COMPOSITION OF PIGMENTS AND COLOUR CHANGES IN GREEN TABLE OLIVES RELATED TO PROCESSING TYPE

Eva Ramírez, Beatriz Gandul-Rojas, Concepción Romero, Manuel Brenes,

Lourdes Gallardo-Guerrero*

Instituto de la Grasa (IG-CSIC), Avda. Padre García Tejero 4, 41012 Seville, Spain

*Corresponding author.

Tel.: +34 954690850; Fax: +34 954691262; E-mail address: lgallardo@ig.csic.es

Running title: Pigments and colour changes in green table olives

ABSTRACT

Brownish colourations in Natural green table olives (non-treated with alkali) make this product less attractive to consumers than Spanish-style green table olives (treated with alkali), which develop a more appreciated bright golden-yellow colour. These colour differences were studied in relation to changes in the composition of chlorophyll and carotenoid pigments, as well as polyphenolic compounds and polyphenol oxidase enzyme (PPO) activity. Natural green olives showed a different chlorophyll profile than Spanish-style. However, all the chlorophyll pigments formed in both processing types were Mg-free derivatives (mostly pheophytins) with similar colourations, ranging from grey to green brownish. In the carotenoid fraction no appreciable differences were found between both processing types. The fruit's brownish colour was mainly due to polymeric substances with a size of >1,000 daltons and polyphenolic nature, resulting from an enzymatic oxidation by PPO of the *o*-diphenolic compounds present in the fresh fruits.

Keywords: table olives; *o*-diphenol oxidation; chlorophyll pigments; carotenoids; polyphenol oxidase; colour.

1. Introduction

Table olives have been a component of the Mediterranean diet for centuries and their consumption is increasing worldwide because of their nutritional and palatable characteristics. Among the different types of commercial table olives, Spanish-style green olives are the most popular, whose processing consists of treating the fruits with a dilute NaOH solution to remove their bitterness, followed by washings with tap water and placing the olives in brine where spontaneous lactic acid fermentation takes place (Montaño, Sánchez, & de Castro, 1993). Nevertheless, there are other trade preparations of fermented green olives that involve the direct brining of olives without any alkaline treatment, which are known as Natural green olives, and are also highly appreciated by consumers in the Mediterranean region. For preparing both types of table olives the fruits are harvested with a green/yellow surface colour. However, after processing, the table olive colour is noticeably different for both Spanish-style and Natural green olives, with a much preferred golden-yellow colour in the first case, while in most of the cases of Natural green olives, the fruits turn their colour from green to brownish tones.

The colour changes that the fruits undergo during processing are due to the transformation of their pigments. At the early ripening stages of the olive fruit, from intense green to green-yellow, the colour of the olives is due to the presence of chlorophylls *a* and *b*, and the typical chloroplastic yellow carotenoids (Mínguez-Mosquera & Garrido-Fernández (1989). During the processing of table olives as Spanish-style, the chlorophylls degrade to several Mg-free derivatives (Mínguez-Mosquera & Gallardo-Guerrero, 1995; Mínguez-Mosquera, Gandul-Rojas, & Mínguez-Mosquera, 1994), with grey-brownish colours, while in the carotenoid fraction only those ones with 5,6-epoxide groups in their molecule are transformed to their corresponding derivatives with 5,8-furanoxide groups (Mínguez-Mosquera & Gallardo-

Guerrero, 1995; Mínguez-Mosquera & Gandul-Rojas, 1994), with lighter yellow colours than their precursors. As a consequence of all these pigment transformations, the Spanish-style table olives show the characteristic golden-yellow colour. Recently, Gallardo-Guerrero, Gandul-Rojas, Moreno-Baquero, López-López, Bautista-Gallego and Garrido-Fernández, (2013) have studied the changes in the chloroplastic pigments related to freshness during storage at different conditions and packing of cracked Aloreña table olives, which is a seasoned green table olive specialty. However, there is no knowledge about the pigment transformation during the processing of Natural green table olives and its relation to the final yellow/brown colour of the fruits. These brownish tones could be related to chlorophylls and carotenoid pigment transformations but also to the oxidation of polyphenolic compounds.

