Available online at www.sciencedirect.com
ScienceDirect
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

María Roca, Beatriz Gandul-Rojas, Mª Isabel Minguez-Mosquera *

Chemistry and Biochemistry Pigments Group, Food Biotechnology Department, Instituto de la Grasa, Consejo Superior de Investigaciones Científicas (CSIC), Avda. Padre García Tejero, 4, Sevilla 41012, Spain

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.

© 2006 Elsevier B.V. All rights reserved.

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

* Corresponding author. Tel.: +34 954 691054; fax: +34 954 691262.
E-mail address: minguez@cica.es (M.I. Minguez-Mosquera).

0925-5214/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
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-

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 and return to initial conditions in 7 min. Sequential detection was performed with a photodiode array detector at 410, 430, 450 and 666 nm. Data were collected and processed with a LC HP ChemStation (Rev.A.05.04). Pigments were identified by co-chromatography with authentic samples and from their spectral characteristics. The on-line UV–vis spectra were recorded from 350 to 800 nm with the photodiode–array detector.

2.5. Acetone powder preparation

The method was an adaptation of that used by Terpstra and Lambers (1983). De-stoned and sliced olive fruit (25 g, approximately 21 fruits) were homogenized with 20 volumes of acetone at −20 °C (500 mL). The supernatant was removed by filtration and the residue was treated again with 8 volumes of acetone (200 mL). This operation was repeated until the supernatant was colorless (generally four washes were sufficient). Finally, the precipitate was collected by vacuum filtration and left to dry at ambient temperature (20–25 °C). From each gram of fruit approximately 0.09 g of acetone powder was obtained. Two different acetone powders were obtained for each ripening stage.

2.6. Preparation of enzyme extracts

Extraction of the enzyme was carried out according to Johnson-Flanagan and Thiagarajah (1990). The acetone powder (0.5 g) was extracted with 15 mL of 5 mM sodium phosphate buffer (pH 7), containing 50 mM KCl and 0.24% (w/v) Triton X-100. The contents were stirred for 1 h at 20 °C. The extract was filtered through four layers of cotton gauze and the filtrate was centrifuged at 12,000 × *g* for 10 min. The supernatant was used as a crude enzyme extract.

2.7. Substrate preparation

Chlorophyll *a* was isolated from fresh spinach leaves by pigment extraction with acetone (Holden, 1976) followed by TLC separation on Silicagel GF_{254} (0.7 mm thickness) plates using petroleum ether (65–95 °C)–acetone–diethylamine (10:4:1, v/v/v) as developer. After separation, the chl *a* band was scrapped off, eluted with acetone and again rechromatographed by TLC in the same solvent system for purification (Mínguez-Mosquera and Garrido-Fernández, 1989). Chlorophyll *a* obtained by this procedure was about 98% pure judged by HPLC. The other substrates were obtained as described in Standard pigments for HPLC.

2.8. Measurement of chlorophyllase activity (EC: 3.1.1.14)

The technique followed was, in general, that described by Mínguez-Mosquera et al. (1994). The standard reaction mixture (1.1 mL) contained about 0.1 μmol of chl *a* in acetone, 100 mM Tris buffer (pH 8.5) containing 0.24% (w/v) Triton-X-100 and solubilized enzyme in a 1:5:5 ratio. The results are expressed as units of enzyme activity per kg acetone powder (ap). One unit of enzyme activity, the catal (cat), is defined as the amount of enzyme that catalyzes the formation of one mole of product per second. Data are means of duplicate analyses.

