Phenolic Compounds and Antioxidant Activity of *Olea europaea* L. Fruits and Leaves

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Extracts of leaves, fruits and seeds of olive tree cultivars of Trás-os-Montes e Alto Douro (Portugal) were analysed by reverse phase HPLC with diode array detection and mass spectrometry (MS). This methodology allowed the identification of some common phenolic compounds, namely, verbascoside, rutin, luteolin-7-glucoside, oleuropein and hydroxytyrosol. Moreover the hyphenation of HPLC with MS enabled the identification of nüzhenide in olive seeds. An oleuropein glucoside was also detected in olive tree leaves. The total phenolic content was determined with the Folin Denis reagent and the total antioxidant activity with the ABTS$^+$ method. There is a correlation between total antioxidant activity and total phenolic content with the exception of the seed extracts analysed. The apparent high antioxidant activity of seed extracts may be due to nüzhenide, a secoiridoid that is the major phenolic component of olive seeds. These results suggest a possible application of olive seeds as sources of natural antioxidants.

Key Words: phenolic compounds, *Olea europaea* L., antioxidant activity, nüzhenide

INTRODUCTION

Phenolic compounds are plant secondary metabolites, which play important roles in disease resistance (Servili and Montedoro, 2002; Antolovich et al., 2000), protection against pests and species dissemination. The interest on these compounds is related with their antioxidant activity and promotion of health benefits (Ryan et al., 2002).

Phenolic compounds are a complex but important group of naturally occurring products in plants and are present in the Mediterranean diet which includes table olives and olive oil (Manna et al., 1999; Servili et al., 2004; De la Torre-Carbot et al., 2005).

The interest on phenolic compounds has raised attention for its study in olive fruits and other parts of the olive tree. Virgin olive oil is an important dietary oil, rich in natural antioxidants. These substances have a remarkable pharmacological action and low toxicity.

Traditionally, olive tree leaves have been used as a folk remedy for combating fevers and other diseases, such as malaria (Benavente-Garcia et al., 2000; Soler et al. 2000). Previous investigations carried out on olive leaf extracts have demonstrated hypotensive, hypoglycaemic, hypouricaemic, antimicrobial and antioxidant activities (Benavente-Garcia et al., 2000). The antiviral activity of olive leaves extracts against HIV-1 infection and replication has also been referred (Lee-Huang et al., 2003). The antioxidant activity of olive wood extracts was reported by Altarejos et al., 2005. In addition, it has been shown that oleuropein, a typical secoiridoid of the olive tree, has hypocholesterolemic and hypoglycaemic activities (Romani et al., 1999) and is a potent antioxidant with anti-inflammatory properties (Benavente-Garcia et al., 2000). Hydroxytyrosol, a degradation product of oleuropein, also share some of the latter biological properties such as antimicrobial, hypoglycaemic, hypolipidaemic, hypocholesterolic, antioxidant and free radical-scaveging actions. The association of these properties with the prevention of several diseases such as atherosclerosis and heart disease has raised interest in these phenolic compounds (Cardoso et al., 2005).

Olives contain high concentrations of phenolic compounds ranging between 1–3% of the fresh pulp weight. The main classes of phenolic compounds present in olives are phenolic acids, phenolic alcohols, flavonoids and secoiridoids. Hydroxytyrosol and tyrosol are the most abundant phenolic alcohols in olives. The flavonoids include flavonol glycosides such as luteolin-7-glucoside and rutin as well as anthocyanins. Verbascoside, a hydroxycinnamic acid derivative, is also found in these samples. While phenolic acids, phenolic alcohols and flavonoids occur in many fruits and vegetables belonging to various botanical families, secoiridoids are present exclusively in plants belonging to the *Oleaceae* family which includes *Olea europaea* L. (Robards et al., 1999; Servili and Montedoro, 2002; Servili et al., 2004; De la Torre-Carbot et al., 2005).

Secoiridoids are produced from the secondary metabolism of terpenes as precursors of various indole alkaloids, and are usually derived from the glucoside oleosides, which are characterised by a combination of elenolic acid and a glucosidic residue. It could be stated that these compounds proceed from the acetate/mevalonate pathway (De la Torre-Carbot et al., 2005). The phenolic compounds classified as secoiridoids are characterised by the presence of either elenolic acid or its derivatives in their molecular structures. Oleuropein, demethyloleuropein, ligstroside and nüzhenide are the most abundant secoiridoids in olives (Servili and Montedoro, 2002).

Oleuropein and hydroxytyrosol are naturally occurring phenolic compounds in olive fruits. While oleuropein is present in high amounts in unprocessed olive fruit, hydroxytyrosol is more abundant in the processed fruit and olive oil. Oleuropein is responsible for the bitter taste of immature and unprocessed olives (Tan et al., 2003). This compound amounts up to 14% of the dry weight in unripe olives but, during maturation, undergoes hydrolysis and yields several simple molecules like hydroxytyrosol and oleuropein aglycone (Tan et al., 2003). It is noteworthy that olives contain complex phenols, as glycosides, present in a rather polar and hydrophilic form, whereas the oil contains their aglyconic form, which is the most lipid-soluble part of the molecule (Visioli et al., 2002).

There are differences in levels and type of phenolic compounds in *Olea europaea* L. leaves, fruits and seeds (Ryan et al., 2002). There are also significant changes in the phenolic composition of fruits and leaves during the maturation period (Briante et al., 2002).

