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Influence of pH on the antioxidant phenols solubilised from hydrothermally treated olive oil by-product (alperujo).

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ABSTRACT

The application of a novel industrial 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. In this study the effect of pH on phenol extraction with ethyl acetate from the aqueous phase of hydrothermally treated alperujo at 160ºC/60 min (without modification, pH 4.5, and adjusted to pH 2.5) was evaluated, beside the increase of hydroxytyrosol during the storage. The variation of the concentration of phenolic compounds in each extract was analyzed by HPLC. The phenolic extract obtained at pH 4.5 presented a higher proportion of total and individual phenols and better antioxidant capacity *in vitro* than the extract obtained at pH 2.5. The use of lower pH values enhances the concentration of hydroxytyrosol in the liquid diminishing the storage times.

Keywords: alperujo; hydroxytyrosol, phenols; antioxidant; olive oil wastes; ethyl acetate extract.

1. Introduction.

The Mediterranean diet, characterized by a high consumption of olive oil, fruits, vegetables, grains and legumes, reduces the incidence of major cardiovascular events (Estruch et al., 2013; De Lorgeril, Salen, Martin, Monjaud, Delaye, & Mamelle, 1999) and is associated with a lower risk of peripheral artery disease (Ruíz-Canela, Estruch, Corella, Salas-Salvado & Martínez-González, 2014). High concentrations of free radical-scavenging like polyphenols and flavonoids have been attributed as an important contributory factor for the health benefits of the Mediterranean diet. Virgin olive oil is rich in unsaponifiable minor components such as sterols, tocopherols, and polyphenols. The polyphenols are natural antioxidants that not only contribute to the stability of the oil, but also have anti-inflammatory and anti-atherosclerotic properties (González-Santiago et al., 2006). After olive oil extraction, only a low percentage of the total phenolic compounds present in the olive fruits are found in the virgin olive oil. The remaining phenolics (98-99 %) end up in alperujo, a by-product of the modern two-phase processing technique used in olive oil production (Fernández-Bolaños, Rodríguez, Gómez, Guillén, Jiménez, Heredia, & Rodríguez, 2004). As such, this material should be considered as an important source of polyphenols. Several studies have evaluated the extraction capacity of different solvents to obtain phenolic extracts from olive oil by-products. Allouche, Fki, and Sayadi (2004) compared various polar solvents such as methyl isobutyl, acetone, methyl ethyl ketone, diethyl ether and ethyl acetate in the extraction of phenolic compounds from the alperujo by a continuous extraction system. The results showed that extraction with ethyl acetate yields more enriched extract phenols than the others solvents. Obied, Allen, Bedgood, Prenzler, and Robards (2005) conducted a study on different solvents to obtain an extract enriched in

phenols from the alperujo. Acetate ethyl and various aqueous mixtures of methanol, ethanol, n-propanol, acetonitrile, and acetone were tested, and the results showed that acetate ethyl extraction is selective for small and medium molecular weight phenols.

The pH of extraction with ethyl acetate used was highly variable among the different studies, with some studies utilising ethyl acetate acidified with HCl to pH 3 to extract phenols from olive oil solid residues (Lesage-Meessen et al., 2001; Obied, et al., 2005; Mulinacci et al., 2005), and others using ethyl acetate at pH 4.5 (Capasso, Cristinzio, Evidente, & Scognamiglio, 1992) and pH 2 (El-Abbassi, Kiai, & Hafidi, 2012) to extract phenol from olive mill waste water.

A hydrothermal treatment has been developed at industrial scale (Patent Nº ES2374675) to facilitate the extraction of valuable compounds and the liquid-solid separation into two phases (solid and liquid) from two-phase olive waste. This process produces a high solubilisation of simple phenolic compounds in the liquid phase due to their breakdown from complex molecules. Its industrial implementation will produce a considerable amount of liquid rich in sugars and phenols, making necessary studies to recover these valuable compounds. The liquid phase is industrially stored in evaporation ponds, in which hydrolysis reactions can release simple phenols like hydroxytyrosol from their conjugated forms, but during long storage undesirable compounds and odours are formed by fermentation. The use of lower pH values during the storage could help to prevent it, beside to enhance the concentration of simple phenols.