2.9. Measurement of peroxidative assays (EC 1.11.1.7)

The standard reaction mixture (1080 μL) consisted of 450 μL of crude enzyme extract, 10 μM chl *a* dissolved in acetone and 100 mM sodium phosphate containing 50 mM KCl and 0.24% Triton X-100 (w/v). The reaction mixture was buffered at pH 6 and included 5.1 mM 2,4-dichlorophenol (DCP) in methanol and 2.45 mM H_2O_2 (Gandul-Rojas et al., 2004). The concentration of organic solvent in the mixture was <9% in all cases. The enzyme assay was carried out under dim green light at 25 °C. The reaction was stopped by adding 150 μL into 600 μL of acetone and vortexing immediately. The mixture was centrifuged at 13,000 × *g* for 5 min and the supernatant was used for HPLC analysis. In each assay, controls were included. These consisted of the reaction mixture without enzyme and another reaction mixture with the crude enzyme extract boiled. Activity was expressed in terms of moles of 13^2 OH chl per s (catal) and per kg acetone powder. Data are means of duplicate analyses.

2.10. Statistical analysis

Significant differences were measured by Duncan's tests, using the program STATISTICA for Windows (Statsoft Inc., 1984–1999).

3. Results

The chl fraction of the fruit of both varieties comprised essentially chl *a* (75%) and chl *b* (16%). The other 9% included chl catabolites that are a reflection of the metabolic stage of the fruit at each time stage; they included chlorophyllide *a*, pheophorbide *a*, pyrochlorophyllide *a*, 13^2-OH-chl (both series a and *b*) and 15^1 lactone chlorophyll (both series a and *b*). Chlorophyllide *b* was not detected at any stage, although its presence during fruit growth has been reported in other olive varieties (Mínguez-Mosquera and Gallardo-Guerrero, 1996). Table 1 summarizes the main spectroscopic and chromatographic characteristics of the chl derivatives studied in the present work and Fig. 1 shows their structure.

Table 2 shows a comparison of the changes in the chl fraction in fruit of the two olive varieties at the same stages of ripening, as determined visually by the fruit color. The concentration of total chls in all the stages was always higher in 'Arbequina', and decreased with ripening in both varieties. The initial differences in chl content between varieties increased as the green fruit color changed. At the light green stage, the chl concentration for 'Blanqueta' (36.09 mg/kg dry weight) was similar to that of 'Arbequina' fruit at the purple stage (38.16 mg/kg dry weight).

In relation to the dephytylated chl derivatives detected *in vivo* in the two olive varieties, there was accumulation of chlorophyllide and pheophorbide, indicating that in ripening fruit

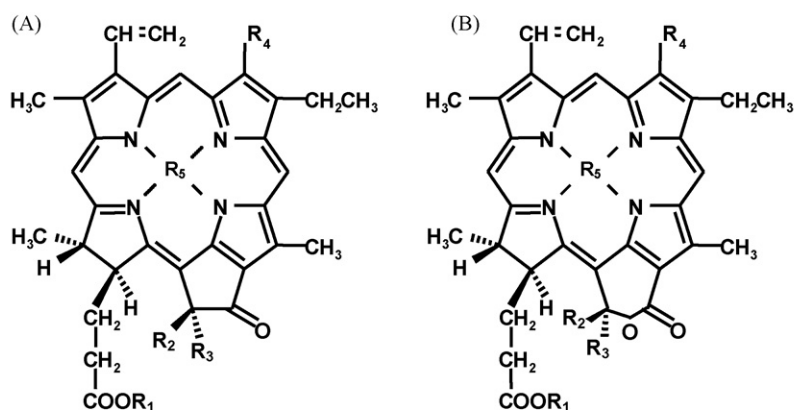
Table 1
Chromatographic and spectroscopic properties of the chlorophyll derivatives

Peak no.	Pigment	K[a]	Position of peak (nm)			Peak height relationship[b]
			I	II	III	
1	Chlorophyllide a	0.87	432	616	664	1.30
1′	Chlorophyllide a′	1.20	432	616	664	1.28
2	Pyrochlorophyllide a	2.06	432	616	664	1.30
3	Pheophorbide a	2.79	410	608	666	2.48
4	15^1–OH-lactone chl b	6.40	454		634	4.42
5	13^2–OH-chlorophyll b	6.87	462	600	650	2.94
6	Chlorophyll b	7.45	466	600	650	2.80
7	15^1–OH-lactone chl a	7.92	420	614	656	1.77
8	13^2–OH-chlorophyll a	8.17	432	616	666	1.20
9	Chlorophyll a	8.90	432	616	666	1.27
9′	Chlorophyll a′	9.40	432	616	666	1.14

