Thylakoid peroxidase activity responsible for oxidised chlorophyll accumulation during ripening of olive fruits (*Olea europaea* L).

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Abstract

Type III peroxidase (EC 1.11.1.7) (POX) is the enzyme direct responsible of the 13^2 OH chlorophyll formation on oxidative catabolism of chlorophylls (chls). Despite the higher content of oxidised derivatives of chlorophylls (ox-chls) in fruits of the Arbequina variety compared to Hojiblanca, the evolution of total chlorophyll oxidative peroxidase activity (POX-chl) showed that this activity levels were higher in fruits of Hojiblanca compared with Arbequina variety. Subsequently, a deepened study on the subcellular distribution of POX-chl activity from mesocarp and epicarp cells of olive fruit of both varieties was made, founding that the POX-chl activity located in thylakoid fraction (the only fraction in direct contact with chls *in vivo*), was in Arbequina fruits higher than in Hojiblanca ones and involved more than 50% of the membranous POX-Chl activity. It has been demonstrated also, that the evolution of the POX-chl activity in thylakoid membranes enriched fraction throughout the whole life cycle, was parallel with the formation and accumulation of ox-chls in olive fruits. Data allowed to conclude that the formation of ox-chls during the chl catabolism is mediated by a POX-chl activity localized in thylakoid fraction and allow to hypothesize that the high percentage of POX activity found in the soluble cell fraction, estimated at 99.8%, may be involved in the loss of pigmentation by oxidation occurring during fruit processing for obtaining olive oil**.**

Keywords: *Olea europaea* L.; Olive peroxidase; Chlorophyll oxidative peroxidase; Subcellular distribution; Thylakoid fraction; Chlorophyll catabolism; Fruit ripening.

1. INTRODUCTION

Chlorophyll pigments are highly appreciated as functional components in fruits and vegetables both for its green colouring properties as its health benefits for the human consumption derived from their biological properties (Ferruzzi & Blakeslee, 2007). In addition, the ripening process of fruits or technological treatment for food production is associated with chemical and/or enzimatic specific transformations of these pigments making them quality indicators of end products and demonstrating a potential applicability as a tool for traceability the processing (Gandul-Rojas, Roca & Mínguez-Mosquera, 2000).

The fruit of the olive tree (*Olea europaea* L.) is mainly used to obtain olive oil, a natural food that is obtained from the olive fruit solely by mechanical or physical processes. The fundamental steps of the process are crushing, malaxation and phase separation by solid/liquid centrifugation systems, being malaxation the step in oil extraction that especially modifies their qualitative and quantitative composition. After the crushing step, with the broken plant tissue, and released chloroplasts and thylakoid membranes, the lipophylic chlorophyll pigments, will transfer to the oil phase during malaxation, giving different colours to olive oil. In this process the chlorophylls undergo a series of structural changes mainly due to breakage of cellular structures that allow the release of acid as well as greater accessibility of their natural substrates for endogenous enzymes. Different reactions can occur as pheophytinization, phytol hydrolysis via chlorophyllase and chemical and enzymatic oxidative reactions that lead to the formation of coloured oxidized intermediates and non coloured final products. Chlorophyllic pigment content of olive fruits is cultivar dependent and negatively correlated to fruit ripening (Mínguez-Mosquera & Garrido-Fernández, 1989; Roca & Mínguez-Mosquera, 2003) and the ocurrence of lipophylic chlorophyll compounds in virgin olive oil can be related to the activities of several endogenous enzymes present in the olive fruit such as POX promoting chlorophyll oxidation (Gandul-Rojas, Roca & Mínguez-Mosquera, 2004). So that the study of catabolic processes involved in degradation of these pigments is particularly relevant this field of food technology to know biochemical fundamentals that define the profile of these intrinsic minor components of virgin olive oil, potentially applicable as a tool for olive oil traceability. There currently exists a general consensus regarding the succession of enzymatic reactions implicated in the catabolism of the chlorophyll *a* (Chl) molecule which takes place during leaf senescence. First step is the

removal of Mg by Mg-dechelating substance (MCS) (Shioi, Tomita, Tsuchiya & Takamiya, 1996), producing pheophytin *a* followed by the removal of phytol and the formation of pheophorbide *a* in a reaction catalized by pheophytin pheophorbide hydrolase (PPH) (Schelbert, Aubry, Burla, Agne, Kessler, Krupinska & Hörtensteiner, 2009). Then pheophorbide is degraded to fluorescent chl catabolites (FCCs), which are primarily colorless catabolites, via the enzymatic system feoforbida *a* oxigenasa (PaO) and red Chl catabolite reductase (RCCR). Finally, outside the chloroplast, the FCCs are exported to the cytosol where they are modified with different functional groups until no coloured chl catabolites (NCCs), considered as the final catabolites of chls (Hörtensteiner & Kräutler, 2011).

There is much less research regarding the degradation systems which are involved in the chlorophyllic catabolism of the fruit. It could not be demonstrated the functionality of PPH, checking (Shemer, Harpaz-Saad, Belausov, Lovat, Krokhin, Spicer, Standing, Goldschimidt & Eyal, 2008) that the pathway starts with sequential action of chlorophyllase (CHLASE) that catalyzes the phytol hydrolysis in chl *a* resulting chlorophyllide *a* (Amir-Shapira, Goldschmidt, & Altman, 1987) and MCS that becomes to pheophorbide *a* (Suzuki & Shioi, 2002). Besides these main steps of the PaO pathway, additional/alternative reactions of Chl breakdown have been described. These were (mostly) inferred from the identification of different types of Chl degradation products, such as pyropheophorbide *a* (Suzuki, Amano & Shioi, 2006), Chl-derived monopyrroles (Suzuki and Shioi, 1999), urobilinogenoidic catabolites (Losey & Engel, 2001), and various oxidized chl derivatives with an intact porphyrin ring, that they could not be located within PaO route.

