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CHLOROPHYLL CATABOLISM IN OLIVE FRUITS (var. Arbequina and Hojiblanca) DURING MATURATION.

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ABSTRACT

The central reaction of chlorophyll (chl) breakdown pathway occurring during olive fruits maturation is the cleavage of the macrocycle pheophorbide *a* to a primary fluorescent chl catabolite (pFCC) and it is catalized by two enzymes: pheophorbide *a* oxygenase (PaO) and red chl catabolite reductase (RCCR). In subsequent steps, pFCC is converted to different fluorescent chlorophyll catabolites (FCCs) and nonfluorescent chlorophyll catabolites (NCCs). This work demonstrated that RCCR activity of olive fruits is type II. During the study of evolution of PaO and RCCR activities through the olive fruits maturation in two varieties: Hojiblanca and Arbequina, a significant increase in PaO and RCCR activity was found in ripening stage. In addition, the profile and structure of NCCs present in epicarp of this fruit was studied using HPLC/ESI-TOF-MS. Five different NCCs were defined and for the first time the enzymatic reactions implied in chlorophyll degradations in olive fruits elucidated.

Keywords: *Olea europaea* L.; olive fruits; Hojiblanca; Arbequina; chlorophyll catabolism; pheophorbide *a* oxigenase; RCC reductase, NCCs.

1. INTRODUCTION

Chlorophyll pigments are highly appreciated as functional components in fruits and vegetables both for their 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 for food production is associated with chemical and/or enzymatic 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 beginning of the maturation and senescence in fruits and leaves involves a cessation in the photosynthetic activity, the dismantling of the photosynthetic apparatus and the beginning of catabolism of the pigments responsible for the photosynthesis: chlorophylls. The biochemical pathway involved in the degradation of chlorophylls during the senescence and maturation is delineated. Nevertheless, there remain yet big questions that are stressed in the case of fruit maturation, which apart from differing from foliar senescence, is the least studied case. Therefore, a larger amount of researches dealing with the reactions involved in the degradation of chlorophylls in fruits are necessary. In addition, specifically in the case of olive fruit, it is essential to know the mechanisms responsible for the pigmentation in the fruit, since this composition leads to the coloration of the corresponding olive oils (Roca & Mínguez-Mosquera, 2001). Coloration is a critical parameter in the product marketing. Further, it is a quality and authenticity parameter (Gandul-Rojas et al., 2000) of the product.

The first enzymatic reaction implicated in the catabolism of the chlorophyll *a* (chl) molecule which takes place during leaf senescence is the removal of Mg by Mg-dechelating substance (MCS) (Suzuki & Shioi, 2002), producing pheophytin *a* followed by the removal of phytol and the formation of pheophorbide *a* in a reaction catalyzed by pheophytin pheophorbide hydrolase (PPH) (Kräutler & Hörtensteiner, 2013). However, the occurrence of these reactions during the fruit ripening is not clear, mainly because there is much less research regarding the degradation systems which are involved in the chlorophyllic catabolism in fruits. In fact, there are contradictory results: Guyer, Schelbert, Christ, Silvestre, Rossi & Hörtensteiner (2014) have proved PPH functionality in tomatoes (*Solanum lycopersicum*). Nonetheless, other authors (Shemer et al. 2008) in citrus fruits have checked that the pathway starts with sequential action of chlorophyllase (CHLASE) catalyzing the phytol hydrolysis in chl *a* resulting chlorophyllide *a*

(Amir-Shapira, Goldschmidt & Altman, 1987) followed by the removal of Mg by Mg-dechelating substance (MCS) (Suzuki & Shioi, 2002).

Independently of the pathway, pheophorbide *a* is degraded to primarily fluorescent chl catabolites (pFCCs), via the enzymatic system pheophorbide *a* oxygenase (PaO) in conjunction with red chl catabolite reductase (RCCR) (Figure 1). This reaction constitutes the cleavage of the tetrapyrrole and up to now, the only reaction regulated, being consequently the central step in the chlorophyll catabolism pathway (Kräutler & Hörtensteiner, 2013). In thylakoid membranes, PaO and RCCR work as a coupled enzymatic system that depends on the presence of ferredoxin (Fd) reduced as an electron donor to sequentially transform pheophorbide *a* into RCC and this into pFCC (Rodoni, Vicentini, Schellenberg, Matile & Hortensteiner, 1997). *In vivo*, Fd is reduced in the presence of light by means of Photosystem I (PSI). However, *in vitro*, a reducing power in the form of NADPH is needed for the formation of pFCC from pheophorbide *a* (Rodoni et al., 1997). PaO is a monooxygenase dependent on oxygen, cloned in 2003 (Pružinská, Tanner, Anders, Roca & Hörtensteiner, 2003), which is located in the inner membrane of chloroplasts (Kräutler & Hörtensteiner, 2013). Regarding RCCR, it is a soluble protein located in the stroma of chloroplast and it can produce two pFCC stereoisomers at C-1, (pFCC-1 and pFCC-2) (Mühlecker, Ongania, Krautler, Matile & Hortensteiner, 1997; Mühlecker & Krautler, 2000) depending on the plant species. The replacement of phenylalanine by valine in the amino acid sequence of RCCR is responsible for the stereospecificity (Pružinská et al. 2007).

Following, pFCCs are exported to the cytosol where they are modified with specific functional groups in three fixed positions generating fluorescent chl catabolites (FCCs). The first position is at C8² (Figure 1), where hydroxylation, glucosylation and/or malonylation could take place. The second modifiable position is O13⁴ where a reaction of demethylation is possible, allowing the occurrence of esterified or de-esterified possible structures. This enzymatic modification of pFCC is carried out by MES16, the unique enzyme involved on pFCC pathway described so far. Finally at C3, FCCs can be dihydroxylated or not at the vinyl group of pyrrole A (Figure 1). Combinations of these modifications generate a series of different FCCs that own a common structure consisting of a tetrapyrrole structure with an oxygenolytic opening of the porphyrin macrocycle (Kräutler & Hörtensteiner, 2013).