The phenolic compounds are responsible for the characteristic bitterness of the olive fruit and most of them are also involved in the colour changes in the olives. It is well know that the enzymatic and chemical oxidation of *o*-diphenolic compounds may form dark coloured compounds (Cilliers & Singleton, 1989; Zawistowski, Biliaderis, & Eskin, 1991). Sciancalepore and Lognone (1984) showed a direct correlation between the polyphenol oxidase (PPO) activity and the rate of browning of a crude homogenate of fruit in five Italian olive varieties. This browning has also been correlated with the oleuropein content in ten olive cultivars (Goupy, Fleuriet, Amiot, & Macheix, 1991). Also, the existence of a coordinate response between PPO and the concentration of total phenols in four Spanish olive varieties has been shown (Ortega-García & Peragón, 2009).

The main phenolic compound in olives is oleuropein, a bitter glucoside. The key step in the processing of table olives is the elimination of this bitter component to obtain a more palatable product. This process has to be more or less intense depending on the

variety of olive used, the ripening stage and the fruit format (whole or broken). Therefore, there is a broad range of processing styles. Oleuropein is hydrolyzed by the NaOH treatment during the Spanish-style green olive processing or by the acidic and enzymatic conditions in Natural green olives. In both cases, the *o*-diphenol hydroxytyrosol is released, which is a potential substrate for oxidative enzymes present in the fruits. Moreover, the PPO activity has been studied in depth in fresh olives to explain the browning reaction which results from mechanical injury during the olive harvesting (Ben-Shalom, Kahn, Harel, & Mayer, 1977; Sánchez, Romero, Ramírez, & Brenes, 2013; Segovia-Bravo, Jarén-Galán, García-García, & Garrido-Fernández, 2007). It has been hypothesized that the mechanism of the browning reaction in olives consists first of an enzymatic release of hydroxytyrosol from oleuropein due to the action of β-glucosidase and esterase enzymes. Then, this *o*-diphenol is oxidized by the PPO, forming brown compounds (Segovia-Bravo, Jarén-Galán, García-García, & Garrido-Fernández, 2009). However, it has been shown that oleuropein can be directly oxidized by PPO in green olives preserved in acidified brine during their debittering by the overpressure of oxygen (García, Romero, Medina, García, de Castro, & Brenes, 2008).

The present study was aimed at explaining the differences in colour between non-treated green table olives (Natural olives) and olives treated with alkali (Spanish-style), related to changes in the composition of either chlorophyll and carotenoid pigments or polyphenolic compounds and PPO activity, since brownish colourations in Natural green table olives make this product less attractive to consumers than Spanish-style green table olives. The study was carried out with Manzanilla and Hojiblanca olive varieties because they are found among the most prominent table olive varieties at the international trade level (Rejano, Montaño, Casado, Sánchez, & de Castro, 2010).

2. Materials and methods

2.1. Raw material

Fruits of the Manzanilla and Hojiblanca varieties (*Olea europaea* L.) in the ripening stage corresponding to the green-yellow colour on the surface were supplied by local farmers. The study was carried out first with the Manzanilla olives, in mid-September, and around one month later it was with the Hojiblanca olives. Manzanilla is an early olive variety, and reaches the proper ripening stage for processing as green table olives about 20-30 days sooner than Hojiblanca.

2.2. Olives processing

2.2.1. Green table olives

Olive fruits were processed at laboratory scale as Spanish-style and Natural green table olives (Garrido-Fernández, Fernández-Díaz, & Adams, 1997). For Spanish-style processing, olives were put into 3 L PVC vessels and covered with a 1.9% (w/v) NaOH solution during 7 h until the lye had penetrated two-thirds the way to the pit of them. Subsequently, fruits were washed with tap water for 14 h and then covered with a 11% (w/v) NaCl solution. At the same time, for the processing of Natural green olives, similar amounts of the fruits were also placed in PVC vessels and covered with a brine of 10% NaCl and 0.4% acetic acid. All PVC vessels contained 1.7 kg of olives and 1.2 L of liquid and a spontaneous anaerobic fermentation was carried out covering the surface of the brines with a floating cap. The experiments were run at ambient temperature (22–28 ºC) in duplicate during 6 months.