There is evidence (Maunders, Brown & Woolhouse, 1983; Yamauchi, Akiyama, Kako, & Hashinaga, 1997; Takahashi, Adachi, Furuta, Yamamoto, Kurata, Azuma, Miyoshi & Shimokawa, 2001; Roca, Gandul-Rojas & Mínguez-Mosquera, 2007) which suggests the hypothesis of the coexistence of a specifically oxidative metabolism in parallel to the general catabolic route (Janave, 1997), peroxidase (POX) being the enzyme which has been most clearly linked with this metabolism (Gandul-Rojas, Roca & Mínguez-Mosquera, 2004; Huff, 1982; Matile, 1980; Roca et al., 2007).

Aljuburi, Huff & Hshieh (1979) were the first to describe an enzyme which exhibits a oxidising and decolourizing action on the chlorophyll in the flavedo of oranges in the presence of H_2O_2 and a phenol. However, Matile (1980) was the first to suggest that POX

100 is responsible for that activity by observing an *in vitro* modification to chl in the presence
101 of H_2O_2, 2,4-dichlorophenol (2,4-DCP) and horseradish peroxidase (HRP). These results
102 were confirmed by Huff (1982), ascertaining that the chl *a* molecule was degraded by
103 POX extracted from oranges (*Citrus sinensis* L.), in presence of various phenols, even
104 determining the activity with various chl substrates and estimating the corresponding
105 kinetic parameters (K_m and V_{max}).

106 Until recently it was assumed that peroxidase mediates *in vitro* Chl degradation
107 only in the presence of phenolic compounds, being the highest enzyme activities in plant
108 systems found with *p*-coumaric acid, apigenin and naringenin, which have a hydroxyl
109 group at the *p*-position (Kato & Shimizu, 1985; Yamauchi & Eguchi, 2002; Yamauchi &
110 Minamide, 1985). Hynninen, Kaartinen, & Kolehmainen (2010) have defended the
111 opposing hypothesis demonstrating that, *in vitro*, HRP can oxidise chl *a* in the absence of
112 phenols, and for this reason propose a direct reaction between the chlorophyll molecule
113 and POX, in which chl *a* would act as a reducer substrate, rendering the presence of a
114 second electron donor substrate totally unnecessary. Recently, Vergara-Domínguez,
115 Roca & Gandul-Rojas, (2013) demonstrated that POX is capable of directly oxidising chl
116 *a* in the absence of another electron donor, however the presence of a cosubstrate donor
117 like 2,4-DCP accelerates the oxidation rate of the chlorophyllic substrate catalyzed by
118 POX.

119 Due to the multitude of isoforms of POX described in the bibliography (Akiyama &
120 Yamauchi, 2001; Funamoto, Yamauchi, & Shigyo, 2003; Gandul-Rojas et al. 2004;
121 Saraiva, Nunes & Coimbra, 2006), the study of the intracellular distribution of the POX-chl
122 activity was approached from different perspectives. Some papers demonstrate the
123 existence of this activity in thylakoid membranes (Martinoia, Dalling & Matile, 1982,
124 Johnson-Flanagan & Spencer, 1996; Gandul-Rojas et al., 2004); however, data exist to
125 demonstrate the presence of this activity in other subcellular fractions (Akiyama &
126 Yamauchi, 2001; Funamoto et al., 2003).

127 Akiyama & Yamauchi (2001), studying the distribution of different enzymes
128 involved in the degradation of chlorophylls and applying differential centrifugal techniques
129 demonstrated that, of all the POX-chl activity present in the cotyledon cells of the radish
130 (*Raphanus sativus* L.), only a percentage of less than 1 % were located in the
131 chloroplasts, whereas the rest was found in other cellular organelles. This fact was
132 understood as evidence that the majority of the POX-chl present in the cell is found on

the exterior of the chloroplast and that only a minority fraction is found in the interior of this organelle, which would be the part potentially involved in the oxidative degradation of chlorophyll that takes part *in vivo*. Similar results were obtained by Funamoto et al. (2003) on measuring POX-chl activity in different subcellular fractions of broccoli florets (*Brassica oleracea* L.). It was established that the microsomal and cytosolic fraction made up approximately 95 % of the entire POX-chl activity present in the cell, whereas the activity measured in the chloroplast only represented 1 %.

To date, there is no information regarding the distribution of POX-chl activity in fruit. Nevertheless, the bibliography contains studies of the distribution of the POX activity relating to the oxidation of phenols (POX-phe), although the data obtained are contradictory. On the one hand, Civello, Martínez, Chaves & Añón, (1995), studying the distribution of the POX activity against guaiacol in *Fragaria ananassa (Duch.),* found that the specific POX-phe activity of the fraction linked to membranes was far higher than that measured in the soluble fraction and was equivalent to approximately 95% of the total POX-phe activity. On the other hand, Estrada, Bernal, Díaz, Pomar & Merino (2000), studying the distribution of this activity in *Capsicum annuum,* (L.) from an organelle homogenate, found that the POX activity measured over 4-methoxy-R-naphthol (4MN) in the soluble fraction was always higher than that obtained in the rest of the subcellular fractions (ionic bonding to membranes, ionic bonding to cellular wall and covalent bonding to cellular wall) and that it increased from 77% to 96% with ripening.

