New Phenolic Compounds Hydrothermally Extracted from the Olive Oil Byproduct Alperujo and Their Antioxidative Activities

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Abstract

The application of a novel process based on the hydrothermal treatment of olive oil waste (alperujo) led to a final liquid phase that contained a high concentration of simple phenolic compounds. This study evaluated the effects of time (15−90 min) on the composition of the phenolic compounds isolated at a fixed temperature of 160 °C. Phenolic compounds were extracted with ethyl acetate. Both qualitative and quantitative HPLC analyses of the extracts showed variation of the concentrations of phenolic compounds with time. In addition, new phenols that were not present in the untreated control have been characterized. The antioxidant activities of different phenolic extracts were measured by various assays conducted *in vitro*: antiradical capacity (using DPPH and ABTS radicals), ferric reducing power (PR), inhibition of primary and secondary oxidation in lipid systems, and other tests, such as inhibition of tyrosinase activity. The results show that the phenolic extracts inhibited oxidation in aqueous and lipid systems to a significantly greater extent than the untreated control, and they performed as well as or better than vitamin E in this capacity.

KEYWORDS: alperujo, olive oil wastes, phenols, antioxidant, hydroxytyrosol, tyrosinase, ethyl acetate extract

INTRODUCTION

The olive oil industry is important in the Mediterranean region, and Spain produces >30% of the world's olive oil. Traditionally, the three-phase mill uses large volumes of water to aid the separation of oil and generates two byproducts. The first one is a liquid waste, which is known as olive mill wastewater, vegetation water, or alpechin. The second byproduct, a solid waste, is a combination of olive pulp and stones and is called pomace or orujo. The use of a modern two-phase processing technique, in which no water is added, generates a new byproduct called alperujo, which is a combination of liquid and solid waste. This two-phase centrifugation process is used for the separation of the oil from the vegetable material, which includes all of the mineral celluloses, hemicelluloses, pectins, gums, tannins, and polyphenols. In Spain, a massive change from the traditional three-phase process to the new two-phase process has taken place, and large volumes of waste, approximately 3.5−6 million tons/year, are generated.[1]

The Mediterranean diet has been documented by a large number of epidemiological studies.[2] It has been shown that the consumption of virgin olive oil, with high content in monounsaturated fatty acids and with an unsaponifiable fraction rich in minor components such as polyphenols, sterols, and tocopherols, leads to an increase in the total phenolic content of low-density lipoprotein (LDL) to prevent the LDL oxidation in the arterial intima.[3] Phenolic compounds from virgin olive oil may delay the progression of atherosclerosis by this mechanism. The antioxidative effects of phenolic compounds present in olive products also may contribute to the prevention of chronic diseases such as cardiovascular disease and cancer.[4] These phenolic compounds protect organisms against oxidative damage and prevent the deterioration of food by inhibiting lipid oxidation. The dialdehydic form of one of the secoiridoids, deacetoxy-ligstroside

aglycone (oleocanthal), was also recently found in the olive oil. Oleocanthal has the ability to inhibit cyclooxygenase (COX-1 and COX-2), showing an anti-inflammatory effect similar to that seen with ibuprofen.[5]

After the extraction of oil, 98−99% of the phenolic compounds present in the fruit of the olive remain in the alperujo. To explore the possibility of obtaining simple phenolic compounds in high yield from two-phase olive waste, a series of hydrothermal treatments was performed. A process that allows for easy separation of the solid and liquid phases of alperujo has been developed. The process also allows for the recovery of value-added compounds in the water-soluble fraction. In this treatment, which has been patented (PCT/ES2011/070583), an autohydrolytic process occurs, resulting in the solubilization of the alperujo. Usually, whenever a lignocellulosic material is treated with water or steam at temperatures from 160 to 240 °C, an autohydrolysis process occurs.[6] Depending on the conditions used, there may be a depolymerization of polysaccharides (mainly of hemicelluloses) and a breaking of lignin structures, resulting in the solubilization of lignin fragments of low weight.

As a consequence of our hydrothermal treatment, the byproduct was partially solubilized. The simple phenols, such as hydroxytyrosol, tyrosol, and 3,4-dihydroxyphenylglycol, increased in concentration as a result of the breakdown of complex molecules, such as oleuropein, demethyloleuropein, verbascoside, and others, that contain them in their structure.[7]

Interest in antioxidants for the prevention and treatment of human diseases has been sustained for at least two decades. It has been suggested that the consumption of certain foods that contain bioactive compounds, including fruits, vegetables, and red wine, may help to prevent cardiovascular diseases.[8] Several studies conducted in vitro have shown that this beneficial effect may be explained in part by the presence of polyphenols.[2]

The first objective of this work was to determine the composition of the different phenolic extracts obtained from treated alperujo and to study the influence of reaction time on the composition of the phenolic compounds extracted. The second objective was to characterize the specific antioxidative potentials of individual extracts obtained from treated alperujo and to compare their antioxidative capacities with those of

untreated alperujo and antioxidants such as hydroxytyrosol, 3,4-dihydroxyphenylglycol, and vitamin E.

MATERIALS AND METHODS

Materials. The sample of alperujo (a semisolid residue composed of olive peels, pulp, seeds, and ground stones) was obtained in March of 2009 from Picual olives processed at a Spanish oil mill (Almazara Experimental, Instituto de la Grasa, Seville). The alperujo was processed in the pilot reactor without removal of the stones.

Standard Compounds. Hydroxymethylfurfural, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, p-coumaric acid, caffeic acid, chlorogenic acid, 2,6-dihydroxybenzoic acid, 4-methylcatechol, syringic acid, vanillin, and 3,4-dihydroxyphenylglycol were obtained from Sigma-Aldrich (Deisenhofer, Germany). Tyrosol and 3,4-dihydroxyphenylacetic acid were obtained from Fluka (Buchs, Switzerland). Oleuropein, verbascoside, and luteolin-7-O-glucoside were obtained from Extrasynthese (Lyon Nord, Geney, France). Hydroxytyrosol was obtained according to the method described by Fernández-Bolaños et al.[9]

Thermal Treatment. The hydrothermal treatment used has been patented (PCT/ES2011/070583). It was performed using a prototype designed by our research group at the Instituto de la Grasa (Seville, Spain). The reactor has a stainless-steel reservoir (100 L capacity) that can operate at temperatures between 50 and 190 °C and at a maximum pressure of 1.2 MPa.

Either 10 or 20 kg of alperujo was loaded into the reactor, according to the treatment. Fresh alperujo samples were treated for 15−90 min at 160 °C. Then the wet material was centrifuged at 4700g (Comteifa, S.L., Barcelona, Spain) to separate the solids and liquids. After centrifugation, 10 L of the liquid phase of each treatment was concentrated to 1 L by rotary evaporation in a vacuum at 30 °C.

Phenol Extraction. The liquid portions obtained after treatment were washed with hexane to remove the lipid fraction: 1 L of liquid was mixed with 500 mL of hexane; the

mixture was shaken vigorously, and then the phases were separated by decantation and washed twice.