2.2.2. Aseptic brining of olives

In order to eliminate interferences from the activity of microorganisms and exogenous oxidative enzymes, olives of the Manzanilla variety were elaborated in aseptic conditions in accordance with Medina, Brenes, Romero, García, and de Castro (2007). The fruits were selected to remove those with blemishes, cuts, and insect damage. After washing thoroughly with tap water to remove impurities, half of the fruits were pasteurized at 90 ºC for 30 min to inactivate the oxidative enzymes and subsequently check the effect caused on the fruit brownish colourations. With that aim, all the olives, pasteurized and non-pasteurized fruits, were placed in a sodium hypochlorite solution (50 mg/L active chlorine) at 35 ºC for 2 min and then they were washed with sterilized water twice to remove chlorine. Subsequently, 190 g of fruits were introduced into autoclaved bottles (250 mL capacity) and covered with a 5% NaCl and 0.5% acetic acid sterile solution. These manipulations were carried out in a laminar flow cabinet. Finally, the bottles were sealed and stored at room temperature for a month. After this time, the bottles were opened and checked for microbial growth by visual appearance and plate counts, and microorganisms were not detected in any aseptic brine. Then, fruits were exposed to air during 24 h. All the experiment was carried out in duplicate.

2.3. Chemical parameters

The concentration of NaCl was analyzed by titration with a 0.1 N silver nitrate solution, using potassium chromate solution as indicator. The pH of storage solutions was measured in a Beckman model 45 pH-meter. Free acidity was measured by titration using a Metrohm 670 Titroprocessor (Herisau, Switzerland) up to pH 8.3 with 0.2 M NaOH and expressed as % (w/v) of lactic acid.

163

164 *2.4. Colour analyses*

165 Colourimetric measurements on olives were performed using a BYK-Gardner
166 Model 9000 Colour-view spectrophotometer, equipped with computer software to
167 calculate the CIE L* (lightness), a* (redness), b* (yellowness), C (chroma) and h (hue
168 angle) parameters by scanning the surface from 400 to 700 nm. The chroma was
169 obtained as $(a^2 + b^2)^{1/2}$ and the hue angle was calculated from arctg(b* / a*) (McLaren,
170 1980). Interference by stray light was minimized by covering samples with a box which
171 had a matt black interior. The data of each measurement were the average of 20 olives.

172 Olive juice was obtained from pitted olives as described elsewhere (Sánchez, de
173 Castro, Rejano, & Montaño, 2000) and its colour was measured by scanning solutions
174 in 1 mm path length quartz cells from 400 to 700 nm in a Cary 1E UV-vis
175 spectrophotometer (Varian, Mulgrave, Australia), which was equipped with a computer
176 software program (Varian) to calculate the CIELAB parameters. In order to remove
177 polymeric substances, the olive juice of Natural Hojiblanca olives preserved for 6
178 months in acidified brine was also filtered through a 10,000 and 1,000 daltons cut off
179 DIAFLO ultrafiltration membrane using a magnetically stirred 50-ml Amicon cell
180 operated under N_2 pressure (Amicon Corp, Danners, MA, USA), and the colour
181 measured as described above.

182

183 *2.5 Analysis of chloroplastic pigments*

184 Pigment extraction was performed from 10 g of olive sample taken from a
185 homogenized triturate, prepared from 15 to 20 pitted fruits. All procedures were
186 performed under dimmed green light to avoid any photooxidation of chlorophylls and
187 carotenoids. The method of Mínguez-Mosquera and Garrido-Fernández (1989), slightly

188 modified as previously described by Gandul-Rojas, Roca and Gallardo-Guerrero (2012),
189 was used. The technique is based on extraction with N,N-dimethylformamide (DMF)
190 saturated with $MgCO_3$ followed by the selective separation of components between
191 DMF and hexane. Hexane phase carried over lipids and carotenes, whereas DMF phase
192 retained chlorophylls and xanthophylls. β-carotene was directly quantified in the hexane
193 phase by absorbance measurement at 450 nm.

194 The pigments from DMF phase were later transferred to ethyl ether,
195 concentrated to dryness, and the dry residue dissolved in 1.5 mL acetone for pigment
196 analysis by HPLC. Pigment separation was carried out using a stainless steel column
197 (20 x 0.46 cm i.d.), packed with a multifunctional end-capped deactivated octadecylsilyl
198 (C18) Mediterranea™ Sea18, 3 μm particle size (Teknokroma, Barcelona, Spain). The
199 column was protected by precolumn (1 x 0.4 cm i.d.) packed with the same material.
200 Solutions of pigment extract were centrifuged 10 min at 13000 g prior to injection (20
201 μL) into the chromatograph (HP 1100 Hewlett-Packard, Palo Alto, CA) fitted with an
202 automatic injector and diode array detector). Separation was performed using an elution
203 gradient (flow rate 1.250 mL min^{-1}) with the mobile phases (A) water/ion pair
204 reagent/methanol (1/1/8, v/v/v) and (B) methanol/acetone (1/1, v/v). The ion pair
205 reagent was 0.05 M tetrabutylammonium and 1 M ammonium acetate in water. The
206 gradient scheme was a modification of that of Mínguez-Mosquera, Gandul-Rojas,
207 Montaño-Asquerino and Garrido-Fernández (1991), and briefly was initially 75% A and
208 25% B, then changed to 25% A in 8 min, isocratic 2 min, changed to 19% A in 1 min,
209 then to 14% A in 2 min, 11% A in other 2 min, and 10% A in 3 min. Isocratic 3 min,
210 and later changed to 8% A in 1 min, and 6% A in 0.5 min. Then 100% B in 0.5 min,
211 isocratic 12 min, and returned to initial conditions in 5 min. Spectrophotometric
212 detection of pigments was performed at 410, 430, 450 and 666 nm. The on-line UV-Vis

