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

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Abstract

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

1. Introduction

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

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

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

2. Materials and methods

2.1. Materials

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

2.2. Standard compounds

Hydroxymethylfurfural, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, p-coumaric acid, caffeic acid and 3,4-dihydroxyphenylglycol were obtained from Sigma-Aldrich (Deisenhofer, Germany). Tyrosol and 3,4-dihydroxyphenylacetic acid were obtained from Fluka (Buchs, Switzerland). Oleuropein, verbascoside and luteolin-7-O-glucoside were obtained from Extrasynthese (Lyon Nord, Geney, France). (±)-α-Tocopherol ≥95% purchased from Sigma-Aldrich was used as reference compound. Hydroxytyrosol was obtained using the method described by Fernández-Bolaños et al. (2002).

2.3. Thermal treatment

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

2.4. Determination of the total phenolic content

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

2.5. Phenol extraction

The liquid portions (1 L) obtained after treatment were washed with hexane (500 mL) to remove the lipid fraction. The mixture was shaken vigorously, and the phases were then separated by decantation and washed twice (De Marco, Savarese, Paduano, & Sacchi, 2007).
Phenolic compounds were extracted with ethyl acetate (500 mL per 200 mL of sample) using a continuous extraction at 77 °C for 8 h. The organic phase was rotary-evaporated under vacuum at 30 °C.

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

Approximately 12 g of EtOAc extract was dissolved in 60 mL of H_2O:MeOH (80:20). The extract was passed through an open column of 3.5 cm in diameter and 40 cm in height packed with the non-ionic resin Amberlite XAD16 (Vivaqua, Spain). The elution was performed using 1 L of H_2O, EtOH 30% (v/v), EtOH 50% (v/v) and EtOH 95% (v/v); ten fractions of 100 mL each were collected and analyzed using HPLC, and those with similar compositions were mixed, concentrated to 50 mL and passed through a second column. This second column was 3.5 cm in diameter and 45 cm in height and filled

with polyamide (particle size = 50-160 mm, Fluka Analytica). The elution was performed using 500 mL of H2O, MeOH 25% (v/v), MeOH 50% (v/v), MeOH 75% (v/v) and MeOH 100% (v/v). Ten fractions of 50 mL each were collected and monitored using HPLC. Fractions with similar composition were combined (Fig. 1). In each fraction, the phenols were identified using HPLC-DAD and HPLC-DAD-MS, and quantified using HPLC-DAD.

2.7. Isolation of compounds of interest

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

2.8. HPLC-DAD

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

2.9. HPLC-DAD-MS

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

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

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

2.11. Antiradical activity: 2,20-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)

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

2.12. Reducing power

The reducing power assay was performed according to the procedure described in a previous study (Rodríguez et al., 2005). The assay was calibrated using 6-hydroxy 2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and the results were expressed in mg/mL TE (Trolox equivalent). To express the results, a calibration curve was created by plotting A490 against the known concentration of Trolox (0.059-0.56 mg/mL [correlation coefficient (R) = 0.9936].

2.13. Inhibition of primary oxidation

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

2.14. Inhibition of secondary oxidation

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

2.15. Statistical analysis

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

3. Results and discussion

3.1. Hydrothermal treatment and extraction with ethyl acetate

We have previously characterized the antioxidant extracts recovered with ethyl acetate from autohydrolysis liquids of alperujo steam-treated at a fixed temperature of 160 °C for 15-90 min. The results indicated that the phenol extracts were effective antioxidants to a significantly greater extent than the untreated control and comparable or better than a-tocopherol (Rubio-Senent et al., 2012). The present study was performed to further characterize the antioxidants present in the most active extract, which corresponds to alperujo treated at 160 °C/60 min and extracted for 8 h with ethyl acetate. The yield of the phenol extract was 7.1 g/kg of fresh alperujo with a total phenolic content of 1.74 g gallic acid equivalent/kg of fresh alperujo. In an initial approach a fractionation method was used for characterization (Fig. 1) and the antioxidant activity was evaluated for each fraction.

3.2. Fractionation and characterization of the phenolic extract

Fractionation of the ethyl acetate extract was performed using a system easy to scale up for industries that consist of resin Amberlite XAD-16 and further desorption with water and followed by increasing the concentration of ethanol. Five fractions (1-5) were collected, which were characterized by HPLC analysis on a C18 reverse column. Fig. 2 showed the absorbance profiles of the 5 fractions obtained after fractioned the ethyl acetate extract through the XAD resin to two different wavelengths; 280 nm, where the phenolic compounds absorbed, and 254 nm, where secoiridoids derivatives absorbed. In the first fraction, a very polar compound was identified as 3,4-dihydroxyphenylglycol (DHPG), which was present almost exclusively (Fraction 1). The fractions 2-4 had were further fractionated in a polyamide resin. Six (2A-2F), nine (3A-3I) and seven fractions (4A-4G) were collected from subfractions 2, 3 and 4 respectively. Fraction 5 was eluted with 100% ethanol and represents a new polymeric phenol fraction (PPF) constituted by several unseparated peaks (30-48 min).

The identification of the phenols in each fraction was performed using HPLC-DAD-MS, the retention time and UV spectra (Table 1). Table 2 summarizes the concentrations of the phenolic compounds of each of the fractions and the mg dry extract/mL of the fraction. The analysis of the ethyl acetate extract demonstrated the wide structural diversity of the compounds (Fig. 3).

Among the phenolic alcohols detected, hydroxytyrosol was the main compound. The thermal treatments caused the rupture of the more complex molecule that contained simple phenols and enhanced their solubilization (Fernández-Bolaños et al., 2002). Hydroxytyrosol has been associated with important antioxidant, anti-inflammatory, antiproliferative and antifungal activities (Granados-Principal, Quiles, Ramirez-Tortosa, Sanchez-Rovira, & Ramirez-Tortosa, 2010). Other phenolic alcohol compounds such as DHPG, tyrosol and hydroxytyrosol acetate were also detected.

