetate extract (EtOAc extract)

Approximately 12 g of EtOAc extract was dissolved in 60 mL of $H_2O$:MeOH (80:20). The extract was passed through an open column of 3.5 cm in diameter and 40 cm in height packed with the non-ionic resin Amberlite XAD16 (Vivaqua, Spain). The elution was performed using 1 L of $H_2O$, EtOH 30% (v/v), EtOH 50% (v/v) and EtOH 95% (v/v); ten fractions of 100 mL each were collected and analyzed using HPLC, and those with similar compositions were mixed, concentrated to 50 mL and passed through a second column. This second column was 3.5 cm in diameter and 45 cm in height and filled with polyamide (particle size = 50–160 μm, Fluka Analytica). The elution was performed using 500 mL of $H_2O$, MeOH 25% (v/v), MeOH 50% (v/v), MeOH 75% (v/v) and MeOH 100% (v/v). Ten fractions of 50 mL each were collected and monitored using HPLC. Fractions with similar composition were combined (Fig. 1). In each fraction, the phenols were identified using HPLC-DAD and HPLC-DAD-MS, and quantified using HPLC-DAD.

### 2.7. Isolation of compounds of interest

The compounds that are not commercially available were purified on a silica gel preparative TLC (Merck 60F254) and eluted with a mixture of chloroform and methanol 8:2 (v/v). The different bands were identified by their absorption at 254 nm and 366 nm.

### 2.8. HPLC-DAD

Quantitative evaluation of phenols content was carried out as described by Rubio-Senent et al. (2012). Quantification was performed in triplicate using external standards. Standards curve were linear in all cases ($R^2 > 0.99$).

### 2.9. HPLC-DAD-MS

The phenolic compounds were separated by HPLC as described above and identified by the electrospray ionization mass data collected on a quadrupole mass analyzer (ZMD4, Micromass, Waters Inc.; Manchester, U.K.). Electrospray ionization (ESI) mass spectra were obtained at ionization energies of 50 eV and 100 eV in negative mode and 50 eV in positive mode; the capillary voltage was 3 kV, the desolvation temperature was 200 °C, the source temperature was 100 °C, and the extractor voltage was 12 V. The flow was maintained at 1 mL/min$^{-1}$ in split mode (UV detector MS) for each analysis.

### 2.10. Antiradical activity: 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The free radical-scavenging capacity was measured using the DPPH method described in a previous study (Rodríguez et al., 2005) and expressed as $EC_{50}$ (effective concentration, mg/mL), calculated from a calibration curve using linear regression for each antioxidant.

### 2.11. Antiradical activity: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)

The ABTS assay was performed according to the method of Gonçalves et al. (2009), with some modifications described in a previous work (Rubio-Senent et al., 2012). The results were expressed in terms of the Trolox equivalent antioxidant capacity (TEAC) in mg/mL.

### 2.12. Reducing power

The reducing power assay was performed according to the procedure described in a previous study (Rodríguez et al., 2005). The assay was calibrated using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and the results were expressed in mg/mL TE (Trolox equivalent). To express the results, a calibration curve was created by plotting $A_{490}$ against the known concentration of Trolox (0.059–0.56 mg/mL) [correlation coefficient ($R$) = 0.9936].

### 2.13. Inhibition of primary oxidation

The method is based on the ferric thiocyanate (Sánchez-Moreno, Larrauri, & Saura-Calixto, 1999) with some modifications described in a previous work (Rubio-Senent et al., 2012). The accumulation of hydroxyperoxides due to the oxidation of linoleic acid was measured at 490 nm. The results are expressed as $EC_{50}$ (mg/mL).

### 2.14. Inhibition of secondary oxidation

The evaluation of the inhibition of secondary oxidation was based on thiobarbituric acid method of Sánchez-Moreno et al. (1999) with modifications. This assay was based on thiobarbituric acid-reactive substances (TBARS) to measure the antioxidant ability of the tested samples with a lipid peroxidation inducer. The results are expressed as $EC_{50}$ (mg/mL).

### 2.15. Statistical analysis

Three replicates were performed for each assay. STATGRAPHICS® Plus software was used for statistical analysis. The