Extraction of phenolic compounds was carried out with ethyl acetate (500 mL per 200 mL of sample). The liquid−liquid extraction was performed with ethyl acetate (refluxed at 77 °C) in a continuous extractor of a heavier liquid (water) by lighter liquid (ethyl acetate) for 8 h. The aqueous and organic phases were separated, and the organic phase was rotary-evaporated under vacuum at 30 °C, producing a viscous dark brown extract.

Phenolic Extract Obtained from Control by Conventional Extraction. Phenols were extracted from the alperujo control to assess the effects of heat treatment on the composition of the extracted phenolic compounds. We used the method reported by Obied et al.,[10] with some modifications. Alperujo was extracted with aqueous methanol (80% v/v; 15 mL/10 g of alperujo) for 30 min at ambient temperature. The extraction was repeated twice more using 10 mL/10 g of alperujo. The combined liquid fraction was filtered, and then the phenols were extracted with ethyl acetate under the above-mentioned conditions.

Determination of the Total Phenolic Content. The phenolic content was measured according to the Folin−Ciocalteu method[11] and expressed as grams of gallic acid equivalents per kilogram of fresh alperujo.

Chromatographic Fractionation of the Ethyl Acetate Extracts. Approximately 12 g of each extract was dissolved in 60 mL of H_2O/MeOH (80:20). First, the extracts were passed through a column 3.5 cm in diameter and 40 cm in height filled with Amberlite XAD16. The elution was performed with 1 L of H_2O, 30% EtOH (v/v), 50% EtOH (v/v), and 95% EtOH (v/v). Ten fractions of 100 mL each were collected. Fractions were analyzed by HPLC, and those with similar compositions were mixed, concentrated to 50 mL, and passed through a second column. The second column was 3.5 cm in diameter and 45 cm in height, and it was filled with polyamide (particle size = 50−160 μm, Fluka Analytica). The elution was performed with 500 mL of H_2O, 25% MeOH (v/v), 50% MeOH (v/v), 75% MeOH (v/v), and 100% MeOH (v/v). Ten fractions of 50 mL each were collected and monitored by HPLC. Fractions with similar compositions were combined. Only a few

fractions were further passed through a third column to purify their components, helping their identification. The third column was 3.0 cm in diameter and 40 cm in height, and it was filled with reverse phase Amberchrom CG161 M (particle size = 75 µm, pore size = 150 Å, Rohm and Haas). The elution was performed with 100 mL of H_2O and 100 mL of 5% EtOH in water (v/v) increased from 5 to 100%, at 5% intervals, with 100 mL each. Ten fractions of 10 mL each were collected in a collector of fractions RadiFrac (Pharmacia Biotech). In each fraction, the different phenols were identified by HPLC-DAD and HPLC-MS, and they were quantified by HPLC-DAD.

Isolation of Compounds of Interest. Compounds of interest that are not commercially available were purified. The fractions containing these compounds were purified on 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 different wavelengths (visible light, 254 and 366 nm).

HPLC-DAD. The different phenols were quantified using a Hewlett-Packard 1100 liquid chromatography system with a C-18 column (Teknokroma Tracer Extrasil ODS-2, 250 mm × 4.6 mm i.d., 5 µm). The system was equipped with a diode array detector (DAD; the wavelengths used for quantification were 254, 280, and 340 nm) and Rheodyne injection valves (20 µL loop). The mobile phases were 0.01% trichloroacetic acid in water and acetonitrile utilizing the following gradient over a total run time of 55 min: 95% A initially, 75% A at 30 min, 50% A at 45 min, 0% A at 47 min, 75% A at 50 min, and 95% A at 52 min until the run was completed. Quantification was carried out by integration of peaks at different wavelengths with reference to calibrations made using external standards. The linearity of standards curve was expressed in terms of the determination coefficients plots of the integrated peaks area versus concentration of the same standard. These equations were obtained over a wide concentration range in accordance with the levels of these compounds in the samples. The system was linear in all cases ($r > 0.99$). Three replicates on the same day were carried out.

HPLC-MS. The phenolic compounds present in the different fractions were identified by electron impact mass data collected on a quadrupole mass analyzer (ZMD4, Micromass,

Waters Inc., Manchester, U.K.). Electron spray ionization (ESI) mass spectra were obtained at ionization energies of 50 and 100 eV in negative mode and of 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. A Tracer Extrasil ODS-2 column (250 mm × 4.6 mm i.d., 5 μm) (Teknokroma, Barcelona, Spain) was used. The mobile phases were 0.01% trichloroacetic acid in water and acetonitrile utilizing the gradient used in HPLC-DAD.

Antiradical Activity: 2,2-Diphenyl-1-picrylhydrazyl (DPPH). Free radical-scavenging capacity was measured using the DPPH method described in a previous work.[12] The method is based on the measurement of the free radical-scavenging capacity of the antioxidant against the stable radical DPPH•. An iMark microplate absorbance reader model 550 (Bio-Rad, Hercules, CA) was used for the absorbance measurements. DPPH• has an absorption band at 515 nm, which disappears upon reduction by antiradical compound. For each test compound and standard, the decrease in absorbance (expressed as a percentage of the initial absorbance) was plotted against the concentration of the antioxidant in the reaction mixture. The radical-scavenging capacity of each antioxidant was expressed as the EC_{50} (effective concentration, mg/mL), which is the amount of antioxidant necessary to decrease the initial absorbance by 50%, calculated from a calibration curve by linear regression for each antioxidant. The values of parameters for the regression analyses of the extracts were as follows: control ($y = -8.51x + 97.66$, $R^2 = 0.969$), 160 °C/15 min ($y = -32.25x + 102.78$, $R^2 = 0.993$), 160 °C/30 min ($y = -28.68x + 92.65$, $R^2 = 0.990$), 160 °C/45 min ($y = -22.08x + 74.45$, $R^2 = 0.990$), 160 °C/60 min ($y = -24.76x + 80.43$, $R^2 = 0.896$), 160 °C/75 min ($y = -24.76x + 76.34$, $R^2 = 0.985$), and 160 °C/90 min ($y = -26.64x + 95.95$, $R^2 = 0.988$).