[a] Retention factor $(K) = (t_r - t_m)/t_m$, where t_r is the retention time of the pigment peak and t_m is the retention time of an unretained component.
[b] Peak ratio I/III.

chlorophyllase and Mg dechelatase were active. The content of chlorophyllide was similar in both varieties but the maximums were displaced in time. In 'Arbequina' this maximum happened at the end of the ripening, whereas in 'Blanqueta' fruit, the highest levels occurred in the light green state. With respect to pheophorbide, 'Blanqueta' fruit in the first stages of ripening showed a greater modification of the substrate, later pheophorbide decreased and attained values similar to those from 'Arbequina'. In this latter variety the maximum accumulation of pheophorbide was at the yellow stage and its concentration remained more or less constant until the end of ripening. But in addition to these dephytylated compounds, in these two varieties, there was an accumulation of pyrochlorophyllide (Fig. 1). Although its concentration was very low, detection of this compound in 'Blanqueta' fruit during the whole of ripening and in the last stages of ripening of 'Arbequina' fruit, also confirmed the qualitative similarity between both varieties in relation to dephytylated compounds, and also their different appearance with time.

Table 2 also shows the contribution of the oxidized chlorophylls, during chl catabolism *in vivo*, where two different compounds, 13^2 OH chlorophyll and 15^1 OH lactone chlorophyll were identified. The concentration of 13^2 OH chlorophyll was always 10 times greater in 'Arbequina' than in 'Blanqueta' fruit and decreased in both varieties with the progress of the ripening. However, the concentration of 15^1 OH lactone was important solely in 'Blanqueta' fruit, since in 'Arbequina' fruit, that corresponding to the series *a* was absent for all the experi-



Trivial Name	R_1	R_2	R_3	R_4	R_5	Fig.
Chl a	Phytol	H	COOMe	CH_3	Mg	A
Chld a	H	H	COOMe	CH_3	Mg	A
Pheide a	H	H	COOMe	CH_3	2H	A
Pyrochld a	Phytol	H	H	CH_3	Mg	A
13^{2-}OH-chl a	Phytol	OH	COOMe	CH_3	Mg	A
15^{1-}OH-lactone chl a	Phytol	OH	COOMe	CH_3	Mg	B

Fig. 1. Structures of the main chlorophyll derivatives. The replacement of the CH_3 group in R_4 by a CHO group forms chl b derivatives.

Table 2
Chlorophyll metabolites in 'Arbequina' and 'Blanqueta' fruit during ripening (mg/kg, dry weight)

Ripening stages	Arbequina					Blanqueta				
	Gr[a]	L gr	Yell[b]	Mottled	Purple	Gr	L gr	Yell	Mottled	Purple
Chl a	47.45	43.48	40.69	36.29	29.02	30.98	26.97	14.01	11.96	4.50
Chld a	nd[c]	0.22	0.20	0.90	0.27	0.12	0.56	0.37	0.19	0.02
Pyrochld a	nd	nd	0.04	0.02	0.02	0.02	0.05	0.04	0.10	0.02
Pheide a	0.13	0.36	1.18	0.81	1.19	0.90	1.48	0.84	0.78	0.87
13^2 OH chl a	7.94	2.96	2.81	1.70	0.97	0.79	0.28	0.22	0.19	0.06
15^1 lact.chl a[d]	nd	nd	nd	nd	nd	0.75	0.25	0.25	0.23	0.12
Chl b	9.93	9.30	8.67	7.41	6.57	7.41	6.42	3.25	2.82	1.09
13^2 OH chl b	0.41	0.24	0.12	0.16	0.10	0.16	nd	nd	nd	nd
15^1 lact. chl b	nd	nd	nd	0.03	0.02	0.50	0.08	0.09	0.07	0.03
Total chls	65.86	56.56	53.71	47.32	38.16	41.47	36.09	19.07	16.34	6.71

S.D. ≤ 10% in all cases.
[a] Gr, green; L gr, light green; Yell, yellow.
[b] Yellow is the general color for olive fruit ('Arbequina' variety) in this period, except for 'Blanqueta' fruit that is white.
[c] nd, non detected.
[d] 15^1 lact.chl, 15^1 OH lactone chlorophyll.