Finally, FCCs are transported up to the vacuole where, due to a non-enzymatic tautomerization by the action of acid pH in the vacuole, are transformed into nonfluorescent chl catabolites (NCCs) considered as the final catabolites of chlorophylls (Kräutler & Hörtensteiner, 2013). Fifteen different combinations have been described in the nature, all of them with similar structures and spectral properties (Berghold, Breuker, Oberhuber, Hörtensteiner & Kräutler, 2002; Berghold, Eichmüller, Hörstenstainer & Kräutler, 2004; Berghold, Müller, Ulrich, Hörtensteiner & Kräutler, 2006; Kräutler, Jaun, Amrein, Brortlik, Schellenberg & Matile, 1992; Losey & Engel, 2001; Mühlecker & Kräutler, 1996; Müller, Oradu, Ifa, Cooks, & Kräutler, 2011a; Müller, Rafelsberger, Vergeiner, & Kräutler, 2011b; Müller, Ulrich, Ongania & Kräutler, 2007; Pružinská et al., 2005; Scherl, Müller & Kräutler, 2012; Rios, Pérez-Gálvez & Roca, 2014a; Ríos, Roca & Pérez-Gálvez, 2014b). So far, NCCs have only been described in five fruits: pears (*Pyrus communis*, L.) and apples (*Malus sylvestris* L. Mill.) (Müller et al., 2007), bananas (*Musa acuminata* Colla) (Moser, Müller, Holzinger, Lütz & Kräutler, 2012), quince (*Cydonia oblonga* Miller) (Ríos et al., 2014a) and loquat (*Eriobotrya japonica* Lindl.) (Ríos et al., 2014b).

Today, there currently exists a general consensus regarding the succession of enzymatic reactions implicated in the catabolism of the chl *a* molecule which takes place during leaf senescence. It has been demonstrated that PaO activity is mainly present during the foliar senescence (Ginsburgn, Schellenberg & Matile, 1994). Nevertheless, Pružinská et al. (2003) have found certain PaO activity in presenescent tissues of *Arabidopsis* leaves. PaO expression undergo an increase that correlates with the activity of this enzyme in early stages of foliar senescence, though PaO activity decreases in the final stages, in contrast to the expression (Pružinská et al. 2005). On the contrary, RCCR seems to be constitutively active, so that it is not regulated during foliar senescence (Kräutler & Hörtensteiner, 2013). On the other hand, there is little knowledge about the role played by PaO and RCCR in the chlorophyll catabolism that occurs during fruit maturation. Exclusively, there have been found increases in PaO activity during tomato and pepper maturation (Moser & Matile, 1997; Akhtar, Goldschmidt, John, Rodoni, Matile & Grierson, 1999). Both enzymes level of involvement in the chlorophyll degradation that happens during the maturation of these fruits has not been determined though.

Olive fruits are an ideal model to study the catabolism of chlorophylls occurring in fruits, since there are large varietal differences in relation to chlorophyll metabolism inside the same

species. Roca & Mínguez-Mosquera, (2001) have described significant differences in the chlorophyll profile of the varieties Arbequina and Hojiblanca, as well as the degradation rate of these pigments. Specifically, there is very different involvement of CHLASE in both varieties (Roca & Mínguez-Mosquera, 2003). This research aim to deeply analyze the enzymatic mechanisms responsible for the different chlorophyll degradation in both varieties: Arbequina (low pigmentation variety, with presence of dephytylated chlorophyll derivatives) and Hojiblanca (high pigmentation variety, without dephytylated chlorophyll derivatives). With that purpose, the involvement of the enzymatic system composed of PaO and RCCR will be studied in olive fruits for the first time, as well as the identification of the NCCs present in the mature fruit. In a parallel way, this research will enlarge the little knowledge about chlorophyll catabolism in fruits.

2. MATERIALS AND METHODS

2.1. Plant material

The study was carried out with olive fruits (*Olea europaea* L.) of Hojiblanca and Arbequina varieties from the farm "La Platosilla", in Marchena (Seville, Spain). The fruits were collected every month from August to December. A total of 2 Kg of fruits were collected from six olive trees randomly chosen. The olive fruits were taken from the branches that were as high as the extended arms, throughout all the perimeter of the tree, and from the outer and inner areas. For the FCCs and NCCs standards extraction, senescent leaves of rape (*Brassica napus* L.) and spinach (*Spinacea oleracea* L.) and pepper fruits (*Capsicum annum* L.) were processed. Rape seeds were germinated in vermiculite (3-5 days) until the apparition of the first cotyledons. Leaves of spinach were bought at local market. The senescence induction of rape cotyledons and spinach leaf took place under dark conditions in Petri dishes on moistened filter discs during 5-7 days at 25 °C, until the prevailing coloration was yellow (Roca, James, Pruzinská, Hörtensteiner, Thomas & Ougham, 2004). The pepper fruits in a single advanced maturity stage (red colour) were acquired in a supermarket.

2.2. Extraction, separation and quantification of chlorophylls

Samples were taken from a homogenized triturate, prepared from 100 de-stoned fruits (ca. 40g) of the most representative size by accurately weighing from 4 to 15 g for each analysis depending on the degree of ripeness of the fruits. Pigment extraction was performed with N,N-

dimethylformamide and the chlorophyll fraction was purified and removed of the lipid compounds and the carotenes by liquid-liquid extraction with hexane (Roca & Mínguez-Mosquera, 2003). All analyses were performed in triplicate under a green light. The pigments were separated by reverse phase HPLC using a Hewlett-Packard HP 1100 liquid chromatograph. A Mediterranea Sea18 column (20 cm x 0.46 cm, 3 μ m) was used (Teknokroma, Barcelona, Spain); protected by a guard column (1 cm x 0.46 cm) packed with the same material. Separation was performed using an elution gradient (Roca & Mínguez-Mosquera, 2003) 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. 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 (Vergara-Domínguez, Roca & Gandul-Rojas, 2014) and from their spectral characteristics. The on-line UV-Vis spectra were recorded from 350 to 800 nm with the photodiodearray detector.