In previous work, the effect of the length of hydrothermal treatment on the composition of phenolic extracts and their properties was studied (Rubio-Senent, Rodríguez-Gutíerrez, Lama-Muñoz, & Fernández-Bolaños, 2012). In this work a study of the increase of hydroxytyrosol during the storage at lower pH and different temperatures was determined in the liquid after the thermal treatment. The influence of

the pH of the ethyl acetate extraction on the composition and antioxidant characteristics of phenolic extracts has been also evaluated, for two different pHs. These two different pHs have been selectioned considering the most employed by different authors. So usually two different actions were realized when an extraction with ethyl acetate was carried out, or the sample was acidified at pH 2-3 with HCl, or the pH is not modified leaving the sample with its original value of pH (4.5). Furthermore, we confirmed the reproducibility of the hydrothermal treatment together with the phenolic extraction.

2. Materials and methods.

2.1. Raw material.

The sample of alperujo (a semi-solid residue composed of olive peels, pulp, seeds, and ground stones) was obtained in February of 2011 (end of the olive oil season) from Picual olives processed at a Spanish oil mill (Experimental Mill, Instituto de la Grasa, Seville). The alperujo was processed in the pilot reactor without removal of the stones.

2.2. Standard compounds.

Hydroxymethylfurfural, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, 4-methylcatechol, catechol, 3,4-dihydroxyhydrocinnamic acid, homovainillic acid and 3,4-dihydroxyphenylglycol were obtained from Sigma-Aldrich (Deisenhofer, Germany). Tyrosol was obtained from Fluka (Buchs, Switzerland). Oleuropein, hydroxytyrosol acetate and hydroxytyrosol was obtained from Extrasynthese (Lyon Nord, Geney, France).

The compound (E)-3-(1-Oxobut-2-en-2-yl)glutaric acid that are not commercially available was 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.

2.3. Thermal treatment.

The hydrothermal treatment (Patent Nº ES2374675) was performed using a prototype designed by our research group at the Instituto de la Grasa (Seville, Spain). 10 kg of fresh alperujo were treated for 60 min at 160 ºC, and the wet material was centrifuged at 4700 g (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 ºC. The process was realized in duplicate (termed process A and process B).

2.4. Phenol extraction.

The liquid portions obtained after treatment were washed with hexane to remove the lipid fraction. Half of the sample was subjected to extraction with ethyl acetate without modifying the pH (4.5), while the other half was acidified with HCl to pH 2.5. The same method was performed for the samples from process A and process B. Extraction of phenolic compounds was carried out with ethyl acetate (500 mL per 200 mL of sample), following the process described by Rubio-Senent et al. (2012), where the aqueous and organic phase were separated.

2.5. Determination of the total phenolic content.

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

2.6. Determination of hydroxytyrosol concentration during the storage.

Samples of the liquid fraction obtained after the thermal treatment (60 min at 160 ºC) were acidified with HCl to pH 2.5 and stored at four temperatures, 8, 25, 35 and 50 ºC. A sample without acidification was used as a control (pH 4.5) stored at room temperature. Aliquots of each sample were taken on different days over a period of 220 days (close to the maximum time of storage in the industry pounds) and filtered through 0.45μm for the analysis of the concentration of hydroxytyrosol by HLPC.

2.7. Chromatographic fractionation of the ethyl acetate extracts

For facility determination and quantification of phenols in each extract a fractionation had been realized. Each extract 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. In each fraction, the different phenols were identified and quantified by HPLC-DAD.

2.8. HPLC-DAD.

The method used was described by Rubio-Senent et al., (2012). The different phenols were quantified using a Hewlett-Packard 1100 liquid chromatograph system with a C-18 column (Teknokroma Tracer Extrasil ODS-2, 250 mm x 4.6 mm i.d. 5 μm).