spectra were recorded from 350 to 800 nm with the photodiode–array detector. Data were collected and processed with a LC HP ChemStation (Rev.A.05.04). Pigments were identified by co-chromatography with the corresponding standard and from the spectral characteristics as has been described in detail elsewhere (Mínguez-Mosquera et al., 1991; Mínguez-Mosquera & Gandul-Rojas, 1995; Aparicio-Ruiz, Riedl, & Schwartz, 2011). Pigments were quantified using external standard calibration curves prepared with purified standards of each pigment. The analyses for fresh fruit were performed in triplicate. In the case of processed olives, analyses were run in quadruplicate (samples processed in duplicated and each one analyzed subsequently in duplicated)

2.6. Analysis of phenolic compounds

The extraction of phenolic compounds from the olive skin was based on the methodology proposed elsewhere (Sánchez et al., 2013). Around 0.1 g of olive skin from pasteurized and non-pasteurized fruits were mixed with 0.5 mL dimethyl sulfoxide (DMSO), vortexed for 1 min and sonicated for 5 min. After 30 min resting contact, the mixture was centrifuged at 6000*g* for 5 min (22 ºC), and 0.25 mL of the supernatant was diluted with 0.5 mL of DMSO and 0.25 mL of 0.2 mM syringic acid in DMSO (internal standard). The analysis of phenolic compounds in brine was carried out mixing 0.25 mL of brine, 0.25 mL of internal standard (2 mM syringic acid in water), and 0.5 mL of deionized water.

All samples were filtered through a 0.22 μm pore size nylon filter and an aliquot (20 μL) was injected into the chromatograph. The chromatographic system consisted of a Waters 717 plus autosampler, a Waters 600 E pump, a Waters column heater module, and a Waters 996 photodiode array detector operated with Empower software (Waters Inc.). A 25 cm x 4.6 mm i.d., 5 μm, Spherisorb ODS-2 (Waters Inc.) column, a flow rate

of 1 mL/min and a temperature of 35 ºC were used in all experiments. Separation was achieved by gradient elution using (A) water (pH 2.5 adjusted with 0.15% phosphoric acid) and (B) methanol. Initial composition was 90% A and 10% B. The concentration of B was increased to 30% over 10 min and was maintained for 20 min. Subsequently, B was raised to 40% over 10 min, maintained for 5 min, and then increased to 50%. Finally, B was increased to 60%, 70%, and 100% in 5-min periods. Initial conditions were reached in 10 min. Chromatograms were recorded at 280 nm (Medina et al., 2007). The evaluation of each compound was performed using a regression curve with the corresponding standard. Hydroxytyrosol-1-glucoside and caffeoyl ester were quantified using the response factors of hydroxytyrosol and caffeic acid, respectively. Analyses of the olive skin were run in quadruplicate. In the case of brine analyses were performed in duplicated

2.7. Analysis of polyphenol oxidase activity

The enzyme extraction was carried out from a protein precipitate as described elsewhere (Sciancalepore & Longone, 1984). Acetone powders were obtained from 50 g of olive pulp homogenized with 100 mL of cold acetone (−30ºC) containing 2.5 g of polyethylene glycol. The residue was re-extracted three times with 100 mL of cold acetone, obtaining a white powder that was dried overnight at room temperature to remove residual acetone. The acetone powder (0.5 g) was suspended in 20 mL of 0.1 M phosphate buffer, containing 1 M KCl and the pH was adjusted at 6.2 units with NaOH. The suspension was stirred at 4 ºC for 30 min and then centrifuged at 15,550*g* for 20 min at 4 ºC. The pellet was discarded and the supernatant divided in two aliquots; one was used as the active crude enzymatic extract, and the other was boiled for 30 min to obtain the denatured enzymatic extract.