2.3. Extraction, separation and identification of NCCs

Following previous methodology (Mühlecker & Kräutler, 1996), fresh tissue was extracted with potassium phosphate buffer pH 7.0/methanol (1:3, v/v), centrifuged and concentrated by SPE column (Bakerbond C18 SPE, 500 mg/6 mL, J.T. Baker, Deventer Holland). NCCs were analyzed with a Dionex Ultimate 3000RS U-HPLC (Thermo Fisher Scientific, Waltham, MA, USA). The eluents were (A) 0.1% (v/v) formic acid in water and (B) 0.1% (v/v) formic acid in methanol, following a gradient previously published (Ríos et al. 2014a), and with 1 mL/min as flow rate. The HPLC column was a 3 μ m C18 Spherisorb ODS-2 (Teknokroma, Barcelona, Spain) (20 $\times$ 0.46 cm i.d.). The MS system was a microOTOF-QII High Resolution Time-of-Flight mass spectrometer (UHR-TOF) with qQ-TOF geometry (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) interface. The method included to scan from m/z 50 to 1200, always at positive mode and the mass spectra were registered in MS fullscan mode. For a deep characterization, MS² analysis were also acquired in Auto-MS/MS.

The software Bruker Daltonics TargetAnalysis™ 1.2 allowed the identification of a compound in base to mass accuracy and the isotopic pattern calculated with the SigmaFit™ algorithm. The maximum limits for both parameters were established at 5 ppm and 50 (Ríos, Roca

& Pérez-Gálvez, 2015). For the screening, we have developed a NCC library which includes monoisotopic masses, elemental composition, retention time and characteristic fragment ions, for all the NCCs already identified (Berghold et al., 2002; Berghold et al., 2004; Berghold et al., 2006; Kräutler et al., 1992; Losey & Engel, 2001; Mühlecker & Kräutler, 1996; Müller et al., 2011a; Müller, et al., 2011b; Müller et al., 2007; Pružinská et al., 2005; Scherl et al., 2012, Rios et al., 2014a; Ríos et al., 2014b). In addition, for the study of the MS² data we used the SmartFormula3D™ module.

2.4. Measurement of PaO/RCCR activity

Based on Hörtensteiner, Vicentini & Matile (1995), 2 gr of cutted fresh material were homogenized with buffer (0.4 M sorbitol, 25 mM Tricina-KOH pH 8, 2mM Na-EDTA, 1 mM MgCl₂, 0.1 % BSA, 5 mM PEG 4000 and 10 mM cisteamina-HCl) on ice bath (14000 rpm 7 s) and spin (7200 x g, 4° C, 4 min). The pellet was dissolved in buffer 0.4 M sorbitol, 25 mM Tricina-KOH pH 8, 5 mM PEG 4000 and 10 mM cisteamina-HCl, spin (6000 x g, 4°C, 4 min) and finally the pellet was frozen until -80 °C). The thylakoid membranes were dissolved in buffer Tris-Mes pH 8, and spin (20800 x g, 5 min, 4° C). The supernatant includes the soluble proteins, enriched in RCCR. The pellet was washed several times with buffer Tris-Mes pH 8, spin at 20800 x g, 5 min, 4° C, and the final pellet was dissolved in buffer Tris-Mes pH 8 with 1% Triton X-100 shaking during 30 min at 4° C in darkness. 25 µl aliquots include proteins associated with membranes and source of PaO. The assay contained the RCCR fraction, PaO fraction and supplements (glucose-6-P 50 mM, NADPH 50 mM, glucose-6-P dehydrogenase 50 U/mL, ferredoxin 2 U/mL and ferredoxin-NADPH-oxidoreductase 2 U/mL dissolved in Tris-Mes buffer pH 8) and pheophorbide 1 µg/µl in Tris-Mes buffer pH 8 with 1% Tritón X-100 and proceded during 1 hour. The activity is measured in base to the formation of pFCCs (Roca et al. 2004). The separation was carried out adapting the methodology described by Ginsburg & Matile (1994), through HPLC in reverse phase with a Mediterranea Sea18 column (20 cm x 0.46 cm, 3 µm) acquired in Teknokroma (Barcelona), protected by a precolumn (1 cm x 0.46 cm) packed with the same material. The elution was carried out with two solvent mixtures: A.- potassium phosphate buffer at 50 mM pH 7 and B.- deionised water, Pi-K buffer at 50 mM pH 7 and methanol on a scale of 1:1:8; isocratically 20% A - 80% B (Roca et al. 2004). FCCs were quantified with the use of fluorescence units (FU)

(Hörtensteiner et al., 1995). FCCs were identified by means of a fluorescence detector (320/450 nm), retention times and co-chromatography with authentic standards.

3. RESULTS AND DISCUSSION

3.1. Characterization of the RCCR activity of olive fruits

RCCR is a stereospecific enzyme, able to produce two epimeric *p*FCCs, *p*FCC1 (Mühlecker et al., 1997) or *p*FCC2 (=1-*epi-p*FCC) (Mühlecker et al., 2000), depending on the position of H at C1. This stereospecificity is plant species-inherent (Pruzinská et al., 2003) and is defined by a small protein domain that can be reversed by a single Phe-to-Val exchange (Pruzinská et al., 2007). This way, two RCCR types are defined: RCCR I and RCCR II. Only limited plant species have the type of RCCR identified and olive fruits are not one of them. To define the RCCR type, enzymatic systems of well-characterized plant species were taken as patterns: rape cotyledons, which contain RCCR I (Rodoni et al., 1997) and pepper, containing RCCR II (Moser & Matile, 1997). While assaying the RCCR activity, rape cotyledons produced *p*FCC1, with a retention time of 11 min (Figure 2a), pepper enzymatic extract produced *p*FCC2, which eluted in 13 min (Figure 2b). By using a chromatographic gradient similar to that published, the retention times obtained were similar (Rodoni et al. 1997; Moser & Matile, 1997). The absorption spectra of both *p*FCCs exhibit the maximums characteristic of FCCs: 320 nm due to the α -formyl pyrrole moiety at ring B and 360 nm due to a chromophore extension over rings C and D (Mühlecker et al., 1997).

