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Cultivar effect on the phenolic composition and antioxidant potential of stoned table olives

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ABSTRACT

Stoned green table olives "*alcaparras*" prepared from five different cultivars (*Cv.* Cobrançosa, Madural, Negrinha de Freixo, Santulhana and Verdeal Transmontana) were investigated concerning their phenolic composition and antioxidant potential. From each cultivar, five independent lots were prepared. The phenolic profile was determined by HPLC/DAD at 280 nm, and antioxidant potential measured using the reducing power and scavenging effect on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals assays. Twelve phenolic compounds were identified, being hyrdoxytyrosol the most abundant one, followed by verbascoside and tyrosol. *Cv.* Cobrançosa and Santulhana reported higher content of phenolic compounds, with 165.76 and 163.66 mg/kg of fresh "*alcaparras*" table olives, respectively. Regarding antioxidant activity, *Cv.* Santulhana and Cobrançosa showed higher EC_{50} values, lower than 1.40 and 0.48 mg/mL for reducing power and DPPH methods, respectively. Significant negative correlations were obtained between olive phenolics and EC_{50} values from the antioxidant activity. The direct contact of the pulp with water, characteristic of this processing method, eliminates important hydrossoluble compounds, being the cultivar used an important determinant for the final "*alcaparras*" composition in terms of ingested phenolic compounds and antioxidant activity.

1. Introduction

Olea europaea L. products, mainly olive oil and table olives, are very important components of the Mediterranean diet (Boskou et al., 2006). Their postulated health benefits seem to be intrinsically linked to the high monounsaturated fat content (Bianchi, 2003) and to minor constitutes like tocopherols and phenolic compounds (Montaño et al., 2005).

Phenolic compounds are of great importance for the olive fruit, being responsible for important characteristics and properties, such as color, taste and texture (Marsilio et al., 2001). Several reports also highlight their important antioxidant capacity (Ben Othman et al., 2009), antimicrobial activity (Sousa et al., 2006), and protection against micotoxins effects (Beekrum et al., 2003).

Several phenolic compounds have been identified in table olives, including oleuropein and hydroxytyrosol (Briante et al., 2002), tyrosol (Briante et al., 2002), rutin (Boitia et al., 2001), quercetin (Obied et al., 2007), as well as caffeic (Papadopoulos and Boskou, 1991), vanillic and σ- and ρ-coumaric acids (Brenes et al., 1999), among others. Olives phenolic composition, however, is highly variable in both quality and quantity (Uccella, 2001; Vinha et al., 2005), in the dependence of several factors: processing method (Romero et al., 2004), irrigation regimes (Patumi et al., 2002), cultivar (Romani et al., 1999), and maturation degree (Ryan et al., 1999). For instance, important changes are reported to occur in the phenolic fraction during olive fruit development, with depletion of oleuropein and increasing of tyrosol and hydroxytyrosol concentrations (Esti et al., 1998; Ferreira et al., 2002; Piga et al., 2001).

Three kinds of table olives are more representative in the international market: Spanish-style green olives in brine, Greek-style naturally black olives in brine, and Californian black ripe olives (Blekas et al., 2002; Sabatini et al., 2009). All processing methods influence the phenolic composition of table olives reducing its content by different ways. In the Spanish-style green olive processing, Brenes et al. (1995) studied the changes in phenolic compounds and noticed that the NaOH treatment hydrolyzed oleuropein into hydroxytyrosol and elenolic acid glucoside, and that caffeic acid, oleuropein, and *p*-coumaric acid contents reduce during fermentation period, while tyrosol concentration remained constant (Brenes et al., 1995). Marsilio et al. (2001) showed that Californian-style ripe olive processing also influences the phenolic composition. In particular, vanillic acid and oleuropein content decreased while tyrosol and hydroxytyrosol increased. Although the bacterial metabolism in the fermenting brine seems to play an important

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role, the washing step to remove the excess of NaOH (Marsilio et al., 2001) was also the most implicated processing step. Romero et al. (2004) demonstrated that the main phenolic compounds before fermentation naturally black olives (Greek-style) were hydroxytyrosol-4-β-glucoside, oleuropein, hydroxytyrosol, tyrosol, salidroside, and verbascoside, while after 12 months the main phenolic was hydroxytyrosol, followed by hydroxytyrosol acetate, tyrosol, and tyrosol acetate.