Phenolic acid and oleuropein family compounds were also identified in the extract. The concentration of the oleuropein and oleuropein derivatives also is low, possibly because the treatment promotes the breakdown of oleuropein into simpler molecules.

Three elenolic acid derivatives were tentatively identified in the extract being their spectral characteristics and m/z values coincident with those given in the literature (Obied, Bedgood, Prenzler, & Robards, 2007). The first, termed A (R_t = 8.5 min), is characterized by a maximum absorbance in the UV-vis spectrum at 230 nm. In its mass spectrum, a possible molecular ion is present at m/z 423 that has not been identified; however, its fragmentation in negative mode produces a profile of fragments associated with the elenolic acid structure, with the presence of the fragment at m/z 241 [elenolic acid-H]$^-$ and the fragments at m/z 197 [elenolic acid-COO]$^-$ and m/z 137 [197-$COOCH_3$]$^-$. This same signal at m/z 241 was identified in the mass spectrum of the derivative B (R_t = 30.1 min); this derivative is present in a minor amount and is characterized by the presence of absorbance maxima at 210 and 264 nm. The derivative C (R_t = 21.2 min) has a UV-vis spectrum characteristic of elenolic derivatives, with a maximum absorbance at 242 nm. This molecule produces fragmentation, in positive mode, with a profile signal similar to elenolic acid, as described by De Nino et al. (1999), with fragments at m/z 243 [elenolic acid]$^+$, m/z 211 [elenolic acid-CH_3-H_2O]$^+$ and m/z 151 [211-CH_3COOH]$^+$.

Verbascoside and the flavones apigenin-7-O-rutinoside, luteolin-7-O-glucoside and luteolin 7-O-rutinoside previously reported in olive fruit (Ryan et al., 2002) were also detected in the ethyl acetate extract.

The molecular ion of 1-phenyl-6,7-dihydroxyisochroman ($[MH]^-$ at m/z 241) was detected together with two characteristic fragments, at m/z 211 and 136, in the mass spectrum (Bianco, Coccioli, Guiso, & Marra, 2001). This molecule is not present in olive fruit; however, it is formed during the storage of olive oil. It has not been previously detected in alperujo although in this study has been found in very low amounts. Because of the hydrothermal treatment conditions (high temperature and pressure) of the alperujo and the abundant presence of free HT, the aldehyde formation and the acidity condition of the extract could promote the formation of this compound.

3.3. Antioxidant activity

The antioxidant value potential of each phenolic fraction obtained after the fractionation was compared with the values for the unfractionated phenolic extract and with the values of other known antioxidant compounds such as HT, DHPG, a-tocopherol and Trolox, the water-soluble α-tocopherol analogue.

3.3.1. Free radical scavenging capacity (DPPH and ABTS assays)

Data available in the literature on DPPH and ABTS assays indicate that they are not always well correlated and they don't often give the same results because it deals with two different action mechanisms using two different radicals. It is for this reason that both assays were considered. Individual regression equations were used to calculate the antiradical activity of each sample. Statistical analyses of the results indicate that the dose response adjusted to a linear model for two of the assays, with $R2 \geq 0.90$. Lower EC50 values for DPPH assay and higher Trolox equivalent values for ABTS assay mean high antioxidant activity.

The results of the antiradical activity against the DPPH radical (Fig. 4a) indicate that fractions rich in DHPG, HT, tyrosol, protocatechuic acid, vanillic acid, hydroxytyrosol acetate, caffeic acid, comsegoloside, verbascoside, and polymeric phenolic fraction, have a higher antiradical capacity and do not exhibit significant differences compared with the three standards (HT, DHPG and α-tocopherol). The fractions 4A and 3B with a high percentage of elenolic acid derivative A (91 and 99%, respectively) exhibit very minimal activity.

The results of antiradical activity against the ABTS radical (Fig. 4b) indicate that fractions 2B, 4F and 4G have a high capacity for ABTS antiradical activity, with capacities between those of HT and DHPG. The fraction 2B is characterized by a high percentage of HT (96.14%), the fraction 4F contains HT and other compounds such as vanillic acid (23.82%) and verbascoside (65.11%), and the fraction 4G is characterized by a high percentage of comsegoloside (91.61%). All of these compounds are most likely responsible for the antiradical activity. However, fractions 2C, 3C, 3E, 4C, 4D and 4E have ABTS antiradical activity similar or greater than that of a-tocopherol because of the presence of compounds such as HT, tyrosol, protocatechuic acid, vanillic acid, hydroxytyrosol acetate and polymeric phenolic fraction (PPF). The activity of fraction 3E with a high percentage of elenolic acid derivative A is not attributable to the

compound because fraction 4A and 3B, which are among the richest in this compound, exhibit no activity.
The different comportment between fraction 1, composed almost exclusively of the phenol DHPG, and the standard DHPG is most likely explicable by the low purity of fraction 1 of only approximately 18% of total phenol/weight total. The same result was observed in the reducing power and secondary oxidation assays discussed above.

3.3.2. Reducing power

The reducing power results (Fig. 4c) indicate that there are two fractions, 2B rich in HT (96.14%) and 2C is composed exclusively of protocatechuic acid (100%), whose reducing values are higher than Trolox. There is a group of fractions rich in HT, tyrosol, verbascoside, protocatechuic acid, vanillic acid and hydroxytyrosol acetate (3D, 3E, 3F, 4C, 4D and 4F) with Trolox equivalent values between 0.8 and 1. In fraction 3D, the major component is the elenolic acid derivative A (89.03%); however, this species cannot be attributed to the reducing power because fractions 3B and 4A are also rich in this species (99.64 and 91.18%, respectively) and have very low reducing power. The dose response adjusted to a lineal model ($R^2 \geq 0.90$).