Antiradical Activity: 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic Acid) (ABTS). The ABTS assay was performed according to the method of Gonçalves et al.[13] This assay is based on the scavenging of ABTS radical ($ABTS^{\bullet+}$) by antioxidants compared to the antioxidant potency of Trolox that is used as standard (water-soluble α-tocopherol analogue). The concentration of ABTS radical was adjusted with ethanol 80% (v/v) to an

initial absorbance of 0.700 ± 0.020 at 734 nm. Aliquots of 13 µL of each phenolic extract, the standards (hydroxytyrosol (HT), 3,4-dyhydroxyphenyl glycol (DHPG), and vitamin E), and their dilutions were added to 187 µL of the ABTS•$^{+}$ solution in a 96-well microplate in triplicate. For each sample, a blank with ethanol instead of ABTS•$^{+}$ solution was included. A delay of 30 min was programmed into the reader before readings at 414, 655, and 750 nm. The results were expressed as the average of the ratios of the slopes of the lines obtained for each sample with Trolox calculated for each of these three wavelengths. The results were expressed in terms of Trolox equivalent antioxidant capacity (TEAC) in mg/mL. The values of parameters for the regression analyses of the extracts were as follows: control ($y = 302.19x + 6.81$, $R^2 = 0.951$), 160 °C/15 min ($y = 727.07x + 6.22$, $R^2 = 0.991$), 160 °C/30 min ($y = 521.01x + 8.78$, $R^2 = 0.907$), 160 °C/45 min ($y = 705.56x + 3.88$, $R^2 = 0.992$), 160 °C/60 min ($y = 722.82x + 4.14$, $R^2 = 0.989$), 160 °C/75 min ($y = 589.39x + 7.90$, $R^2 = 0.964$), and 160 °C/90 min ($y = 721.13x + 4.30$, $R^2 = 0.992$).

Reducing Power. The reducing power assay was carried out according to the procedure described in a previous work.[12] The antioxidative potentials of the extracts were estimated for their ability to reduce $FeCl_3$. All of the analyses were made using a microplate reader, and the absorbance was measured at 490 nm. The values of parameters for the regression analyses of the extracts were as follows: control ($y = 0.13x + 0.02$, $R^2 = 0.994$), 160 °C/15 min ($y = 0.45x - 0.01$, $R^2 = 0.998$), 160 °C/30 min ($y = 0.35x - 0.01$, $R^2 = 0.990$), 160 °C/45 min ($y = 0.47x - 0.02$, $R^2 = 0.968$), 160 °C/60 min ($y = 0.50x - 0.01$, $R^2 = 0.995$), 160 °C/75 min ($y = 0.44x - 0.01$, $R^2 = 0.997$), and 160 °C/90 min ($y = 0.48x - 0.01$, $R^2 = 0.964$). The assay was calibrated using Trolox. To express the results, a calibration curve was established by plotting A_{490} against known concentrations of Trolox (0.059−0.56 mg/mL). Reducing power (PR) was expressed as Trolox equivalents (TE) in mg/mL from the equation as determined from linear regression: $PR = 0.1932 \times A490 - 177.48$ (correlation coefficient $R^2 = 0.994$).

Inhibition of Primary Oxidation. Evaluation of the inhibition of primary oxidation of lipid was based on the ferric thyocianate (FTC) method of Sánchez-Moreno et al.[14] with modifications. Twenty-five microliters of each fraction of phenolic extracts, reference

compounds (HT, DHPG, and vitamin E) and their dilutions, 5 µL of 0.07 M 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP) (water-soluble free radical initiator), and 2 mL of 2.6 mM linoleic acid (LA) emulsion (0.1 M of SDS in aqueous solution of 0.01 M Na_2HPO_4, pH 7.4) were placed in different tubes in triplicate. For each sample, a blank without LA was included. The absorbance of LA without antioxidant solution was used as the standard for 100% oxidation. Test tubes were incubated at 50 °C for 15.5 h. After cooling at room temperature, 100 µL of 30% NH_4SCN in water and 100 µL of 0.02 M $FeCl_2$ in 3.5% HCl were added to each tube. Accumulation of hydroxyperoxides due to the oxidation of LA was measured at 490 nm. The results, calculated from the regression curves, are expressed as EC_{50} (mg/mL). The values of parameters for the regression analyses of the extracts were as follows: control ($y = 81.71 - 35.46\sqrt{x}$, $R^2 = 0.886$), 160 °C/15 min ($y = 81.26 - 31.96\sqrt{x}$, $R^2 = 0.846$), 160 °C/30 min ($y = 89.52 - 36.96\sqrt{x}$, $R^2 = 0.897$), 160 °C/45 min ($y = 88.42 - 39.69\sqrt{x}$, $R^2 = 0.873$), 160 °C/60 min ($y = 93.89 - 41.28\sqrt{x}$, $R^2 = 0.886$), 160 °C/75 min ($y = 82.20 - 31.96\sqrt{x}$, $R^2 = 0.896$), and 160 °C/90 min ($y = 84.19 - 33.42\sqrt{x}$, $R^2 = 0.795$).

Inhibition of Secondary Oxidation. Evaluation of the inhibition of secondary oxidation was based on the method of Moon and Shibamoto[15] with some 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, using LA emulsion as lipid-rich media. Twenty-five microliters of each fraction of phenolic extracts, reference compounds (HT, DHPG, and vitamin E) and their dilutions, 5 µL of 0.07 M ABAP, and 1 mL of 5.2 mM LA emulsion (see primary oxidation) were placed in different tubes in triplicate. For each sample, a blank without LA was included. The absorbance of LA without antioxidant solution was used as the standard for 100% oxidation. Test tubes were vortexed and then incubated at 50 °C for 24 h. One hundred microliters of each reaction mixture were mixed with 100 µL of 2.8% (w/v) trichloroacetic acid in water and 100 µL of 0.8% thiobarbituric acid in 1.1% SDS. This mixture was vortexed and then heated at 90 °C for 20 min. After cooling at room temperature, 0.5 mL of 1-butanol was added, and the samples were stirred and centrifuged at 8050g for 3 min. The absorbance of the butanol layer was measured at 540 nm. The results are expressed as EC_{50} (mg/mL). The values of parameters for the

regression analyses of the extracts were as follows: control ($y = 87.54 - 28.01\sqrt{x}$, $R^2 = 0.940$), 160 °C/15 min ($y = 105.62 - 46.03\sqrt{x}$, $R^2 = 0.988$), 160 °C/30 min ($y = 99.11 - 42.89\sqrt{x}$, $R^2 = 0.984$), 160 °C/ 45 min ($y = 82.38 - 31.52\sqrt{x}$, $R^2 = 0.915$), 160 °C/60 min ($y = 77.90 - 28.46\sqrt{x}$, $R^2 = 0.876$), 160 °C/75 min ($y = 102.22 - 44.50\sqrt{x}$, $R^2 = 0.974$), and 160 °C/90 min ($y = 104.56 - 42.66\sqrt{x}$, $R^2 = 0.985$).

Inhibition of Tyrosinase Activity. Inhibition was tested according to the method of Prasad et al.[16] with some modifications. Polyphenol oxidase (PPO) catalyzes two successive reactions involving molecular oxygen; the hydroxylation of monophenols leads to the formation of o-diphenols, followed by their subsequent oxidation into o-quinones, which are in turn polymerized into brown, red, or black pigments. The inhibition in enzyme activity is followed by a decrease in the formation of products that absorb radiation at 490 nm. The decrease in absorbance is indicative of a decrease in the product formed and hence a greater inhibition of enzyme activity.

