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Postharvest Biology and Technology 44 (2007) 150–156

Postharvest Biology and Technology

www.elsevier.com/locate/postharvbio

Varietal differences in catabolic intermediates of chlorophylls in *Olea europaea* (L.) fruit cvs. Arbequina and Blanqueta

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Received 31 July 2006; accepted 7 December 2006

Abstract

A comparative study of the chlorophyll catabolism in fruit of *Olea europaea*, cvs. Arbequina and Blanqueta during the ripening, has demonstrated a temporal disparity in chlorophyll disappearance between varieties. In 'Blanqueta' fruit, the early cleavage of the macro-ring of the chlorophyll molecule implies a fast loss of chlorophylls before the synthesis of anthocyanins. The displacement in the time of this process agrees in each variety with the maximum levels of *in vivo* chlorophyllide and chlorophyllase activity (EC 3.1.1.14). The temporary difference in the activation of chlorophyllase and the rest of enzymes implied in the pheophorbide *a* oxygenase pathway is responsible for the step to colorless products. In addition, the different involvement of minor oxidized chlorophylls in the varieties implies a different participation of chlorophyll catabolic oxidatives enzymes. The greater oxidative activity in the fruit of the 'Blanqueta' variety can indirectly have an influence on the lower oxidative stability of corresponding oils.

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Keywords: Arbequina; Blanqueta; Chlorophyll catabolism; *Olea europaea* (L.); Olive fruits; Variety

1. Introduction

The natural phenomenon of loss of green color during leaf senescence and fruit ripening has been the subject of research for many years, and the colored catabolites have been cataloged in detail (Mínguez-Mosquera and Gallardo-Guerrero, 1995; Matile et al., 1999). It has been established that during leaf senescence, chlorophyll (chl) is degraded to non-colored linear tetrapyrroles, denominated non-colored chl catabolites (NCC), following the "PaO degradation pathway" (Matile et al., 1999). Two consecutive reactions, catalyzed by chlorophyllase and Mg dechelatase, initiate this pathway starting from chl *a*, eliminating both the phytol chain and the Mg atom, and finally generating pheophorbide *a*. Subsequently, the porphyrin ring is oxygenolytically opened by pheophorbide *a* oxygenase (PaO) between carbons 4 and 5. The product, known as red chlorophyll catabolite (RCC), is not accumulated *in vivo*, as it is rapidly converted into a primary non-colored chl catabolite.

In parallel, oxidized chl catabolites such as 13^2-OH-chl *a* and other allomerized chls have been found both in senescent leaves (Maunders et al., 1983) and during on-tree ripening of olives (Mínguez-Mosquera and Gallardo-Guerrero, 1996; Roca and Mínguez-Mosquera, 2003) and other fruit (Yamauchi et al., 1997). The compound 13^2-OH-chl *a* has been identified as the principal product of Chl *a* oxidation by H_2O_2 catalyzed by peroxidase (Kaartinen et al., 1985). As a consequence of chl allomerization (oxidation at C-13^2 by triplet oxygen ($^3O_2$)), 13^2-OH-chl *a* is produced, and according to the free-radical mechanism proposed for this reaction by Hynninen (1991), peroxidase would assist in producing chl 13^2-radicals, accelerating the allomerization mechanism. Janave (1997) reported *in vitro* evidence for chl degradation by an oxidative enzyme in Cavendish banana (*Musa cavendishi*) apart from the dephytylating chlorophyllase pathway. Recently, a peroxidative activity has been found in solubilized thylakoid membranes of olives, *Olea europaea* cv. Hojiblanca that catalyses degradation of chloroplast pigments to 13^2-OH-chl *a,* 13^2-OH-chl *b* and oxidized carotenoids (Gandul-Rojas et al., 2004).

'Arbequina' and 'Blanqueta' are two of the 24 main olive cultivars in Spain. Morphologically, their fruit are very similar: spherical, small in size, around 2.0 g, and with a high yield of

Abbreviations: Chl, chlorophyll; DMF, *N*,*N*-dimethylformamide; PaO, pheophorbide *a* oxygenase; ap, acetone powder

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doi:10.1016/j.postharvbio.2006.12.001

oil (around 20 or 22%). These two varieties are characterized by their low pigment content (Roca and Mínguez-Mosquera, 2001a) and by the specific color changes in their fruit before ripening starts. In the variety ‘Blanqueta’, the surface color of the fruit becomes whitish before the start of ripening, while ‘Arbequina’ is known for its yellowish fruit, implying carotenogenesis (Roca and Mínguez-Mosquera, 2001b; Criado et al., 2007), also before the synthesis of anthocyanins.

Preliminary studies in five varieties of olive fruit showed that the rate of degradation of chlorophylls and carotenoids during the ripening process differs depending on variety, with fruit of ‘Blanqueta’ having the highest values and those of ‘Arbequina’ the lowest (Roca and Mínguez-Mosquera, 2001a). Although both varieties have low pigmentation and are morphologically very similar, physiologically, as mentioned above, there are certain differences during chl catabolism.