3.3.3. Inhibition of lipid oxidation

In the primary oxidation assay the response was not linear but depended on the square root of x ($y = a + bx^2$), with $R^2 \geq 0.882$. In the secondary oxidation assay (TBARS), all of the samples are also described by a “square root x” regression model ($R^2 \geq 0.807$).
In fraction 1, 2B and 3C, the positive effect shown in the primary oxidation assay (Fig. 5a) may be due to the high concentrations of HT and DHPG. The fraction 4C is rich hydroxytyrosol acetate (95.5%), the antioxidant effect is most likely due to this compound, which is the hydroxytyrosol derivative more liposoluble according to Trujillo et al. (2006). In other cases, the activity may be due the presence of vanillic acid, verbascoside, comsegoloside and PPF, as in the previous cases. If we compare the fraction composition of 3F, 3E and 2C, we see that 2C consists exclusively of protocatechuic acid, whereas 3E also contains vanillic acid (11.46%) and 3F also contains p-hydroxybenzoic acid (6.63%) and is most active with respect to 2C inhibition primary oxidation, most likely because these species act synergistically.
The antioxidant properties of these fractions were also tested with a peroxidation inducer by thiobarbituric acid-reactive substance (TBARS) assay (Fig. 5b). Almost all of the fractions exhibit a value of inhibition of secondary oxidation similar to that observed for HT and DHPG, and, in some cases, even better than that of a-tocopherol. The fractions that exhibited less activity are rich in tyrosol and hydroxymethylfurfural that should not have an excess capacity to inhibit the secondary oxidation. However, fractions 3B, 4A and 4B are high in elenolic acid derivatives (99.64, 91.18 and 97.36%, respectively), which should not inhibit the secondary oxidation.

3.4. Conclusions

The present study demonstrated the great diversity and complexity of the mixture of phenolic compounds present in the ethyl acetate extract of steam-treated alperujo. Among all of the compounds detected, hydroxytyrosol was observed in the highest concentration, followed by the elenolic acid derivatives produced during the process, which possess the core structure of secoiridoid compounds. Tyrosol, 3,4-dihydroxyphenylglycol, hydroxytyrosol acetate, comsegoloside, protocatechuic acid, along with a new polymeric phenolic fraction (PPF), must also be considered because its concentration and its contribution to the antioxidant/free radical-scavenging activity. A significant contribution was made by PPF, exhibiting a strong antioxidant activity in a lipid system as an emulsion, similar to a-tocopherol.

It was noted that there were a number of fractions whose behavior in all tests were quite good, these fractions are the 2B, 2C, 3C, 3D, 3E, 3F, 4C, 4D, 4E, 4F and 4G, which are rich in DHFG, hydroxytyrosol, tyrosol, protocatechuic acid, verbascoside, 4-hydroxybenzoic acid, vainillic acid, hydroxytyrosol acetate, polymeric phenolic fraction, comsegoloside or/and p-coumaric acid. Furthermore, it was observed that there are also a number of fractions to the test whose results were bad in all cases studied; these fractions were 3A, 3B, 4A and 4B rich in hydroxymethylfurfural, elenolic acid derivative A and/or elenolic acid derivative B.

These findings might be helpful for the production of a natural antioxidant extract for use in the food, cosmetic or pharmaceutical industries. Although further studies about their toxicity, in vivo activity and bioavailability are necessary. Thus, hydrothermal treatment, a technology being scaled to the industrial level, along with ethyl acetate extraction and the use of adsorbent resin, may enable the use of alperujo, which should not be considered a polluting residue but a precious and inexpensive source of natural antioxidants.

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References

Bianco, A., Coccioli, F., Guiso, M., & Marra, C. (2001). The occurrence in olive oil of a new class of phenolic compounds: hydroxy-isochromans. Food Chemistry, 77, 405-411.

Covas, M. I., de la Torre, K., Farré-Albaladejo, M., Kaikkonen, J., Fitó, M., López-Sabater, C., et al. (2006). Postpandrial LDL phenolic content and LDL oxidation are modulated by olive oil phenolic compounds in humans. Free Radical Biology and Medicine, 40, 608-616.

De la Torre-Carbot, K., Jáuregui, O., Gimeno, E., Castellote, A. I., Lamuela-Raventós, R. M., & López-Sabater, M. C. (2005). Characterization and quantification of phenolic compounds in olive oil by solid-phase extraction, HPLC-DAD, and HPLC-MS/MS. Journal of Agricultural and Food Chemistry, 53, 4331-4340.

De Marco, E., Savarese, M., Paduano, A., & Sacchi, R. (2007). Characterization and fractionation of phenolic compounds extracted from olive oil mill wastewaters. Food Chemistry, 104, 858-867.

De Nino, A., Mazzotti, F., Morrone, S. P., Perri, E., Raffaelli, A., & Sindona, G. (1999). Characterization of Cassanese olive cultivar through the identification of new trace components by ionspray tandem mass spectrometry. Journal of Mass Spectrometry, 34, 10-16.

De Nino, A., Mazzotti, F., Perri, E., Procopio, A., Raffaelli, A., & Sindona, G. (2000). Virtual freezing of the hemiacetal-aldehyde equilibrium of the aglycones of oleuropein and ligstroside present in olive oils from *Carolea* and *Coratina* cultivars by ionspray ionization tandem mass spectrometry. Journal of Mass Spectrometry, 35, 461-467.

Fernández-Bolaños, J., Rodríguez, G., Rodríguez, R., Heredia, A., Guillén, R., & Jiménez, A. (2002). Production in large quantities of highly purified hydroxytyrosol from liquid-solid waste of two-phase olive oil processing or "alperujo". Journal of Agricultural and Food Chemistry, 50, 6804-6811.

