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Isolation of a powerful antioxidant from *Olea Europea* fruit-mill waste: 3,4-dihydroxyphenylglycol.

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ABSTRACT

Nutritional and antioxidant properties of phenolic compounds are important in relation to human health and palatability of products. 3,4-Dihydroxyphenylglycol (DHPG) is a strong antioxidant found in small amounts in virgin olive oil and table olives, with an antioxidant activity even higher than that the powerful hydroxytyrosol. The origin of this antioxidant is completely unclear since has never been reported as a free plant metabolite. In this respect possible precursors of DHPG have also been discussed in this study. The presence of soluble compounds that either contain DHPG in their molecular structure or act as substrates for its synthesis has been showed for the first time. The quantities of DHPG recovered in olive drupe tissue by thermal treatment exceed widely the values indicated in the literature, showing the release or formation of additional DHPG from precursors after heating. In addition, DHPG obtained under certain extraction conditions from fresh solid waste of two-phase olive oil extraction systems (alperujo) is its most important phenolic compound. Therefore, the solid olive waste is a good source of this simple monomer phenol. The chemical structure, purity and racemic nature of isolated DHPG were also analysed for the first time by NMR experiments.

Keywords: Liquid-solid two-phase olive waste (Alperujo); Hydrothermal treatments; Hydroxytyrosol; 3,4-Dihydroxyphenylglycol; HPLC analysis; Antioxidant; Chromatography; NMR spectroscopy

INTRODUCTION

Recently, there has been a growing interest in the impact of daily food intake on disease. Olive oil and table olives are typical Mediterranean products with well known nutritional and economic importance. Indeed, olive and olive oil consumption has been shown to be associated with a variety of health benefits including a lower incidence of heart disease and certain types of cancer. These beneficial effects have been attributed not only to a low saturated/monounsaturated fatty acid ratio, but also to other additional molecules present in minor concentration, particularly antioxidant phenolic compounds (Pérez-Jiménez, Ruano, Pérez-Martínez, López-Segura & López-Miranda, 2007). Olive phenols exert antioxidant activity by scavenging peroxy radicals, hydroxyl radicals, superoxide anions, and through metal chelation. Phenols prevent or reduce the effect of the oxidative stress associated with the pathogenesis of various diseases including atherosclerosis, cancer, diabetes mellitus, and inflammatory and neurodegenerative disease (Deiana, et al., 2007; Bogani, Galli, Villa & Visioli, 2007). Besides, recent studies indicate that olive phenols have a profound effect on cell survival/death genes and signal translation (Fabiani, Rosignoli, De Bartolomeo, Fuccelli, & Morozzi, 2008).

Olive fruits contain a wide variety of phenolic compounds that play an important role in the chemical, organoleptic and nutritional properties of virgin olive oil and table olives. These compounds prevent the deterioration of food by inhibition of lipid oxidation and improve the health-promoting properties when they are added. Therefore, it would be desirable to develop a process for the extraction of the antioxidant components of olive-based starting materials. After the mechanical processing of olives for olive oil extraction, less than 1% of the phenolic compounds are found in the olive oil with over 98% being located in the olive-mill waste (liquid and solid waste). Consequently, this residue could be considered a promising source for these compounds.

Hydroxytyrosol, 2-(3,4-dihydroxyphenyl)ethanol (HT), is one of the main and the most interesting phenolic compounds present in the olive fruit, with known high levels of pharmacological and antioxidant activities (Schaffer, Podstawa, Visioli, Bogani, Müller & Eckert, 2007). This compound can be found in the waste generated during olive processing, in free, as monomer, and bound forms, as oleuropein, acteoside (=verbascoside) or glucosides (Obied, Karuso, Prenzler & Robards, 2007). In a recent study, we developed a hydrothermal process to increase free HT concentration in water from semisolid waste known as alperujo (Fernández-Bolaños, Rodríguez, Rodríguez, Heredia, Guillén & Jiménez, 2002). Further, we have developed a new, simple, practical, and economical system for the purification of this natural antioxidant, which has made commercial production possible (Fernández-Bolaños, Heredia, Rodríguez, Rodríguez, Jiménez & Guillén, 2005).

Another simple phenol, 3,4-dihydroxyphenylglycol (DHPG), structurally similar to HT but with an additional hydroxyl group, was also described as a component of the vegetation water of the olive fruit (Limiroli, Consonni, Ranalli, Bianchi & Zetta, 1996). Recently, it was also detected in olive oil at a concentration below 35μmol/kg (Medina, de Castro, Romero & Brenes, 2006). This concentration is however, higher than the corresponding flavone concentration. Also, although the final concentration of the total phenols in the table olive depends on the method of processing, the presence of DHPG was confirmed in the final product (Marsilio, Seghetti, Iannucci, Russi, Lanza & Felicioni, 2005). This substance has never been reported as a free plant metabolite, but may be of interest in the fields of nutrition and pharmacology due to its powerful antioxidant properties. It is the main metabolite produced by deamination of the human neurotransmitter noradrenaline (norepinefrine). Therein, we demonstrated for the first time that the antioxidant efficiency of DHPG in water is 2-3 times higher than that of ascorbic acid or hydroxytyrosol, whereas in

lipidic medium it is comparable to that of vitamin E despite its high polarity (Rodríguez, Rodríguez, Jiménez, Guillén & Fernández-Bolaños, 2007).