When olive fruit PaO-RCCR activity was assayed, the quantity of formed product (*p*FCC) during the reaction was not enough to be detected under the analytical conditions employed, even though fluorescence detection was used (Figure 2c). Initial attempts to optimize the measurement were unsuccessful. High fat content of olive fruit impeded the increase of the sample size or the selection of more suitable maturity stages. Assays addressed to diminish the potential inhibitor effect of polyphenols (including PVPP in the homogenization buffer) on the enzymatic activities were void. This result indicated a low level of PaO-RCCR activity present in the olive fruit, compared to the unique fruit in which both activities had been determined, pepper (Moser & Matile, 1997). Taking advantage of the fact that the PaO/RCCR measurement is a coupled enzymatic system, and based on a similar previous approximation (Roca et al., 2004), an

alternative measurement system was developed, allowing to amplify the enzymatic activity, including an auxiliary PaO or RCCR source of enzyme with elevated activity as pepper (Moser & Matile, 1993). For that, several combinations of the cell fractions necessary for PaO-RCCR coupled enzymatic reaction were made, for one of the enzyme sources to always come in the incubation from the pepper fruit (Table 1). Only in that case, PaO-RCCR activity in olive fruit could be measured, and obviously, much less activity than in the case of pepper or rape. This new optimized measurement system allowed to measure, for the first time, olive fruits PaO and RCCR activities independently. When pepper extract was used as RCCR source (Figure 2b), pFCC2 was produced, as expected (regardless of the PaO source), but in much less quantity (Table 1). In the reaction mixtures with enzymatic extracts of different species, the rate of final formation of catabolite (pFCC) was exclusively limited by olive fruit PaO or RCCR activity. Finally, by measuring activity with an RCCR source of olive fruit, it was observed that a catabolite that eluted in 13 min (Figure 2b and Table 1) was formed. It was identified as pFCC2 through cochromatography with the corresponding standard from pepper. Consequently, RCCR activity of the species *Olea europaea* L. was classified as type II. This result was confirmed in both varieties (Table 1).

3.2. Evolution of PaO and RCCR activities in olive fruits

Considering that the maturity stage of olives determines the coloration of the corresponding oils (Roca & Mínguez-Mosquera, 2001), it is essential to analyze the evolution of the enzymatic systems involved in the degradation of chlorophylls. Following the analytic procedure described in the previous section, the evolution of PaO and RCCR activities in Arbequina and Hojiblanca olive fruits during the growing and maturation processes (Table 2) between the months of August (green immature fruit) and December (purple/black mature fruit) was studied. It must be mentioned that, for this research, PaO and RCCR extracts of the pepper fruit were always taken in a unique maturity stage (red mature) to avoid variables. Only the olive fruits used to obtain the enzymatic extract were taken in different maturity stages. It was not possible to quantify PaO or RCCR activity (Table 2) in Arbequina and Hojiblanca olive fruits in the first maturity stages (August-September), due to the low activity levels of both enzymes during this period. Nonetheless, in the months of full maturation (October-December), when olive fruits are harvested for olive oil extraction, PaO and RCCR activities were detected, until very advanced

maturity stages (December/black), when the chlorophyll quantity present in tissues is very low, and no activity was detected. Similar results for PaO activity have been described in *Brassica napus* cotyledons (Ginsburg & Matile, 1994), *Festuca pratensis* leaves (Vicentini, Hörtensteiner, Schellenberg, Thomas & Matile, 1995) and *Arabidopsis* leaves (Pruzinská et al., 2003, 2005 y 2007), where low levels of PaO activity are described in the first senescence days, followed by a fast and sharp increase in this activity and, finally, an activity decrease in advanced senescence stages. With regards to RCCR levels, in the only work carried out so far, it has been demonstrated that protein expression and abundance and RCCR activity remain constant during *Arabidopsis* leaf senescence (Pruzinská et al., 2005). This would suggest that RCCR was not regulated during foliar senescence. Nevertheless, the data obtained in this study show that RCCR activity in olive fruits is subject to certain modulation during maturation (Table 2). It is not known at what level pre- or post-transcriptional regulation occurs, but its activity is not constant in these fruits.

In olive fruits (Table 2), the maximum values of PaO activity were much lower than the maximum levels of RCCR. PaO activity in both varieties during the month of December is concomitant of the highest chlorophyll degradation rates. This suggests that in PaO-RCCR system, the oxygenolytic opening of pheophorbide a catalyzed by PaO is the limiting step to the global reaction. This hypothesis has been proposed by Pruzinská et al., (2005) after verifying that the increase in PaO activity in *Arabidopsis* leaves was concomitant with the accumulation of FCCs and NCCs. The involvement of the PaO-RCCR enzymatic system in the degradation of chlorophylls during the maturation of both varieties of olive fruits is evident, and special attention must be paid to the bigger influence of PaO activity in the final reaction.

3.3. Identification of nonfluorescent chlorophyll catabolites (NCCs) in olive fruits

NCC extracts obtained in both varieties of olive fruits were analyzed by HPLC/ESI-TOF-MS. The collected data were processed by means of the software Target Analysis™. For the automatic identification in the database, it was created a library based in the bibliography, containing all the information relative to the elemental composition, accurate mass and fragmentation ions of all NCCs identified and described so far (see section 2.3). On the basis of the data obtained after the analysis by HPLC/ESI-TOF-MS, the identification was based on the accurate mass as well as the elemental composition and the co-injection with patterns.

As can be observed in Figure 3, two NCCs were detected in mature fruits of Arbequina variety, while in Hojiblanca variety, however, three NCCs were identified. All of them showed the same absorption spectrum with the characteristic maximum at 320 nm (Mühlecker et al., 1997). Following the nomenclature proposed by Ginsburg & Matile (1993), in which the prefix indicates the initials of the plant species in which they are identified, and the number makes reference to the order of elution in the chromatographic conditions of analysis, these new catabolites were named Oe-NCC1 and Oe-NCC4 (Table 3). In supplementary data 1, the MS spectra of each of them are shown.