Table 3
Chlorophyll loss, total dephytylated and oxidized chl and enzyme activities in 'Arbequina' and 'Blanqueta' fruit during ripening

Apparent color	Chll loss (%)				Total dephytylated (mg/kg, dry weight)		Chlorophyllase activity (ncat/kg, acetone powder)		Total oxidized chl (mg/kg, dry weight)		Peroxidase activity (ncat/kg, acetone powder)	
	Consecutive stages		Accumulated		Arbeq	Blanq	Arbeq	Blanq	Arbeq	Blanq	Arbeq	Blanq
	Arbeq[a]	Blanq	Arbeq[a]	Blanq								
Green	nd	nd	nd	nd	0.13	1.62	255.85	238.19	8.35	2.04	6.28	7.52
Light green	14.12	12.97	14.12	12.97	0.58	1.51	232.41	370.86	3.20	0.61	8.52	14.23
Yellow[b]	5.04	47.16	18.45	54.01	1.42	1.25	324.97	349.74	2.93	0.56	11.08	17.89
Mottled	11.90	13.09	28.15	60.60	1.73	1.07	415.62	383.49	1.89	0.49	21.12	10.43
Purple	19.36	58.94	42.06	83.82	1.48	0.91	567.21	408.26	1.09	0.21	8.71	11.69

[a] Arbeq, 'Arbequina' variety; Blanq, 'Blanqueta' variety.
[b] As in Table 2.

mental period and that to series *b* was only detected in last stages of ripening. The presence of 15^1 OH lactone in the fruit of 'Blanqueta' indicated more oxidation than required for the formation of 13^2 OH chlorophyll.

By calculating the percentage loss of total chls between consecutive stages of ripening (Table 3), it can be seen that parallel patterns did not exist between the two varieties: the clear inflection point of chl disappearance in 'Blanqueta' fruit was not concomitant with the beginning of ripening associated with anthocyanin synthesis. In 'Arbequina' the decrease in chls in the first assays was slow and constant (10%), until the mottled stage (20%), while in 'Blanqueta' fruit, during the same period, the losses were sudden and sharp, around 45% in the step to white color. Thus, similar stages of development or ripening determined by apparent color did not necessarily denote homologous physiological states. In fact the accumulated losses of chls at the end of the ripening period amounted to 42% for 'Arbequina' fruit whereas for 'Blanqueta' fruit it was 84%.

With regard to the enzymes responsible of the *in vivo* substrate modification, the measured levels (300–400 ncat/kg acetone powder) of chlorophyllase activity were of the same order between varieties. In general, this enzyme activity followed the same pattern during ripening, reaching significant differences (Duncan's test, $p < 0.05$) at the end of ripening, (the values were generally slightly higher in 'Arbequina' fruit). In the case of 'Blanqueta', chlorophyllase activity was significantly higher only in the fruit with a light green color.

The measurement of peroxidative activity (Fig. 2 and Table 3), responsible for the oxidized chl formation, showed slightly higher values for 'Blanqueta' than for 'Arbequina' fruit, and this activity increased with ripening up to a maximum and

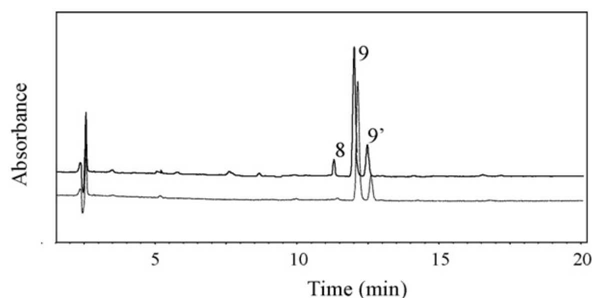


Fig. 2. HPLC separation of chl degradation products obtained by incubation with peroxidase extracts from olives. Discontinuous outline: initial time and continuous outline: after incubation. Peaks: $8 = 13^2$-OH-chlorophyll *a*, 9 and $9' =$ chlorophyll *a* and *a*′.