An interesting model for the study of the physiological involvement of POX in chl metabolism is provided by certain varieties of olive given that differences in the oxidised chl profile for this fruit based on the variety have already been detected (Roca et al., 2007). In general, in olive fruit varieties the qualitative composition of pigments is basically the same, and is not subject to modification with the advancement of maturity in the fruit (Roca & Mínguez-Mosquera, 2001). In contrast, in the variety Arbequina an accumulation of 13^2 OH chl *a* has been detected during the transition from the periods of growth through to maturity, reaching up to 15% of the chl fraction (Roca & Mínguez-Mosquera, 2003), while in other varieties such as Hojiblanca and Picual the presence of this compound represents 1% of the total chl fraction during the complete fruit life cycle.

The study of the involvement of POX-chl activity in the metabolism of chls in the Hojiblanca variety has revealed that there is a correlation between the formation and/or build-up of certain oxidized chl derivatives and the total POX-chl activity levels measured

from protein precipitated (Vergara-Domínguez, Gandul-Rojas & Roca, 2011). However, in varieties with a high level of chlorophyllase (CHLASE) activity, such as Arbequina, it was not possible to establish this correlation, and an *in vitro* competence between POX and CHLASE was revealed towards the chl *a* substrate. Subsequently, (Vergara-Domínguez et al., 2013), in an optimisation of the *in vitro* measurement of the POX-chl activity established new measurement conditions that permitted the displacement of the competence between CHLASE and POX towards the chl *a* substrate, in favour of the latter.

These new measurement conditions permitted the study of the involvement of POX-chl activity in the oxidation metabolism for chls in fruits with high CHLASE activity to be studied, in order to establish distinctions between varieties with different oxidative chl profiles (Arbequina vs. Hojiblanca). Consequently, in this paper a study has been made of the subcellular distribution of the POX-chl activity in olives from both varieties and this compared with the total measured for this activity, based on a protein precipitated. Subsequently, the POX-chl activity specifically obtained in the enriched fraction in the thylakoid membranes was monitored, to look for a correlation between the enzymatic activity and the build-up of oxidized chl derivatives.

2. MATERIAL AND METHODS

2.1 Plant material

The work was carried out with two different varieties of fruit from the olive tree (Olea europaea L.), Hojiblanca and Arbequina, from two plantation of young olive trees in "Cortijo Carcahueso" (Aznalcóllar, Sevilla, Spain) and Hacienda Guzmán (Aceites del Sur-Coosur S.A.). Six olive trees per variety were selected during the 2010–2011 and 2011-2012 seasons, and they were sampled every two weeks from mid-July (immature fruit) to mid-December (mature fruit). The sampling was performed at the same time each day, by randomly collecting 2 kg of fruit from branches within arm's reach and around the circumference of the tree, both the outer and the interior.

2.2 Chemicals and standards.

Tetrabutylammonium acetate and ammonium acetate were supplied by Fluka (Zwijndrecht, TheNetherlands). HPLC reagent grade solvents were BDH-Prolabor (VWR International Eurolab, Barcelona, Spain). For the preparation, isolation, and purification of chl pigments, analytical grade solvents and reagents were used (Scharlau, Microdur,

198 Sevilla, Spain). The deionized water used was obtained from a Milli-Q 50 system
199 (Millipore Corp., Bedford, MA).

200 Chl *a* and *b* were isolated from fresh spinach leaves by means of acetone
201 extraction and transfer to diethyl ether, and subsequently were separated and isolated by
202 TLC on silicagel GF254 using the developing mixture light petroleum ether 65–
203 95 ºC/acetone/diethylamine (10:4:1 v/v/v). Bands for chl *a* and *b* were scraped off at Rf
204 0.51 and 0.54 respectively, and eluted in acetone (Mínguez-Mosquera & Garrido-
205 Fernández, 1989). Pheophytin (Phy) *a* or *b* was prepared from pure chl *a* or *b* solution in
206 diethyl ether, by acidification with a few drops of HCl 10% (v/v) (Sievers & Hynninen,
207 1977). The resulting phy *a* or *b* was transferred to diethyl ether, washed with distilled
208 water, dried over anhydrous Na_2SO_4, evaporated and dissolved in acetone. The 13^2 OH
209 chl *a* and *b* were obtained by selenium dioxide oxidation of related chl (*a* or *b*) at reflux-
210 heating for 4 h in pyridine solution under argon (Laitalainen, Pitkänen & Hynninen, 1990).
211 15^1 OH lactone chl *a* and *b* were obtained by alkaline oxidation in aqueous medium. For
212 this purpose, solid and chromatographically pure chl (*a* or *b*) was dissolved in acetone
213 and mixed with 0.5% NaOH and exposed to atmospheric oxygen at room temperature for
214 10 min. The resulting oxidation products were transferred to diethyl ether by addition of
215 water saturated with NaCl, and 15^1 OH lactone chl (*a* or *b*) was isolated by NP-TLC and
216 semi-preparative HPLC (Mínguez-Mosquera, Gandul-Rojas & Garrido-Fernández, 1996).