Oe-NCC1 (Figure 1; Table 3, supplemental data 1) presents a m/z of 631.2762 and an elemental composition of $C_{34}H_{38}N_4O_8$. Such NCC has been previously identified in *Brassica napus* L. senescent cotyledons (*Bn*-NCC3, Mühlecker & Kräutler, 1996), in *Spinacea oleracea* L. senescent leaves (*So*-NCC3, Berghold et al. 2002), and in *Arabidopsis thaliana* L. senescent leaves (*At*-NCC2, Pružinská et al. 2005). To confirm the identification of Oe-NCC1, a *Bn*-NCC3 from an extract of NCCs of *Brassica napus* (L.) senescent cotyledons was used as standard (Mühlecker & Kräutler, 1996). In both NCCs (Oe-NCC1 and *Bn*-NCC3), the retention time, accurate mass, elemental composition (Table 3) and fragmentation profile (supplementary data 2) were the same. In the analysis of MS/MS of Oe-NCC1, such profile coincides with the ions previously described (Mühlecker & Kräutler, 1996): molecular ion and loss of ring A (Supplementary data 2). Finally, it was confirmed that Oe-NCC1 of olive and *Bn*-NCC3 co-eluted with the same retention time. Of the three positions able to be modified with different functional groups, Oe-NCC1 presents a vinyl group at C3, an OH group at C8² and is not esterified at O13⁴ (Figure 1). In relation to the original substrate, pheophorbide *a*, besides the oxygenolytic opening at C4-C5, Oe-NCC1 only presents the group CH₃ of O13⁴ replaced by an H.

The molecular ion of Oe-NCC2 (Figure 1; Table 3, supplemental data 1) was observed at m/z 645.2919 in the high-resolution ESI-MS with mass error and mSigma values within the threshold limit. This compound presents an elemental composition ($C_{35}H_{40}N_4O_7$) that fits with the structural configuration of an NCC previously described in some plant species: *So*-NCC4 in *Spinacea oleracea* L. senescent leaves (Berghold et al. 2002), *Cj*-NCC1 in *Cercidillium japonicum* Siebold & Zucc. senescent leaves, (Oberhuber Berghold, Breuker, Hörtensteiner & Kräutler, 2003), *Pc*-NCC2 and *Md*-NCC2 in mature pears and apples (Müller et al. 2007) and *Sw*-NCC58 in

Spatiphyllum wallissii senescent leaves (Kraütler et al. 2010). So-NCC4 was obtained from spinach senescent leaves and was used as standard. It was verified that retention time, accurate mass, elemental composition and fragmentation profile (Supplemental data 2) coincided with the description above (Berghold et al. 2002). Specifically, for Oe-NCC2, apart from the molecular ion, loss of ring A (Supplemental data 2) and loss of group CH₃OH took place. These are characteristics losses of NCCs and, in particular, of this compound (Berghold et al. 2002, Oberhuber et al. 2003, Müller et al. 2007, Kraütler et al. 2010). Oe-NCC2 co-elutes with So-NCC4 and presents a vinyl group at C3, an hydroxyl group at C8² and is esterified at O13⁴ (Figure 1).

Oe-NCC3 (Figure 1; Table 3, supplemental data 1) shows an accurate mass of m/z of 629.2970 and a predicted elemental composition of C₃₅H₄₀N₄O₇. Such compound has been previously identified in senescent leaves of spinach (So-NCC5) (Berghold et al. 2002) and to Cj-NCC2 in *Cercidillium japonicum* senescent leaves, Siebold & Zucc. (Oberhuber et al. 2003). So-NCC5 was obtained from spinach senescent leaves and was used as standard. Likewise, it was tested that retention time, accurate mass, elemental composition and fragmentation profile (Supplemental data 2) coincide with what has been previously described (Berghold et al. 2002). For Oe-NCC3, the following ions were obtained: molecular ion and loss of group CH₃OH and ring A simultaneously (Supplemental data 2). These losses are characteristic of NCCs. Oe-NCC3 co-eluted with So-NCC5. The structure of this NCC is simpler than those aforementioned: a vinyl group at C3, H at C8² and is esterified at O13⁴ (Figure 1).

Lastly, Oe-NCC4 (Figure 1; Table 3) with a m/z of 615.3177 and an elemental composition of C₃₅H₄₂N₄O₆ has also been identified in *Brassica napus* L. senescent cotyledons (*Bn*-NCC4) and in *Arabidopsis thaliana* L. senescent leaves (At-NCC5, Pružinská et al. 2005). To confirm its identification, *Bn*-NCC4 was obtained from rape senescent cotyledons and taken as standard; retention time, accurate mass, elemental composition and fragmentation profile were measured (Müchlecker & Kraütler, 1996, supplemental data 2). Oe-NCC4 fragmentation ions coincide with those previously described (Berghold et al. 2002) for this compound: molecular ion, loss of a water molecule and loss of ring A (Supplemental data 2). These losses are characteristic of NCCs. *Bn*-NCC4 co-elution with Oe-NCC4 confirmed its identification. The structure of this NCC presents a vinyl group at C3, H at C8² and H at O13⁴.

The molecular structure of NCCs identified in the olive fruit differentiated Hojiblanca and Arbequina varieties, which only showed an only NCC catabolite in common: Oe-NCC2 (Table 3). Oe-NCC1 was only found in Arbequina variety. In contrast, Oe-NCC3 and Oe-NCC4 were only detected in Hojiblanca variety (Figure 3 and table 3). This is the first time that the NCC profiles are compared between varieties belonging to the same species. Therefore, it remains unknown whether this varietal differentiation is an isolated or generalized case in the plant kingdom. Nonetheless, in the light of the areas obtained in the chromatograms through UV visible detection at 320 nm (Table 3), Oe-NCC2 is the most abundant NCC for both varieties.