later decreased. In 'Arbequina' fruit, the values of activity were two-fold in relation to 'Blanqueta' only in the mottle stage. Nevertheless, these values of activity were not correlated with the oxidized chl levels present in the fruit of both varieties, since the *in vivo* modification of the substrate was approximately four times greater in the 'Arbequina' fruit.

4. Discussion

'Arbequina' and 'Blanqueta' are considered to be "low pigmentation" varieties, since in the developed green fruit the total content of chls is around 40–65 mg/kg dry weight, significantly lower than that of other varieties considered "high pigmentation" varieties, such as 'Hojiblanca' or 'Picual', which at a similar stage of development contain from 200 to 350 mg of chls per kg dry weight (Roca and Mínguez-Mosquera, 2001a). In 'Arbequina' fruit, chlorophyllase activity is of the order of 80–100-fold greater than that measured in olive varieties of high pigmentation and low chlorophyllase activity, and in which no evidence has been found of *in vivo* transformation of the substrate by this enzyme (Roca and Mínguez-Mosquera, 2003). Consequently, it was proposed that varietal differences in chl levels were due to the fact that during the period of net synthesis, the catabolic pathway (turnover) would be potentiated over the anabolic one in varieties of low pigmentation, thus assuming that chlorophyllase is active during the turnover of chls during growth. The results obtained in the present study of the chlorophyllase activity in fruit of the Blanqueta variety confirm this hypothesis, which is consistent with the proposal of Eckhardt et al. (2004) in that chl catabolism takes place initially at a basal level during turnover and increases during ripening.

Nevertheless, in spite of this similarity, the different patterns of change in colored chlorophyllic intermediates demonstrate metabolic differences during chl catabolism. The early and fast chl degradation to colorless products (almost 50%) that occurs in 'Blanqueta' fruit in the first stages of ripening before the synthesis of anthocyanins, distinguish this variety, not only from the common olive varieties, but also from 'Arbequina'.

The displacement in the time of the breakdown of the chl macro cycle between fruit of both varieties, agrees in each variety with the maximum levels of chlorophyllide *in vivo* and the chlorophyllase activity measured. It is possible then to suggest that not only a temporary difference in chlorophyllase activation but also in the rest of enzymes in the PaO is responsible for these differences. The parallel between chlorophyllase activity, accumulation of dephytylated compounds, and loss of chls could be extended to the whole life cycle in the fruit of both varieties. The fact that in the variety 'Blanqueta', high levels of chlorophyllase activity do not correlate *in vivo* with substantial accumulation of dephytylated components is due to chlorophyllase being a latent enzyme (Matile et al., 1999). The *in vitro* measurement of its activity should correlate with the *in vivo* modification of the substrate, showing that the enzyme is active. In the case of 'Blanqueta' fruit, at that time of ripening there is formation of dephytylated compounds, although not as much, possibly for lack of substrate. Although chlorophyllase was cloned in 1999 (Jacob-Wilk et al., 1999; Tsuchiya et al., 1999), and its expression is constitutive, the mechanisms that regulate its activity are not yet known; only ethylene activation is clear (Jacob-Wilk et al., 1999). The physical separation of the substrate and its intrinsic nature as a membrane protein make it difficult to fully explain the activation of the enzyme in physiological conditions.