Many different substrates, system compositions and analytical methods have been employed in screening tests to evaluate the effectiveness of antioxidants. The antioxidant tests available in foods could be classified in two groups: assays used to measure free radical-scavenging ability in aqueous media and assays used to evaluate lipid peroxidation, in which a lipid substrate under standard conditions is used and the degree of oxidation inhibition is measured (Sánchez-Moreno C., 2002).

The purpose of this work was to study the main phenolic compounds present in olive tree leaves, fruits and seeds obtained from *Olea europaea* L. of Portuguese cultivars, using reverse phase liquid chromatography with diode array detection and MS. The total antioxidant activities of these samples were evaluated by the extent of their capabilities to scavenge the $ABTS^+$ radical cation and to compare those activities with total phenolic contents.

MATERIALS AND METHODS

Materials

Reagents

Acetonitrile, methanol, sodium carbonate and tannic acid were from Riedel-de-Häen (Seelze, Germany). Phosphoric and formic acids were from Panreac (Barcelona, Spain). Hexane was purchased from LabScan (Dublin, Ireland) and sodium metabisulphite from BDH laboratories (Darmstadt, Germany).

The Folin Denis reagent was from Fluka (Steinheim, Germany).

Water purified by means of Milli-Q from Millipore (Bedford, USA).

Oleuropein, luteolin, luteolin-7-glucoside and tyrosol were from Extrasynthese (Genay, France). Rutin was purchased from Merck (Seelze, Germany) and hydroxytyrosol from Cayman (Michigan, USA).

All chemicals were analytical grade except acetonitrile and methanol that were HPLC grade.

Standard Solutions

Stock solutions, 1,000 mg/L, were prepared in methanol:water (80:20, v/v), with the exception of tannic acid that was prepared in water to a concentration of 100 mg/L. Diluted solutions were prepared in water:methanol (80:20, v/v) to concentrations from 0.5 up to 100 mg/L.

Samples and Sample Pre-treatment

Olives and leaves of ten olive tree cultivars (*Olea europaea* L.) from the region of Trás-os-Montes e Alto Douro (Portugal) were studied: 'Bical', 'Borrenta', 'Cobrançosa', 'Coimbreira', 'Lentisca', 'Madural', 'Negrinha de Freixo', 'Redondal', 'Santulhana' and 'Verdeal Transmontana'. Olives were randomly picked at optimum ripening stages for production of olive oil, according to their skin colour, in crop 2002/2003 (January, 2003). Leaves were also randomly collected at the same time as the fruits.

Olives from the cultivars studied were ground in a grinder to obtain pastes. Pulps were prepared removing the stone from the fruits and homogenised using a blender. The stones were broken in order to remove the intact seed and the tegument was also isolated.

Fresh leaves were dried in a ventilated oven for 48 h at 40°C.

Commercial dried leaves were purchased from a herbalist shop, for comparison.

Pastes (5 g), pulps (5 g), seeds (5 g), teguments (5 g), fresh leaves (5 g) and dried leaves (2.5 g) were extracted by solid-liquid extraction. The difference between the weight of fresh and dried leaves is justified with the water content of the former. Three successive hexane extractions (3×50 mL) applied to pulps and pastes allowed removal of pigments and most lipids. The phenolic compounds were extracted by a mixture methanol:water (80:20, v/v) after adding 10 mL of sodium metabissulphite 2% to the samples. After three extractions (3×50 mL), the final extract was used for HPLC analysis.

Infusions were prepared from dried leaves (2.5 g) and boiling water (75 mL). After 10 min of contact the infusion was separated by decantation and filtration.

All samples were filtered through a 0.45 μm filter Acrodisc® and stored at −20°C until needed.

Methods

HPLC with diode array detector (DAD)

The HPLC system (Thermofinnigan) consisted of a pump, an autosampler and a diode-array detector. Data acquisition and remote control of the system were done by Chromquest version 4.0 (Thermofinnigan).

HPLC was performed with a LiChrospher (Merck) C18 column (5 μm, 250 mm × 4 mm i.d. with a C18 pre-column). All separations were conducted at 35°C.

Samples and standards were injected (20 μL) directly on the HPLC column and eluted with an aqueous gradient (flow rate of 0.7 mL/min) prepared from a mixture of water (99.9%) and phosphoric acid (0.1%) as solvent A, water (59.9%), acetonitrile (40%) and phosphoric acid (0.1%) as solvent B. The solvent gradient started with 100% solvent A, reaching 80% after 15 min, 30% after 70 min and 0% after 85 min, followed by an isocratic step for 30 min and a return to initial conditions. Diode array detection was done using the following conditions: scan 200–600 nm, scan rate 1 Hz and bandwidth 5 nm. Chromatograms at 254, 280 and 320 nm were used and those were acquired with 10 Hz rate and 11 nm bandwidth.

HPLC Coupled with Atmospheric Pressure Chemical Ionisation (APCI) MS

The MS system was a LCQ ion trap mass spectrometer (Thermofinnigan) equipped with APCI source and run by Xcalibur (Thermofinnigan) version 1.3 software.

The HPLC conditions mentioned above were used but the solvents were prepared replacing phosphoric acid (0.1%) by formic acid (0.5%), and the injection volume was 50 μL.

The following conditions were used in experiments with APCI source in negative mode: vaporiser temperature, 465°C; discharge current, 10 μA; temperature of the heated capillary, 250°C.