217 **2.3 Pigment extraction**

218 Samples were taken from a homogenised triturate, prepared from 100 de-stoned
219 fruits (ca. 40 g) of the most representative size by accurately weighing from 4 to 15 g for
220 each analysis depending on the degree of ripeness of the fruits. Pigments were extracted
221 with N,N-dimethylformamide (DMF) saturated with $MgCO_3$ (Mínguez-Mosquera &
222 Garrido-Fernández, 1989). The solid residue was collected by vacuum filtration and the
223 extraction repeated until filtrates were colourless. The extracts combined in a funnel were
224 repeatedly treated with hexane. Chls, chl derivatives and xanthophylls were retained in
225 DMF phase. The hexane phase contained lipids and carotenes was discarded. The DMF
226 phase was treated with 10% (w/v) NaCl solution at 0 ºC and the chls and xanthophylls
227 transferred to 100 ml of a mixture of diethyl ether/hexane (1:1 v/v). The aqueous layer
228 was washed with diethyl ether and finally discarded, eliminating polyphenols and other
229 water-soluble compounds. The combined organic phases were filtrated through
230 anhydrous Na_2SO_4 and evaporated to dryness under vacuum at a temperature below 30

ºC. The dry residue was dissolved in 1.5 ml acetone prior to HPLC. Analysis was immediate or followed storage at -20 ºC not more 18 h. All analyses were performed under green light.

2.4 Obtaining the crude enzyme extract

Acetone powders were obtained (Terpstra & Lambers, 1983) from 25 g pitted and chopped fruit mixed with 20 volumes of acetone at -20 ºC, which was ground with a Polytron homogeniser on an ice bath. The solid residue was recovered by vacuum filtration and treated similarly with 8 volumes of acetone (200 mL). This process was repeated until colourless extracts were obtained (4 washes were usually sufficient). Finally the precipitate was collected, dried at room temperature, weighed and stored at -20 ºC until used (Mínguez-Mosquera, Gandul-Rojas & Gallardo-Guerrero, 1994). The crude enzyme extract was obtained by modifying the method developed by Johnson-Flanagan & Thiagarajah (1990). The acetone powder (0.2 g) was mixed with 6 mL of extraction buffer (5 mM Na-Pi pH7, 50 mM KCl and 0.24%(w/v) Triton X-100), and magnetically stirred at room temperature for 1 h (Roca et al., 2007). The resulting mixture was filtered through four layers of cotton gauze and centrifuged at 4500 x g for 5 min at 4 ºC. The supernatant was the crude enzyme extract.

2.5 Obtaining subcellular fractions

The different subcellular fractions were obtained by cold (4ºC) differential centrifugation, similar to the specific methodology used for olives established by Salas, William, Harwood & Sánchez, (1999) (Figure 1).

Between 8 and 10 g of pitted olives were immediately immersed in a 50 mM K-Pi pH 7.0 buffer solution with 0.1% of ascorbate to prevent oxidation of the tissues. After removing the excess buffer solution from the tissue, the sample was crushed four times in the presence of 15 ml of a 50 mM Tricine-KOH pH 7.6 buffer solution, with 330 mM sorbitol, 20 mM KCl, 2 mM $MgCl_2$, 5mM EDTA, 7 mM 2-mercaptoethanol, 3 mM dithioerythritol, 2 mM metabisulphite, 0.1 % ascorbate and 10 % glycerol, to which 5 g of polyvinyl polypyrrholidone washed with acid was added. Next it was filtered through 2 layers of Miracloth and centrifuged at 100 x g for 3 min. The resultant pellet, which contained traces of cellular tissue and nuclei, was rejected and the supernatant subjected to repeated centrifugations at 4200 x g for 10 min, 15,000 x g for 10 min (15 K) (enriched fraction in low density membranes), 40,000 x g for 20 min (40 K) (enriched fraction in high density membranes) and 150,000 x g for 80 min (150 K) (microsomal fraction). The

pellets resulting from the 15 K, 40 K and 150 K centrifugations were resuspended in 300 µL of incubation buffer solution, 100 mM Na-Pi pH 7, and quickly frozen in N_2 and stored at -80 ºC. The supernatant obtained after the 150 K centrifugation was considered as the soluble fraction.

The pellet from the centrifugation at 4200 x g was resuspended with 1.5 ml of resuspension buffer solution (50 mM Tricine-KOH pH 7.6, with 330 mM sorbitol, containing 10 % glycerol) and centrifuged again at 6800 x g for 4 min. The supernatant was rejected and the pellet was considered as the chloroplast and thylakoid-enriched fraction (7K). From this fraction, following the methodology described by Hörtensteiner, Vicentini, & Matile, (1995), the thylakoid membranes-enriched fraction (7K*) was obtained by resuspending the 7K fraction in a 100 mM Na-Pi pH 7 buffer solution and then centrifuging at 20800 x g for 5 min. The supernatant was rejected whereas the pellet formed the thylakoid membrane enriched fraction (7K*) which was frozen in liquid N_2 and stored at -80ºC.

The entire procedure took place in a chamber at 4 ºC.

2.6 Determination of POX-chl enzymatic activity

To measure the POX-chl activity, a standard reaction mixture (0.36 mL) was used containing 80 µL crude enzyme extract, 239 µL incubation buffer (100 mM Na-Pi pH 7), 15 µL chl *a* (4 mM) dissolved in acetone and 15 µL 2,4-dichlorophenol (DCP) (2%, w/v) dissolved in acetone. The reaction was started by adding 11 µL H_2O_2 (0.2%, w/v).

To determine the subcellular distribution of the POX-Chl activity, the pellets from the corresponding membrane protein fractions (fraction 7K*, 15K, 40K and 150K) obtained by differential centrifugation, were resuspended in 239 µl of incubation buffer solution whereas one aliquot of the soluble protein solution (80 µl) was mixed with 159 µl of the same buffer solution. In both cases, the reaction mixture was obtained by adding to each corresponding protein fraction of 239 µl, 80 µl of extraction buffer solution *(5* mM Na-Pi pH7, 50 mM KCl and 0.24% (w/v) Triton X-100) and the different substrates (chl a, DCP and H_2O_2) in the aforementioned volumes (Table 1).