"*Alcaparras*" are a kind of stoned green table olives processed by a traditional method in Trás-os-Montes region, highly appreciated and commercialized in local markets. For their production healthy green or yellow-green olive fruits are used, and, are broken to remove the stone. The pulp is immersed in water to remove natural bitterness being changed daily until achieve edible grade. Commercial "*alcaparras*" table olives, a blend of several olive cultivars, were already studied for their phenolic composition, with three flavonoidic compounds identified: luteolin 7-O-glucoside, apigenin 7-O-glucoside, and luteolin (Sousa et al., 2006). They have also showed antioxidant properties and antimicrobial activity (Sousa et al., 2008). Nevertheless, important variations were observed in their composition and sensorial attributes (data not published), highlighting the importance of a more dedicated work on the factors involved. Therefore, the present paper aimed to study the effects of olive cultivar on the phenolic composition and antioxidant activity of "*alcaparras*" produced by the traditional method in Trás-os-Montes region (Northeast of Portugal).

2. Material and methods

2.1. Reagents and standards

Methanol, 2,2-diphenyl-1-picrylhydrazyl and iron (III) chloride were obtained from Sigma–Aldrich (St. Louis, USA). Methanol (HPLC grade), sodium dihydrogen phosphate dihydrate, potassium hexacyanoferrate (III), formic acid 98–100% were purchased from Merck (Darmstadt, Germany). Hydrochloric acid and di-sodium hydrogen phosphate 2-hydrate were obtained from Panreac (Barcelona, Spain). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA). Hydroxytyrosol, tyrosol, chlorogenic acid, vanillic acid, syringic acid, verbascoside, luteolin 7-O-glucoside, oleuropein, rutin, apigenin 7-O-glucoside, quercetin and luteolin standards, used for phenolic profile identification were obtained from Extrasynthèse (Genay, France).

2.2. Stoned table olives "Alcaparras" sampling and preparation

For this study, five of the most representative olive cultivars (*Cv.* Cobrançosa, Madural, Negrinha de Freixo, Santulhana and Verdeal Transmontana) from Trás-os-Montes region were collected in September and October of 2006 from different olive groves subjected to similar agro-climatic and agronomic conditions. From each cultivar, five independent lots of olives, approximately of 5 kg each, were collected from several trees and immediately transported to the laboratory. At the laboratory, approximately 2 kg of stoned table olives were prepared from each lot. Only green or yellow-green healthy olive fruits were used, being manually broken to separate the pulp from the stone. The pulp was immersed in water during a week, daily changed, to remove olives bitterness. After the treatment, "*alcaparras*" table olives were frozen at −20 °C and freeze dried (Ly-8-FM-ULE, Snijders) prior analysis.

2.3. Extraction preparation

For each sample, three freeze dried powdered sub-samples (~5 g; 20 mesh) were extracted with 250 mL of boiling water for 45 min and filtered through Whatman No. 4 paper. The aqueous extracts were weight, frozen, and lyophilized and again dissolved in water in concentrations ranging from 0.01 and 5 mg/mL for antioxidant activity assay and 50 mg/mL for phenolic profile evaluation.