Nitrogen was used as sheath gas and auxiliary gas. The sheath and auxiliary gas flow rates were 80 and 20 arbitrary units, respectively.

HPLC-MS was performed in the full scan mode from m/z 130 to 1,400. The collision energies used in MS^2 and MS^3 fragmentation experiments conducted by HPLC-MS were chosen after analyses of individual standard solutions of the phenolic compounds studied by direct injection in the mass spectrometer. All the fragmentation experiments were done with 40% collision energy. This value was applied in HPLC-MS^n experiments with standards and samples.

Total Phenolic Content

A diluted sample extract (2 mL) or phenolic standard was mixed with the Folin Denis reagent (2.5 mL) and aqueous sodium carbonate 20% (5 mL), in a 50 mL-volumetric flask. After 45 min the volume was made up with water and the total phenolic content was determined colorimetrically at 760 nm. The standard curve was prepared using 1, 2, 3, 4 and 5 mg/L solutions of tannic acid in water.

Total Antioxidant Activity

The spectrophotometric technique used measured the relative abilities of antioxidants to scavenge the 2,29-azinobis(3-ethylbenzothiazoline-6-sulphonate) radical cation ($ABTS^+$) in comparison with the antioxidant activity of standard amounts of Trolox(6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). The radical cation $ABTS^+$, produced by the ferrylmioglobin radical generated from metmyoglobin (chromogen) and H_2O_2 in the presence of peroxidase is a blue/green chromogen with characteristic absorption at 600 nm. The determination of the total antioxidant activity (TAA) was carried out using a RANDOX kit (Crumlin, UK). Twenty microlitres of extract diluted four-fold, was added to 1 mL chromogen solution previously incubated at 37°C. Keeping the temperature at 37°C, the absorbances were measured at 600 nm at the start of the reaction and after 3 min. Total antioxidant activity was calculated against a standard curve using solutions 1.0, 1.3, 1.5 and 1.7 mM of Trolox.

RESULTS AND DISCUSSION

Chromatographic Analysis

Chromatographic profiles obtained for the extracts of leaves, fruits (pastes, pulps, teguments) and seeds of ten olive tree cultivars were compared: 'Cobrançosa' and 'Negrinha de Freixo' were the cultivars with highest concentrations of phenolic compounds (Figure 1).

Hydroxytyrosol, rutin, luteolin-7-glucoside, luteolin-4'-glucoside and ligstroside were detected in paste extracts. A major difference observed in the chromatogram of the seed extracts was the high level of nüzhenide, which is in accordance with data reported by Servili (Servili et al., 1999). The characterisation of phenolic compounds of olive seeds was achieved by HPLC-APCI-MS (Silva, 2004) with fragmentation of ions detected, and by comparison with literature data (Servili et al., 1999; Durán et al., 1994) as described below.

Paste and pulp extracts analysed for each of the ten cultivars studied showed similar chromatographic profiles. However, there were some components that were detected in higher levels in pastes than in pulps, which can be related to the contribution of phenolic compounds from the seeds. For most cultivars studied, the levels of hydroxytyrosol in pastes were higher than in pulps. Hydroxytyrosol was not detected in olive seeds, and therefore it was concluded that the concentration of this component increased due to enzymatic degradation of oleuropein present in fruits. There is a temperature increase when the paste is prepared with a mill and therefore some enzymatic reactions are favoured.

Olive Seed Extracts

High levels of nüzhenide were detected in the seed extracts of all cultivars analysed. According to Servili (Servili et al., 1999) nüzhenide is located mainly in olive seed.

Analysing the absorption spectra of the detected compounds we concluded that the majority of them could belong to the same phenolic group, excluding flavonoids, since they do not absorb at 300 nm but lower in the 250–280 nm wavelength range (Figure 2). Most of the detected peaks in the TIC chromatogram corresponded to more than one ion as can be seen in the mass spectra. This observation may be explained by the co-elution of several compounds for the same retention time and also to the formation of adducts with formic acid from the mobile phase. For example, the ions at m/z 731 and 1,117 are $[M + HCOO]^-$ adducts of nüzhenide (molecular mass 686) and nüzhenide oleoside (molecular mass 1,072). Nüzhenide oleoside was identified by Maestro-Durán in olive seeds (Durán et al., 1994). Ions 715 and 771 and the respective formic acid adducts (m/z 761 and 817) were also detected in the extract analysed (Figure 2) and it was suggested that they could be derivatives of nüzhenide oleoside (Silva, 2004). The ion m/z 685 was detected at several retention times as shown in Figure 3, however its highest signal intensity was at 55 min retention time. However, the detection of the same ion with the same fragmentation pattern at other retention times suggest the presence of nüzhenide isomers, which is in accordance with data published by He (He et al., 2001). Those authors studied fruits from *Ligustrum lucidum*, also an *Oleaceae* family plant.
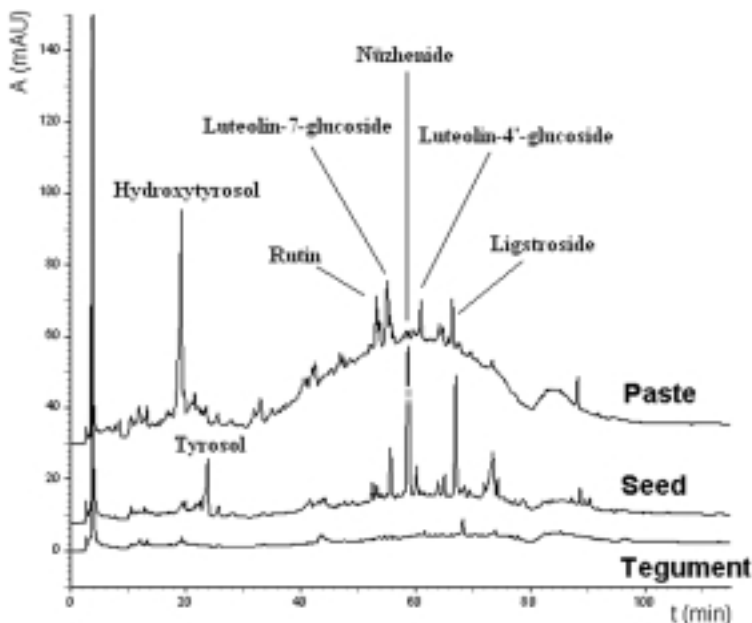