Based in their chemical structures it is important to emphasize that DHPG and HT are part of the same family of compounds known as the phenylpropanoid glycosides or acteosides (**Figure 1**). They are widely found as secondary metabolites in many plant species and found in certain medicinal plants used in traditional Chinese, Japanese, and Korean medicine. These plants belong to the *Oleacea, Plantaginaceae, Orobanchaceae, Laminaceae, and Compositae* families, which are used as extracts against different diseases (Nishibe, 2002; Li, Tsao, Yang, Liu, Young & Zhu 2008). Recently, biological and medicinal studies have furthered our understanding of the phytochemical constituents of these plants. The presence of phenylpropanoid glycosides confers a variety of novel biological activities that can be rationalized by their high antioxidant activity. These compounds present antiproliferative activities (Ohno, Inoue, Ogihara & Saracoglu, 2002) and could be potential candidates for the treatment of neurodegenerative disorders (Koo, Kim, Oh & Kim, 2006). They inhibit both 5-lipoxygenase and cAMP-phosphodiesterase, block the cyclooxygenase metabolic pathway, and present complement-inhibitor activity. These properties could be valuable therapeutics in a variety of inflammatory or degenerative diseases (Díaz, Abad, Fernandez, Silvan, De Santos & Bermejo, 2004; Schneider & Bucar, 2005; Apers et al., 2002). In these oligoglycosidic compounds, both phenolic substituents (the caffeoyl and phenylethyl moieties) are required for the indicated activities. Some authors have suggested that the phenylethyl group is more important for the activities of acteoside than the caffeoyl group (Tozuka, Ota, Kofujita & Takahashi, 2005). As such, it is important to note that the scavenging activity of hydroxyacteoside was higher than that of acteoside due to hydroxylation (Harput, Çaliş, Saracoğlu, Dönmez & Nagatsu, 2006).

In the present report, we demonstrated that olive waste is a good source of DHPG which may help to protect certain organisms against lipid oxidation induced by oxidative stress. Its introduction into foods increases their health-promoting properties, and is a promising field. Besides, obtaining isolated DHPG with high degree of purity will facilitate the study of its compound bioactivity, bioavailability, toxicology, stability and interactions with other food ingredients. Another possibility for the application of these compounds is the emerging and promising role of antioxidants as therapeutic tools against neurodegenerative diseases such as Alzheimer and Parkinson diseases (Ramassamy, 2006). Well-controlled *in vivo* studies can assess the level of brain penetration of these compounds, and their subsequent impact on the progression of these neurodegenerative disorders. Likewise, the utilization of a purified and cheap DHPG may aid in developing new routes for the preparation of these biologically active products, helping to produce new drugs more inexpensively.

As a continuation of our previous investigations into the use of solid waste from two-phase oil olive extraction systems, known as "alperujo," the present research addresses a suitable strategy to treat this waste for maximum recovery of free DHPG. The effect of hydrothermal treatment that allows the recovery of the HT (Fernández-Bolaños et al., 2002) and other value-added compounds (Fernández-Bolaños et al., 2004) on the level of free DHPG recovered was also evaluated. Particular emphasis has been placed on the study of DHPG stability over a long storage period at different temperatures.

Additionally, in this paper we will mention a system for the purification of DHPG that is not described due to a patent pending status. However, the final balance of DHPG purification, which allows for the assessment of the real production level, is described. Further, the enantiomeric purity of recovered DHPG is estimated.

MATERIALS AND METHODS

Raw material and pre-treatment. Samples of alperujo, a wet solid waste from two-phase decanters, were supplied by the Oleícola El Tejar oil extraction factory in Córdoba, Spain, after certain storage period. These waste samples were partially de-stoned, partially de-oiled (after secondary centrifugal processing to obtain the residual olive oil), and had a high water content (70 % of moisture). These samples were obtained from the same source as those previously employed for the recovery of hydroxytyrosol (Fernández-Bolaños et al., 2002) and other compounds of interest (Fernández-Bolaños et al., 2004; Rodríguez et al., 2007). Alperujo was sampled at three different dates in the two olive oil production seasons (2004/2005 and 2005/2006). The first sample was taken in November, at the beginning of the olive oil production season, the second was taken in January, at the halfway mark of the season, and the third was taken in March, at the end of the season.

Fresh alperujo samples (not stored) were obtained after oil extraction from olive fruits in an experimental mill of the Instituto de la Grasa in Seville, Spain. Samples of wet alperujo were taken from the output of the horizontal centrifuge of the two-phase process.

Thermal treatment between 50 and 90 ºC for 1–4 hour was performed in a laboratory bath with slow agitation. 25 g of wet feedstock was used and water was added at 1/5 (w/v) solid/liquid ratio. Also the alperujo was autoclaved at 121 ºC for 1 hour.

More extreme steam treatment of the raw material was carried out in a custom-built batch pilot unit based on Masonite technology, and equipped with a 2 L reaction vessel designed to reach a maximum operating pressure of 42 kg/cm^{2}. The reactor was charged with 250 g of moist sample and heated to 180–240 ºC with saturated steam for 5 min. The treated material was recovered by a quick-opening ball valve. Temperature and reaction time of pretreatment used in the present study were selected based on hydroxytyrosol release (Fernández-Bolaños et al., 2002).

Additionally, the effects of prior acidification of the substrate were tested. The alperujo was acidified by soaking in 1.0, 1.5, 2.0 and 2.5% v/v of sulphuric or phosphoric acid solutions, based on the water content of the sample.

After each hydrothermal treatment, the wet material was filtered through filter paper, using a Buchner funnel or centrifuged (25000 g/10 min), for solid and liquid recovery.

HPLC analysis. An aliquot from the water soluble fraction obtained after hydrothermal treatment was filtered through a 0.45 μm membrane and used for direct HPLC determination of DHPG. HPLC was performed on a Hewlett-Packard Series 1100 liquid chromatograph system equipped with an ultraviolet-visible detector and a Rheodyne injection valve (20 μL-loop). A Spherisorb ODS-2 column (250 x 4.6 mm i.d.; particle size = 5 μm) (Tecnokroma, Barcelona, Spain) was used at room temperature. Gradient elution was performed at a flow rate of 1.0 mL/min, using a mobile phase of trifluoracetic acid in water (pH= 2.5) and acetonitrile, from 5 to 25% of acetonitrile over 30 min. Chromatograms were recorded at 280 nm.