149 The system was equipped with a diode array detector (DAD; the wavelengths used for
150 quantification were 254, 280 and 340 nm) and Rheodyne injection valves (20 μL loop).
151 The mobile phases were 0.01% trichloroacetic acid in water and acetonitrile utilizing
152 the following gradient over a total run time of 55 min: 95 % A initially, 75 % A at 30
153 min, 50 % A at 45 min, 0 % A at 47 min, 75 % A at 50 min, 95 % A at 52 min until the
154 run was completed. Quantification was carried out by integration of peaks at different
155 wavelengths with reference to calibrations made using external standards.
156
157 2.9. Antioxidant activities.
158
159 *2.9.1. Antiradical capacity: 2,2-diphenyl-1-picrylhydrazyl (DPPH).*
160 The free radical-scavenging capacity was measured using the DPPH method
161 described in a previous study (Rubio-Senent et al., 2012) and expressed as EC50
162 (effective concentration, mg/mL), calculated from a calibration curve using linear
163 regression for each antioxidant.
164
165 *2.9.2. Reducing power*
166 The reducing power assay was performed according to the procedure described in
167 a previous study (Rubio-Senent et al., 2012). The assay was calibrated using 6-hydroxy
168 2,5,7,8- tetramethylchroman-2-carboxylic acid (Trolox), and the results were expressed
169 in mg/mL TE (Trolox equivalent). To express the results, a calibration curve was
170 created by plotting A_{490} against the known concentration of Trolox (0.059e0.56 mg/mL)
171 [correlation coefficient (R) ¼ 0.9936].
172
173 2.10. Statistical analysis.

Results were expressed as mean values ± standard deviations. To assess the differences between samples a comparison was performed using the Statgraphics Plus program version 2.1. Multivariate analysis of variance (ANOVA), followed by Duncan's comparison test was performed. Results were considered statistically significant for $P < 0.05$.

3. Results and discussion.

3.1. Hydroxytyrosol increase during the storage.

The concentration of one of the most active simple phenol present in olive was measured at different days as **Figure 1** shows. Despite the initial concentration of HT was high (2.10 mg/mL) an increase of the HT content was observed for all the samples except for the higher storage temperature (50 ºC). The acidification leads to increase fast the HT concentration up to a maximum value over 3.1 g/L for 25 and 35 ºC and 126 days, but not the final value at 220 days in comparison with the control. The concentration of HT increased slowly in the control without maximum, appearing only in this sample undesirable odours from the 126 days, approximately. A loss of HT concentration was observed using 50 ºC or in the acidified samples after 126 storage days. The causes of the degradation of HT by chemical or biological oxidation are well known as the formation by the hydrolysis from its conjugated forms like oleuropein or their glycosides derivatives (Fernández-Bolaños et al., 2008). Beside the formation of HT by their precursors, some authors have been proposed the loss or the release of phenols like HT should be linked to condensation reactions or certain interactions

among phenols, polysaccharides, and glycoproteins during the malaxation of the olive paste (Fernández-Bolaños et al., 2002).

3.2. Hydrothermal treatment and phenolic extract.

The results obtained after hydrothermal treatment of fresh alperujo at 160ºC/60 min and extraction of the aqueous fraction with ethyl acetate at pH 4.5 or adjusted to 2.5 are shown in **Table 1**. Double the amount of phenolic extract (organic fraction evaporated to dryness) per kg of fresh alperujo was obtained when the extraction was performed at pH 2.5 (30 g for pH 2.5 vs. 15 g for pH 4.5). However, the phenolic content in the extracts showed that at pH 4.5 the extracts contained 62% of phenol while at pH 2.5 only 46% of the extract had phenolic nature, and therefore contained many other compounds with no phenolic nature (16 g for pH 2.5 vs 6 g for pH 4.5). The total phenols determined by colorimetric method have been used to quantify the maximum quantity of phenols, the free ones and the phenols linked to other compounds.

Extraction with ethyl acetate at pH 4.5 caused a small increase of phenols in the aqueous fraction (g/ 100 g aqueous extract) compared to extraction at pH 2.5. Previous studies conducted by our research group have shown that in the aqueous fraction at pH 4.5 the phenolic compounds present were bound to oligosaccharides, and others were identified as phenolic glycosides (i.e. glucosides of hydroxytyrosol and tyrosol), verbascoside and secoiridoids (Rubio-Senent, Rodríguez-Gutíerrez, Lama-Muñoz, & Fernández-Bolaños 2013a).