The mushroom tyrosinase used for this bioassay was purchased by Sigma (St. Louis, MO) and was used without further purification. Tyrosinase functions as both a monophenolase and an o-diphenolase. In the current experiment, L-tyrosine (monophenolase assay) was used as a substrate. For each sample, a blank without L-tyrosine was included. The absorbance without a sample solution was used as the standard for 100% oxidation. Fifty microliters of 5 mM L-tyrosine were added to 100 µL of phosphate buffer (0.05 M, pH 6.6) in a 96-well microplate. After 10 min of incubation at 30 °C, 50 µL of the sample solution and 50 µL of the aqueous solution of the mushroom tyrosinase (50 units/mL) were added to the mixture solution. The absorbance at 490 nm was recorded every 20 s for 15 min using the microplate reader. For the inhibition of tyrosinase activity, a time point of 300 s was fixed. Percent of anti-tyrosinase activity was calculated using the following formula: optic density (OD) of blank − OD of sample/OD of blank × 100.

Statistical Analysis. Statgraphics Plus software was used for statistical analysis. Correlation coefficients were determined using regression analysis at the 95% confidence level. Comparisons among samples were made with one-way analysis of variance (ANOVA) and the LSD method at the same confidence level.

RESULTS AND DISCUSSION

Hydrothermal Treatment. The study of the hydrothermal processing of alperujo was undertaken at temperature of 160 °C for treatment times of 15, 30, 45, 60, 75, and 90 min. This process led to a solution that contained a high quantity of compounds that are easily solubilized (including carbohydrates, organic acid, phenols, polyphenols) or that are formed from thermal degradation (including hydroxymethylfurfural).

As can be seen from Table 1, different amounts of alperujo were introduced into the reactor, providing different volumes of liquid fractions. Ten liters of each liquid fraction was collected and concentrated to 1 L. The extraction of phenols was carried out with ethyl acetate. The amounts of phenolic extract obtained per kilogram of fresh alperujo are shown in Table 1 for different treatments and the control.

The amount of phenolic extract increased with increasing treatment time, reaching a maximum at 75 min of 11.2 g/kg fresh alperujo that doubles the amount found in the control. At 90 min, the amount of phenolic extract was slightly lower than the maximum, possibly due to degradation or polymerization reactions as a result of such prolonged treatment.

Three groups of results were obtained. In the first group (the control and treatments of 15 min), the amount of extract was 4−5 g/kg of fresh alperujo; in the second group (treatments of 30, 45, and 60 min), the amount of extract was 7−8 g/kg; and in the third group (treatments of 75 and 90 min), the amount of extract was 9−11 g/kg.

Fractionation and Characterization of the Phenolic Extracts. The extracts, which are a highly diverse and complex mixture of phenolic compounds (Figure 1), need to be fractionated with different resins to purify their components for further identification (Figure 2). The preliminary identification of phenolic compounds presents in the phenolic extracts (control and treatments at 160 °C of 30, 60, and 90 min) was performed by HPLC-DAD; the relative retention times and UV spectra were compared against standard solutions. The final identification was made by HPLC-MS; for those compounds that lacked pure standards, identification was based on the search for pseudomolecular $[M - H]^-$ ions, using ion mass chromatograms, together with the

interpretation of collision-induced dissociation (CID) fragments. When standards were available, identification was carried out by comparing retention times and mass spectra with those standards.

The concentrations of phenolic compounds in the extracts were calculated by comparing HPLC-DAD peak areas with the corresponding external standards (Table 2). The HPLC-DAD equipment was calibrated using external standards for those species not available commercially but proceeded to their isolation.

The results obtained for the different extracts are shown in Tables 3 and 4. The hydrothermal treatment caused a significant increase in the concentration of phenolic alcohols. This is mainly due to increased concentrations of hydroxytyrosol and 3,4-dihydroxyphenylglycol in free form. The treatments caused the rupture of more complex molecules that contain simple phenols, and the treatments enhanced their solubilization.[17] Tyrosol, which is found in olive seeds, also increased in concentration because the treatment caused the rupture of complex molecules containing tyrosol and their solubilization.[7]

Increased concentrations of hydroxymethylfurfural were obtained for longer treatment times. This species has not been previously described in alperujo. It formed in the reactor due to the degradation of hexoses during the treatment. Phenolic acids increased slightly in concentration with respect to the control. In the extracts obtained after 30 and 60 min of treatment, protocatechuic acid was obtained in high concentrations; after 90 min, chlorogenic acid and 3,4-dihydroxyphenylacetic acid were obtained in high concentrations.

The concentration of oleuropein decreased over time due to its breakdown into simpler molecules. The concentration of demethyloleuropein, which comes from the degradation of oleuropein by loss of a methyl group, a process favored in the treatment, increased. Overall, the extract obtained after 30 min of treatment contained the highest concentrations of derivatives of oleuropein. This may be because the shorter treatment favors the solubilization of these compounds without degrading them.

Elenoic acid derivatives decreased in concentration, compared with the control, with increasing temperature, probably because the treatment promotes the breakdown of elenoic acid into simpler molecules.

The treatment also favors the formation of demethylligstroside, as happened with the oleuropein and demethyloleuropein.

In the extract were also identified two lignans, 1-acetoxypinoresinol (Figure 3a) and pinoresinol, which were not present in the extract control. These species were identified in the oil due to their liposoluble natures.[18] These molecules have been described in alperujo only in one recent paper,[19] probably due to their low concentration. These compounds can be used as antioxidative, antiviral, antibacterial, insecticidal, and fungicidal agents.[20]

The treatments promoted the formation of 1-phenyl-6,7-dihydroxyisochroman (Figure 3b). This molecule is part of the phenolic composition of olive oil, and it is formed spontaneously during storage of the oil by the reaction of benzaldehyde and hydroxytyrosol, with oleic acid acting as a catalyst.[21] The high pressure and temperature used together with the abundant presence of free hydroxytyrosol and the aldehyde formation in acid medium make possible the synthesis of the species during the treatment. This molecule acts as an inhibitor of platelet aggregation and helps prevent cardiovascular disease.[8]

The concentration of comsegoloside decreased compared with the control with regard to the treatment time because the molecule breaks down into simple structures such as caffeic acid.

In addition, the ethyl acetate extraction process after the hydrothermal treatment favors the formation of a polymeric phenolic fraction, the concentration of which increases with increasing treatment time. This fraction has the ability to absorb hydroxytyrosol. The results of research on this new polymeric phenolic fraction will be shown in a forthcoming paper.

Antiradical Activity and Reducing Power. Statistical analyses of the results show that the dose response adjusted to a linear model for three of the assays, with $R^2 \geq 0.98$ (data not shown).

Individual regression equations were used to calculate the antioxidative capacities of each sample. The values for each phenolic extract obtained after the different treatments (160 °C for 15, 30, 45, 60, 75, and 90 min) were compared to the values for the phenolic extract control (untreated) and to the values of species that have potent

antioxidant activity, such as HT, DHPG, and vitamin E. Antiradical activity was evaluated with radicals from two different sources, ABTS and DPPH (Table 5). Antiradical activity against the DPPH radical is expressed as EC_{50}, and antiradical activity against the ABTS radical is expressed as TEAC. The results were similar in both cases; all of the phenolic extracts obtained after the treatments had activities similar to that of vitamin E, somewhat lower in the case of DPPH assay, and to each other, and all had higher activities than the control extract.