For system calibration, individual stock solutions of DHPG (Sigma-Aldrich Química, Madrid, Spain) were prepared with deionised water in volumetric flask. Known concentration aliquots of the compound (0.02–0.4 mg/mL) were used for standard curve preparation, and for determination of the linearity of the peak-height response. This analysis was performed in triplicate.

Identification of DPGH . After purification, DHPG was identified by direct comparison with commercial DHPG by HPLC/UV analysis using retention-times and absorption spectra in the 200–380 nm, and by NMR spectroscopy. NMR spectra were recorded at 303 K on a Bruker Avance 500 spectrometer operating at 500.13 MHz (^{1}H). Samples of commercial and natural DHPG were examined as ca. 0.1 M solutions in DMSO-d_6 or acetone-d_6. Chemical

shifts are reported in parts per million (ppm) downfield from the tetramethylsilane (TMS) signal reference (0.00 ppm). To confirm the assignments of NMR signals, extensive 2D experiments were carried out. ^{1}H-NMR data for DHPG (acetone-d_6): δ 6.88 (1H, *d*, $^{4}J_{4,8}$ = 2.0 Hz, H_4), 6.75 (1H, *d*, $^{3}J_{7,8}$ = 8.0 Hz, H_7), 6.70 (1H, *dd*, H_8), 4.56 (1H, *dd*, $^{3}J_{1a,2}$ = 4.0 Hz and $^{3}J_{1b,2}$ = 8.0, H_2), 3.56 (1H, *dd*, $^{2}J_{1a,1b}$ = 10.9 Hz, H_{1a}), 3.47 (1H, *dd*, H_{1b}).

Europium tris((3-trifluoromethylhydroxymethylene)-(+)-camphorate) [$Eu(tfc)_3$] was used as a chiral shift reagent for enantiomeric purity assays. Appropriate quantities (typically 11.2 mg, 0.25 eq) of this reagent were added to 0.5 mL of the above mentioned samples solutions.

The purity of DPGH was determined by both HPLC and NMR analyses, and verified using a gravimetric method, % Purity = 100× (mg HPLC/ mg dry weight).

RESULTS AND DISCUSSION

Previously, we have examined the extraction process of HT from a semisolid residue obtained in the olive-oil industry, called “alperujo”. We proposed a hydrothermal treatment (steam explosion) as a quantitative method for evaluation of total HT content in this complex matrix. A significant retention in this solid phase is primarily responsible for the supposed loss of phenols during olive oil processing (Fernández-Bolaños et al., 2002). Extraction time and temperature, together with the use of an acid catalyst, were important variables in the extraction of this important antioxidant, comprised of both free-form phenol and phenol linked to the secoiridoidic nucleus. In the present work, the release of DHPG obtained from this natural matrix by the application of several procedures has been evaluated. Although this compound has been documented in the olive fruit (Marsilio et al., 2005), vegetation water (Limiroli et al., 1996), and recently in olive oil (Medina, de Castro, Romero & Brenes, 2006),

it has not been studied in depth, despite the higher antioxidant properties *in vitro* of this molecule relative to HT (Rodriguez et al., 2007).

The steam conditions resulting in adequate HT recovery were applied in the current case. **Figure 2** shows that DHPG was efficiently recovered by steam treatment without acidification. However, when the temperature of the reaction was increased, a notable decrease in glycol content took place. Under these conditions, in the absence of any catalyst, an autohydrolysis process of alperujo, with a decrease of pH, has been observed (Fernández-Bolaños et al., 2002). At temperatures between 220 and 240 ºC for 5 minutes, very low concentrations were detected, although complete disappearance never occurred. Similar low values were obtained in experiments conducted in the presence of an acid catalyst (H_2SO_4, 2.5%), about 0.31–0.37 g/kg of dry alperujo for each of the three different samples analysed during both studied seasons, for all temperatures (between 180 and 240 ºC). This observation contrast with the results obtained for HT in which use of an acid catalyst in the steam treatment facilitated compound liberation, even under mild conditions of temperature and pressure. A better recovery of DHPG was achieved with catalytic phosphoric acid, a less corrosive and less oxidizing catalyst compared to sulphuric acid (**Figure 3**). This result suggests that this phenol has sensitivity to high temperature and chemical treatment.

In order to account for the influence of temperature, a milder thermal treatment was conducted with fresh alperujo, without using the steam explosion reactor. Temperatures of 50, 70 and 90 ºC and treatment times of 1 and 2 hours were assayed. As expected, the amounts of DHPG were increased markedly with respect to their content in the sample control after the extraction (**Table 1**). The control fraction was recovered after a simple agitation with water, at room temperature, followed by filtration (time zero). This finding suggests that the temperature at which the alperujo is treated should be considered as one of the factors responsible of solubilisation and/or hydrolysis of DHPG in water. When the experimental

conditions were rendered more severe (90 ºC for 3 and 4 hours of treatment, autoclaved 121 ºC for 1 hour, steam-exploded at 165 and 185 ºC for 5 min), a clear decrease in the recoveries of DHPG from fresh alperujo was observed, likely due to the thermal instability previously discussed previously.