3.3. Characterization of phenolic extract.

The compositions of phenolic extracts from the organic fraction were analyzed by chromatographic fractionation using Amberlite XAD-16 (Rubio-Senent et al., 2012),

223 and the different phenols identified and quantified by HPLC-DAD in each extract are
224 listed in **Table 2** and show in a representative chromatogram at 280 nm in **Figure 2**.
225 Firstly the data show that there are not important differences between the two
226 replicates for each of the treatments.
227 Some differences were found in the phenolic composition of extracts depending
228 on the pH of extraction with ethyl acetate. Changing the pH did not cause a significant
229 difference in the concentration of some compounds, such as protocatechuic acid, p-
230 hydroxybenzoic acid, vainillic acid, oleuropein derivatives and the polymeric phenolic
231 fraction (PPF). However, many other compounds quantified showed significant
232 differences in concentration as a function of pH. The phenolic alcohols (3,4-
233 dihydroxyphenylglycol, hydroxytyrosol, tyrosol), the phenolic acid 3,4-
234 dihydroxyhydrocinnamic acid, oleuropein, and the elenolic acid derivatives, including
235 the (E)-3-(1-Oxobut-2-en-2-yl)glutaric acid, showed a higher concentration in the
236 extract obtained at pH 4.5 than at pH 2.5. This last compound, and the second most
237 abundant, is a novel elenolic derivative whose structural determination and nature have
238 been recently presented by our group (Rubio-Senent et al., 2015). Other species, such as
239 catechol, 4-methylcatechol, hydroxytyrosol acetate and homovainillic acid were found
240 in greater amounts in the extract obtained at pH 2.5 than at pH 4.5. In addition, the
241 extract obtained at pH 2.5 had higher amounts of degradation products of sugars
242 (hydroxymethylfurfural). These differences in concentration may be due to the
243 instability of some compounds at the lower pH. Alternatively, the degradation of some
244 compounds may cause the release of different units that form their structures, as for the
245 case of oleuropein, which is more concentrated in the extract at pH 4.5 than at pH 2.5.
246 The concentration of hydroxytyrosol acetate and hydroxytyrosol showed an inverse
247 relationship between the two extracts at different pH, probably because the acetylation

for the formation of hydroxytyrosol acetate is favored in the presence of ethyl acetate at low pH (2.5).

With respect to the total balance, although the method of Folin-Ciocalteu quantified more total phenols in the extract obtained at pH 2.5 in comparison with the extract obtained at pH 4.5 (14 g or 11.5 g phenol per kg of fresh alperujo, respectively), a greater amount of individual phenols were identified and quantified by HPLC-DAD in the extract obtained at pH 4.5 (7.4 g vs. 5.9 phenol per kg of fresh alperujo).

In relation to PPFs, which were formed during the ethyl acetate extraction process and have been studied in a previous work (Rubio-Senent, Lama-Muñoz, Rodríguez-Gutiérrez & Fernández-Bolaños, 2013b), there were no significant differences between the amounts of PPF obtained at the different pHs of extraction. However, an important difference in the composition of PPF was detected. The PPF obtained at pH 2.5 presented in their composition only 10.5% of total phenols, whereas the PPF obtained without modification of pH (4.5) presented in their composition 70 % of total phenols. Therefore, the extract obtained with modification of pH was enriched in other components different to phenols that affected the composition of the PPF fraction. This observation is consistent with the properties of adsorption of the phenol of PPF (Rubio-Senent et al., 2013b) that reaches its maximum at pH 4.5, or at least these phenols adsorbed are those quantitatively detectable.

3.4. Antiradical activity and reducing power.

The results obtained for the antiradical activity and reducing power of the organic and aqueous fractions from the liquid-liquid extraction with ethyl acetate at the two pH tested were related with phenol content. **Figure 3A** shows the antiradical capacity against DPPH radicals (EC_{50}), and **Figure 3B** the reducing power (expressed as Trolox

equivalents in mg/mL) for the fractions from each hydrothermal treatment in duplicate (Treatment A and B).

Both the antiradical capacity (**Figure 3A**) and reducing power (**Figure 3B**) showed a positive relationship with the total phenolic content in the fractions studied. Interestingly, the organic fractions obtained at pH 4.5, which had a higher proportion of total and individual phenols (**Tables 1 and 2**) than at pH 2.5, presented an antiradical capacity similar to widely known natural antioxidants such as HT, DHPG and α-tocopherol, as deduced from the EC_{50} value. The reducing power values, expressed as Trolox equivalents in mg/mL, indicated that the organic extract obtained at pH 4.5 exhibited higher activity than at pH 2.5, although they presented lower reducing power values than for HT and DHPG, although they were more effective than α-tocopherol.