The results for reducing power (Table 5) are expressed as Trolox equivalents in mg/mL; high concentrations indicate high activity. In this case, all of the phenolic extracts obtained after treatments had similar activities, and all were more effective reducing agents than vitamin E. Thus, the hydrothermal treatment promotes the solubilization/formation of compounds that increase antiradical activity against the two types of radicals studied, ABTS and DPPH, also increasing reducing power.

Inhibition of Lipid Oxidation. In the primary oxidation assay, the accumulation of hydroxyperoxide in the oxidation of linoleic acid was measured. These were unlikely to display antiradical activity or reducing power, and the regression analyses of the dose−response lines confirmed that they were described not by a lineal model but by a "square root of x" ($y = a + bx^{-2}$), with $R^2 \geq 0.91$. In the secondary oxidation assay, all of the samples are described by a "square root of x" regression model ($R^2 \geq 0.94$). The results of both assays are expressed as EC_{50} (Table 6). The capacities of all extracts to inhibit the primary oxidation were 1.1−1.9-fold higher than the control and showed less activity than HT, DHPG, and vitamin E. The results obtained for the inhibition of secondary oxidation showed that the extracts had values similar to the control and lower than the reference standards.

Theoretical Activity of HT and DHPG in Phenol Extracts. The theoretical contribution of HT and DHPG to reducing power, antiradical activity (ABTS), and inhibition of primary and secondary oxidation was calculated on the basis of the concentrations of free-form HT and DHPG in each of the extracts. These values were compared with the experimental values. The concentrations (in mg/mL) of HT and DHPG present in 1 mg/mL of extract were determined, and an estimate of activity for these concentrations was

established from the graphs obtained for standards (HT and DHPG) related to the activity with a given concentration in milligrams per milliliter of HT and DHPG. The sum of the activities obtained for HT and DHPG provides theoretical activity (theor) without considering possible synergistic or antagonistic effects. This value was compared with the activity provided by 1 mg/mL of each extract, giving the experimental value (exptl). The theoretical and experimental results were compared (Table 7).

The results show that the extracts at 60 and 90 min had more reducing power than the individual standards, as the experimental Trolox equivalents are higher than the theoretical ones. This indicates that there are species in the extract that may promote this activity, and/or there is a synergistic effect between HT, DHPG, and other components. The theoretical values for HT and DHPG for antiradical activity (DPPH) of the three extracts were higher than the experimental values, but not for the case of the control. This may be due to antagonistic effects between the HT, DHPG, and other phenolic species present in the extract, which lowered the activity. For primary oxidation, the data show that the extracts, including control, had a greater ability to inhibit primary oxidation than the standards (theor), except in the extract obtained at 30 min. Therefore, some species in the extracts promote this activity, and/or there is a synergistic effect between HT, DHPG, and other components. Finally, values from the inhibition of secondary oxidation showed that both HT and DHPG (theor) were similar to the experimental value in the case of extract control but had higher capacities for inhibition of secondary oxidation than in the case of the extracts. This may be due to possible antagonistic effects between the HT, DHPG, and other phenolic species present in the extracts.

Inhibition of Tyrosinase Activity. Tyrosinase activity (monophenolmonoxygenase, EC 1.14.18.1) was measured using L-tyrosine as the substrate. All phenolic extracts obtained after hydrothermal treatment showed similar inhibition capacities after 300 s (Figure 4) in a concentration-dependent manner (Table 8), and all were higher than that of the control. This untreated extract control did not inhibit tyrosinase at the concentrations tested. From a concentration of 0.75 mg/mL of extract the activity was enhanced. The same test was conducted to determine the ability to inhibit the enzyme by the HT. To obtain results similar to those of the treated extracts, the HT concentration

must be at least 316 mg/mL. Therefore, the phenolic extracts hydrothermally treated were more effective at inhibiting tyrosinase activity than HT. However, kojic acid, a reference inhibitor, showed a strong tyrosinase inhibitory activity with 50% inhibition for 0.01 mg/mL.

General Comments. The results of the present work showed liquors obtained from a new hydrothermal process of alperujo and extracted with ethyl acetate that have higher phenolic content and stronger antioxidant/free radical-scavenging and anti-tyrosinase activities than the control obtained with conventional extraction method. In addition, the great diversity and complexity of the natural mixtures of phenolic compound in the extracts was indicated. This thermal treatment promotes the solubilization and/or formation of new compounds not present in untreated alperujo, such as 1-acetoxypinoresinol, pinoresinol, and 1-phenyl-6,7-dihydroxyisochroman, that contribute significantly to the health benefits associated with the consumption of virgin olive oils. Of all treatments and compounds determined, hydroxytyrosol was found in the highest concentration, and it was mainly responsible for the in vitro antioxidant activity of the extracts. However, antagonist or synergistic interaction between phenolic components of extracts should be considered. In addition, the positive results prove this low-cost procedure could be an alternative to the conventional extraction method for obtaining antioxidant phenolic extracts from alperujo. These antioxidants could be used as food additives or for applications in the pharmaceutical and cosmetic industries, revaluing the byproduct of virgin olive oil processing. However, the biological properties of these compounds in vivo will depend on the extent to which they are absorbed and metabolized in the gastrointestinal tract and even the interaction with other food components.

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Figures

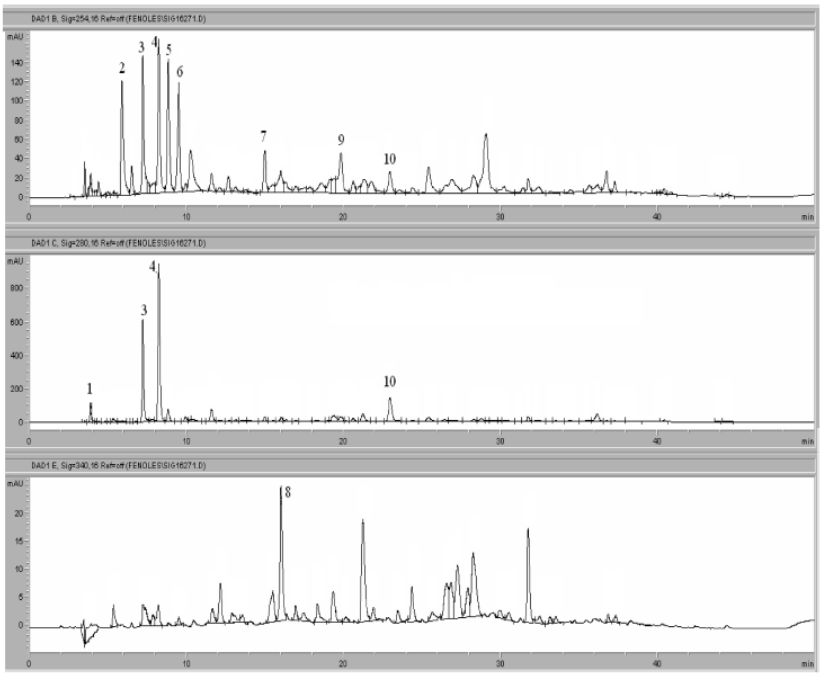

Figure 1. Representative HPLC-DAD chromatograms (at 254, 280, and 340 nm) of phenolic extracts obtained from steam-treated alperujo at 160 °C for 60 min. Identified compounds: (1) 3,4-dihydroxyphenylglycol; (2) unknown; (3) hydroxymethylfurfural; (4) hydroxytyrosol; (5) protocatechuic acid; (6) elenoic acid derivative; (7) vanillic acid; (8) caffeic acid; (9) elenoic acid derivative; (10) hydroxytyrosol acetate.