Fu, S., Arráez-Román, D., Menéndez, J. A., Segura-Carretero, A., & Fernández-Gutiérrez. (2009). Characterization of isomers of oleuropein aglycon in olive oils by rapid-resolution liquid chromatography coupled to electrospray time-off light and ion trap tandem mass spectrometry. Rapid Communications in Mass Spectrometry, 23, 51-59.

Fu, S., Segura-Carretero, A., Arráez-Román, D., Menéndez, J. A., De la Torre, A., & Fernández-Gutiérrez, A. (2009). Tentative characterization of novel phenolic compounds in extra virgin olive oils by rapid-resolution liquid chromatography coupled with mass spectrometry. Journal of Agricultural and Food Chemistry, 57, 11140-11147.

Gonçalves, B., Falco, V., Moutinho-Pereira, J., Bacelar, E., Peixoto, F., & Correia, C. (2009). Effects of elevated CO_2 on grapevine (*Vitis vinifera* L.): volatile composition, phenolic content, and in vitro antioxidant activity of red wine. Journal of Agricultural and Food Chemistry, 57, 265-273.

Granados-Principal, S., Quiles, J. L., Ramirez-Tortosa, C. L., Sánchez-Rovira, P., & Ramírez-Tortosa, M. C. (2010). Hydroxytyrosol: from laboratory investigations to future clinical trials. Nutrition Review, 68, 191-206.

Obied, H. K., Bedgood, D. R., Prenzler, P. D., & Robards, K. (2007). Chemical screening of olive biophenol extracts by hyphenated liquid chromatography. Analytical Chimica Acta, 603, 179-189.

Pérez-Jiménez, F. (2005). International conference on the healthy effect of virgin olive oil. European Journal of Clinical Investigation, 35, 421-424.

Rodríguez, R., Jaramillo, S., Rodríguez, G., Espejo, J. A., Guillén, R., Fernández-Bolaños, J., et al. (2005). Antioxidant activity of ethanolic extracts from several asparagus cultivars. Journal of Agricultural and Food Chemistry, 53, 5212-5217.

Romero, C., García, P., Brenes, M., García, A., & Garrido, A. (2002). Phenolic compounds in natural black Spanish olive varieties. European Food Research and Technology, 215, 489-496.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., & Fernández-Bolaños, J. (2012). New phenolic compounds hydrothermally extracted from the olive oil byproduct alperujo and their antioxidative activities. Journal of Agricultural and Food Chemistry, 60, 1175-1186.

Ryan, D., Antolovich, M., Herlt, T., Prenzler, P. D., Lavee, S., & Robards, K. (2002). Identification of phenolic compounds in tissues of the novel olive cultivar Hardy's Mammoth. Journal of Agricultural and Food Chemistry, 50, 6716-6724.

Sánchez-Moreno, C., Larrauri, J. A., & Saura-Calixto, F. (1999). Free radical scavenging capacity and inhibition of lipid oxidation of wines, grape juices and related polyphenolic constituents. Food Research International, 32, 407-412.

Savarese, M., De Marco, E., & Sacchi, R. (2007). Characterization of phenolic extracts from olives (*Olea europaea* cv. *Pisciottana*) by electrospray ionization mass spectrometry. Food Chemistry, 105, 761-770.

Singleton, V. L., & Rossi, J. A., Jr. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture, 16, 144-158.

Suárez, M., Macià, A., Romero, M. P., & Motilva, M. J. (2008). Improved liquid chromatography tandem mass spectrometry method for the determination of phenolic compounds in virgin olive oil. Journal of Chromatography A, 1214, 90-99.

Suárez, M., Rometo, M. P., Ramo, T., Macià, A., & Motilva, M. J. (2009). Methods for preparing phenolic extracts from olive cake for potential application as food antioxidants. Journal of Agricultural and Food Chemistry, 57, 1463-1472.

Trujillo, M., Mateos, R., Collantes de Terán, L., Espartero, J. L., Cert, R., Jover, M., et al. (2006). Lipophilic hydroxytyrosol esters. Antioxidant activity in lipid matrices and biological systems. Journal of Agricultural and Food Chemistry, 54, 3779-3785.

Figures

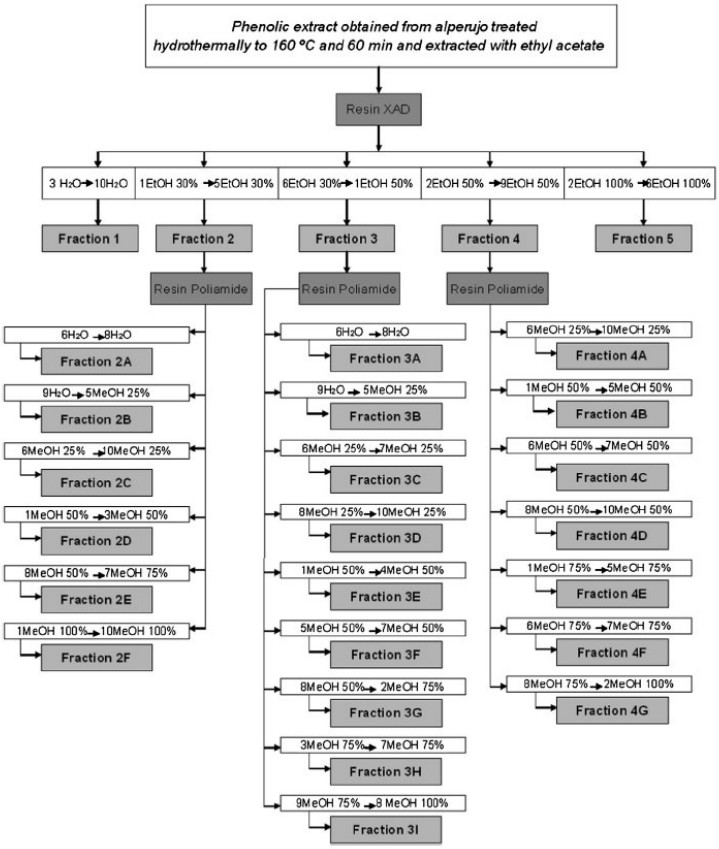

Fig. 1. Steps of fractioning of ethyl acetate extract. The fractions corresponding to the elution through the fist resin Amberlite XAD with one liter of H_2O, EtOH 30% (v/v), EtOH 50% (v/v) and EtOH 95% (v/v) divided in ten fractions of 100 mL each one, and elution through the second resin polyamide with 500 mL of H_2O, MeOH 25% (v/v), MeOH 50% (v/v), MeOH 75% (v/v) and MeOH 100% (v/v) divided in ten fractions of 50 mL each one. The intervals of the eluted fractions and collected fractions were specified.