In relation to oxidized chl catabolites, Hynninen (1991) has demonstrated that the mechanism of peroxidase action is via free radicals, the first intermediate being 13^2- OH-chl and later 15^1 lactone chl. Janave (1997), studying chl degradation in bananas, in addition to the PaO pathway, proposed that another oxidative catabolic pathway that finally would initiate decoloration exists in parallel. In this pathway, 13^2 OH chl and 15^1 OH lactone chl would form sequentially. It has already been verified in olives that the *in vitro* measurement of peroxidase from olive thylakoids (variety 'Hojiblanca') yields the corresponding derivative hydroxylated on $C13^2$ as the intermediate product (Gandul-Rojas et al., 2004). Nevertheless, the measurement of peroxidase activity in the fruit of 'Arbequina' and 'Blanqueta' is not correlated with the accumulated chl allomerized levels *in vivo*. The slightly higher peroxidase activity measured in 'Blanqueta' fruit is correlated with the exclusive *in vivo* accumulation of 15^1 lactone chl a and b, suggesting an increase in oxidation level of the chl molecule, present always in 'Blanqueta' fruit and only in small amounts in the final stages of ripening in 'Arbequina' fruit. The interpretation of these results is more difficult when bleaching activity by peroxidase enzyme is considered. Bleaching activity during the incubation of peroxidase is only positive in 'Blanqueta' fruit in the light green stage, agreeing in time with the greater formation of colorless products.

Altogether, the results show that peroxidase can be responsible to a certain degree for the oxidation that happens *in vivo* in the chl molecule, but is not responsible for the differential accumulation of 13^2 OH chl between varieties. 'Blanqueta' fruit have greater contents of linoleic acid 'Arbequina' fruit (Uceda and Hermoso, 1996), and since this cofactor is necessary for chlorophyll-oxidase activity, it will contribute to the major oxidation levels in 'Blanqueta' fruit, and consequently to the lower stability of 'Blanqueta' chlorophylls and oils (Cert et al., 1996; García et al., 1996). If we can assume that there is a bleaching action for peroxidase, it would explain the smaller presence of oxidized compounds in this variety and therefore the lack of correlation between accumulation of 13^2 OH chl and the measured enzyme activity.A similar correlation can be made between varieties of high pigmentation ('Picual' and 'Hojiblanca') and those of low pigmentation ('Arbequina' and 'Blanqueta'). Although the oxidative stability of oils varies with the ripening stages of the fruit from which they are extracted, a high oxidative stability is typical of varieties of high pigmentation (Gutierrez et al., 1999; Roca and Mínguez-Mosquera, 2003). The oxidative stability is correlated with high levels of polyphenols, ortodiphenols α-tocopherol and relation oleic/linoleic acid (Aparicio et al., 1999), as well as a greater pigment level. In fact, chlorophyll pigments are also well known for an antioxidant role in the stability of virgin olive oils (Gutiérrez et al., 1992; Psomiadou and Tsimidou, 2002). For that reason, the stability and the properties that affect stability are higher in varieties of high pigmentation ('Hojiblanca' and 'Picual') than in the varieties of low pig-

mentation ('Arbequina' and 'Blanqueta') (García et al., 1996; Gutierrez et al., 1999; Uceda and Hermoso, 1996).

Acknowledgments

We are sincerely grateful to the Comision Interministerial de Ciencia y Tecnología of the Spanish Government (CICYT) for supporting this research project, AGL 2003-0383, and to the Junta de Andalucía for supplementary financing. We thank AGRA, S.A. for supplying the samples. M.R. was supported by a Ministerio de Ciencia y Tecnología contract (Juan de la Cierva).

References

Aparicio, R., Roda, L., Albi, M.A., Gutiérrez, F., 1999. Effect of various compounds on virgin olive oil stability measured by rancimat. J. Agric. Food Chem. 47, 4150–4155.

Cert, A., Alba, J., León-Camacho, M., Moreda, W., Pérez-Camino, M.C., 1996. Effects of talc addition and operating mode on the quality and oxidative stability of virgin olive oils obtained by centrifugation. J. Agric. Food Chem. 44, 3930–3934.

Criado, M.N., Motilva, M.J., Goñi, M., Romero, M.P., 2007. Comparative study of the effect of the maturation process of the olive drupe on the chlorophyll and carotenoid fractions of drupes and virgin oils from Arbequina and Farga cultivars. Food Chem. 100, 748–755.