Figure 2. Flow diagram of the fractionation protocol followed for the ethyl acetate extracts from the liquid phase generated during hydrothermal treatment of alperujo.

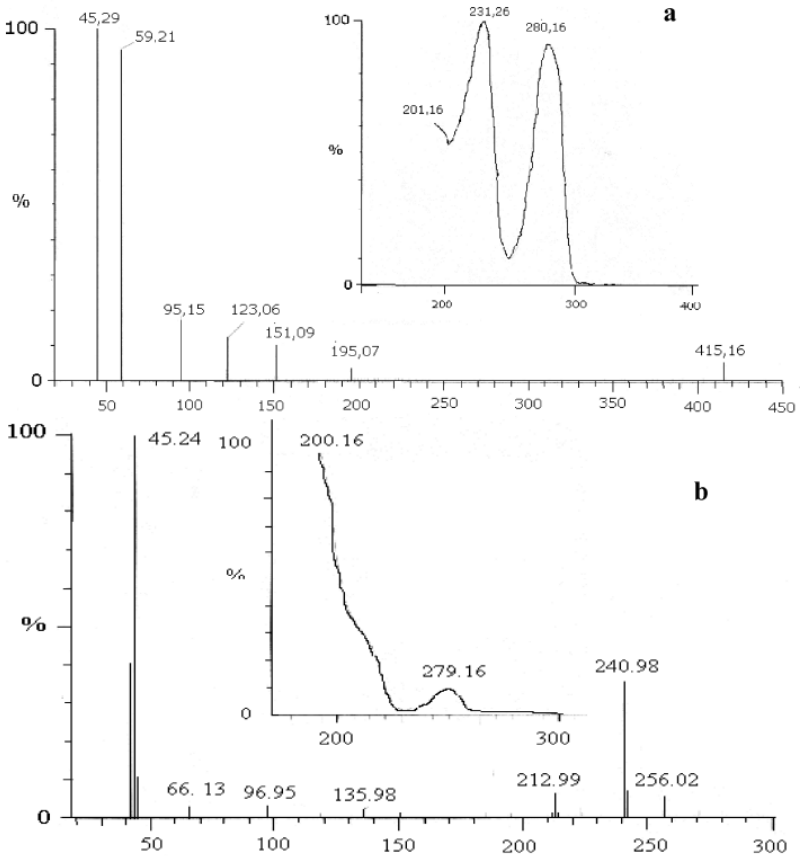

Figure 3. Mass spectra in negative ionization mode m/z^- and absorption (nm) spectra of 1-acetoxypinoresinol (a) and 1-phenyl-6,7-dihydroxyisochroman (b).

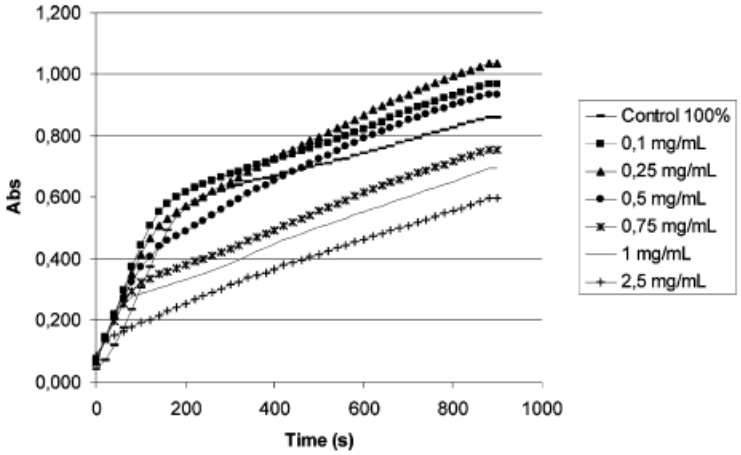

Figure 4. Progress curves for the inhibition of mushroom tyrosinase by different concentrations of phenolic extract (dissolved in a solution of H_2O and MeOH (8:2)) obtained after treatment at 160 °C for 60 min. All analyses were run in quadruplicate, and the average values are presented, the coefficient of variation being <10% in all cases.

Tables

Table 1. Yield of Phenolic Extracts and Total Phenolic Content Obtained by Hydrothermal Treatment of Alperujo at 160 °C for Increasing Treatment Time and Comparison with an Untreated Control

		hydrothermal treatment at 160 °C					
control	0 min	15 min	30 min	45 min	60 min	75 min	90 min
amount of alperujo treated (kg)	2.7	10	10	20	20	20	10
liquid volume fraction (L)	9.4	16.5	21	42	51	63	29
g of phenolic extract/kg fresh alperujo	5.8	4.1	7.0	8.3	7.1	11.2	9.1
total phenolic content (gallic acid equivalent (g/kg fresh alperujo)	0.65	1.62	2.03	3.06	1.74	3.43	2.21

Table 2. Wavelength of the Maximum Absorption, Concentration Range of Quantification, and Regression Equation of the Calibration Curve for Standard Compounds Identified in Extracts

compound	λ quantification (nm)	concentration range (mg/mL)	lineal regression[a]	R^2[b]
3,4-dihydroxyphenylglycol	280	0.40−0.01	$y = 16415x + 49.66$	0.999
hydroxytyrosol	280	0.63−0.032	$y = 14298x - 54.05$	0.999
tyrosol	280	1−0.01	$y = 12520x + 120.95$	0.999
vanillin	280	0.50−0.05	$y = 72734x - 125.89$	0.999
4-methylcatechol	280	2.50−0.1	$y = 19637x - 1060.70$	0.999
hydroxymethylfurfural	280	0.10−0.01	$y = 145360x + 52.66$	0.999
3,4-dihydroxyphenylacetic acid	280	5−0.20	$y = 16433x - 391.64$	0.999
protocatechuic acid	254	0.25−0.01	$y = 63627x + 125.13$	0.999
caffeic acid	340	1−0.05	$y = 65738x + 60.02$	0.997
4-hydroxybenzoic acid	254	0.20−0.01	$y = 109834x + 79.53$	0.999
p-coumaric acid	280	0.20−0.01	$y = 91146x + 5.75$	0.999
chlorogenic acid	340	2.50−0.25	$y = 41912x + 49.78$	0.999
syringic acid	280	5−0.50	$y = 40581x - 1265.60$	0.999
vanillic acid	254	0.20−0.01	$y = 65622x - 22.722$	0.999
oleuropein	280	0.75−0.05	$y = 48117x + 437.26$	0.994
elenoic acid derivative A	254	4.40−0.18	$y = 2517.4x + 5.99$	0.999
elenoic acid derivative B	254	10.70−0.54	$y = 2538.1x - 152.17$	0.994
luteolin-7-*O*-glucoside	340	0.10−0.01	$y = 352117x - 556.12$	0.998
hydroxytyrosol acetate	280	4.50−0.45	$y = 11161x - 1397.10$	0.999
1-phenyl-6,7-dihydroxyisochroman	280	4.50−0.23	$y = 8271.9x - 40.04$	0.998
verbascoside	280	0.10−0.01	$y = 19632x - 16.42$	0.999
comsegoloside	280	2.68−0.13	$y = 15167x + 461.21$	0.999

[a] y, peak area of standard compound; x, mg standard compound. [b] R, correlation coefficient.