Taking into consideration the potential structural modifications of NCCs, the structures identified in olive fruits are the simplest possible (Figure 1). Of the three possible positions where modifications in the NCCs have been described, the vinyl group at C3 (or dihydroxy-vinyl), O13⁴ (CH₃ or H) and C8² (H, OH, O-malonyl, O-glucopyranosyl or O-malonyl-glucopyranoside), the NCCs present in olive fruits only exhibit modifications at O13⁴ (CH₃ or H) and C8² (H and OH). Taking into account these structures, the area determined and the reactions in the chlorophyll degradation pathway, an NCC formation pathway in olive fruits (Figure 1) can be suggested. From pheophorbide *a* as the common necessary catabolite, the NCC directly originated would be Oe-NCC3. Apart from the common and ever-present oxygenolysis at C4-C5, Oe-NCC3 has not experienced any additional modifications in the potential functional groups. It should be reminded that these modifications of the substituents of the tetrapyrrole structure occur at the level of FCC, through enzymatic reactions in the cytoplasm. Subsequently, the corresponding NCCs originate from the FCCs through a non-enzymatic tautomerization in the vacuole. Oe-NCC3 (So-NCC5/Cj-NCC2) is considered to be the simplest NCC, since it comes directly from pheophorbide *a*, without any refuncionalizations. Consequently, it is one of the least abundant NCC in general terms. Oe-NCC2 presents, regarding the molecule of pheophorbide *a*, a hydroxylation at C8². This reaction is verified in senescent leaves and mature fruits of pears and apples (Müller et al., 2007) and is catalyzed by an enzyme that has not been identified so far. This reaction seems to be a necessary condition for the subsequent substitution of this functional group (-OH) by other and more complex substituents (Berghold et al., 2002). These modifications are so far more common in the NCCs described in the bibliography. That is why Oe-NCC2 is the most abundant NCC in the olive fruit. In addition, MES16 (Christ et al. 2012), catalyze the specific demethylation at O13⁴, generating

Oe-NCC4 or Oe-NCC1 (Figure 1). This enzyme, recently identified in *Arabidopsis* senescent leaves, belongs to the protein family of methylesterases. This enzyme is thus active in olive fruits, though its role does not seem to be very important.

4. CONCLUSIONS

Measurement of the enzymatic activities involved in the general chlorophyll catabolic pathway, PaO and RCCR, , as well as the identification of the final catabolites of this pathway (NCCs) in olive fruits, show that the general chlorophyll catabolic pathway in senescent leaves initially described, is also active in these fruits. Likewise, the study of the NCC structures present in both varieties of olives analyzed show that, though the main pathway for NCC formation is the same, certain reactions are more prone to occur than others depending on the variety. Consequently, the NCC profile generated is different depending on the variety. This is the first evidence of varietal differentiation in the profile of NCCs found in the plant kingdom.

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5. REFERENCES

- Akhtar, M. S., Goldschmidt, E. E., John, I., Rodoni, S., Matile, P., & Grierson, D. (1999). Altered patterns of senescence and ripening in *gf*, a stay-green mutant of tomato (*Lycopersicon esculentum* Mill.). *Journal of Experimental Botany*, 50(336), 1115–1122.
- Amir-Shapira, D., Goldschmidt, E. E., & Altman, A. (1987). Botany Chlorophyll catabolism in senescing plant tissues: In vivo breakdown intermediates suggest different degradative pathways for Citrus fruit and parsley leaves. *Proc. Natl. Acad. Sci.*, 84, 1901–1905.
- Berghold, J., Breuker, K., Oberhuber, M., Hörtensteiner, S., & Kräutler, B. (2002). Chlorophyll breakdown in spinach: on the structure of five nonfluorescent chlorophyll catabolites. *Photosynthesis Research*, 74, 109–119.

409 Berghold, J., Eichmüller, C., Hörtensteiner, S., & Kräutler, B. (2004). Chlorophyll breakdown in
 410 Tobacco: On the structure of two nonfluorescent chlorophyll catabolites. *Chemistry &*
 411 *Biodiversity*, 1, 657–668.

412 Berghold, J., Müller, T., Ulrich, M., Hörtensteiner, S., & Kräutler, B. (2006). Chlorophyll breakdown
 413 in maize: On the structure of two nonfluorescent chlorophyll catabolites. *Monatshefte Für*
 414 *Chemie*, 137, 751–763.

415 Christ, B., Schelbert, S., Aubry, S., Süssenbacher, I., Müller, T., Kräutler, B., & Hörtensteiner, S.
 416 (2012). MES16, a member of the methylesterase protein Family, specifically demethylates
 417 fluorescent chlorophyll catabolites during chlorophyll breakdown in *Arabidopsis*. *Plant*
 418 *Physiology*, 158, 628–641.

419 Ferruzzi, M. G., & Blakeslee, J. (2007). Digestion, absorption, and cancer preventative activity of
 420 dietary chlorophyll derivatives. *Nutrition Research*, 27, 1–12.

421 Gandul-Rojas, B., Roca, M., & Mínguez-Mosquera, M. I. (2000). Use of chlorophyll and carotenoid
 422 pigment composition to determine authenticity of virgin olive oil. *Journal of the American Oil*
 423 *Chemists' Society*, 77, 853–858.

424 Ginsburg, S., & Matile, P. (1993). Identification of catabolites of chlorophyll-porphyrin in senescent
 425 rape cotyledons. *Plant Physiology*, 102, 521–527.

426 Ginsburg, S., Schellenberg, M., & Matile, P. (1994). Cleavage of chlorophyll-porphyrin-
 427 requirement for reduced ferredoxin and oxygen. *Plant Physiology*, 105, 545–554.

428 Guyer, L., Hofstetter, S. S., Christ, B., Lira, B. S., Rossi, M., & Hörtensteiner, S. (2014). Different
 429 mechanisms are responsible for chlorophyll dephytylation during fruit ripening and leaf
 430 senescence in tomato. *Plant Physiology*, 166, 44–56.

431 Hörtensteiner, S., Vicentini, F., & Matile, P. (1995). Chlorophyll breakdown in senescent
 432 cotyledons of rape, *Brassica-napus* L.: enzymatic cleavage of pheophorbide a in vitro. *New*
 433 *Phytologist*, 129, 237–246.

434 Kräutler, B., Banala, S., Moser, S., Vergeiner, C., Müller, T., Lütz, C., & Holzinger, A. (2010). A
 435 novel blue fluorescent chlorophyll catabolite accumulates in senescent leaves of the peace
 436 lily and indicates a split path of chlorophyll breakdown. *FEBS Letters*, 584, 4215–4221.