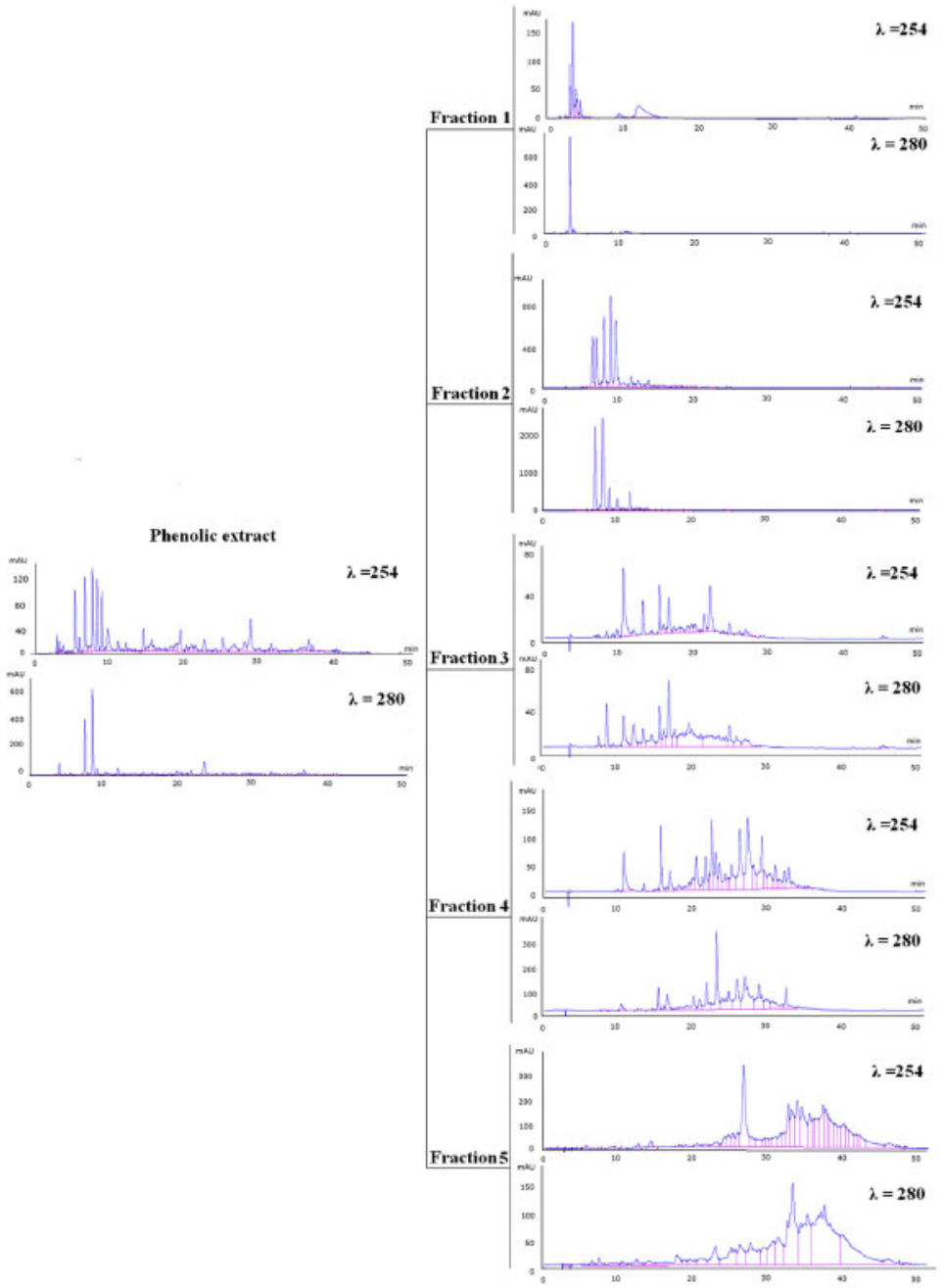

Fig. 2. HPLC-DAD chromatographic profile at 254 and 280 nm of the ethyl acetate extract collected from steam-treated alperujo and the different fractions obtained after the first step using the XAD resin.