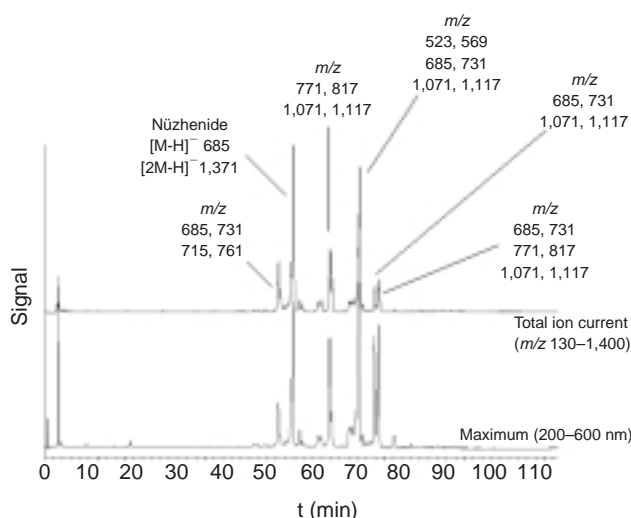**Figure 1.** Chromatograms at 280 nm of paste, seed and tegument extracts of 'Cobrançosa' cultivar.

Figure 2. Maximum absorption and total ion current chromatograms for seed extract of 'Cobrançosa' cultivar.
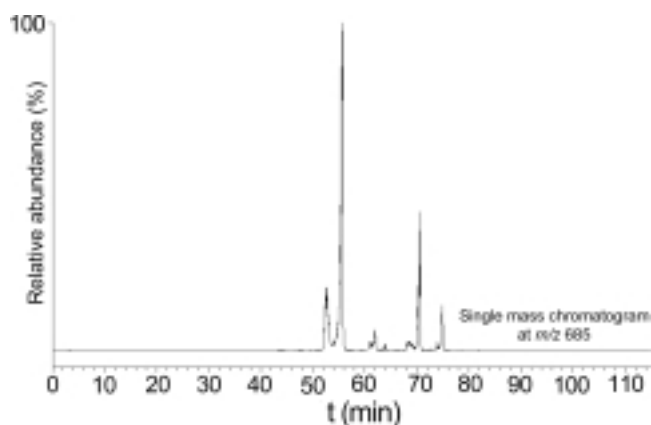

Figure 3. Single mass chromatogram at *m/z* 685 for seed extract of 'Cobrançosa' cultivar.

The ions detected in the mass spectrum: *m/z* 685 and 731, for 55 min chromatographic peak, are shown in Figure 4. The MS[2] spectrum obtained by fragmentation of the ion *m/z* 685 (Figure 5) presented the following *m/z* values: 299, 421, 453 and 523. The molecular structure of nüzhenide with the suggested fragmentation scheme for *m/z* 299 and 523 is presented in the same figure. The fragmentation of the [M − H]⁻ ion of nüzhenide at *m/z* 685 yields the corresponding aglycone with *m/z* 523, by neutral loss of 162 mass units. The ion *m/z* 299 is formed by the loss of the elenolic acid glucoside unit. The ions at *m/z* 421 and 453 are fragments of ion *m/z* 523, as shown in MS[3] spectrum (Figure 6).
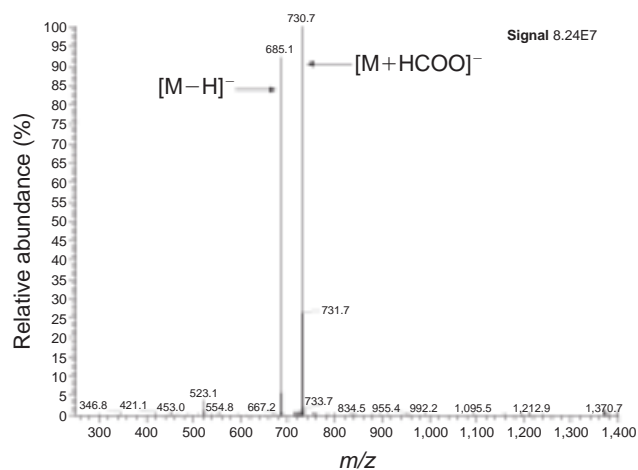

Figure 4. Mass spectrum of 55 min TIC chromatographic peak in seed extract of 'Cobrançosa' cultivar.