Eckhardt, U., Grimm, B., Hörtensteiner, S., 2004. Recent advances in chlorophyll biosynthesis and breakdown in higher plants. Plant Mol. Biol. 56, 1–14.

Davies, B., 1976. Carotenoids. In: Goodwin, T.W. (Ed.), Chemistry and Biochemistry of Plant Pigments, II. Academic Press, London, pp. 38–166.

Gandul-Rojas, B., Roca, M., Mínguez-Mosquera, M.I., 2004. Chlorophyll and carotenoid degradation mediated by thylakoid-associated peroxidative activity in olives (*Olea europaea*) cv. Hojiblanca. J. Plant Physiol. 49, 499–507.

García, J.M., Seller, S., Pérez-Camino, M.C., 1996. Influence of fruit ripening on olive oil quality. J. Agric. Food Chem. 44, 3516–3520.

Gutiérrez, F., Garrido, J., Gallardo, L., Gandul, B., Mínguez, M.I., 1992. Action of chlorophylls on the stability of virgin olive oil. J. Am. Oil Chem. Soc. 69, 866–871.

Gutierrez, F., Jímenez, B., Ruíz, A., Albi, M.A., 1999. Effect of olive ripeness on the oxidative stability of virgin olive oil from the varieties Picual and Hojiblanca and on the different components involved. J. Agric. Food Chem. 47, 121–127.

Holden, M., 1976. Analytical methods—chlorophylls. In: Goodwin, T.W. (Ed.), Chemistry and Biochemistry of Plant Pigments, II. Academic Press, London, pp. 1–37.

Hynninen, P.H., 1991. Chemistry of chlorophylls: modifications. In: Scheer, H. (Ed.), Chlorophylls. CRC Press, Boca Raton, FL, pp. 145–209.

Jacob-Wilk, D., Holland, D., Goldschmidt, E.E., Riov, J., Eyal, Y., 1999. Chlorophyll breakdown by chlorophyllase: isolation and functional expression of the Chlase1 gene from ethylene-treated Citrus fruit and its regulation during development. Plant J. 20, 653–661.

Janave, M.T., 1997. Enzymic degradation of chlorophyll in cavendish bananas: *In vitro* evidence for two independent degradative pathways. Plant Physiol. Biochem. 35, 837–846.

Johnson-Flanagan, A., Thiagarajah, M., 1990. Degreening in canola (*brassica napus*, cv westar) embryos under optimum conditions. J. Plant Physiol. 136, 180–186.

Kaartinen, V., Kolehmainen, E., Hynninen, P., 1985. Peroxidase catalized oxidation of chlorophyll *a*: ^{1}H NMR spectroscopic characterization of the principal products as (R)-and 10 (S)-hydroxychlorophyll a. In: Abstracts of the 9th National NMR Symposium, University of Joensuu, Finland, pp. 10–12.

Laitalainen, T., Pitkänen, J., Hynninen, P., 1990. Diastereoselective 13^2-hydroxylation of chlorophyll a with SeO_2. In: IUPAC (Eds.), Abstracts of the 8th International IUPAC Conference on Organic Synthesis, Helsinki, p. 246.

Matile, P., Hörtensteiner, S., Thomas, H., 1999. Chlorophyll degradation. Ann. Rev. Plant Physiol. Plant Mol. Biol. 50, 67–95.

Maunders, M., Brown, S., Woolhouse, H., 1983. The appearance of chlorophyll derivatives in senescing tissue. Phytochemistry 22, 2443–2446.

Mínguez-Mosquera, M.I., Garrido-Fernández, J., 1989. Chlorophyll and carotenoid presence in olive fruit (*Olea europaea* L.). J. Agric. Food Chem. 37, 1–7.

Mínguez-Mosquera, M.I., Gandul-Rojas, B., Montaño-Asquerino, A., Garrido-Fernández, J., 1991. Determination of chlorophylls and carotenoides by HPLC during olive lactic fermentation. J. Chromatogr. 585, 259–266.