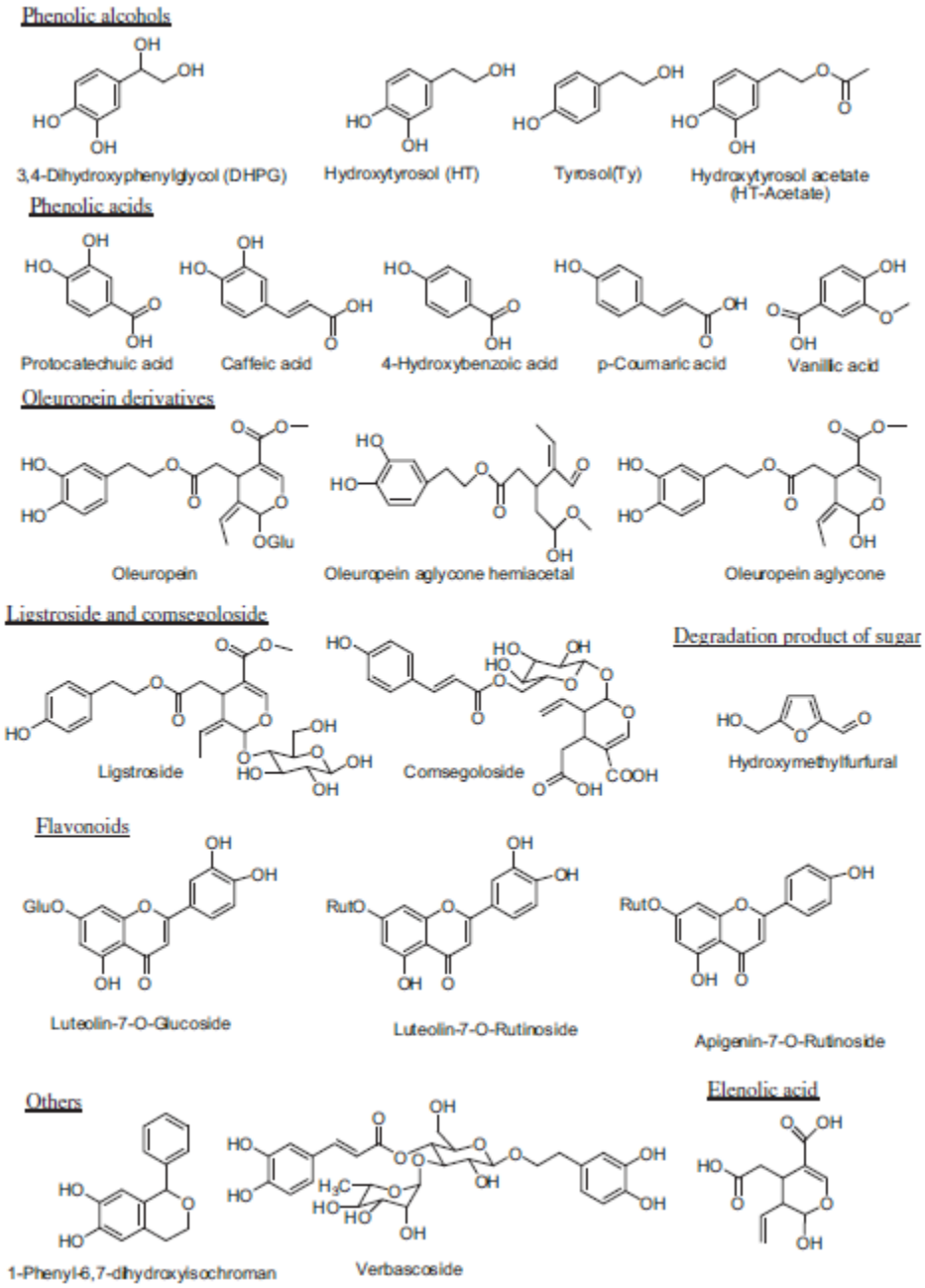

Fig. 3. Structures of the species identified.

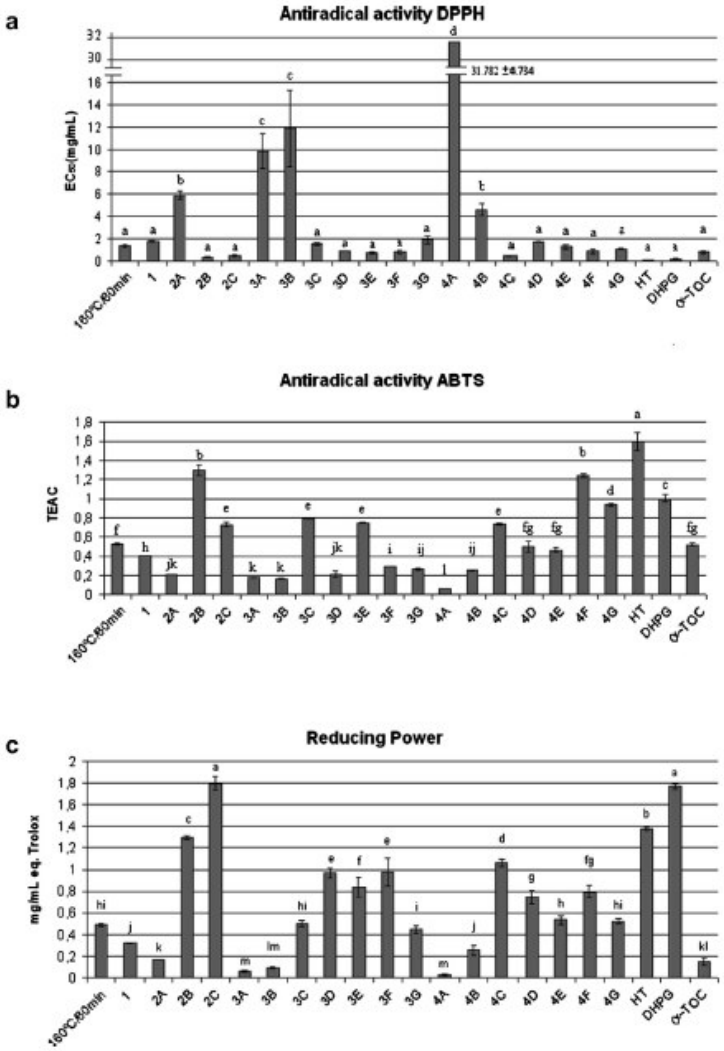

Fig. 4. Results for the different fractions obtained after the hydrothermal treatments for the fractionated, unfractionated and standard samples. (a) and (b) Antiradical activity values expressed as EC_{50} and TEAC for the DPPH and ABTS, respectively. (c) Reducing power values expressed as mg/mL Trolox. The data are presented as means ± SDs. The different letters indicate significantly different results ($p < 0.05$).