Currently, the main use of this by-product is the electrical power generation. In order to improve this use the alperujo is pre-treated to reduce its moisture content. A mild heat treatment allows recovery of a new class of olive mill waste-water with the most readily extracted polyphenols. In this work, we investigated this new liquor obtained on industrial level as a cheap source of natural antioxidants from the stored alperujo. The presence of DHPG was verified in these new liquors (**Figure 4**), and appeared in significant amounts. The initial concentrations of DHPG in two different samples were 432 mg/L and 235 mg/L. These differences likely arose from variabilities inherent to the alperujo at the industrial level. Both samples were stored in vessels at a pH of 4.5, without initial correction of pH in the case of the first sample and with pH lowered to 2.9 in the second sample. Subsequently, the evolution of DHPG was followed. In the first sample, after a 9-month storage time at ambient temperature (9–37 ºC) in an industrial warehouse, the concentration of DHPG remained nearly constant, at a concentration of 450 mg/L. In the second sample, which liquor was stored under more acidic conditions and at different temperatures, changes in the concentration of DHPG were observed (**Figure 5)**. The concentrations of this compound increased by 35% with respect to initial concentration for all temperatures over the first few days, and decreased slowly during the storage process. After 126 days of storage at 8 ºC and 25 ºC, the DHPG concentration remained constant, whereas at 35 ºC and 50 ºC, the concentration continued to diminish to levels below the initial concentration. The increase in DHPG that occurred at the beginning of the storage for all temperatures was most interesting, and warrant comment. This change suggests the presence of one or more soluble compounds

containing the DHPG core skeleton in their molecular structure or that some type of reaction implied in the DHPG formation takes place. The hydrolysis of these compounds or the mentioned reaction would likely happen during storage, thereby releasing additional DHPG. As we are aware, these observations have not yet been reported.

We successfully verified the presence of this DHPG precursor and the formation of free DHPG upon analysis of the changes in the fractions solubilised in the extraction process, under mild thermal treatment above described, from fresh alperujo. **Table 1** illustrates the important raises that occurred in the sample control and the treated samples at 50, 70, and 90 ºC for 1–2 h, and when their liquid extracts were post-treated at 90 ºC for 2 h. Such conditions proved to be optimal for DHPG release. The amount of glycol in post-treated samples was increased relative to non-post-treated samples. A simple extraction of alperujo with water (sample control) allowed us to obtain water-soluble substances such as free DHPG (0.47 g/kg of dry alperujo) and its promoters, that were later hydrolysed or transformed during post-treatment (90 ºC/2 h) up to reach 1.67 g/kg, increasing by about 237 %. The solubility of DHPG and its precursors was enhanced by increasing the extraction temperature, and the data obtained indicated a marked increase in the recovery of free DHPG from 50 ºC for 1 h, and posterior heating of the aqueous fraction to 90 ºC for 2 h (> 250% respect to non-post-treated). Quantitatively, the best conditions for extraction of free DHPG employed a temperature of extraction of 90 ºC for 2 h, and posterior heating of the solubilised fraction for 90 ºC for 2 h, reaching up to 2.69 g/kg of dry alperujo.

Additional aqueous samples of fresh and stored alperujo were treated at 90 ºC for 2 h to confirm the above increases (**Table 2**). An initial water extraction from fresh alperujo followed by a posterior treatment at 90 ºC for 2 h, resulted in a double increase of DHPG concentration and represents 87.7% and 78 % of free total DHPG. The second extraction with water with a post-treatment at 90 ºC for 2 h recovered the remaining 12.3% and 22%

respectively. These DHPG quantities exceed widely the values indicate in the literature (Marsilio et al., 2005; Limiroli et al., 1996; Medina, de Castro, Romero & Brenes, 2006), showing for the first time the release or formation of additional DHPG from precursors after heating.

The initial concentrations of DHPG in the aqueous fraction of the stored alperujo recovered to the industrial level, also called olive mill waste-water, were similar to the above samples. When these fractions were heated to 90 ºC for 2 h, an increase also occurred though there was some variability among samples. This is likely due to differences among olive cultivars, maturation of fruits, and time of storage of alperujos (fermentation time). The release of DHPG could be produced chemically by hypothetical hydrolytic reaction between the phenolic compound and the rest of the water-soluble substances. This fact is supported by the liquor acidity (pH ~4.5-5.0) and the disruption of any enzymatic process (e.g. hydrolysis) by the high temperature, time of extraction, and post-treatment.

Figure 6 shows the variation in the chromatographic profile of the phenolic compounds present in the aqueous fraction (50 ºC/1 h) as a consequence of post-treatment at 90 ºC for 2 h. The noted increase in DHPG concentration did not coincide with the decrease in the concentration of other known compounds (i.e. HT, tyrosol or their glucosides), the levels of which remained nearly constant. This observation rules out the HT-glycol or tyrosol-glycol dimers as possible DHPG precursors, isolated and identified in olive mill waste-waters (DellaGreca, Previtera, Temussi & Zanelli, 2004), because there are not increase of HT and tyrosol that coincide with the increase of DHPG by rupture of these dimers. Also, the hypothesis that DHPG results from HT oxidation, with the initial formation of an *o*-quinone and posterior addition of water giving an hydroxylation of its side chain carbon (Roche, Dufour, Mora & Dangles, 2005), was ruled out. A derivative of hydroxycinammic acid, similar to verbascoside but with DHPG in place of HT, has been isolated from plants of

Oleaceae family (Nishibe, 2002) and recently identified in *O. europeae*, as two diastereoisomers of β-OH-acteoside (Innocenti, la Marca, Malvagia, Giaccherini, Vincieri & Mulinacci, 2006). Also recently, the presence of 2”-hydroxyoleuropein, with the hydroxyl group located at the phenylethanolic moiety, has been discovered in olive tissues for the first time (Di Donna, Mazzotti, Napoli, Salerno, Sajjad & Sindona, 2007). We thus hypothesized that these two compounds may be good candidates to explain the increase in DHPG concentration with the post-treatment.