The reaction was carried out with strong stirring, at 30 ºC and in conditions of darkness, taking aliquots at the initial time and after 50 min of reaction (for enzyme solubilized from acetone powder) or 6 hours (for subcellular fractions). The reaction was concluded with the addition of 300 µL of acetone to an aliquot portion of 75 µL of the

296 reaction mixture. After vigorous vortex stirring, it was immediately frozen at -30 ºC until
297 carrying out of a quantitative analysis of the reaction products by HPLC.

298 **Table 1**

299 Composition of reaction mix prepared to quantify the POX-chl activity in different
300 subcellular fractions of olive fruits[a].

301

	Sample	Extraction buffer[b] (µL)	Incubation buffer[c] (µL)
7K* fraction[d]	pellet	80	239
15K, 40K, 150 K fractions	239 µL	80	-
Soluble fraction	80 µL	80	159

302 [a]All reaction mixtures also contained 11 µL of hydrogen peroxide at 0.2% (v/v), 15 µL of
303 2,4-dichlorophenol at 2% in methanol (w/v) and 15 µL of 4 mM chlorophyll *a* in acetone in
304 a total volume of 360 µL. [b]Extraction buffer: 5 mM Na-Pi pH 7 with 50 mM KCl and 0,24
305 % (p/v) of Tritón X-100; [c]Incubation buffer: 100 mM Na-Pi pH 7. [d]The chloroplast fraction
306 (7K) was resuspended in 100 mM Na-Pi pH 7 and centrifuged for 5min at 20,800xg to
307 obtain a enriched fraction in thylakoid membranes (7K*) (Hörtensteiner et al.1995).

308

309 Measurements from crude enzyme extracts were made in quadruplicate and
310 measurements from subcellular fraction were made in duplicate, both with their
311 corresponding blank tests with deactivated enzyme (15 min at 100 ºC), in order to
312 evaluate the possible chemical autoxidation of the chl pigments.

313 The peroxidase activity was defined as the formation of oxidised chl derivatives
314 expressed in nkatals in relation to the amount of protein in the crude extract enzyme or
315 proteic fraction. The Katal unit of activity is defined as the quantity of enzyme necessary
316 to form a mole of oxidised chl derivatives per second.

317 **2.7 Determination of protein concentration**

318 Measurements of the amount of proteins were made in crude enzyme extracts
319 and subcellular fractions. The amount of protein was determined using a comercial
320 bicinchoninic acid kit for protein determination (Sigma-Aldrich) based on the Lowry´s
321 method (Lowry, Rosebrough, Farr & Randall, 1951).

322 **2.8. Statistical data analysis**

323 Data were expressed as mean values ± standard deviation (SD) and were
324 analyzed for differences between means using one-way analysis of variance (ANOVA).
325 The analyses were performed using Statistica for Windows (version 6, StatSoft, 2001)

and the t-Student test was used as a post hoc comparison of statistical significance (p values < 0.05).

3. RESULTS AND DISCUSSION

3.1. Evolution of the total POX-chl activity

Difficulties in measuring POX-chl activity in plant tissues with a high level of CHLASE activity (Vergara-Domínguez et al., 2011) resulted in the need to define a methodology which, on using DCP as a cosubstrate reducer (Vergara-Domíguez et al. 2013), would permit the elimination of the competence between POX-chl and CHLASE**.**

The evolution of the POX-chl activity levels in the Arbequina variety with the new methodology in the absence of CHLASE activity (Figure 2, continuous line), displayed a relatively high period of activity in the months of August and September, coinciding with the higher levels of CHLASE activity (Vergara-Domínguez et al., 2011), followed by a period of declining levels of activity. The improvement in the determination of the POX-chl activity was evident when compared with the evolution of POX-chl determined using the previous methodology (Figure 2, discontinuous line), where for this period of ripening, the POX-chl activity was minimum (approximately 1 nkat/g protein), due to the clear interference of CHLASE and demonstrating the infra-valuation of the activity that was taking place to date.

In the Hojiblanca variety, the evolution of the POX-chl activity, measured in the absence of CHLASE activity (Figure 2), did not display any significant differences to the evolution of the levels of said activity, previously measured in the presence of this enzymatic competence (Vergara-Domínguez et al., 2011), as in the fruit from this variety the levels of CHLASE activity are not significant in comparison with the levels of POX-chl.

Comparing varieties, the values of POX-chl activity in the Arbequina variety were lower than those found in the Hojiblanca variety. The new measure of POX-chl activity permitted a more exact quantification of this activity in the Arbequina variety, by eliminating interference from the CHLASE activity (Vergara-Domínguez et al., 2013). Nevertheless, the results obtained did not provide an explication for the higher build-up of oxidized chl derivatives appearing in fruit from this variety (13%) with respect to fruit from Hojiblanca (3%) (Vergara-Domíguez et al. 2011), making it necessary to adopt a new approach to find an answer to this problem.

Given the wide distribution of POX in the different cellular compartments, the compartmentalisation of POX-chl activity in the different fractions from the mesocarp and epicarp cells of olives was studied in order to find, where applicable, possible differences between the Arbequina and Hojiblanca varieties, that would justify the differences existing in the profile of oxidized chl derivatives.