The virgin olive oils obtained from these two varieties are very much appreciated for their organoleptic characteristics, although both of them have low stability in comparison to other mono-varietal virgin olive oils (García et al., 1996; Gutierrez et al., 1999).

The present work seeks a physiological interpretation of the difference between the rate of disappearance of chls in varieties that are apparently similar, as are ‘Arbequina’ and ‘Blanqueta’, but which show biochemical differences.

2. Materials and methods

2.1. Plant material

The study was carried out on olives (*Olea europaea* L.) cv. Blanqueta, from Gandía (Valencia) and cv. Arbequina from Cabra (Córdoba) during fruit ripening that begins with the appearance of reddish spots of anthocyanins on the skin of the fruit, a stage designated “mottled.” As ripening progresses, the synthesis of anthocyanins increases, and they gradually cover the whole skin, which becomes purple. In all cases, the starting material was developed fruit, with stable pit and pulp weight, and evaluations were carried out on homologous stages of ripening pre-established visually by the color: green, light green, yellow (white for ‘Blanqueta’), mottled and purple (Walalí et al., 1984). The white color of ‘Blanqueta’ fruit, and the yellow of ‘Arbequina’ fruit at the same stage of ripeness, are found only in these two varieties.

2.2. Pigment extraction

Samples were taken from a homogenized triturate, prepared from 100 de-stoned fruit (ca. 40 g) of the most representative size by accurately weighing from 4 to 15 g for each analysis depending on the degree of ripeness of the fruit. Pigments were extracted with *N*,*N*-dimethylformamide (DMF) saturated with $MgCO_3$ according to Mínguez-Mosquera and Garrido-Fernández (1989). The solid residue was collected by vacuum filtration and the extraction repeated until filtrates were colorless. The extracts combined in a funnel were repeatedly treated with hexane (3 mL × 70 mL). Chlorophylls, chl derivatives and xanthophylls were retained in the DMF phase. The hexane phase contained lipids and carotenes. The DMF phase was treated with 10% (w/v) NaCl solution at 0 °C and the chls and xanthophylls transferred to 100 mL of a mixture of diethyl ether/hexane (1:1, v/v). The aqueous layer was washed with diethyl ether and finally discarded, eliminating polyphenols and other water-soluble compounds. The combined organic phases were filtered through anhydrous Na_2SO_4 and evaporated to dryness under vacuum at a temperature below 30 °C. The dry residue was dissolved in 1.5 mL acetone prior to HPLC. Analysis was immediate or followed storage at −20 °C for not more than 18 h. Data are means of triplicate analyses.

2.3. Standard pigments for HPLC

Chlorophyll *a* and *b* were purchased from Sigma. Chlorophyllide was formed by enzymatic de-esterification of chl. The reaction mixture contained 100 mM Tris-HCl (pH 8.5) containing 0.24% (w/v) Triton X-100, chl *a* dissolved in acetone and crude enzymatic extract from *Ailanthus altissima* (Mill.) leaves in a 5:1:5 ratio (Mínguez-Mosquera et al., 1994). C-13 epimer of chl a was prepared by treatment with chloroform (Watanabe et al., 1984). The 13^2-OH-chl *a* and *b* was obtained by selenium dioxide (37 mg, 0.34 mmol) oxidation of chl *a* at reflux-heating for 4 h in pyridine (5 mL) solution under argon (Laitalainen et al., 1990). 15^1-OH-lactone chls a and b were obtained by alkaline oxidation in aqueous medium. For this purpose, solid and chromatographically pure chl (a and b) was dissolved in acetone and mixed with 0.5% NaOH and exposed to atmospheric oxygen at room temperature for 10 min. The resulting oxidation products were transferred to diethyl ether by addition of water saturated with NaCl, and 15^1-OH-lactone chls were isolated by NP-TLC and semi-preparative HPLC according to Mínguez-Mosquera et al. (1996). Pyrochlorophyllide *a* was obtained from the respective chlorophyllide by reflux-heating at 100 °C in collidine (Schwartz et al., 1981). All Mg-free derivatives were obtained from the corresponding chl parent dissolved in diethyl ether by acidification with 2–3 drops of 5 M HCl (Sievers and Hynninen, 1977). All standards were purified by NP- and RP-TLC (Davies, 1976; Mínguez-Mosquera et al., 1991; Mínguez-Mosquera et al., 1993).

2.4. Analysis of chl and chl catabolites by HPLC

The separation and quantification of chl degradation products were carried out by HPLC using a HP 1100 Hewlett-Packard liquid chromatograph fitted with a HP1100 automatic injector HPLC. A stainless steel column (25 cm × 0.46 cm i.d.), packed with 5 μm C_{18} Spherisorb ODS-2 (Teknokroma, Barcelona, Spain) was used. The column was protected by a precolumn (1 cm × 0.4 cm i.d.) packed with the same material. Separation was performed using an elution gradient (flow rate 2 mL min^{-1}) with the mobile phases: water/ion pair reagent/methanol (1:1:8, v/v/v) and 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 has been described in detail by Mínguez-

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