In order to investigate the presence of these water-soluble substances, a detailed examination of the HPLC-DAD data of the phenolic compounds was performed. These compounds were solubilised from fresh alperujo and subsequently post-treated at 90 ºC for 2 h. The chromatographic profile made identification of DHPG precursors difficult, because there were no peaks that changed proportionally with that of the DHPG peak. Therefore, further research was necessary. DHPG was one of the main compounds detected in the analysed fraction from fresh alperujo under the above mentioned extraction conditions. This fact was supported only by Bianchi & Pozzi (1994) who considered the DHPG as a major component of the olive phenolic fraction. It is an unexpected event, although it placed great significance on fresh pulp of the olive fruits. The release of DHPG was clearly defined during the extraction and hydrolysis processes, while minimal variation was observed in the quantities of HT, tyrosol or glucoside of HT after post-treatment. Nevertheless, if the alperujo is stored at the industrial level, and thus not fresh, HT was determined to be the main compound detected, with concentrations approximately ten times higher than DHPG concentrations (**Figure 4**). Certain reactions that facilitate HT release appear to occur during the alperujo storage. Since this is the first report on the isolation in large pure DHPG quantity from alperujo (fresh or stored) or any other natural source, we propose this extraction

procedure, carried out on this scale, as a viable system to achieve a high level of this free phenolic compound.

Additional analysis of the phenolic profile of the liquor from fresh alperujo allowed for direct observation of a major DHPG peak at 280 nm. At 330 nm (**Figure 7**), two distinct peaks were also identified in the UV spectrum, identical to those described in natural black olives (Romero, García, Brenes, García & Garrido, 2002) and recently identified by mass spectra and NMR data as the caffeoyl ester of secologanoside (Innocenti et al., 2006) and p-coumaroyl ester of secologanoside (comselogoside) (Obied, Karuso, Prenzler & Robards, 2007).

From the aqueous fractions generated from two-phase olive waste and studied in the present work, it has been possible to obtain large quantities of highly purified DHPG. The purification was performed using a simple chromatographic system, which is currently under patent. 29% pure DHPG was isolated in an overall 40 % yield, and 96% pure product was obtained in a 21 % yield. Thus, the final yield of this process on a 1000 kg sample of wet alperujo (300 kg of dry matter), would provide an initial extraction of 807 g of soluble DHPG under the best operating conditions (2.69 g/kg of dry alperujo), and would subsequently produce 323 g or 170 g of purified DHPG for each step. This simple and inexpensive method is the first to be applied to the production of this antioxidant from natural sources, and this system will allow for its production at industrial scale at very low cost.

The identification of recovered DHPG was based on comparison of UV absorbance spectra of compound with commercial standard. The chemical structure and purity of isolated DHPG was confirmed by ^{1}H-NMR spectroscopy (**Figure 8**). Besides, enantiomeric purity of naturally occurring DHPG was also determined by NMR (Parker, 1991). For this purpose, $Eu(tfc)_3$ was used as the chiral shift reagent, which it is known to form diastereoisomeric complexes *in situ* with substrate enantiomers. Commercial DHPG was used as a standard for

the adjustment of conditions as described previously (Sweeting, Crans & Whitesides, 1987). As it can be seen in **Figure 9A**, a well resolved set of doublet for methine (H_2) and methylene (H_{1a} and H_{1b}) protons were observed after addition of the chiral shift reagent. Similar results were obtained with natural DHPG (**Figure 9B**), showing the racemic nature of the recovered DHPG. These results are in agreement with the presence of the epimers, at C-2" of 2-hydroxy-1-(3,4-dihydroxyphenyl)ethanol moieties, (2"R)-2"-hydroxyoleuropein and (2"S)-2"-hydroxyoleuropein in very similar proportions (3.9 and 3.5 mg respectively) as found in *Fraxinus Americana* of the family Oleaceae (Takenaka, Tanahashi, Shintaku, Sakai, Nagakura & Parida, 2000).

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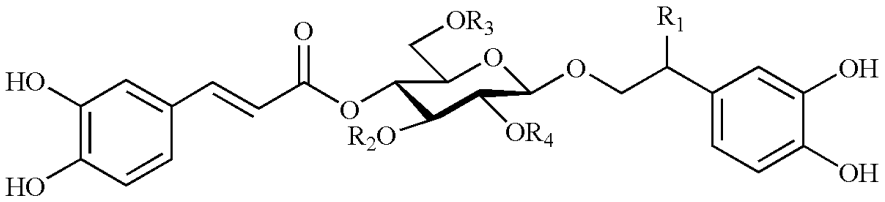
(1) .



β-Hydroxyacteoside	R_1 = OH	R_2 = rhamnosyl	R_3 = H	R_4 = H
Suspensaside	R_1 = OH	R_2 = H	R_3 = rhamnosyl	R_4 = H
Hellicoside	R_1 = OH	R_2 = glucosyl	R_3 = H	R_4 = H
Orobanchoside	R_1 = OH	R_2 = H	R_3 = H	R_4 = rhamnosyl
Wedelosin	R_1 = OH	R_2 = rhamnosyl-apiofuranosyl	R_3 = H	R_4 = H
Acteoside (= Verbascoside)	R_1 = H	R_2 = rhamnosyl	R_3 = H	R_4 = H

Figure 1. Structures of the phenylpropanoid glycosides (acteoside family)

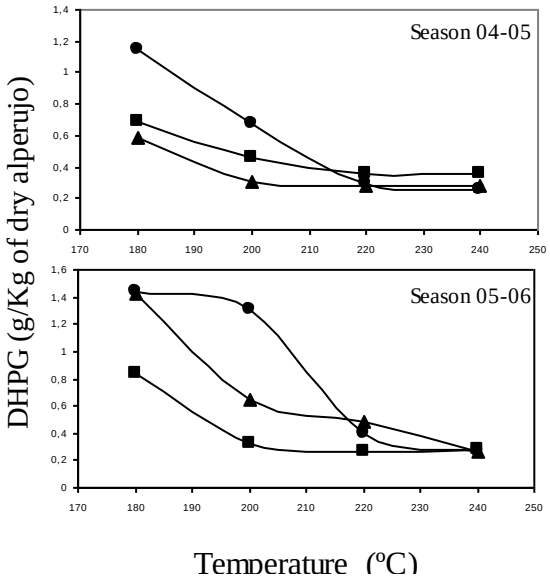


Figure 2. Evolution of released DHPG for three samples (first, ●; second, ■; third, ▲) in two seasons for the two-phase olive waste treated by steam over 5 minutes at different temperatures.