437 Kräutler, B., & Hörtensteiner, S. (2013). Chlorophyll breakdown: chemistry, biochemistry, and
 438 biology. In G. C. Ferreira, K. M. Kadish, K. M. Smith, & R. Guilard (Eds.), *Handbook of*
 439 *Porphyrin Science* (pp. 117–185). World Scientific Publishing, USA.

440 Kräutler, B., Jaun, B., Amrein, W., Bortlik, K., Schellenberg, M., & Matile, P. (1992). Breakdown
 441 of chlorophyll: constitution of a secoporphinoid chlorophyll catabolite isolated from
 442 senescent barley leaves. *Plant Physiology and Biochemistry*, 30(3), 333–346.

443 Losey, F. G., & Engel, N. (2001). Isolation and characterization of a urobilinogenoidic chlorophyll
 444 catabolite from *Hordeum vulgare* L. *Journal Biological Chemistry*, 276, 8643–8647.

445 Moser, D., & Matile, P. (1997). Chlorophyll breakdown in ripening fruits of *Capsicum annuum*.
 446 *Journal of Plant Physiology*, 150, 759–761.

447 Moser, S., Müller, T., Holzinger, A., Lütz, C., & Kräutler, B. (2012). Structures of Chlorophyll
 448 catabolites in bananas (*Musa acuminata*) reveal a split path of chlorophyll breakdown in a
 449 ripening fruit. *Chemistry European Journal*, 18, 10873 – 10885.

450 Mühlecker, W., Kräutler, B., Moser, D., Matile, P., & Hörtensteiner, S. (2000). Breakdown of
 451 chlorophyll: A fluorescent chlorophyll catabolite from sweet pepper (*Capsicum annuum*).
 452 *Helvetica Chimica Acta*, 3, 278–286.

453 Mühlecker, W., Ongania, K.-H., Kräutler, B., Matile, P., & Hörtensteiner, S. (1997). Tracking down
 454 chlorophyll breakdown in plants: Elucidation of the constitution of a “fluorescent” chlorophyll
 455 catabolite. *Angewandte Chemie-International Edition*, 36, 401–404.

456 Müller, T., Oradu, S., Ifa, D. R., Cooks, R. G., & Kräutler, B. (2011). Direct plant tissue analysis
 457 and imprint imaging by desorption electrospray ionization mass spectrometry. *Analytical*
 458 *Chemistry*, 83(14), 5754–5761.

459 Müller, T., Rafelsberger, M., Vergeiner, C., & Kräutler, B. (2011). A dioxobilane as product of a
 460 divergent path of chlorophyll breakdown in Norway maple. *Angewandte Chemie -*
 461 *International Edition*, 50(45), 10724–10727.

462 Müller, T., Ulrich, M., Ongania, K.-H., & Kräutler, B. (2007). Colorless tetrapyrrolic chlorophyll
 463 catabolites found in ripening fruit are effective antioxidants. *Angewandte Chemie-*
 464 *International Edition*, 46, 8699–8702.

465 Oberhuber, M., Berghold, J., Breuker, K., Hörtensteiner, S., Kräutler, B., & Eschenmoser, A. J.
 466 (2003). Breakdown of chlorophyll: A nonenzymatic reaction accounts for the formation of
 467 the colorless nonfluorescent" chlorophyll catabolites. *Proc. Natl. Acad. Sci.*, 100(12), 6910–
 468 6915.

469 Pružinská, A., Anders, I., Aubry, S., Schenk, N., Tapernoux-Lüthi, E., Müller, T., Kräutler, B.,
 470 Hörtensteiner, S. (2007). In vivo participation of Red Chlorophyll Catabolite Reductase in
 471 chlorophyll breakdown. *The Plant Cell*, 19, 369–387.

472 Pružinská, A., Tanner, G., Anders, I., Roca, M., & Hörtensteiner, S. (2003). Chlorophyll
 473 breakdown: Pheophorbide a oxygenase is a rieske-type iron–sulfur protein, encoded by the
 474 accelerated cell death 1 gene. *Proc. Natl. Acad. Sci.*, 100(25), 15259–15264.

475 Pružinská, A., Tanner, G., Aubry, S., Anders, I., Moser, S., Mü, T., Ongania, K.H., Kräutler, B.,
 476 Youn, J.-Y., Liljegren, S.J., Hörtensteiner, S. (2005). Chlorophyll breakdown in senescent
 477 Arabidopsis leaves. Characterization of chlorophyll catabolites and of chlorophyll catabolic
 478 enzymes involved in the degreening reaction. *Plant Physiology*, 139, 52–63.

479 Ríos, J. J., Pérez-Gálvez, A., & Roca, M. (2014). Non-fluorescent chlorophyll catabolites in quince
 480 fruits. *Food Research International*, 65, 255–262.

481 Ríos, J. J., Roca, M., & Pérez-Gálvez, A. (2014). Nonfluorescent chlorophyll catabolites in Loquat
 482 Fruits (*Eriobotrya japonica* Lindl.). *Journal of Agricultural and Food Chemistry*, 62,
 483 10576–10584.

484 Ríos, J. J., Roca, M., & Pérez-Gálvez, A. (2015). Systematic HPLC/ESI-High Resolution-qTOF-
 485 MS methodology for metabolomic studies in nonfluorescent chlorophyll catabolites pathway.
 486 *Journal of Analytical Methods in Chemistry*, 2015, 1–10.

487 Roca, M. (2012). In vitro digestive stability and uptake by Caco-2 human intestinal cells of
 488 nonfluorescent chlorophyll catabolites. *Food Chemistry*, 130, 134–138.

489 Roca, M., James, C., Pružinská, A., Hörtensteiner, S., Thomas, H., & Ougham, H. (2004).
 490 Analysis of the chlorophyll catabolism pathway in leaves of an introgression senescence
 491 mutant of *Lolium temulentum*. *Phytochemistry*, 65, 1231–1238.

492 Roca, M., & Mínguez-Mosquera, M. I. (2001). Changes in chloroplast pigments of olive varieties
 493 during fruit ripening. *Journal of Agricultural and Food Chemistry*, 49(2), 832–839.

494 Roca, M., & Mínguez-Mosquera, M. I. (2003). Involvement of chlorophyllase in chlorophyll
 495 metabolism in olive varieties with high and low chlorophyll content. *Physiologia Plantarum*,
 496 117, 459–466.