The results show that the aqueous fraction presented lower antioxidant properties than the organic fraction for both pHs tested. These low activities have been related with antioxidant phenols that remain in the aqueous fraction and are bound to oligosaccharides (Fernández-Bolaños, Rubio-Senent, Lama-Muñoz, García & Rodríguez-Gutiérrez, 2014). Furthermore, the aqueous fractions could act as antioxidant soluble fiber with bioactive properties assigned to a phenol moiety.

In addition, we confirmed that there were no significant differences in either the composition or activity of both the organic and aqueous extracts obtained from the two treatments at 160 ºC/60 min (treatment A and B). Consequently, we can conclude that the hydrothermal treatment for the same raw material is reproducible.

4. Conclusions.

The acidification of the liquid phase after the thermal treatment helps to increase the concentration of hydroxytyrosol in free form and to prevent the formation of undesirable odours during the storage. By other hand, the results demonstrate that the pH at which the extraction is performed affects the phenolic extract obtained. The extract obtained at pH 4.5 presented a greater percentage of total phenols and it was composed mainly of hydroxytyrosol and an elenolic acid derivative. In the extract obtained at pH 2.5 however, other molecules such as 4-methylcatechol and hydroxytyrosol acetate were in greater proportion, followed by hydroxytorosol and the elenolic acid derivative. Moreover, the phenolic extract obtained at pH 4.5 showed higher antioxidant/free radical-scavening activity than the extract obtained at pH 2.5, with an activity similar to HT, DHPG and α-tocopherol. The PPF isolated was also more enriched in phenolic compounds at pH 4.5. Therefore, this study reveals that the pH of the extraction with ethyl acetate is critical since it determines both the characteristics and the composition of the extract obtained. We also confirmed that the hydrothermal treatment of alperujo and the liquid-liquid solvent extraction process is reproducible, with no significant differences observed in the composition of the extracts obtained in duplicate in any of the tests performed. Thus, the production of phenolic extracts by organic solvent should be done avoiding long period of storage of the liquid phase or storing at lower pH, and correcting it to the initial one (pH 4.5) just before the extraction.

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Figure Captions.

Figure 1. Concentration of hydroxytyrosol during the storage of the liquid phase obtained from alperujo thermally treated at two pHs (the original at pH 4.5 and the acidified one at pH 2.5) and four different temperatures. The data are presented as means ± SD (Standard Deviation) of three determinations. * Significantly different from control (p >0.05).

Figure 2. Phenolic profile at 280 nm of representative phenolic extract after the thermat treatment (160 ºC, 60 min) at pH 2.5. The identified phenolic compounds are: 1). 3,4-dihydroxyphenylglycol, 2) hydroxymethylfurfural, 3) hydroxytyrosol, 4) (E)-3-(1-Oxobut-2-en-2-yl) glutaric acid, 5) tyrosol, 6) catechol, 7) 3,4-dihydroxyhydrocinnamic acid, 8) protocatechuic acid, 9) p-hydroxybenzoic acid, 10) homovainillic acid, 11) vainillic acid, 12) 4-methylcatechol, 13) hydroxytyrosol acetate, 14) oleuropein and 15) elenoic acid derivatives.

Figure 3. **A.** Radical scavenging capacity (DPPH radical) and **B.** reducing power of the extracts obtained from hydrothermally treated alperujo and extraction with ethyl acetate (organic and aqueous phases) using two different pHs (2.5 and 4.5) and standards (HT, DHFG, and α-tocopherol). Antiradical activity DPPH is expressed as EC_{50} (mg/mL) and reducing power in Trolox equivalent (mg/mL). The data are presented as means ± SD (Standard Deviation) of three determinations. The different letters indicate significantly different results ($p < 0.05$). Faq, aqueous fraction; Forg, organic fraction.