Table 3. Phenolic Compounds Identified in Different Extracts Obtained (Control and Treatments at 160 °C for 30, 60, and 90 min) from Alperujo by HPLC-DAD and HPLC-DAD-MS

compound	retention time (min)	mol wt	λ_{max}	m/z^-	ref
phenolic alcohol					
3,4-dihydroxyphenylglycol	4.3	170	214, 234, and 278	169	10
hydroxytyrosol	8.9	154	214, 234, and 278	153 and 123	22
tyrosol	11.7	138	200, 218, and 276	137	23
vanillin	17.8	152	225 and 320	151	22
4-methylcatechol	19.9	124	236		10
hydroxytyrosol acetate	23.1	196	214, 234, and 278	195	
degradation product of sugar					
hydroxymethylfurfural	7.3	126	194, 228, and 284		
phenolic acid					
3,4-dihydroxyphenylacetic acid	10.2	168	214, 234, and 278	151,123, 109, and 59	10
protocatechuic acid	8.3	154	206, 218, 260, and 294	109 and 45	10
caffeic acid	16.2	180	202, 218, 240, and 324	179, 163, 135, and 45	24
4-hydroxybenzoic acid	14.4	138	194 and 256		10
protocatechuic acid derivative	8.7	154	206, 218, 260, and 294	109 and 45	10
p-coumaric acid	20.5	164	194, 210, 226, and 310	119	19
p-coumaric acid derivative	13.4	536	230 and 310	205, 145, and 117	25
chlorogenic acid	15.2	354	200 and 345	247, 163, 135, 133, and 109	
syringic acid	14.5	198	218 and 276		10
vanillic acid	14.1	168	200, 218, 255, and 298		22
oleuropein derivative					
oleuropein aglycone hemiacetal	17.6	352	198, 224, and 275	707 [2M − H], 351, 137, and 119	26
	29.2	340	214, 234, and 278	539, 337,201, 157, and 139	
oleuropein derivative	36	538	214, 234, and 278	537, 403, 361, 223, 151, and 123	
	36.4	556	214, 234, and 278	555, 381, 245, 201, and 183	
oleuropein	30.7	540	198, 232, and 282	539, 377, 307, 275, and 223	23
demethyloleuropein	17.3	526	242	525, 509, 389, 243, 211, 181, 137, and 123	27
secologanoside	10.4	390	206, 218, 260, and 294	389, 345, 167, 123, and 108	10
oleuropein aglycone derivative	32.6	378	200, 222, and 280	377, 307, 275, 149, and 139	10
10-hydroxymethyloleuropein	29.3	556	214, 234, and 278	555, 223, 151, and 123	28
caffeoyl-6′-*O*-secologanoside	27.1	552	198 and 328	551, 507, 389, 281, 251, 179, and 161	10
6′-*O*-[(2*E*)-2,6-dimethyl-8-hydroxy-2-octenoyloxy]secologanoside	37.6	558	214, 234, and 278	557, 539, 345, and 167	28
elenoic acid derivatives					
elenoic acid derivative A	8.5		230	453, 423, and 241	10
elenoic acid derivative C	21.2		242	243, 211, and 151	29
elenoic acid derivative B	30.1		210 and 264	241 and 251	10
ligstroside derivatives					
ligstroside	32.3	524	220, 224, and 280	523, 361, 291, and 259	24
demethylligstroside	20.4	510	250	509 (m/z^+: 511, 211, and 181)	27
flavonoids					
luteolin-7-*O*-rutinoside	26.6	594	200, 254, and 349	593, 447, 285, and 151	30
apigenin-7-*O*-rutinoside	25.7	578	212 and 264	577, 371, and 269	30
luteolin-7-*O*-glucoside	26.4	448	206, 256, 266, and 350	447 and 285	30
cyanidin-3-*O*-rutinoside	29.1	596	200, 225, and 280	(m/z^+: 597, 521, 405, 345, and 137)	30
lignans					
1-acetoxypinoresinol	38.6	416	200, 230, and 280	415, 151, and 123	18, 19
pinoresinol	37.9	357	200, 225, and 280	357, 151, and 123	18, 19
others					
1-phenyl-6,7-dihydroxyisochroman	10.8	242	200, 230, and 280	241 and 136	10
verbascoside	24.5	624	198 and 328	623, 461, and 161	30
comsegoloside	32.1	536	192, 230, and 312	535, 205, and 145	10
nüzhenide	28.9	686	240 and 280	685, 523, 299, and 223	30
3-hydroxymethyl-2,3-dihydro-5-(methoxycarbonyl)-2-methyl-2*H*-pyran-4-acetic methyl ester	29.1	210	198, 232, and 282	257, 210, 151, and 123	28

Table 4. Concentrations[a] of Identified Phenolic Compounds in the Extracts from Hydrothermally Treated Alperujo and Control Untreated by HPLC-DAD