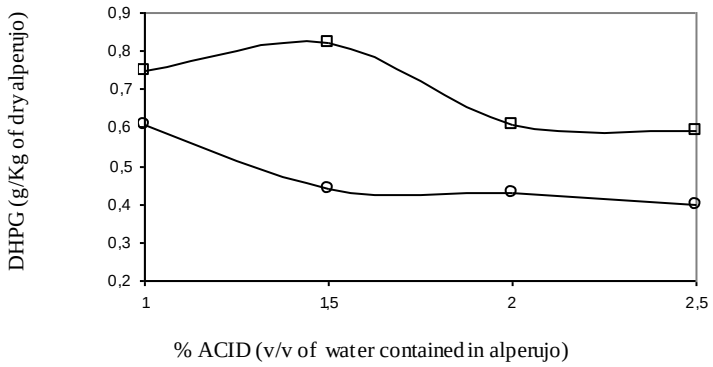


Figure 3. DHPG released after steam treatment (200 ºC, 5 minutes) in presence of an acid catalyst (-□- H_3PO_4 or -◊- H_2SO_4).

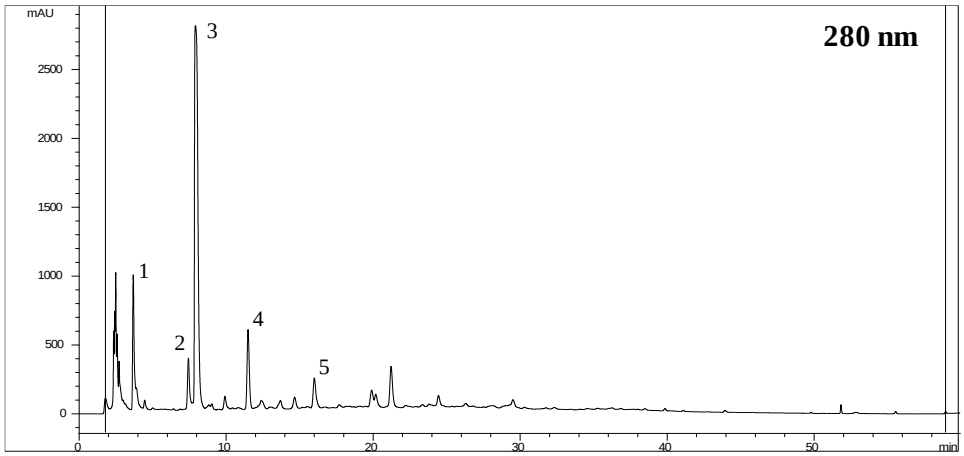


Figure 4. Chromatogram (280 nm) of phenolic compounds in olive mill waste-water from industrially stored alperujo. Peaks: (1) 3,4-dihydroxyphenylglycol; (2) hydroxytyrosol 4-β-D-glucoside; (3) hydroxytyrosol; (4) tyrosol; (5) caffeic acid.

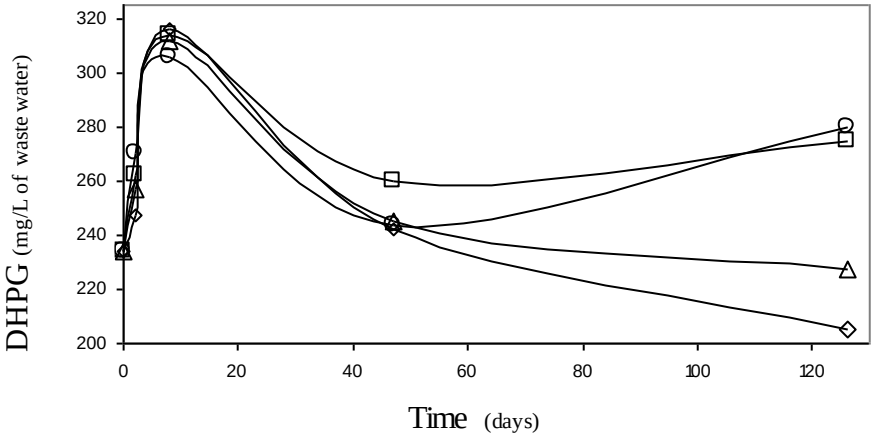


Figure 5. Evolution of the DHPG stored under acidic conditions (pH = 2,9) at different temperatures (○) 8 ºC; (□) 25 ºC; (Δ) 35 ºC and (◊) 50 ºC for 120 days.