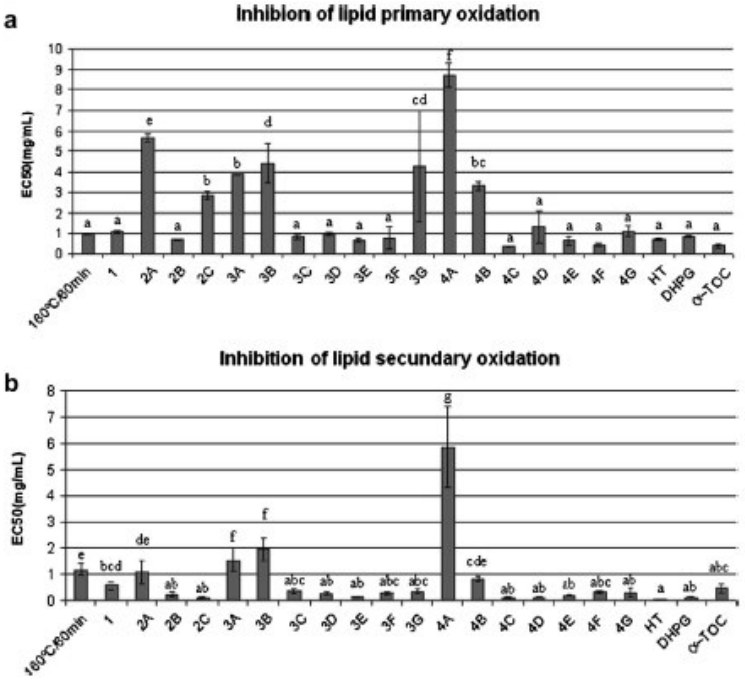

Fig. 5. Inhibition of lipid primary and secondary oxidation of the different extracts, control and standard tested, expressed as EC_{50} (mg/mL). The data are presented as means ± SDs. The different letters indicate significantly different results ($p < 0.05$).

Table 1
Compounds identified in the acetate ethyl extract obtained after the hydrothermal treatments and ethyl acetate extraction.

Compounds	Retention time (min)	Molecular weight	λ_{max} (nm)	m/z (mode negative)	mg/kg fresh alperujo	Reference
3,4-Dihydroxyphenylglycol[a]	4.3	170	214, 234 and 278	169	70.19 ± 0.45	Obied et al., 2007
Hydroxymethylfurfural[a]	7.3	126	194,228 and 284		4.67 ± 0.15	–
Protocatechuic acid[a]	8.3	154	206, 218, 260 and 294	109 and 45	18.35 ± 0.58	Obied et al., 2007
Elenolic acid derivative A[b]	8.5		230	423, 241, 197 and 137	191.29 ± 1.17	Obied et al., 2007
Hydroxytyrosol[a]	8.9	154	214, 234 and 278	153 and 123	776.53 ± 14.68	Suárez, Macià, Romero, & Motilva, 2008
Tyrosol[a]	11.7	138	200, 218 and 276	137	80.62 ± 2.11	De la Torre-Carbot et al., 2005
p-Coumaric acid derivative[c]	13.4	536	230 and 310	205, 145 and 117	0.05 ± 0.01	Romero et al., 2002
Vanillic acid[a]	14.1	168	200, 218, 255 and 298		5.01 ± 0.05	Suárez et al., 2008
4-Hydroxybenzoic acid[a]	14.4	138	194 and 256		1.44 ± 0.01	Obied et al., 2007
Caffeic acid[a]	16.2	180	202, 218, 240 and 324	179, 163, 135 and 45	2.65 ± 0.03	Savarese, De Marco, & Sacchi, 2007
Oleuropein aglycone hemiacetal[d]	17.6	352	198, 224 and 275	707 [2M-H], 351, 137 and 119	0.02 ± 0.01	De Nino et al., 2000
p-Coumaric acid[a]	20.5	164	194, 210, 226 and 310	119	1.49 ± 0.02	Suárez, Romero, Ramo, Macià, & Motilva, 2009
Elenolic acid derivative C[b]	21.2		242	243, 211 and 151 in mode positive	123.56 ± 0.08	De Nino et al., 1999
Hydroxytyrosol acetate[b]	23.1	196	214, 234 and 278	195	60.13 ± 1.27	–
Verbascoside[a]	24.5	624	198 and 328	623, 461 and 161	8.43 ± 0.08	Savarese et al., 2007
Apigenin-7-O-Rutinoside[e]	25.7	578	212, 264 and 350	577, 371 and 269	0.07 ± 0.01	Ryan et al., 2002
Luteolin-7-O-Glucoside[a]	26.4	448	206, 256, 266 and 350	447 and 285	0.18 ± 0.01	Ryan et al., 2002
Luteolin-7-O-Rutinoside[e]	26.6	594	200, 254 and 349	593, 447, 285 and 151	0.58 ± 0.01	Ryan et al., 2002
Elenolic acid derivative B[f]	30.1		210 and 264	241 and 251	0.57 ± 0.01	Obied et al., 2007
Oleuropein[a]	30.7	540	198, 232 and 282	539, 377, 307, 275 and 223	0.46 ± 0.01	Fu, Segura-Carretero, et al., 2009
Comsegoloside[b]	32.1	536	192, 230 and 312	535, 205 and 145	22.68 ± 0.24	Obied et al., 2007b
Ligstroside[d]	32.3	524	220, 224 and 280	523, 361, 291 and 259	0.04 ± 0.01	De la Torre-Carbot et al., 2005
Oleuropein aglycone derivative[d]	32.6	378	200, 222 and 280	377, 307, 275, and 149	0.20 ± 0.01	Fu, Arráez-Román, Menéndez, Segura-Carretero, & Fernández-Gutiérrez, 2009
Oleuropein derivative[d]	36	538	214, 234 and 278	211, 139 and 123	1.39 ± 0.01	Obied et al., 2007
1-Phenyl-6,7-dihydroxyisochroman	38.1	242	200, 230 and 280	241, 211 and 136	n.q.[g]	Bianco et al., 2001
Polymeric phenolic fraction[h]	30–48				77.66	–

[a] The compounds were identified and quantified by corresponding commercial standards.
[b] The compounds were identified with isolated compounds.
[c] The compounds were quantified with calibration of p-coumaric acid.
[d] The compounds were quantified with calibration of oleuropein.
[e] The compounds were quantified with calibration of luteolin-7-O-rutinoside.
[f] The compounds were quantified with calibration of elenolic acid derivative A.
[g] Not quantified.
[h] Quantified by evaporation to dryness.