3.2 Subcellular distribution of the POX-chl activity

Using a homogenisation methodology especially defined for olive pulp by Salas et al., 1992 (Figure 1), followed by differential centrifugation, the following enriched subcellular fractions were obtained: thylakoid fraction (7K*), mitochondrial fraction (15K), high density membrane fraction (40K), low density membrane fraction (150K) and soluble fraction. Table 2 shows the distribution of the POX-chl activity measured in the two varieties of olive under study. It can be seen that the total of POX-chl activities measured in each of the subcellular fractions is significantly higher in the Hojiblanca variety with respect to the Arbequina variety, coinciding with the results described in section 3.1 (Figure 2). In addition, the individualised study of the levels of specific POX-chl activity in each of the subcellular fractions also reveals significant differences between varieties. In the 15 K, 40 K, 150 K and soluble fractions, the values were always significantly higher in the Hojiblanca variety (Table 2), whereas specifically in the thylakoid membrane fraction this relation was inverted, and the Arbequina variety displayed a significantly higher level of POX-chl activity.

Table 2

POX-chl activity associated to subcellular fractions from *O. europea* L. fruits (cvs. Arbequina and Hojiblanca).

	POX-chl activity[a]	
Material	Arbequina variety	Hojiblanca variety
Membranous fractions[b]		
7K*	3.620 ± 0.217[a]	2.880 ± 0.229[e]
15 K	0.860 ± 0.005[b]	2.430 ± 0.071[f]
40 K	0.970 ± 0.038[c]	2.820 ± 0.292[e]
150 K	0.970 ± 0.023[c]	1.750 ± 0.127[g]
Soluble fraction	2632.590 ± 276.790[d]	5450.700 ± 125.150[h]
Total	2639.020 ± 277.073[d]	5460.580 ± 125.750[h]

[a]Data, expressed as nkat/kg of protein, are means of two determinations ± SD. Different letters represent significant differences among materials. Enriched fraction in thylakoid membranes (7K*) obtained as described in table 1.

Expressed as a percentage, in both varieties, the POX-chl activity found in the soluble fraction was approximately 99.8 % of the total activity present in the cell and, therefore, only the 0.2% remaining was distributed in the subcellular fractions associated with the membranes (7K*, 15 K, 40 K and 150 K fractions). These results are similar to those observed in the cotyledon cells of the radish (*Raphanus sativus* L.) (Akiyama & Yamauchi, 2001) and of broccoli florets (*Brassica oleracea* L.) (Funamoto et al., 2003), where it was established that of all the POX-chl activity present in the cells, only a percentage of less than 1 % was located in the chloroplasts, whereas the rest was mainly found in the soluble fraction.

In the processing of the olive fruit for oil extraction, the chl pigment transferring to the oily phase is only partial despite its lipophilic nature. In the industrial processing is estimated that around 60 % may be retained on the solid phase (pomace) and approximately 20% is oxidized to non coloured products, and only 20 % is transferred to the oil (Gallardo-Guerrero, Roca & Mínguez-Mosquera 2002). Thus the final concentration of chl pigment in the virgin olive oil is also strongly affected by processing parameters such as temperature, time or exposure to air, which could modulate the POX-chl activity during the crushing and malaxation processes to obtain virgin olive oil causing chl oxidation. In addition to the high percentage of POX-chl activity that is present in the soluble cell fraction according to our results (99.8%), this oxidation process may also involve the POX-chl from the seed because the fruit is not usually destoned for oil extraction and in accordance with Luaces, Romero, Gutierrez, Sanz & Pérez (2007) olive seed POX would represent more than 99% of total POX in the olive paste used in process for olive oil.

Regarding varietal differences, although our results showed levels of total POX-chl activity in Hojiblanca variety upper than in Arbequina, to date there are not experimental data to estimate the percentage loss of chl pigments by oxidation during the process of virgin olive oil obtaining, in operation comparable conditions for these two varieties of olives.

Nevertheless, there are significant differences between varieties with respect to the distribution of the POX-chl activity in the fractions associated with membranes, and particularly the activity found in the thylakoid fraction (7K*) of both varieties. Whereas in the Arbequina variety, the POX-chl activity located in this fraction was equivalent to 56.39 % of all the POD-chl activity associated with the membranes, in Hojiblanca this percentage was significantly lower and only amounts to 29.14 %. The percentage of

412 POX-chl activity found in the mitochondrial fraction (15K) was of the same order as that
413 associated with the high density membranes (40K), representing a higher percentage in
414 fruit from the Hojiblanca variety in comparison to the same fractions from fruit from the
415 Arbequina variety. The distribution value corresponding to the microsomal fraction (150K)
416 did not display significant differences between the two varieties.

417 Given the thylakoid location of the chls, the most plausible explication is that the
418 POX-chl activity associated with the soluble fraction is not responsible for the formation of
419 the oxidized derivatives of chlorophyll that appear *in vivo* in the mesocarp and epicarp
420 cells of the olive. It is the activity associated with the membranes, more specifically the
421 thylakoid membranes, that is potentially directly linked to the oxidation reactions of chl
422 that appear *in vivo* (Akiyama & Yamauchi, 2001). In this respect, it should be noted that
423 the level of POX-chl activity associated with the thylakoid fraction (7K*) in the Arbequina
424 variety was significantly higher than that found in the Hojiblanca variety (Table 2), in
425 addition to representing more than half of the total percentage of the POX-chl activity
426 associated with the membranes.

427 *In vivo*, the concentration of chls in the Hojiblanca fruit (297.05 ± 14.99 mg/kg dry
428 weight) was far higher than that of the Arbequina fruit (72.43 ± 7.01 mg/kg dry weight) in
429 line with previous results (Roca et al., 2001 & 2003). Given this factor, on expressing the
430 POX-chl activity in relation to the chl concentration present in the fruit, for the Arbequina
431 variety, the activity calculated in the thylakoid fraction was 2.01 nkat/g of chls, whereas
432 for the Hojiblanca variety it was 0,26 nkat/g of chl. Consequently, in relation to the
433 quantity of pigment present in the fruit, the Arbequina variety had 10 times more POX-chl
434 activity in the thylakoid fraction than the Hojiblanca variety.