	Hydroxytyrosol (mg/mL)				
	pH 2,5				pH 4,5
Time (days)	8 ºC	25 ºC	35 ºC	50 ºC	Control
0	2,10 ± 0,04	2,10 ± 0,04	2,10 ± 0,04	2,10 ± 0,04	2,10 ± 0,04
2	2,43 ± 0,06*	2,34 ± 0,02*	2,16 ± 0,03	2,30 ± 0,04*	2,10 ± 0,03
4	2,28 ± 0,03*	2,44 ± 0,03*	2,40 ± 0,03*	2,49 ± 0,05*	2,10 ± 0,03
46	2,60 ± 0,04*	2,76 ± 0,06*	2,71 ± 0,05*	2,49 ± 0,03*	2,15 ± 0,06
126	2,94 ± 0,08*	3,18 ± 0,10*	3,11 ± 0,07*	2,50 ± 0,05	2,51 ± 0,04
220	2,84 ± 0,08	2,77 ± 0,06	2,90 ± 0,06	2,00 ± 0,09*	2,84 ± 0,06

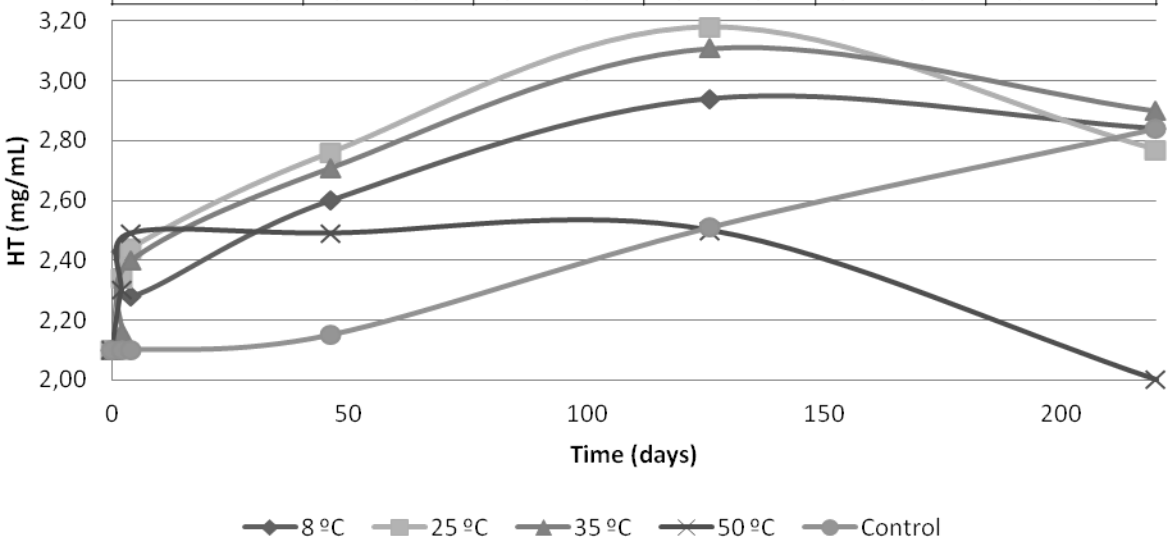

Figure 1.

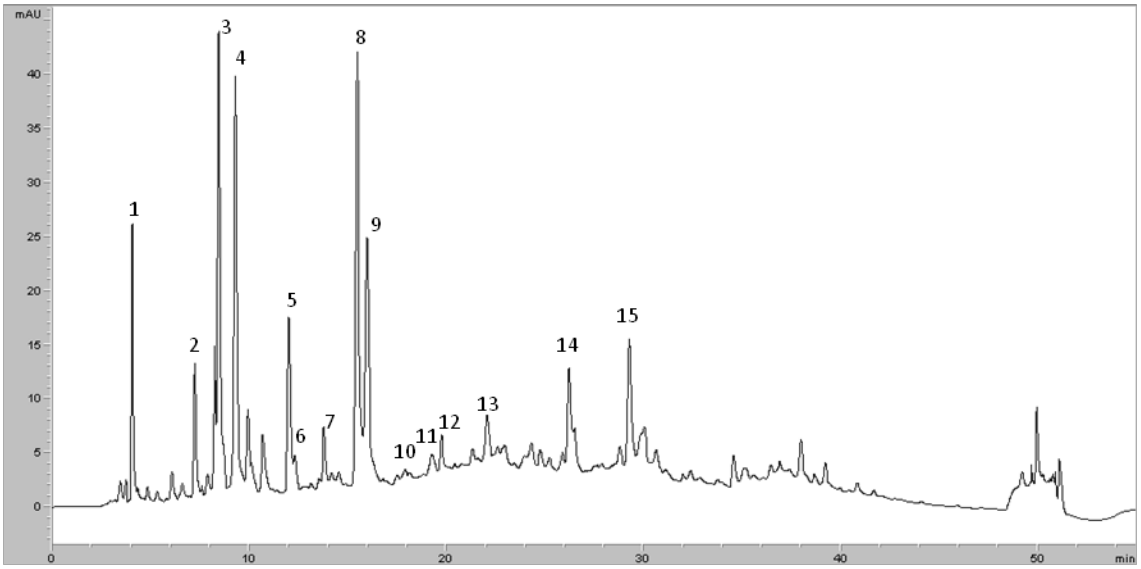
mAU
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Figure 2

