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Original Article

DPPH-scavenging capacity of chloroplastic pigments and phenolic compounds of olive fruits (cv. Arbequina) during ripening

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ABSTRACT

This work studies the antioxidant capacity of chloroplastic pigments (chlorophylls and carotenoids) and phenolic compounds during the growth and ripening of olive fruits *Olea europaea* L. cv. Arbequina, grown under organic and conventional methods. Chloroplastic pigments are lipophilic compounds, while polyphenols are hydrophilic. The antioxidant capacity of lipophilic and hydrophilic fractions was measured by scavenging of the 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH). The lipophilic fraction comprised an acetone solution (containing chlorophylls and xanthophylls) and a hexane solution (including β-carotene), while the hydrophilic fraction corresponded to a methanol solution (containing total phenolic compounds). During the development and ripening phases of fruits, both chloroplastic pigments and total phenolic compounds followed a parallel pattern. Both fractions presented the highest antioxidant capacity during the first weeks of the fruit growing, coinciding with the highest chloroplastic pigments and total phenolic compounds contents. There was a high correlation between the antioxidant capacity of the hydrophilic fraction and total phenolic compounds. The antioxidant capacity of the acetone extract was also positively correlated with the total chlorophyll and xanthophyll content, while lower correlation between the β-carotene content and the antioxidant capacity of the hexane extract was found. All the parameters studied in organic and conventional fruits followed similar evolution patterns, with slight differences.

1. Introduction

The olive tree (*Olea europaea* L.) is one of the most important fruit trees in the Mediterranean basin. The biological properties attributed to the consumption of olive products, table olives and olive oil have been extensively studied (Boskou et al., 2006; Cornwell and Jiyan, 2008). In Spain, table olives are produced from green fruits (Spanish-style green table olives), while virgin olive oil is obtained from ripe fruits. The color of the olive fruit comes from chlorophylls and carotenoids, although a high content of polyphenols is also present. Fruit color changes from intense green to yellow-green as fruit ripens and, finally to purple and then black as the fruit becomes fully ripe. Anthocyanins are the main compounds responsible for the color in the purple and black ripening stages of the olives; however, chlorophylls and carotenoids are still present (Mínguez-Mosquera and Gallardo-Guerrero, 1995).

Olives are a rich source of natural antioxidants which may, by different mechanisms, act as an effective defense against reactive species (Ben Othman et al., 2008). During the last few years, the study of antioxidant capacity has received much attention, mainly due to the growing interest in the efficiency and function of natural antioxidants in food and biological systems. The oxidant reactions of free radicals, which are molecules with unpaired electrons, are thought to contribute to many health problems, including cancer, cardiovascular diseases, inflammatory problems and aging (Kehrer, 1993). Antioxidants are agents that, in one way or another, restrict the deleterious effects of these oxidant reactions, either scavenging free radical (eliminating them without generating more radical-induced damage) or other effects (i.e. preventing radical formation) (DiSilvestro, 2001). Chlorophylls, carotenoids (carotenes and xanthophylls) and polyphenols are bioactive compounds present in olive fruits and processed products which are able of acting as chemical antioxidants and therefore possess the ability to reduce the oxidative damage associated with many diseases (Ferruzzi et al., 2002; Lanfer-Marquez et al., 2005; Pereira et al., 2006). Studies have evidenced chlorophylls as electron donors reducing free radicals such as 2,2-diphenyl-1-picrylhydrazyl (Ferruzzi and Blakeslee, 2007). Carotenoids can be easily oxidized preventing, in this way, other oxidation reactions. Moreover, they may act as direct antioxidants by reacting with active oxygen species (Krinsky, 1989). Phenolic compounds are

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capable of protecting cell membranes against damage induced by reactive free radicals and are able to reduce LDL aggregation (Cadenas and Packer, 2002). Also the need to measure antioxidant capacity is well documented and bibliography reports different methods to measure antioxidant capacity in food (Pérez-Jiménez et al., 2008).

Several studies on the polyphenol content and antioxidant capacity of olives can be found in the literature (Bouaziz et al., 2004; Morelló et al., 2005a). Nevertheless, there have been no studies that consider the possible relationship of chloroplastic pigments to the total antioxidant capacity of olives. Chloroplastic pigments are lipophilic compounds, while polyphenols are hydrophilic. Thus, in this study, the antioxidant capacity of both lipophilic and hydrophilic fractions was measured using the DPPH assay. This method gives a global estimation of antioxidant capacity in foods and, among other methods, it has been routinely assayed in lipophilic and hydrophilic plant extracts (Pérez-Jiménez et al., 2008). The aim of the present work was to evaluate the radical scavenging activity of chloroplastic pigments (chlorophylls and carotenoids) and total phenolic compounds of olive fruits *O. europaea* L. cv. Arbequina during the development and ripening stages. In addition, and because of the growing demand and popularity of organic foods, the study was conducted with organic and conventional cultivated olive fruits. There are some studies comparing virgin olive oil from organic and conventional farming (Gutiérrez et al., 1999; Ninfali et al., 2008); however no study has been found comparing organic and conventional olive fruits.