435 To conclude, although the total level of POX-chl activity in fruit from the Hojiblanca
436 variety was greater than that of the Arbequina fruit, the study of the distribution of this
437 activity in the different subcellular fractions has shown that the thylakoid fraction of the
438 Arbequina fruit has a significantly higher level of POX-chl activity than that found in the
439 Hojiblanca variety. This led to the linking of the formation and build-up of oxidized
440 derivatives of chl that takes place during the ripening of the fruit of this variety to the high
441 levels of POX-chl activity observed in its thylakoid fraction.

442 **3.3 Evolution of the oxidized derivatives of chlorophyll and the POX-chl activity in**
443 **thylakoid membranes.**

The comparison of the POX-chl activity located in the thylakoid fraction (7K*) and the build-up of oxidized derivatives during the lifecycle of the Arbequina fruit (Figure 3) showed that the maximum presence of oxidized derivatives of chl, which occurred in samples 5 and 7, coincided with a clear increase in the POX-chl activity measured in the thylakoid fraction. Nevertheless, between samples 8 and 10, coinciding with very advanced stages of ripeness of the fruit, high levels of POX-chl activity were observed, not concomitant with a high presence of oxidized derivatives in the chl fraction. This may be due to the drop in the chl concentration typical of this stage of ripeness (< 32 mg of chl/kg dry weight), which would imply that although in the thylakoid fraction a high level of POX-chl activity is potentially observed, the low availability of the chl substrate would impede the formation of these oxidized derivatives of chl. The same kinetics is also followed by another chl-degrading enzyme as CHLASE (Roca et al., 2003) in Arbequina fruits. In the Hojiblanca variety, although the presence of oxidized derivatives is much lower than that of the Arbequina variety and does not exceed 3%, the POX-chl activity measured in the thylakoid fraction displayed good correlation to the percentage of these compounds, both parameters exhibiting a maximum at the end of the ripening period (sample 8).

In conclusion, the highest levels of the POX-chl activity measured in the thylakoid fraction (7K*) of the Arbequina and Hojiblanca fruit were concomitant with the greatest rates of formation and build-up of oxidized derivatives of chl. Therefore, these figures suggest that a POX-chl activity located in the thylakoid fraction is directly implicated in the formation of these chl catabolites.

Nevertheless, although the levels of POX-chl activity measured in the thylakoid fraction (7K*) in the Arbequina fruit were higher than those measured in the Hojiblanca fruit, these differences would appear to be insufficient for explaining the wide margin that distinguishes the build-up of oxidized derivatives of chl in both varieties. These results permit the hypothesis of the existence of a regulation at post-transcriptional level of the POX-chl activity that might differentiate the two varieties. This regulation may be more intense in the Hojiblanca variety, such that the increases of POX-chl activity measured *in vitro* in the thylakoid fraction do not correspond to a significant build-up of oxidized derivatives of chlorophyll *in vivo* as occurs in the Arbequina variety.

Information about the enzymes involved in the oxidation of the chlorophyllic pigments in plant tissues *in vivo* is of huge importance in the food technology area for

preventing alterations to their profile during the production, harvest, storage and processing of foods of plant origin. Regardless of varietal differences found, data of the comparison of the POX-chl activity located in the thylakoid fraction and the build-up of oxidized derivatives during the lifecycle of the olive fruits leads to conclude that POX activity localized in chloroplasts is directly involved in the oxidative metabolism of chlorophyll that occurs during fruit ripening. Also, the study of subcellular distribution revealed that a very high percentage of the POX-Chl total activity (99.8%) was localized in the soluble fraction and thus would not be involved in this chl catabolism, because chlorophyll substrate and enzyme activity are in different subcellular compartments. However, this potential activity POX-chl can have a high degree of involvement in the oxidative degradation of these compounds when the loss of cellular compartmentalization happen in the fruit and there is greater accessibility of chlorophyll substrate. This occurs in the last stages of ripening, in senescence processes during storage of the fruit prior to processing or in crushing and malaxation process in olives for olive oil extraction. The importance of the control of these enzymatic oxidation reactions in order to preserve both colouring and functional properties in raw material for olive oil is disclosed.

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638 **Figure 1.** Specific methodology to obtain different subcellular fractions from fruit of mesocarp *O. europaea* L. Based in