2.4. Identification and quantification of phenolic compounds

Phenolic profile was performed by HPLC analysis on a Knauer Smartline separation module equipped with a Knauer smartline autosampler 3800, a cooling system set to 4 °C and a Knauer DAD detector. Data acquisition and remote control of the HPLC system was done by ClarityChrom® software (Knauer, Berlin, Germany). A reversed-phase Spherisorb ODS2 column was used (250 × 4 mm id, 5 μm particle diameter, end-capped Nucleosil C18 (Macherey-Nagel) maintained at 30 °C. The solvent system used was a gradient of water/formic acid (19:1) (A) and methanol (B), which were previously filtered and degasseddegassed and filtered. The flow rate was 0.9 mL/min with the following gradient: 5% B at 0 min, 15% B at 3 min, 25% B at 13 min, 30% B at 25 min, 35% B at 35 min, 40% B at 39 min, 45% B at 42 min, 45% B at 45 min, 47% B at 50 min, 48% B at 60 min, 50% B at 64 min and 100% B at 66 min. For the HPLC analysis the aqueous extracts were dissolved in methanol, in a reason of 50 mg/mL. All samples were filtered through a 0.2 μm Nylon membrane (Whatman) and 10 μL of each solution were injected. Chromatographic data was recorded at 280 nm. Spectral data from all peaks were accumulated in the 200–400 nm range. Phenolic compounds were identified by comparing the retention times and spectrums of the chromatographic peaks with those of authentic standards analyzed under the same conditions. Phenolic compounds quantification was achieved by the absorbance recorded in the chromatograms relative to external standards.

2.5. Scavenging effect assay

The capacity to scavenge the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was monitored according to the method of Hatano et al. (1988). The extract solution (0.3 mL) was mixed with 2.7 mL of methanolic solution containing DPPH radicals (6×10^{-5} mol/L). The mixture was shaken vigorously and left to stand for 60 min at room temperature in dark (until stable absorbance values were obtained). The reduction of the DPPH-radical was measured by continuous monitoring of the absorption decrease at 517 nm.

DPPH scavenging effect was calculated as the percentage of DPPH discoloration using the following equation: % scavenging effect = $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% inhibition (EC_{50}) was calculated from the graph of scavenging effect percentage against extract concentration in the solution.

2.6. Reducing power assay

The reducing power was determined according to a described procedure (Berker et al., 2007). The extract solution (1 mL) was mixed with 2.5 mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After cooling, 2.5 mL of 10% trichloroacetic acid (w/v) were added and the mixture was centrifuged at 1000 rpm for 8 min (Centorion K24OR-2003 refrigerated centrifuge). The upper layer (2.5 mL) was mixed with 2.5 mL of deionised water and 0.5 mL of 0.1% ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm (higher absorbance readings indicate higher reducing power). Extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm against extract concentration in the solution.

2.7. Statistical analysis

A regression analysis, using Excel from Microsoft Corporation, was established between phenolic contents of the different olive cultivars and EC_{50} values obtained from the two antioxidant assays tested. A principal component analysis (PCA) and ANOVA were carried out using SPSS 17.0 software.

An analysis of variance (ANOVA) with Type III sums of squares was performed using the GLM (General Linear Model procedure) of the SPSS software, version 17.0 (SPSS, Inc.). The fulfilment of the ANOVA requirements, namely the normal distribution of the residuals and the homogeneity of variance, were evaluated by means of the Kolmogorov–Smirnov with Lilliefors correction (if $n > 50$), and the Levenés tests, respectively. All dependent variables were analyzed using a one-way ANOVA with or without Welch correction, depending if the requirement of the homogeneity of variances was fulfilled or not. The main factor studied was the effect of olive cultivar on the phenolic compounds profile, EC_{50} values of the two antioxidant assays tested and extraction yield, and, if a statistical significant effect was found, means were compared using Tukeýs honestly significant difference multiple comparison test or Dunnett T3 test also depending if equal variances could be assumed or not. All statistical tests were performed at a 5% significance level.

3. Results and discussion

3.1. Identification and quantification of phenolic compounds

The study of the phenolic composition of "*alcaparras*" table olives produced from different olive cultivars by HPLC/DAD revealed different qualitative and quantitative chemical profiles, in which twelve phenolic compounds were identified and quantified: hydroxytyrosol, tyrosol, chlorogenic acid, vanillic acid, syringic acid, verbascoside, luteolin 7-O-glucoside, oleuropein, rutin, apigenin 7-O-glucoside, quercetin and luteolin (Figs. 1 and 2).