263 The PPO activity was determined spectrophotometrically by using a Cary 1E
264 UV-vis spectrophotometer as described elsewhere (Hornero-Méndez, Gallardo-
265 Guerrero, Jarén-Galán, & Mínguez-Mosquera, 2002). All measurements of PPO activity
266 were carried out with 4-methylcatechol as substrate by measuring the change in
267 absorbance at 410 nm at 25 ºC for 10 min at intervals of 5 s. The incubation mixture
268 contained 0.5 mL of enzyme preparation and 2.5 mL of 0.1 M sodium citrate buffer at
269 pH 5 containing 0.02 M of substrate. The assay mixture with the denatured enzymatic
270 extract served as the control. One unit of enzymatic activity was defined as the amount
271 of the enzyme giving, under the above-mentioned conditions, a change in absorbance of
272 0.05 unit AU/min (e.a.u.). Data were expressed as e.a.u./mL of enzymatic extract. All
273 reactions were carried out in duplicate.
274
275 *2.8. Chemicals and standards*
276 Tetrabutylammonium acetate and ammonium acetate were supplied by Fluka
277 (Zwijndrecht, The Netherlands). Solvents used for chromatography were HPLC grade
278 (Prolabo, VWR International Eurolab, Barcelona, Spain). Analysis grade solvents were
279 supplied by Scharlau (Microdur, Sevilla, Spain). The deionized water was obtained
280 from a Milli-Q® 50 system (Millipore Corporation, Milford, MA). For all purposes,
281 analytical grade (American Chemical Society) reagents were used (Merck, Madrid,
282 Spain).
283 Standards of chlorophylls *a* and *b* and β-carotene were supplied by Sigma
284 Chemical Co. (St. Louis, MO). All other chlorophyll derivatives were prepared in the
285 laboratory from the related chlorophyll (*a* or *b*) extracted from a pigment extract of
286 fresh spinach as is described in Roca, Gallardo-Guerrero, Mínguez-Mosquera and
287 Gandul-Rojas (2010). β-cryptoxanthin was obtained from papaya, while lutein,

violaxanthin, neoxanthin and antheraxanthin were obtained from a pigment extract of fresh spinach saponified and separated by TLC. Luteoxanthin and auroxanthin were prepared from violaxanthin by acidification and subsequent separation by TLC. By the same way, neochrome and mutatoxanthin were prepared from neoxanthin and antheraxanthin, respectively (Mínguez-Mosquera et al., 1991). Hydroxytyrosol, oleuropein, and verbascoside were purchased from Extrasynthese S.A. (Lyon Nord, Genay, France), caffeic acid and 4-methylcatechol from Sigma Chemical Co. (St. Louis, MO). The dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (HyEDA) was obtained by HPLC preparative system as described elsewhere (Brenes, Hidalgo, García, Rios, García, Zamora, & Garrido, 2000).

2.9. Statistical analysis

Data were expressed as mean values ± SD. Statistica software version 7.0 was used for data processing (Statistica for Windows, Tulsa, OK, USA). Comparison between mean variables was made by the Duncan's multiple range tests and the differences considered significant when $p < 0.05$.

3. Results and discussion

3.1. Physicochemical parameters

The chemical parameters of olive brines from the fruits of the Manzanilla and Hojiblanca varieties elaborated as Spanish-style and Natural green olives for 6 months of storage at room temperature are summarize in Table 1. The concentration of salt was something different among treatments, but in the range of 5-6.8% in all cases, which is very common for these types of green table olives (Garrido-Fernández et al., 1997).

Regarding pH and free acidity, big differences were found between olive varieties but not between processes. The brines of the Manzanilla variety had higher values for free acidity and lower pH than those of the Hojiblanca, indicating a clear lactic fermentation for both Spanish-style and Natural green olives of the former variety. Spontaneous lactic acid fermentation currently takes place in the brines of Spanish-style green olives and gives rise to an increase in acidity of up to 0.5-1% thereby decreasing the pH below 4.5 units (Medina, Romero, de Castro, Brenes, & García, 2008). It can be observed that a good fermentation was achieved in the case of Manzanilla olives but not for the Hojiblanca fruits. The development of lactic acid fermentation in the brines of olives non-treated with alkali is more difficult because of the presence of inhibitors, although it also depends on olive variety, salt concentration and other variables (Medina, García, Romero, de Castro, & Brenes, 2009). In our experiments, Natural green Manzanilla olives allowed for lactic acid fermentation but not the Hojiblanca fruits (Table 1).