	mg/kg fresh alperujo			
compound	phenolic extract untreated	160 °C/30 min	160 °C/60 min	160 °C/90 min
3,4-dihydroxyphenylglycol[b]	24.80 ± 0.41	123.44 ± 4.07	70.19 ± 0.45	71.63 ± 3.53
hydroxytyrosol[b]	15.73 ± 0.39	1624.83 ± 6.05	776.53 ± 14.68	1127.53 ± 5.43
tyrosol[b]	14.97 ± 1.22	108.68 ± 1.37	80.62 ± 2.11	167.70 ± 5.97
vanillin[b]	nd	0.09 ± 0.01	nd	11.12 ± 0.04
4-methylcatechol[b]	nd	1.92 ± 0.01	nd	nd
hydroxytyrosol acetate	nd	154.66 ± 0.92	60.13 ± 1.27	174.93 ± 1.09
total phenolic alcohol	**55.50 ± 2.02**	**1858.96 ± 11.51**	**927.34 ± 17.24**	**1377.98 ± 14.97**
hydroxymethylfurfural[b]	nd	1.32 ± 0.02	4.67 ± 0.15	21.29 ± 0.53
degradation product of sugar	**nd**	**1.32 ± 0.02**	**4.67 ± 0.15**	**21.29 ± 0.53**
3,4-dihydroxyphenylacetic acid[b]	nd	15.56 ± 0.38	nd	19.58 ± 0.46
protocatechuic acid[b]	9.17 ± 0.04	24.47 ± 0.46	18.35 ± 0.58	2.74 ± 0.02
caffeic acid[b]	nd	7.01 ± 0.21	2.65 ± 0.03	0.96 ± 0.05
4-hydroxybenzoic acid[b]	0.40 ± 0.06	1.65 ± 0.02	1.44 ± 0.01	1.07 ± 0.02
protocatechuic acid derivative[c]	nd	5.14 ± 0.07	nd	nd
p-coumaric acid[b]	2.93 ± 0.04	nd	1.49 ± 0.02	nd
p-coumaric acid derivative[c]	nd	nd	0.05 ± 0.01	nd
chlorogenic acid[b]	nd	nd	nd	19.52 ± 0.17
syringic acid[b]	nd	nd	nd	1.78 ± 0.10
vanillic acid[b]	3.36 ± 0.09	nd	5.01 ± 0.05	2.09 ± 0.03
total phenolic acid	**15.86 ± 0.23**	**53.83 ± 1.14**	**28.99 ± 0.70**	**47.74 ± 0.85**
oleuropein aglycone hemiacetal[d]	nd	nd	0.02 ± 0.01	nd
oleuropein derivative[d]	nd	8.36 ± 0.13	1.39 ± 0.01	nd
oleuropein[b]	2.74 ± 0.01	0.92 ± 0.02	0.46 ± 0.01	nd
demethyloleuropein[d]	nd	nd	nd	14.50 ± 0.38
secologanoside[f]	nd	7.36 ± 0.06	nd	nd
oleuropein aglycone derivative[d]	nd	nd	0.20 ± 0.01	nd
10-hydroxymethyloleuropein[d]	n.d	4.27 ± 0.12	nd	nd
caffeoyl-6′-*O*-secologanoside[h]	nd	8.75 ± 0.48	nd	0.81 ± 0.06
6′-*O*-[(2*E*)-2,6-dimethyl-8-hydroxy-2-octenoyloxy]secologanoside[d]	nd	2.14 ± 0.06	nd	nd
total oleuropein derivative	**2.74 ± 0.01**	**31.80 ± 0.87**	**2.07 ± 0.04**	**15.31 ± 0.44**
elenoic acid derivative A[e]	1339.95 ± 22.03	664.38 ± 6.59	191.29 ± 1.17	527.40 ± 5.59
elenoic acid derivative C[f]	nd	294.47 ± 3.90	123.56 ± 0.08	nd
elenoic acid derivative B[e]	nd	31.79 ± 0.33	0.57 ± 0.01	9.38 ± 0.45
elenoic acid derivatives	**1339.95 ± 22.03**	**990.64 ± 10.82**	**315.42 ± 1.26**	**536.78 ± 6.04**
ligstroside[d]	nd	nd	0.04 ± 0.01	nd
demethylligstroside[d]	nd	18.71 ± 0.61	2.20 ± 0.01	111.39 ± 0.68
ligstroside derivatives	**nd**	**18.71 ± 0.61**	**2.24 ± 0.02**	**111.39 ± 0.68**
luteolin-7-*O*-rutinoside[g]	nd	1.33 ± 0.01	0.07 ± 0.01	0.12 ± 0.01
apigenin-7-*O*-rutinoside[g]	nd	nd	0.18 ± 0.01	nd
luteolin-7-*O*-glucoside[b]	3.69 ± 0.07	nd	0.58 ± 0.01	nd
cyanidin-3-*O*-rutinoside[g]	nd	0.06 ± 0.01	nd	nd
flavonoids	**3.69 ± 0.07**	**1.39 ± 0.02**	**0.83 ± 0.03**	**0.12 ± 0.01**
1-phenyl-6,7-dihydroxyisochroman[e]	nd	5.09 ± 0.05	39.92 ± 0.08	21.05 ± 0.03
verbascoside[b]	nd	2.02 ± 0.08	8.43 ± 0.08	nd
comsegoloside[e]	184.42 ± 3.47	58.94 ± 0.24	22.68 ± 0.24	5.12 ± 0.16
nüzhenide[d]	nd	2.43 ± 0.06	nd	nd
3-hydroxymethyl-2,3-dihydro-5-(methoxycarbonyl)-2-methyl-2*H*-pyran-4-acetic methyl ester[f]	nd	207.18 ± 13.70	nd	nd
others	**184.42 ± 3.47**	**275.66 ± 14.85**	**71.03 ± 0.40**	**26.17 ± 0.19**
polymeric phenolic fraction[i] (PPF)	**nd**	**515.82 ± 13.47**	**77.66 ± 4.98**	**1286.41 ± 14.72**

[a]Mean ± SD (standard deviation) of three determinations. [b]Compounds were identified and quantified with commercial standards. [c]Compounds were quantified with a calibration of protocatechuic acid and *p*-coumaric acid. [d]Compounds were quantified with a calibration of oleuropein. [e]Compounds were quantified with isolated compounds. [f]Compounds were quantified with a calibration of elenoic acid derivative C. [g]Compounds were quantified with a calibration of luteolin-7-*O*-glucoside. [h]Compounds were quantified with a calibration of comsegoloside. [i]PPF was calculated by gravimetrically from the fraction eluted in the polyamide column with 50% of methanol in water.

Table 5. Radical Scavenging Capacities (DPPH and ABTS Radicals) and Reducing Power of Extracts Obtained from Hydrothermally Treated Alperujo, Control Untreated, and Standards (HT, DHPG, and Vitamin E)[a]

	antiradical activity		reducing power
	DPPH (EC_{50})	ABTS (TEAC)	Trolox equiv (mg/mL)
control	5.59	0.22	0.15
160 °C/15 min	1.64	0.53	0.44
160 °C/30 min	1.50	0.46	0.34
160 °C/45 min	1.11	0.51	0.48
160 °C/60 min	1.23	0.54	0.49
160 °C/75 min	1.06	0.42	0.42
160 °C/90 min	1.72	0.51	0.49
HT	0.11	1.53	1.60
DHPG	0.17	0.99	1.96
vitamin E	0.83	0.50	0.15

[a]The results are expressed as EC_{50} (mg/mL), TEAC (mg/mL), and Trolox equivalent (mg/mL), respectively. The tests were performed in triplicate.

Table 6. Inhibition of Primary and Secondary Oxidation by the Different Extracts Obtained from Hydrothermally Treated Alperujo, Control Untreated, and Standards (HT, DHPG, and Vitamin E) Expressed as EC_{50}[a]

	EC_{50} (mg/mL)	
	primary oxidation	secondary oxidation
control	1.80	1.04
160 °C/15 min	1.46	0.93
160 °C/30 min	1.31	1.19
160 °C/45 min	1.06	0.93
160 °C/60 min	0.96	0.87
160 °C/75 min	1.38	1.07
160 °C/90 min	1.64	1.12
HT	0.68	0.07
DHPG	0.83	0.03
vitamin E	0.52	0.23

[a]The tests were performed in triplicate.