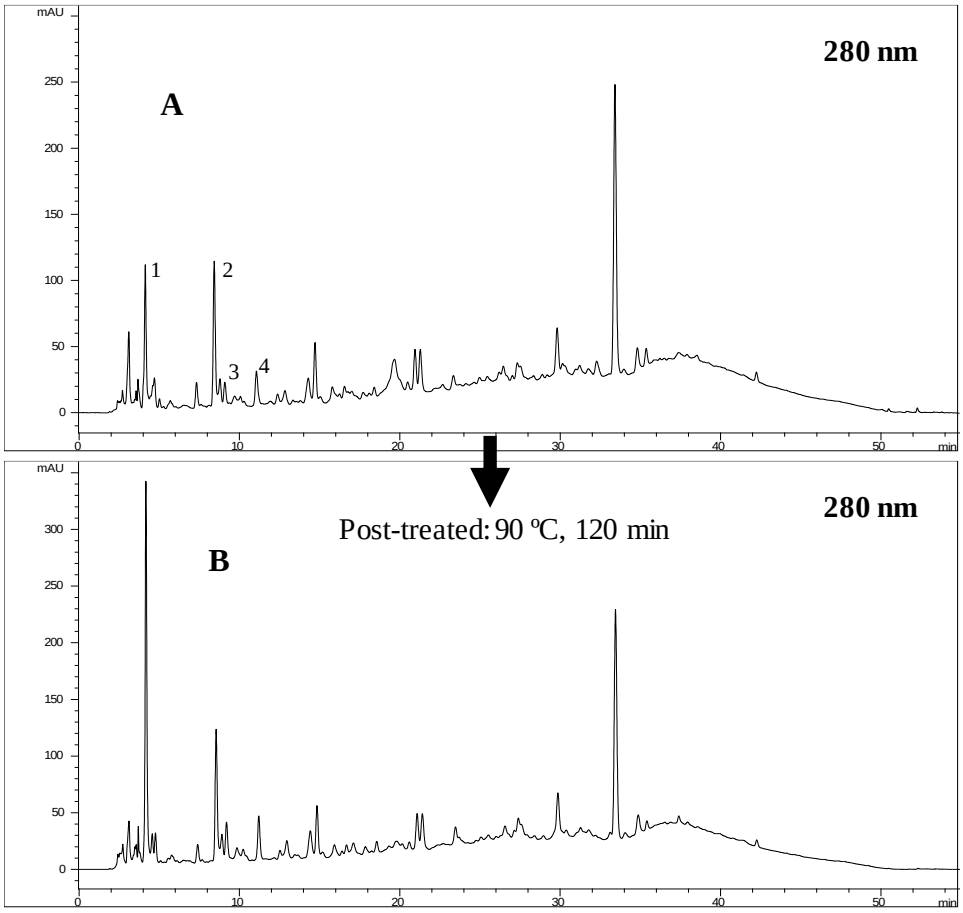


Figure 6. Chromatograms of phenolic compounds in the aqueous fractions recovered from fresh alperujo treated at 50 ºC for 1 hour (A), and after a post-treatment at 90 ºC for 2 hours (B), both at 280 nm. Peaks: (1) 3,4-dihydroxyphenylglycol; (2) hydroxytyrosol 4-β-D-glucoside; (3) hydroxytyrosol; (4) tyrosol.

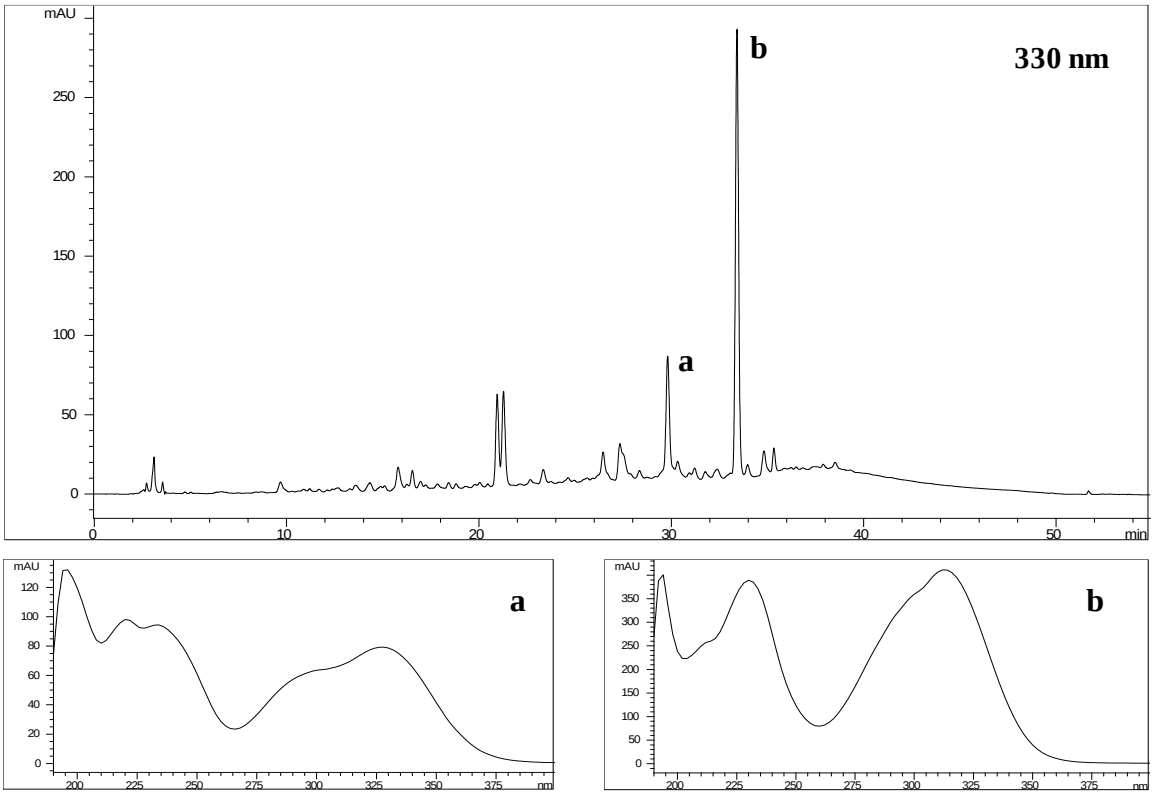


Figure 7. Chromatogram (330 nm) of phenolic compounds in fresh alperujo treated at 50 ºC for 1 hour and UV spectra of "a" and "b" compounds.