The relationship between the pH values and the colour in food is well known (Montaño, Rejano, & Sánchez, 1986). A light colour is associated with a low pH value. Visual assessment and colourimetric measurements in our experiments showed a different colour of olives with a rather similar pH (Table 1), although it was variety dependent. After 6 months of fermentation, the Natural green olives of both varieties showed lower lightness values than the Spanish-style green olives, a difference that was significant for the Manzanilla variety. Likewise, Natural green olives had higher a* values than the Spanish-style green olives, which meant that the former had reddish tones. Also, the lower h values of those olives indicated brownish colouration. For the Manzanilla variety, b* and C parameters were also significantly lower in Natural green olives than in the Spanish-style olive processing. Precisely, consumers appreciate the latter olives because of their bright golden-yellow colour, among other organoleptic

characteristics, while the brown tones developed in Natural green olives make this product less attractive.

3.2. Chloroplastic pigments

Fig. 1 shows the chloroplastic pigment composition found in the fresh fruit of the Manzanilla and Hojiblanca olive varieties, and its changes during processing as Spanish-style or Natural green olives. The mechanism of degradation of the chlorophyll and carotenoid pigments is illustrated in Fig. 2A. The chloroplastic pigment contents of the olive fruits were found to be similar in both varieties, although previous studies found that the Hojiblanca variety had a higher quantity of pigments than Manzanilla at similar ripening stages (Mínguez-Mosquera & Garrido-Fernández, 1989). For an easier interpretation of the results, the allomerized derivatives (AD) of chlorophylls that were detected initially in the fresh fruit (13^2-OH chlorophylls and 15^1-lactone chlorophylls) were presented as the sum of them, both for the chlorophylls of the series *a* and for the series *b*. In general, AD refers to those compounds resulting from the oxidation at C-13^2 in the isocyclic ring (V) of the chlorophyll pigment by molecular triplet oxygen ($^3O_2$) (Hynninen, 1991). Similarly, the epimer isomers of the chlorophyll pigments and the *cis* isomers of carotenoids were quantified with their respective precursors. β-cryptoxanthin is not included as it was found in very small quantities and not detected in all samplings.

The pigment transformations that took place during Spanish-style table olive processing or Natural green olives followed a similar trend for the Manzanilla and Hojiblanca varieties. For the Spanish-style processing, the first sampling was made 3 days after the alkaline treatment. At this time, apart from the chlorophyll pigments initially present in the fresh fruit (chlorophylls *a* and *b*, and some AD and pheophytin *a*), new derivatives were formed, with those with Mg in the porphyrin ring of the

363 molecule but with structure chlorin- (series *a*), and rhodin- (series *b*) type (Mg-15^2-Me-
364 phytol-chlorin e_6 ester and Mg-15^2-Me-phytol-rhodin g_7 ester) (Fig. 2B) being the most
365 prominent. They are also named AD because an oxidation at C-13^2 causes the solvolysis
366 of the isocyclic ring (V), but they will be treated separately throughout the study as
367 chlorophyll derivatives with chlorin or rhodin-type structure. These compounds were
368 also found during the processing of Spanish-style table olives of the Gordal variety and
369 are formed by the alkaline oxidation of the chlorophylls due to the initial treatment of
370 fruits with NaOH (Mínguez-Mosquera & Gallardo-Guerrero, 1995).

371 In this initial sampling, the Mg-free derivative with chlorin-type structure and
372 pyropheophytin *a* had already begun to be detected, as well as an increase in the AD of
373 the chlorophylls *a* and *b*. In the carotenoid fraction no changes in the qualitative
374 composition were found, so that lutein, β-carotene, violaxanthin, neoxanthin,
375 antheraxanthin and β-cryptoxanthin remained present, and only some quantitative
376 variations in the content of minor xanthophylls were observed. As these carotenoids are
377 stable to alkali (Schiedt & Liaaen-Jensen, 1995), this fact could simply be due to the
378 variability of the raw material.