Olive Paste Extracts

The chromatogram obtained for the paste extract of the 'Cobrançosa' cultivar (Figure 1) presented a peak at 54 min identified as the flavonoid luteolin-7-glucoside. The [M − H]⁻ ion at *m/z* 447 was detected and the fragmentation yielded the aglycone luteolin at *m/z* 285, by the neutral loss of 162 mass units. However, an ion with equal fragmentation pattern was detected at 62 min retention time. According to the literature (Cardoso et al., 2005), four luteolin glucosides were detected in olive extracts: luteolin-7-glucoside and its respective three isomers luteolin-4'-glucoside, luteolin-6-glucoside and luteolin-8-glucoside. Luteolin-4'-glucoside is the only compound that elutes after luteolin-7-glucoside, in reverse phase HPLC. Therefore the chromatographic peak detected at 62 min matches with luteolin-4'-glucoside.

Hydroxytyrosol and rutin were easily identified by comparison of retention times and mass spectra with standards. Ligstroside was identified according to data obtained by MS and by literature (Ryan et al., 1999).

Leaf Extracts

The chromatographic profiles of extracts prepared from fresh leaves of the ten cultivars studied were similar, and 'Negrinha de Freixo' cultivar was distinguished by the high levels of phenolic compounds detected (Figure 7). The identification of the detected compounds was achieved using standards and also MS with fragmentation of the ions detected; the following compounds were identified: hydroxytyrosol, rutin, verbascoside, luteolin-7-glucoside, luteolin-4'-glucoside, oleuropein, oleuropein aglycone and ligstroside aglycone. Analysis using HPLC-MS showed that verbascoside co-eluted with luteolin-7-glucoside. However, in
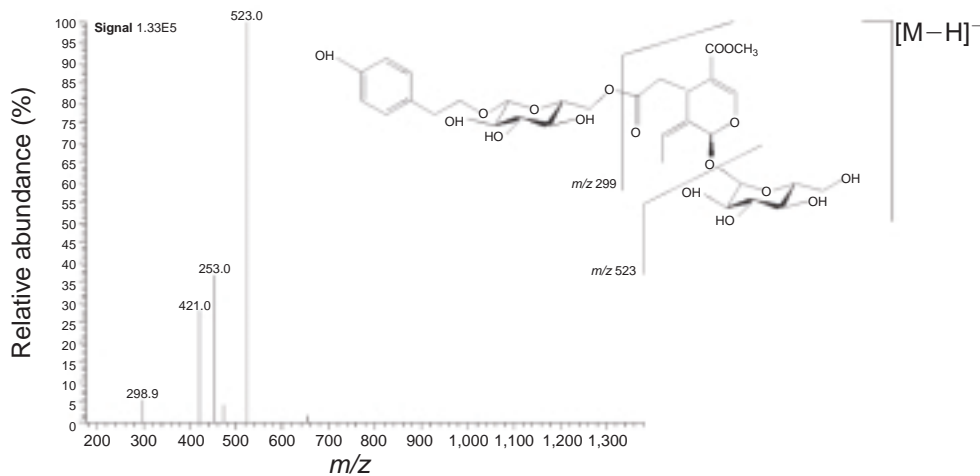
Figure 5. MS² spectrum from precursor ion *m/z* 685, in seed extract of 'Cobrançosa' cultivar.
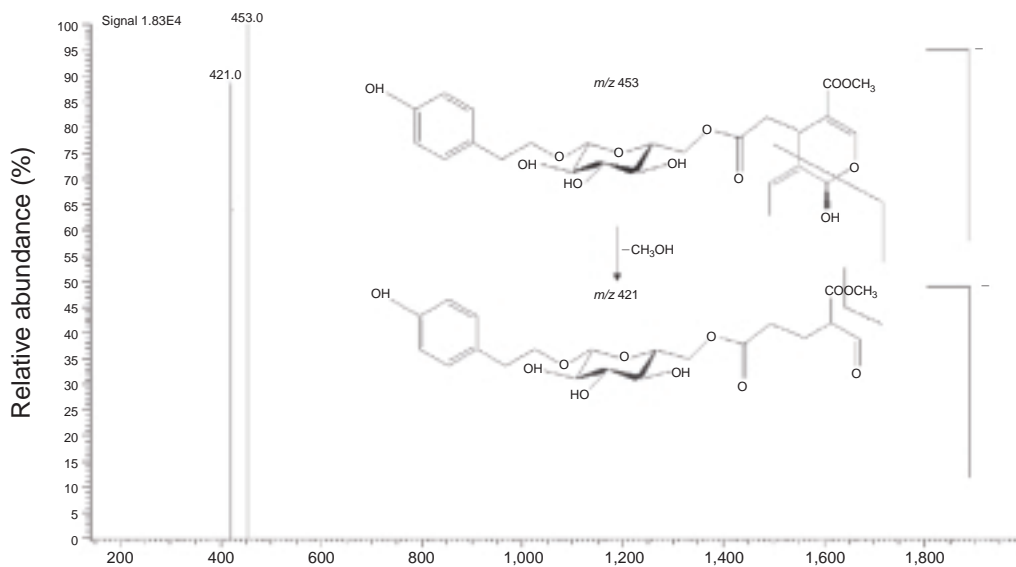

Figure 6. MS³ spectrum obtained from *m/z* 523 MS² fragment, in seed extract of 'Cobrançosa' cultivar.

the mass spectra it was possible to distinguish those substances due to differences in their molecular mass and fragmentation pattern.