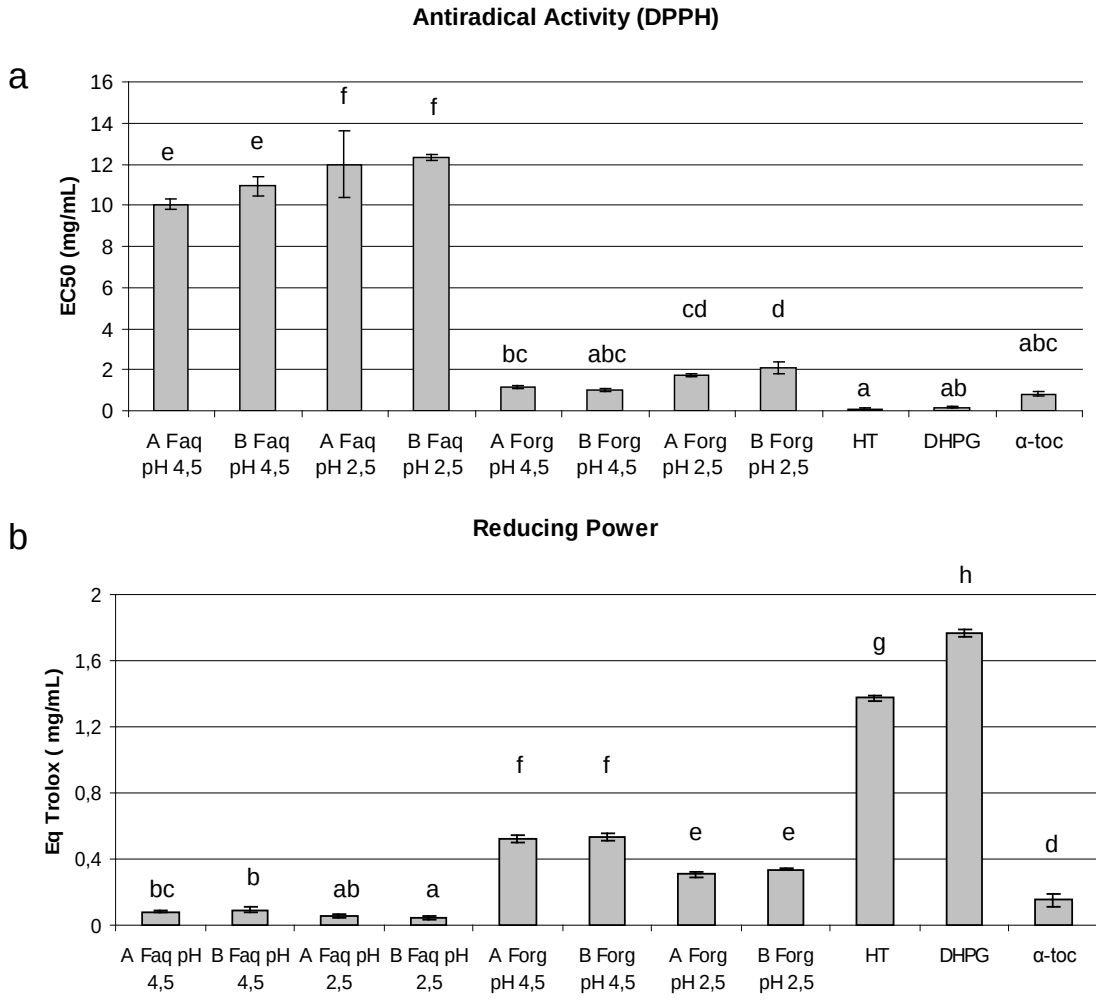

Figure 3

Table 1. Yield of phenolic extracts and phenolic content obtained by hydrothermal treatment of alperujo at 160 ºC/60 min and extracted with ethyl acetate with two different pH (without modification, pH 4.5, and with acidification, pH 2.5).