379 Subsequently, after two months of processing as Spanish-style table olives, a
380 great number of new compounds were found, all of them being Mg-free chlorophyll
381 derivatives (Fig. 1B) and carotenoids with 5,8-epoxide groups (Fig. 1C), which were
382 formed due to the acid pH resulting from the lactic fermentation (Fig. 2B and C). In the
383 first group of pigments the acidic pH leads to the replacement of Mg^{2+} by $2H^+$ in the
384 porphyrin ring of the chlorophyll molecule, which is known as the pheophytinization
385 reaction. In the case of carotenoids with a 5,6-epoxy group in their structure, the acid
386 pH causes the reorganization of the mentioned group to 5,8-furanoid. The main
387 pigments formed were pheophytins *a* and *b*, although the Mg-free derivatives with

388 chlorin or rhodin-type structure and pyropheophytin *a* were also considerable (Fig. 1B).
389 In addition, pyropheophytin *b*, AD of pheophytin a, and dephytylated derivatives
390 (pheophorbides *a* and *b*, and pyropheophorbide *a*) were detected. Similar pigment
391 changes were found until the end of the process (180 days), with the chlorophylls *a* and
392 *b* of the fresh fruit and any other derivative with Mg that were originated during the
393 alkaline treatment of the fruits disappearing almost completely.

394 Although qualitative changes in chloroplastic pigments were essentially the
395 same in both varieties, a significant difference in the percentage composition of the
396 chlorophyll derivatives with chlorin and rhodin-type structure was found. The entire
397 amount of Mg-free compounds with this type of structure meant 8.7% of the total
398 chlorophyll pigments in Manzanilla olives and 15.5% in the case of Hojiblanca at the
399 end of processing as Spanish style green table olives. Their precursor pigments were
400 those initially formed as a result of the alkaline treatment) that were later modified to
401 the Mg-free derivatives due to the acidic pH (Fig. 2A). In this experiment, the alkaline
402 treatment conditions were the same (time and NaOH concentration) for both varieties of
403 olives, thus the differences must be attributed to the morphological characteristics of
404 each variety.

405 When olive fruits were processed as Natural green table olives, the main
406 pigment transformations that took place were those due to the acid pH originated by the
407 fermentation process, and no chlorophyll derivative with chlorin or rhodin-type
408 structure was detected (Fig. 1). Therefore, Spanish-style and Natural green olives
409 showed a different pigment profile, with a smaller number of chlorophyll derivatives
410 being formed in the latter case (Fig. 2A). However, in both cases, all chlorophyll
411 pigments present in the olives at the end of the processes were Mg-free derivatives. The
412 presence of Mg in the chromophoric group of chlorophyll compounds is responsible for

413 the green colourations of these pigments; but when the Mg^{2+} is substituted by $2H^{+}$, the
414 green colourations change to tones ranging from grey (series *a*) to green brownish
415 (series *b*). Therefore, all the Mg-free chlorophyll derivatives show similar colourations
416 even if other structural changes have occurred in the molecule such as the oxidative
417 opening of the isocyclic ring.

418 In the carotenoid fraction no appreciable differences were found between either
419 processing types, although there were slight differences between varieties. In the
420 fraction of acid-sensitive xanthophylls it is worth noting that neoxanthin transformation
421 to neochrome was faster in the Manzanilla variety than in Hojiblanca. In the latter, some
422 neoxanthin remained after 60 days of olive processing both as Spanish-style or Natural
423 green (Fig. 1C). This may be related to a slower fermentation (a higher pH) occurring in
424 the Hojiblanca processes (Table 1).

425 For comparing the chloroplastic pigment contribution to the final colour of table
426 olives processed as Spanish-style or Natural green olives, both for Manzanilla and
427 Hojiblanca varieties, the chlorophyll pigments were grouped as the percentage of those
428 with bright green colours (chlorophylls with Mg in their structure), and those with
429 brownish-grey-tones (Mg-free chlorophyll derivatives) (Fig. 3). The carotenoid group
430 was not included because their changes were similar for both processing types. A
431 similar composition was found for both processes at the same sampling time for each
432 olive variety. In the case of the Manzanilla olives the replacement of Mg^{2+} by $2H^{+}$ in the
433 porphyrin ring of the chlorophyll molecule was almost complete after 2 months of
434 processing as Spanish-style or Natural green olives, reaching the corresponding fraction
435 values of 98% and 95%, respectively, of the total chlorophyll pigments at the end of the
436 processes. In the Hojiblanca olives, the pheophytinization reaction was slower and after
437 two months of the olives processing, around 77% of the chlorophyll pigments were Mg-

438 free derivatives, while 23% remained with the Mg^{2+} ion in their structure both in olives
439 processed as Spanish-style or Natural green. This reaction progressed with time and the
440 Mg-free derivatives became 93% in the first case and 88% in the second, with a higher
441 proportion of Mg-chlorophyll compounds remaining in Hojiblanca than in Manzanilla
442 olives. The lower transformation of the chlorophyll pigments in Hojiblanca olives for
443 both types of processes must be explained by the differences commented in section 3.1,
444 because of the free acidity and pH values between both varieties, just as noted for the
445 5,6-epoxy-xanthophylls.