At 60 min retention time was detected, in the mass spectrum, an ion at *m/z* 701 (corresponding to peak 4, Figure 7). This ion could correspond to a deprotonated form of a compound with molecular mass of 702. Oleuropein glucoside has that molecular mass and, as a diglucoside, it is expected to elute before oleuropein, which has only one glucosidic unit in its structure. Oleuropein glucoside had been detected for the first time in olive pulp by Cardoso (Cardoso et al., 2005) and also in olive tree leaves by De Nino (De Nino et al., 1997). It is interesting to notice the decrease of oleuropein glucoside with the drying process (peak 4,

Figure 7) which is related to an increase of oleuropein (corresponding to peak 6, Figure 7), probably due to the activity of β-glucosidases present in fresh leaves.

Dried Leaf Infusions

Chromatograms of infusions of 'Negrinha de Freixo' and commercial dried leaves were compared (Figure 8). There are phenolic compounds common to both samples like hydroxytyrosol and oleuropein. Verbascoside and luteolin-7-glucoside elute at 58 min retention time. However, in 'Negrinha de Freixo', infusion identified verbascoside at higher levels than luteolin-7-glucoside, as in the corresponding leaves extract.

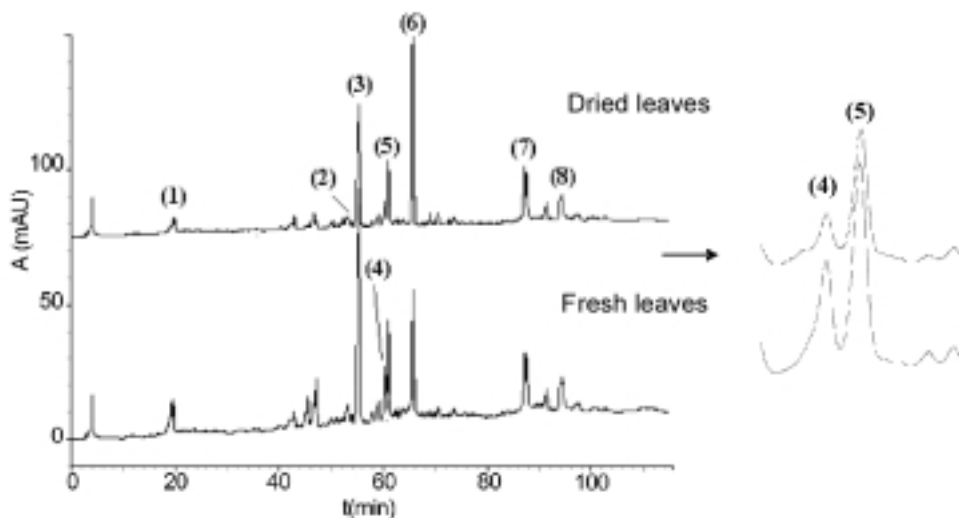Most of the chromatographic peaks detected

Figure 7. Chromatograms at 280 nm of leaves extracts of 'Negrinha de Freixo' cultivar and detail of 58–62 min retention time: **(1)** hydroxytyrosol; **(2)** rutin; **(3)** luteolin-7-glucoside + verbascoside; **(4)** oleuropein glucoside; **(5)** luteolin-4'-glucoside; **(6)** oleuropein; **(7)** oleuropein aglycone; **(8)** ligstroside aglycone.

showed higher intensity signals in 'Negrinha de Freixo' than in commercial infusion. The differences detected among samples could be related with the cultivar, date for collection of leaves, and drying process. It is known that the phenolic compound levels of leaves vary also at the time of fruits maturation (Briante et al., 2002).

The chromatographic profiles of infusion of leaves were very similar to the corresponding extracts. However, infusion had higher levels of phenolic compounds when compared with the respective dried leaves extract. The extraction of dried leaves with hot water increases the extraction of certain phenolic com-

pounds, when compared with methanol:water extraction (Silva, 2004).

The TIC chromatogram obtained for the infusion of dried leaves of 'Negrinha de Freixo' cultivar (Figure 9) displayed the ions detected at several retention times. It is interesting to refer the detection of oleuropein glucoside as $[M - H]^-$ at m/z 701, which is in accordance with results obtained for the leaves extract.

Hydroxytyrosol (m/z 153), hydroxytyrosol glucoside (m/z 315; formic acid adduct m/z 361) and oleoside m/z 389, a non phenolic compound, were also identified according to literature data (Cardoso et al., 2005) and comparison with the hydroxytyrosol standard.