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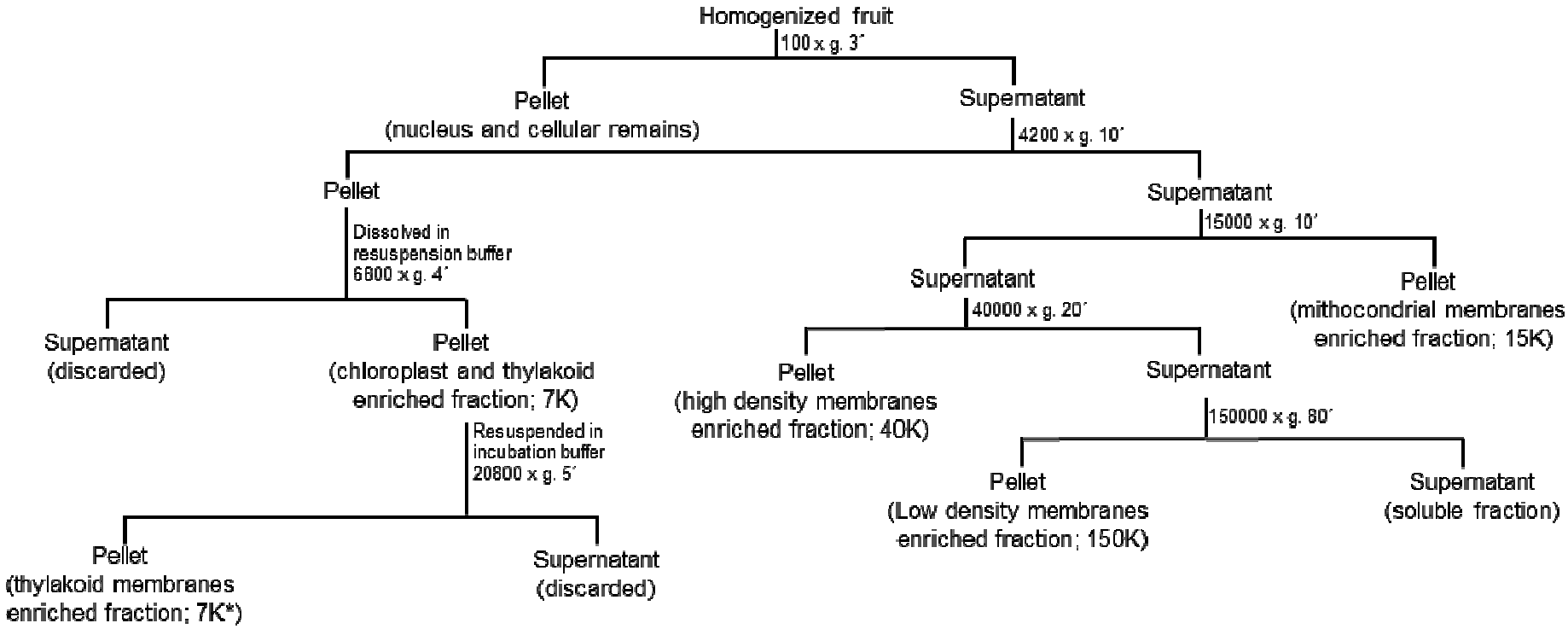
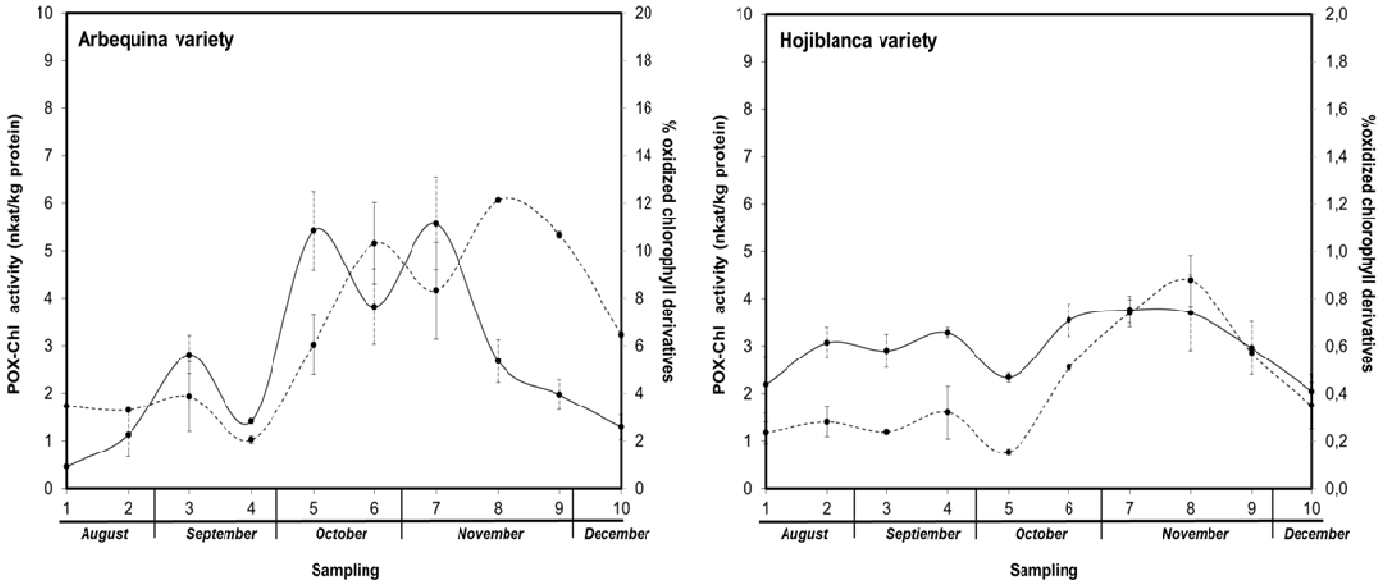


640

641 **Figure 2.** POD-chl activity evolution measured in solubilised enzyme extracts from
642 acetone powder from *O. europaea* L. (cvs. Arbequina and Hojiblanca). In
643 presence of competition with CHLASE activity (Vergara-Domínguez et al., 2011) (-
644 -▲--) and in absence of competition with CHLASE activity (—■—). Mean values a
645 nd standard deviations for n = 4.



646 **Figure 3.** POD-chl activity evolution located in thylakoid fraction (7K*) (—▲—) and
647 percentage of oxidised chlorophyll derivatives(--■--) from *O. europaea* L. fruits
648 (cvs. Arbequina and Hojiblanca). Mean values and standard deviations for n = 2.