497 Rodoni, S., Vicentini, F., Schellenberg, M., Matile, P., & Hörtensteiner, S. (1997). Partial
 498 purification and characterization of Red Chlorophyll Catabolite Reductase, a stroma protein
 499 involved in chlorophyll breakdown. *Plant Physiol*, 115, 677–682.

500 Scherl, M., Müller, T., & Kräutler, B. (2012). Chlorophyll catabolites in senescent leaves of the
 501 Lime Tree (*Tilia cordata*). *Chemistry & Biodiversity*, 9, 2605–2617.

502 Shemer, T. A., Harpaz-saad, S., Belausov, E., Lovat, N., Krokhin, O., Spicer, V., Standing, K.G.,
 503 Goldschmidt, E.E., Eyal, Y. (2008). Citrus chlorophyllase dynamics at ethylene-induced fruit
 504 color-break: A study of chlorophyllase expression, posttranslational processing kinetics, and
 505 in situ intracellular localization. *Plant Physiology*, 148, 108–118.

506 Suzuki, T., & Shioi, Y. (2002). Re-examination of Mg-dechelation reaction in the degradation of
 507 chlorophylls using chlorophyllin a as a substrate. *Photosynthesis Research*, 74, 217–223.

508 Vergara-Domínguez, H., Roca, M., & Gandul-Rojas, B. (2014). Thylakoid peroxidase activity
509 responsible for oxidized chlorophyll accumulation during ripening of olive fruits (*Olea*
510 *europaea* L.). *Food Research International*, 65, 247–254.

511 Vicentini, F., Hörtensteiner, S., Schellenberg, M., Thomas, H., & Matile, P. (1995). Chlorophyll
512 breakdown in senescent leaves identification of the biochemical lesion in a stay-green
513 genotype of *Festuca pratensis* Huds. *New Phytologist*, 129, 247–252.

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Table 1. Formation of *p*FCC in incubation of PaO-RCCR reaction according to source of PaO and RCCR fractions.

Source PaO	Source RCCR	Formation of <i>p</i> FCC	Figure
Rape	Rape	+++ (<i>p</i> FCC1)	2a
Pepper	Pepper	+++ (<i>p</i> FCC2)	2b
Olive fruit (cv. Hojiblanca)	Olive fruit (cv. Hojiblanca)	-	2c
Olive fruit (cv. Arbequina)	Olive fruit (cv. Arbequina)	-	2c
Olive fruit (cv. Hojiblanca/Arbequina)	Pepper	+ (<i>p</i> FCC2)	-
Pepper	Olive fruit (cv. Hojiblanca/Arbequina)	+ (<i>p</i> FCC2)	2d

Table 2.

PaO and RCCR activity (estimated as formation of *p*FCC) from *O. europea* L. fruits (cvs.Arbequina and Hojiblanca).

	Hojiblanca variety		Arbequina variety	
	PaO activity	RCCR activity	PaO activity	RCCR activity
<i>August</i>	-	-	-	-
<i>September</i>	-	-	-	-
<i>October</i>	-	-	-	0,77 ± 0,45
<i>November</i>	-	19,28 ± 1,90	-	24,32 ± 7,82
<i>December</i> ¹	6,35 ± 0,56	11,49 ± 0,31	90,73 ± 9,17	-
<i>December</i> ²	-	-	-	-

^aData were expressed as fluorescent unit/[mg chlorophylls x Kg dry weight⁻¹]. Values show means of two determinations ± SD. Notes: ¹Purple fruits; ²Black fruits.

Table 3. Nonfluorescent chlorophyll catabolites (NCCs) from ripe *O. europea* L. fruits (cvs. Arbequina and Hojiblanca).

Compound	Retention time (min)	Error (ppm)	SigmaFit value	Elemental composition	Exact mass (u) [M+H] ⁺	Area (mUA)
Arbequina variety						
Oe-NCC1 ^a	55,99	1,2	46,9	C ₃₄ H ₃₈ N ₄ O ₈	631,2755	263,9
Oe-NCC2 ^b	58,60	4,9	19,2	C ₃₅ H ₄₀ N ₄ O ₈	645,2887	320,2
Hojiblanca variety						
Oe-NCC2 ^b	59,00	4,8	21,0	C ₃₅ H ₄₀ N ₄ O ₈	645,2888	14424
Oe-NCC3 ^c	59,78	-4,5	49,7	C ₃₅ H ₄₀ N ₄ O ₇	629,2998	5755
Oe-NCC4 ^d	62,87	-2,4	46,9	C ₃₅ H ₄₂ N ₄ O ₆	615,3192	7369

Notes:

^aOe-NCC1 = [*Bn*-NCC3, (Müchlecker et al. 1996); *So*-NCC3, (Berghold et al. 2002); *At*-NCC2, (Pružinská et al. 2005)].

^bOe-NCC2 = [*So*-NCC4, (Berghold et al. 2002); *Cj*-NCC1, (Oberhuber et al. 2003); *Pc*-NCC2 and *Md*-NCC2 (Müller et al. 2007); *Sw*-NCC58, (Kraütler et al. 2010)].

^cOe-NCC3 = [*So*-NCC5, (Berghold et al. 2002); *Cj*-NCC2, (Oberhuber et al. 2003)].

^dOe-NCC4 = [*Bn*-NCC4, (Müchlecker et al. 1996); *At*-NCC5, (Pružinská et al. 2005)].

Figure 2. HPLC chromatograms after PaO-RCCR reaction showing pFCC formation. Detection by fluorescence (λ_{ex} 320nm/ λ_{em} 450 nm). Different chromatograms according to source of PaO and RCCR fractions: . (a) Fractions PaO and RCCR from rape; (b) Fractions PaO and RCCR from pepper; (c) Fractions PaO and RCCR from olive fruit; (d) Fraction PaO from pepper and fraction RCCR from olive fruits.

Figure 3. HPLC analysis of NCCs from olive fruits. Detection by absorbance at 320 nm. (a) Arbequina variety; (b) Hojiblanca variety. *Oe*: *Olea europaea* (L.)