Oleuropein was detected for two retention times indicative of the presence of the isomer of this compound in the sample. The oleuropein isomer, named oleuroside, was identified in olive (Ryan et al., 2001) and olive leaf (Lee-Huang et al., 2003) extracts with reverse phase HPLC, eluting after oleuropein. In the TIC chromatogram of leaves extract analysed, oleuroside was also detected, which is in accordance with the observations mentioned for infusion.
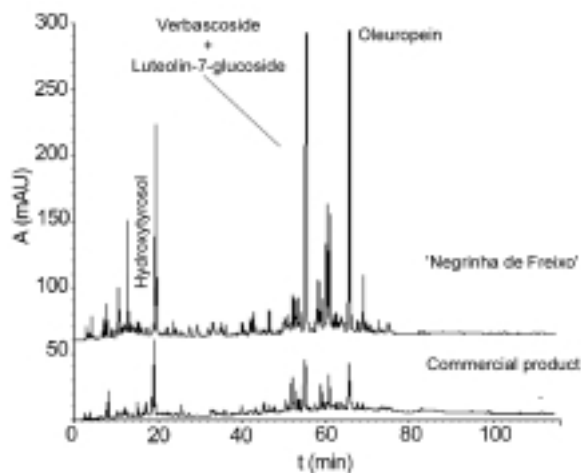
Total Phenolic Content and Total Antioxidant Activity

Total phenolic content, as determined by the Folin-Denis method, was expressed in tannic acid by reference to a standard curve ($y = -0.00276x^2 + 0.101x$, $r^2 = 0.999$). Method validation was carried out in terms of linearity, precision and accuracy. We conclude that for the concentration range studied, 1–5 mg/L, the best calibration fit was a second order function. Linearity studies were accomplished verifying the residual values
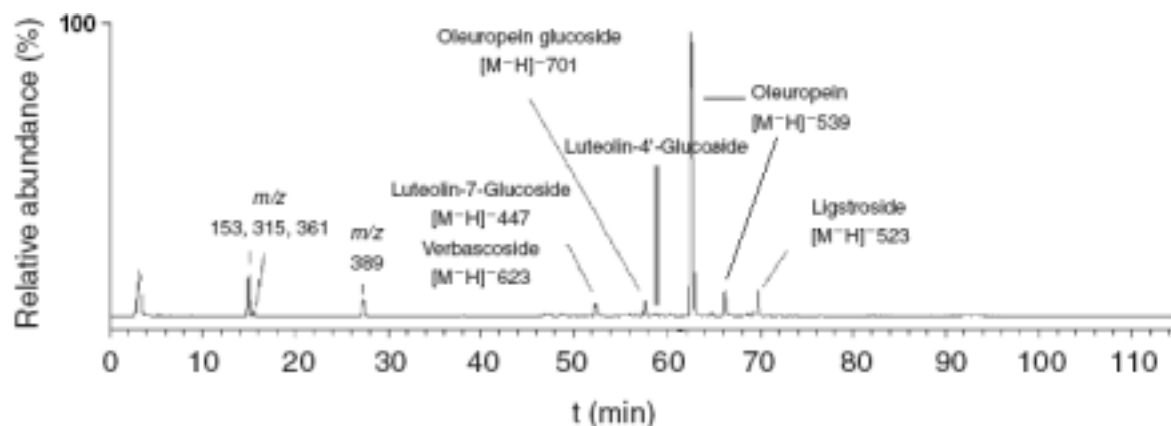

Figure 8. Chromatograms at 280 nm of infusions prepared with dried leaves of 'Negrinha de Freixo' and commercial dried leaves.

Figure 9. TIC chromatogram (*m/z* 130–1,400) obtained for dried leaves infusions of 'Negrinha de Freixo' cultivar.

distribution and applying a statistical test described in ISO 8466–1 (1990). An *f* test was carried out to verify the application of the second order function at confidence level 95%. An explanation for the best fit with a second order function may be related with the complexity of the standard used since there is an excess of Folin-Denis reagent and a negative deviation is observed for higher concentrations of tannic acid.

Precision was calculated on the matrix in terms of repeatability: 'intraday' and 'interday' (intermediate precision) as relative standard deviation (RSD %) at three concentration levels. The results obtained showed good method precision and the highest RSD % values were obtained for the minimum concentration tested (Table 1), as expected.

The matrix effect was investigated by performing spiking recovery experiments in an olive extract matrix for five concentrations (1–5 mg/L), the results were between 93.4–94.5% which is considered acceptable for method precision.

Using the Folin-Denis reagent, we also compared the response of tannic acid with other standards: gallic acid, tyrosol and caffeic acid. The choice of these compounds is related with their frequent use as standards in this type of determination (McDonald et al., 2001; Harwood and Rámon, 2000; Morelló et al., 2004), and

because they have different hydroxyl substitutes which makes them suitable for comparison in the reaction with the Folin-Denis reagent. The regression equations obtained allowed the comparison of the standards studied: gallic acid ($y = 0.106x$, $r^2 = 0.9859$), caffeic acid ($y = 0.105x$, $r^2 = 0.9899$), tannic acid ($y = -0.00101x^2 + 0.0952x$, $r^2 = 0.999$) and tyrosol ($y = 0.0662x$, $r^2 = 0.9867$). Gallic and caffeic acids gave similar responses to the Folin-Denis reagent. The lower reducing power of tyrosol may be explained by its structure containing only one hydroxyl group in the aromatic ring. These experiments were carried out on the same day to enable the comparison of the four standards responses. The equation presented for tannic acid in the beginning of this section was obtained on a different day: small differences observed in the equations' parameters are within the variations allowed for a 95% confidence level.

The values of total phenolic content measured in the samples of ten cultivars varied according with the type of sample (fruit, leaf or seed; Table 2). For seed and tegument samples only the cultivars with higher phenolic content in paste than in pulp were analysed.

Table 1. Precision: repeatability and intermediate precision of total phenolic content method.