Table 2
Composition of each of the fractions that compose the acetate ethyl extract obtained after treating alperujo at 160 °C for 60 min. The nature of the compounds present in each fraction and their concentration in μg/mL are specified.

Fraction	mg/mL (Extract dry)	mg/mL (Total phenol)	Compound	μg Compound/mL fraction	% Individual compound
1	14.24 ± 0.25	2.01 ± 0.18	3,4-Dihydroxyphenylglycol	2457.38 ± 14.83	100
2A	15.17 ± 0.65	2.23 ± 0.13	3,4-Dihydroxyphenylglycol	74.44 ± 0.66	7.99
			Hydroxymethylfurfural	93.02 ± 3.02	9.98
			Hydroxytyrosol	99.88 ± 6.69	10.72
			Tyrosol	664.21 ± 3.84	71.3
2B	44.81 ± 2.32	36.75 ± 1.71	3,4-Dihydroxyphenylglycol	284.16 ± 1.44	0.91
			Hydroxymethylfurfural	53.51 ± 2.32	0.17
			Hydroxytyrosol	29890.67 ± 519.13	96.14
			Tyrosol	861.22 ± 9.27	2.77
2C	2.02 ± 0.02	1.01 ± 0.01	Protocatechuic acid	534.62 ± 21.94	100
3A	7.92 ± 0.57	0.47 ± 0.01	Hydroxymethylfurfural	38.75 ± 0.44	100
3B	15.02 ± 0.65	0.94 ± 0.09	Hydroxymethylfurfural	2.95 ± 0.01	0.08
			Hydroxytyrosol	9.55 ± 0.06	0.26
			Elenolic acid derivative A	3639.06 ± 2.93	99.64
			Oleuropein aglycone hemiacetal	0.76 ± 0.04	0.02
3C	7.32 ± 0.25	3.72 ± 0.07	Hydroxytyrosol	888.20 ± 54.84	29.98
			Elenolic acid derivative A	582.15 ± 9.39	19.65
			Tyrosol	1489.29 ± 66.26	50.27
3D	6.79 ± 0.05	3.45 ± 0.03	Hydroxytyrosol	51.95 ± 2.01	1.76
			p-Coumaric acid derivative	1.96 ± 0.18	0.07
			Tyrosol	224.51 ± 1.26	7.6
			Elenolic acid derivative A	2631.37 ± 29.13	89.03
			1-Phenyl-6,7-dihydroxyisochroman	–	–
			Verbascoside	45.88 ± 0.08	1.55
3E	2.24 ± 0.15	0.82 ± 0.01	Protocatechuic acid	53.51 ± 0.35	6.5
			4-Hydroxybenzoic acid	46.91 ± 0.22	5.7
			Elenolic acid derivative A	625.75 ± 1.35	76
			Luteolin-7-O-Rutinoside	2.91 ± 0.03	0.35
			Vanillic acid	94.31 ± 0.46	11.45
3F	1.32 ± 0.08	0.31 ± 0.01	Protocatechuic acid	150.14 ± 0.84	93.37
			4-Hydroxybenzoic acid	10.66 ± 0.03	6.63
3G	1.82 ± 0.02	0.18 ± 0.01	Caffeic acid	106.86 ± 1.32	100
4A	9.27 ± 0.02	0.19 ± 0.05	3,4-Dihydroxyphenylglycol	5.45 ± 0.66	2.34
			Hydroxytyrosol	12.28 ± 0.01	5.28
			Tyrosol	2.78 ± 0.45	1.19
			Elenolic acid derivative A	211.81 ± 5.03	91.18
4B	4.58 ± 0.25	0.5 ± 0.01	Hydroxytyrosol	78.82 ± 0.24	1.54
			Elenolic acid derivative C	4967.18 ± 32.34	97.36
			Oleuropein derivative	56.11 ± 0.25	1.1
4C	4.56 ± 0.15	3.26 ± 0.29	Hydroxytyrosol	78.82 ± 1.26	3.33
			Hydroxytyrosol acetate	2260.07 ± 50.67	95.48
			Oleuropein	18.55 ± 0.52	0.78
			Ligstroside	1.71 ± 0.56	0.07
			Oleuropein aglycone derivative	7.87 ± 1.14	0.33
4D	1.37 ± 0.08	0.41 ± 0.01	Hydroxytyrosol	64.10 ± 2.44	26.68
			Elenolic acid derivative B	23.19 ± 0.59	9.65
			Hydroxytyrosol acetate	153.01 ± 0.42	63.67
4E	3.78 ± 0.95	0.85 ± 0.02	Polymeric phenolic fraction (PPF)		100
4F	3.65 ± 0.45	1.86 ± 0.16	Hydroxytyrosol	42.64 ± 3.91	9.47
			Vanillic acid	107.21 ± 1.80	23.82
			Apigenin-7-O-Rutinoside	7.19 ± 0.22	1.6
			Verbascoside	293.09 ± 3.19	65.11
4G	2.67 ± 0.18	0.55 ± 0.02	p-Coumaric acid	60.17 ± 0.96	6.04
			Luteolin-7-O-glucoside	23.29 ± 0.07	2.34
			Comsegoloside	911.89 ± 6.62	91.61
5			Polymeric phenolic fraction (PPF)		100