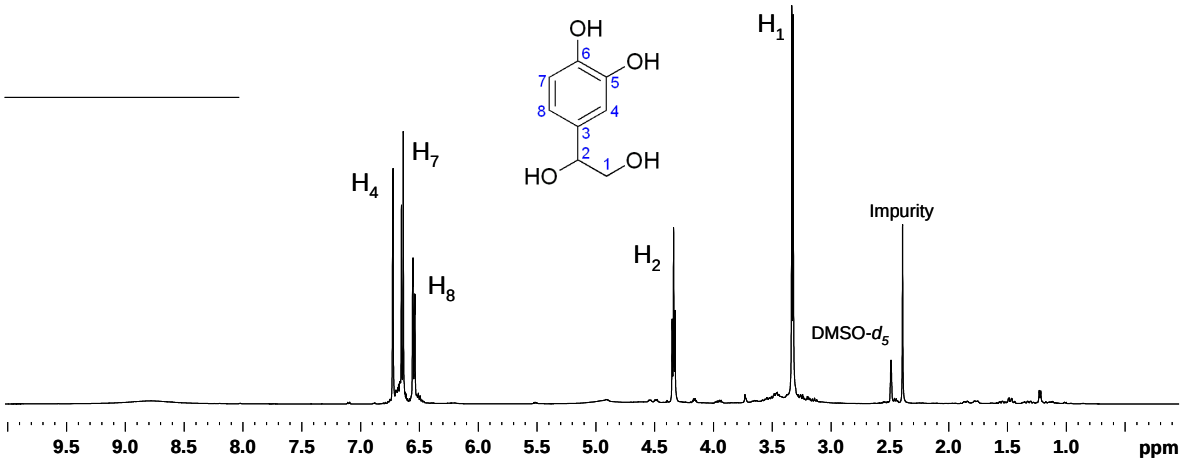


Figure 8: ^{1}H NMR spectrum obtained in DMSO-d_6 for isolated DHPG.

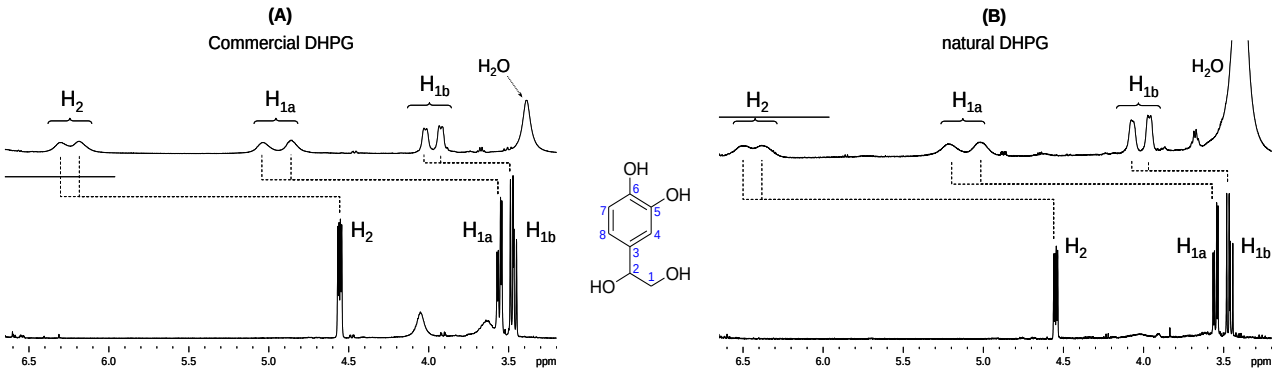


Figure 9: Comparison of ^{1}H NMR spectra obtained in acetone-d_6 for commercial (A) and natural (B) DHPG. In the bottom, a selected region of both is shown, whereas on top, the same region of spectra was recorded after addition of 0.25 eq of $Eu(tfc)_3$.

Table 1. DHPG release under different thermal treatment conditions, and DHPG release after additional post-treatment at 90 ºC for 2 hours.

Temperature (ºC)	Time (minutes)	DHPG (g/kg of dry alperujo)	Post-treatment: 90ºC, 120 min DHPG (g/kg of dry alperujo)
Control	0	0,47 ±0,01[a]	1,67 ± 0,05
50	60	0,57 ± 0,06	2,02 ± 0,07
	120	0,70 ± 0,02	1,97 ± 0,03
70	60	1,40 ± 0,01	2,72 ± 0,08
	120	1,68 ± 0,08	2,50 ± 0,04
90	60	1,51 ± 0,06	2,38 ± 0,04
	120	2,27 ± 0,09	2,69 ± 0,02
	180	1,48 ± 0,10	-
	240	1,08 ± 0,01	-
121[b]	60	1,26 ± 0,04	-
165[c]	5	0,90 ± 0,02	-
185[c]	5	0,76 ± 0,01	-

a) Standard deviation.
b) Autoclaved.
c) Steam explosion treated.

Table 2. DHPG release under treatment condition at 90ºC for 2 h in aqueous samples obtained from fresh alperujo, with a simple and double extraction with water, and from stored alperujo as olive mill waste-water obtained to industrial level.

		DHPG (g/kg of dry alperujo)				
		1st aqueous extration		2nd aqueous extration		
	Samples	Initial	Treatment 90ºC/2h	Initial	Treatment 90ºC/2h	Total
Fresh alperujo	1	0.24±0.01[a]	0.50±0.02 87.7%[b]	0.05±0.01	0.07±0.01 12.3%	0.57±0.03
	2	1.24±0.06	2.34±0.15 78%	0.35±0.01	0.66±0.01 22%	3.00±0.16
		DHPG (mg/L of olive mill waste-water)				
		Initial		Treatment 90ºC/2h		Δ[c] (%)
Stored alperujo	1	284		364		28
	2	305		321		5
	3	203		348		71

a) Standard deviation.
b) Percentage recovered respect to DHPG total.
c) Increasing of DHPG concentration with the heating.