Tannic Acid (mg/L)	Repeatability (n = 5) (RSD %)	Intermediate Precision (n = 15) (RSD %)
1	2.9	4.0
3	1.2	1.4
5	1.2	1.7

Table 2. Total phenolic content range of samples analysed and expressed as tannic acid concentration.

Samples	Total Phenolic Content (g/kg)
Pastes	11.7–24.1
Pulps	13.9–30.5
Seeds[1]	5.9–14.9
Teguments[1]	5.6–7.0
Leaves (fresh)	11.6–17.4
Leaves (dried)	11.7–40.1
Infusions	0.04–0.25 (g/L)

[1]Samples of cultivars: 'Bical', 'Cobrançosa', 'Lentisca', 'Madural', 'Santulhana' and 'Verdeal Transm ontana'.
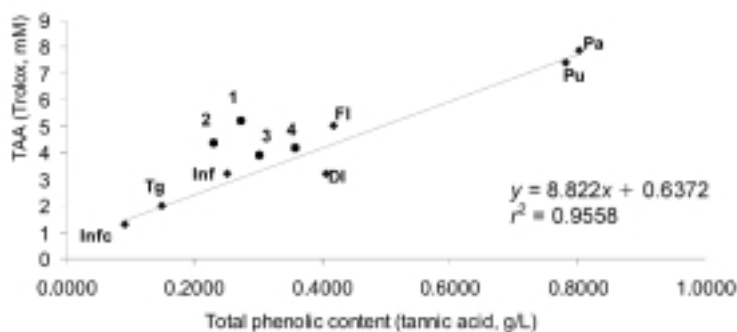
Figure 10. Total phenolic content and total antioxidant activity for samples: **1** 'Cobrançosa' seed; **2** 'Lentisca' seed; '**3** Santulhana' seed; **4** 'Verdeal Transmontana' seed; **Pa** 'Cobrançosa' paste; **Pu** 'Negrinha de Freixo' pulp; **Fl** 'Cobrançosa' fresh leaves; **Dl** 'Cobrançosa' dried leaves; **Inf** 'Negrinha de Freixo' infusion; **Infc** Commercial product infusion; **Tg** 'Santulhana' tegument.

The diversity of phenolic compounds and their different distribution in the plant may explain the different ranges obtained for the total phenolic contents. When the results of fresh and dried leaves were compared it was found that some samples increased its total phenolic content after drying; it should be mentioned that the water content of fresh leaves was taken into account. The observed increase in phenolic content after the drying process could be due to the formation of compounds that react with the Folin-Denis reagent and to the conversion of oleuropein glucoside in oleuropein in the same samples (Figure 7). Therefore, oleuropein could have more reducing power of the Folin-Denis reagent than its glucoside analogue: it was claimed that flavonoid aglycones had more antioxidant activity than their corresponding glycosides, suggesting that the presence of sugar in the molecule decreases the antioxidant activity (Das et al., 1990).

Total phenolic content expressed as tannic acid, for extracts and infusions, presented values in the range of 0.09 to 0.80 g/L (Figure 10). The highest amount was found in fruits (paste and pulp extracts) and the lowest in the infusion of the commercial product.

The total antioxidant activities evaluated were higher in fruit extracts and lower in infusions and in the tegument extracts analysed. The total antioxidant activities of dried and fresh leaves had values between those of infusions and fruits. The seed extracts were excluded from the correlation presented in Figure 10, due to the deviations observed. The seed extracts have high total antioxidant activities and low total phenolic contents: a possible explanation is the existence of several compounds containing a tyrosol unit, like nüzhenide as shown by HPLC-APCI-MS. Compounds with this type of phenolic structure have lower contributions to the total phenolic content measured with the Folin Denis reagent (Silva, 2004).

As far as we know, there are no reports concerning the study of antioxidant activity in olive seeds. Fruit seeds have not received much attention as antioxidant sources. However, there are considerably higher ratios of by-products arising from fruit processing plants as table olives commercialised without a stone. A study relating antioxidant activity and selected fruit seeds, as mango and avocado (Soong and Barlow, 2004) concluded that total antioxidant activity of fruit seeds is significantly higher than in edible portions, showing the interest in studying this part of the fruit.

CONCLUSIONS

The combination of analytical techniques HPLC/DAD/MS is a very useful tool for the analysis of complex mixtures and had been used in the characterisation of phenolic composition of different parts of olive tree cultivars of the Trás-os-Montes e Alto Douro region (Portugal): pastes and pulps of fruits, seeds and leaves. Dried leaf infusions were also prepared. Diode array detection was particularly valuable in confirming the phenolic nature of eluted species from their characteristic spectra. The use of MS allowed the identification of the major phenolic compounds in sample extracts.

The type of phenolic compounds detected in leaf, fruit and seed varied markedly. Hydroxytyrosol and flavonoids like rutin and luteolin-7-glucoside were the major phenolic compounds detected in paste extracts. Verbascoside, luteolin-7-glucoside and oleuropein were abundant in leaves. Nüzhenide was the predominant phenolic secoiridoid compound in seed extracts and may explain the high total antioxidant activities found in these samples.

The method used to evaluate the antioxidant activity of samples ($ABTS^+$ method) showed to be a rapid method for determining this parameter and could be a useful tool to make selection among samples and cultivars in order to obtain high content of natural antioxidants in foods. The high antioxidant activity of seed extracts is due to nüzhenide and related compounds, suggesting the possible application of olive seeds as sources of natural antioxidants.

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