2. Materials and methods

2.1. Plant material and sampling

The study was carried out in olive fruits *O. europaea* L. cv. Arbequina, from trees grown under organic and conventional conditions in Écija (Sevilla, Spain). Olives were picked from the whole perimeter of all six olive trees from both types of growing conditions selected for the study. It began when the growth stage of the fruits was so small that no stone could be distinguished, and finished when ripening had covered the fruit surface with anthocyanins. Sampling was conducted at intervals of 15 days for a period of 6 months (from June to December, 2009).

2.2. Chemicals and reagents

HPLC grade solvents were purchased from Prolabo (VWR International Eurolab, Barcelona, Spain), and HPLC-grade water was obtained with a MilliQ water purifying system from Millipore (Milford, MA, USA). All other reagents used were of analytical grade. Solvents were supplied by Sharlau (Microdur, Sevilla, Spain). Tyrosol, Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH), and Triton X-100 were obtained from Sigma-Aldrich (Barcelona, Spain). Tetrabutylammonium acetate and ammonium acetate were supplied by Fluka (Zwijndrecht, The Netherlands). Other salts, selenium dioxide, and silica gel GF_{254} were obtained from Merck (Barcelona, Spain).

2.3. Standard pigments for HPLC

Chlorophylls *a* and *b* were supplied by Sigma Chemical Co. (St. Louis, MO). Chlorophyllide *a* was formed by enzymatic de-esterification of chlorophyll *a*: a protein precipitate (acetone power) of *Ailathus altissima* (Mill.) leaves was extracted with 50 mM sodium phosphate buffer, pH 7, containing 50 mM KCl and 0.24% Triton X-100 and the substrate dissolved in acetone in a 5:5:1 ratio (Mínguez-Mosquera et al., 1994). 13^2-OH-chlorophylls *a* and *b* were obtained by selenium dioxide-oxidation of chlorophyll (*a* or *b*) at reflux-heating in pyridine solution during 4 h under N_2 (Laitalainen et al., 1990). β-Cryptoxanthin was obtained from red peppers, and lutein, neoxanthin, violaxanthin, and antheraxanthin were obtained from a pigment extract of fresh spinach and separated by TLC with silicagel GF_{254} (0.7 mm) on 20 cm × 20 cm plates using petroleum ether (65–95 °C)/acetone/diethylamine (10:4:1) (Mínguez-Mosquera and Hornero-Méndez, 1993).

2.4. Extraction of chloroplastic pigments

Samples were made from a triturate homogenized from around 50 de-stoned fruits by accurately weighing from 4 to 15 g for each analysis according to the ripeness degree of the fruits. Pigment extraction was performed with N,N-dymethylformamide (DMF) according to Mínguez-Mosquera and Garrido-Fernández (1989). The technique is based on the selective separation of components between DMF and hexane. Hexane phase carried over lipids and carotenes, whereas DMF phase retained chlorophylls and xanthophylls. The DMF phase was later transferred to ethyl ether, concentrated and finally dissolved in 1.5 mL acetone. Both hexane and acetone solutions were used to evaluate the antioxidant capacity of the lipophilic fraction. Analyses were performed in triplicate.

2.5. Determination of chloroplastic pigments

2.5.1. Acetone solution

Chloroplastic pigments present in this solution were separated and quantified by HPLC using a HP 1100 Hewlett-Packard liquid chromatograph fitted with a HP1100 automatic injector HPLC. A stainless steel column (20 cm × 0.46 cm i.d.), packed with 3 μm C_{18} Mediterranea Sea (Teknokroma, Barcelona, Spain) was used. The column was protected by precolumn (1 cm × 0.4 cm i.d.) packed with the same material. Separation was performed using an elution gradient (flow rate 1.250 mL min^{-1}) with the mobile phases: (A) water/ion pair reagent/methanol (1:1:8, v/v/v) and (B) methanol/acetone (1:1, v/v). The ion pair reagent was 0.05 M tetrabutylammonium and 1 M ammonium acetate in water. The column was stored in methanol/water (1:1, v/v). The gradient scheme is a modification of that of Mínguez-Mosquera et al. (1991), and briefly is initially 75% A and 25% B, then changes to 25% A in 8 min, isocratic 2 min, change to 10% A in 8 min, then to 100% B in 5 min, isocratic 15 min, and return to initial conditions in 5 min. Sequential detection was performed with a photodiode array detector at 430 (for chlorophyll *a*) and 450 nm (for chlorophyll *b* and xanthophylls). Data were collected and processed with a LC HP ChemStation (Rev.A.05.04).

The separated pigments in the acetone solution were chlorophylls *a* and *b*, chlorophyllide *a* and 13^2-OH-chlorophylls *a* and *b* (chlorophyllic fraction), and lutein, neoxanthin, violaxanthin, antheraxanthin, β-cryptoxanthin, and lutein epoxide (xanthophyllic fraction). Pigments were individually quantified using external standard calibration curves of HPLC prepared with purified standards of each pigment, except for lutein epoxide. For this pigment, the violaxanthin calibration curve was used. Chloroplastic pigment content in acetone solution was the sum of each individually quantified pigment: chlorophylls *a* and *b*, chlorophyllide *a* and 13^2-OH-chlorophylls *a* and *b* (total chlorophylls), and lutein, neoxanthin, violaxanthin, antheraxanthin, β-cryptoxanthin, and lutein epoxide (total xanthophylls).

2.5.2. Hexane solution

The different hexane phases obtained from the extraction method of chloroplastic pigments were combined in a rotavapor