	Replicate A		Replicate B	
pH	4.5	2.5	4.5	2.5
g of phenolic extract/kg fresh alperujo	13.99	29.58	15.67	30.92
g phenol in organic fraction[a]/100 g extract	60.41±2.43 [b]	44.26±3.44	64.20±5.71	47.43±2.24
g phenol in aqueous fraction[a]/100 g extract	10.70±0.01	8.01±0.59	8.79±0.76	9.09±0.15
g phenol in organic fraction[a]/kg fresh alperujo	11.45±0.34	13.68±1.61	11.63±1.32	14.66±1.31
g phenol in aqueous fraction[a]/kg fresh alperujo	7.66±0.04	5.26±0.14	6.7±0.58	5.25±0.12

[a] Determined by Folin-Ciocalteu's method as gallic acid equivalent.

[b] Mean ± SD (Standard deviation) of three determinations.

Table 2. Concentration of identified phenolic compounds in the extracts (replicate A and B) from hydrothermally treated alperujo with ethyl acetate at different pHs by HPLC-DAD.

	mg/Kg fresh alperujo				
pH	4.5			2.5	
Treatment	A	B		A	B
phenolic alcohol					
3,4-dihydroxyphenylglycol[b]	20.16±1.44[a]	19.43±0.89	*	9.73±0.98	7.50±1.47
Hydroxytyrosol[b]	1508.91±29.01	1412.55±88.22	*	799.82±16.37	832.69±35.60
Tyrosol[b]	196.74±4.33	149.41±1.21	*	115.43±13.23	69.56±4.43
Catechol[b]	39.90±2.55	40.65±3.13	*	66.60±3.60	63.77±1.81
4-methylcatechol[b]	268.26±0.72	222.46±2.26	*	978.44±80.18	936.85±17.48
hydroxytyrosol acetate[b]	686.43±13.75	562.31±22.68	*	866.34±16.37	764.85±4.80
degradation product of sugar					
Hydroxymethylfurfural[b]	36.34±2.42	48.20±0.97	*	59.56±2.31	49.79±6.36
phenolic acid					
protocatechuic acid[b]	40.49±1.83	40.44±1.20		44.38±1.39	38.36±2.32
3,4-dihydroxyhydrocinnamic acid[b]	40.24±1.16	38.78±5.17	*	n.d.	n.d.
homovainillic acid[b]	n.d.	n.d.	*	18.67±0.55	12.56±0.23
p-hydroxybenzoic acid[b]	13.80±0.11	10.29±0.41		7.35±0.58	6.86±0.25
vainillic acid[b]	25.15±0.26	20.56±0.10		23.97±0.26	21.62±0.78
oleuropein derivatives					
Oleuropein[b]	12.96±0.06	14.67±1.68	*	8.47±0.39	7.62±0.32
oleuropein derivatives[c]	36.99±4.55	27.58±2.24		38.12±4.82	39.20±7.41
elenoic acid derivatives					
(E)-3-(1-Oxobut-2-en-2-yl)glutaric acid[b]	1124.38±64.52	950.28±52.14	*	776.48±18.64	770.00±44.16
elenoic acid derivatives[d]	787.60±30.89	895.82±85.93	*	n.d.	n.d.
polymeric phenolic fraction					
polymeric phenolic fraction	2689.00±32.45	2855.00±35.48		2360.00±78.52	2056.00±87.52
total	**7127.36±123.82**	**7243.40±285.93**		**6112.88±238.18**	**5696.09±214.93**

[a] Mean ± SD (Standard deviation) of two determinations. [b] Compounds were identified and quantified with their corresponding standards. [c] Compounds was quantified with a calibration of oleuropein. [d] Compounds was quantified with a calibration of (E)-3-(1-Oxobut-2-en-2-yl)glutaric acid. [e] PPF was calculated by gravimetrically. n.d. not detected. * Significantly different from control (p >0.0