Mínguez-Mosquera, M.I., Gallardo-Guerrero, L., Gandul-Rojas, B., 1993. Characterization and separation of oxidized derivatives of pheophorbide a and b by thin-layer and high-performance liquid chromatography. J. Chromatogr. 633, 295–299.

Mínguez-Mosquera, M.I., Gandul-Rojas, B., Gallardo-Guerrero, L., 1994. Measurement of chlorophyllase activity in olive fruit (*Olea europaea*). J. Biochem. 116, 263–268.

Mínguez-Mosquera, M.I., Gallardo-Guerrero, L., 1995. Disappearance of chlorophylls and carotenoids during the ripening of the olive. J. Sci. Food Agric. 69, 1–6.

Mínguez-Mosquera, M.I., Gallardo-Guerrero, L., 1996. Role of chlorophyllase in chlorophyll metabolism in olives cv. Gordal. Phytochem. 41, 691–697.

Mínguez-Mosquera, M.I., Gandul-Rojas, B., Garrido-Fernández, J., 1996. Preparation of Cu(II) complexes of oxidized chlorophylls and their determination by thin-layer and high-performance liquid chromatography. Chromatography A 731, 261–271.

Psomiadou, E., Tsimidou, M., 2002. Stability of virgin olive oil. 1. Autoxidation studies. J. Agric. Food Chem. 50, 716–721.

Roca, M., Mínguez-Mosquera, M.I., 2001a. Change in chloroplast pigments of olive varieties during fruit ripening. J. Agric. Food Chem. 49, 832–839.

Roca, M., Mínguez-Mosquera, M.I., 2001b. Unusual carotenogenesis in fruits with pronounced anthocyanic ripening (*Olea europaea* var. Arbequina). J. Agric. Food Chem. 49, 4414–4419.

Roca, M., Mínguez-Mosquera, M.I., 2003. Involvement of chlorophyllase in chlorophyll metabolism in olive varieties with high and low chlorophyll content. Physiol. Plant. 117, 459–466.

Schwartz, S., Woo, S., von Elbe, J., 1981. High-performance liquid chromatography of chlorophylls and their derivatives in fresh and processed spinach. J. Agric. Food Chem. 29, 533–535.

Sievers, G., Hynninen, P., 1977. Thin-layer chromatography of chlorophylls and their derivatives on cellulose layers. J. Chromatogr. 134, 359–364.

Terpstra, W., Lambers, J., 1983. Interactions between chlorophyllase, chlorophyll *a*, plants lipids and Mg^{2+}. Bioch. Biophys. Acta 746, 23–31.

Tsuchiya, T., Ohta, H., Okawa, K., Iwamatsu, A., Shimada, H., Masuda, T., Takamiya, K., 1999. Cloning of chlorophyllase, the key enzyme in chlorophyll degradation: Finding of a lipase motif and the induction by methyl jasmonate. Proc. Nat. Acad. Sci. 96, 15362–15367.

Uceda, M., Hermoso, M., 1996. In: Barranco, D., Fernández-Escobar, R., Rallo, L. (Eds.), La calidad del aceite de oliva. El cultivo del olivo, Mundi-Prensa, Madrid, pp. 541–564.

Walalí, D., Chimitah, M., Loussert, R., Mahhou, B., Boulouha, B., 1984. Caracteres morfológicos y fisiológicos de clones de olivo de la variedad Picholine marroqui. Olivae 3, 26–31.

Watanabe, T., Hongu, A., Honda, K., Nakazato, M., Konno, M., Saithoh, S., 1984. Preparation of chlorophylls and pheophytins by isocratic liquid chromatography. Ann. Chem. 56, 251–256.

Yamauchi, N., Akiyama, Y., Kako, S., Hashinaga, F., 1997. Chlorophyll degradation in Wase satsuma mandarin (*Citrus unshiu* Marc.) fruit with on-tree maturation and ethylene treatment. Sci. Hortic. 71, 35–42.