446 The percentage values of each distinguished pigment group resulted in some
447 differences between the Spanish-style and Natural green olives for both the Manzanilla
448 and Hojiblanca varieties. However, although the differences were statistically
449 significant ($p < 0.05$), they did not justify the distinct colour that show the Spanish-style
450 and Natural green table olives. Precisely the latter, which is more brownish (Table 1),
451 retained a higher percentage of green pigments (chlorophylls with Mg) in both olive
452 varieties, thus the brown colour should be due to other fruit components.

453

454 *3.3. Polyphenolic compounds and PPO activity.*

455 A brown colour in olives can also be caused by the enzymatic oxidation of *o*-
456 diphenols. Sciancalepore and Longone (1984) found a positive correlation between the
457 browning rate of fresh fruit and the activity of its own PPO enzyme. Fig. 4 shows the
458 concentration of total *o*-diphenols of the different solutions generated during the
459 processing of Spanish-style and Natural green olives. The NaOH treatment gave rise to
460 a rapid diffusion of polyphenols from the olives to the alkaline solution (lye), wash
461 water and brines, which is equilibrated after 15 days of fermentation, as previously
462 reported (Medina et al, 2008). By contrast, the low permeability of the olive flesh

originated a slow diffusion of these substances in the olives non-treated with alkali (Natural green olives) and less total content of *o*-diphenols was found in the brine until 60 days of fermentation. However, a rather similar concentration was reached for both types of olives after 6 months of fermentation, regardless of the variety.

Polyphenols are currently found in food as simple phenols but also form part of the dark polymerized substances. The juices of Spanish-style and Natural green olives fermented for 6 months were filtered through 10,000 and 1000 daltons pore size, and the absorbance spectra were recorded between 400 and 700 nm (presented in Supplementary material). The absorbance values of the Natural green olive juice were higher than those of Spanish-style; therefore the former juice was darker than the latter. In addition, the colour of both juices decreased when they were passed through 10,000 daltons pore size, and it was particularly reduced with 1,000 daltons pore size where the absorbance at 400 nm was reduced more than 50%. These results meant that the colour of these juices was mainly due to polymeric substances with size larger than 1,000 daltons which could have a polyphenol nature as has been previously reported for the brines of Spanish-style green olives (Brenes, García, & Garrido, 1988).

All these data indicated that the browning observed in Natural green olives was probably the result of an enzymatic or chemical oxidation of the *o*-diphenolic compounds present in the fruits of both varieties. Subsequently, we explored the suspected involvement of PPO in this phenomenon. The PPO activity of the Hojiblanca fresh fruit was 0.872 ± 0.028 e.a.u./mL of enzymatic extract, and this activity was not detected in those fruits treated with NaOH which probably inactivated the enzyme. By contrast, after 15 days of storage, the Natural green olives had a PPO activity of 0.128 ± 0.038 e.a.u./mL of enzymatic extract. However, it was not detected after one month of

storage although the enzymatic oxidation of Natural green olives preserved for 6 months has been reported for the debittering of this fruit (García et al., 2008).

A new experiment was undertaken to clarify the origin of the brown polymers formed during the storage of Natural green olives. The fruits were stored under aseptic conditions to avoid the effect of any microorganism interference. Half of the olives were pasteurized (90 ºC for 30 min) to denature the oxidase enzymes, and then all fruits were put in a 5% NaCl solution. The fresh fruits had PPO activity of 5.822 ± 0.037 e.a.u./mL of enzymatic extract, while no oxidase activity was detected in the pasteurized fruits. After one month of storage, the aseptic bottles were opened and the colour parameters of the skin were immediately evaluated. Subsequently, the olives were exposed to air for 24 hours and the colour was measured again. Statistical differences between the colour parameters at 0 and 24 hours of non-pasteurized olives were found (Table 2). After the exposition of olives to air for 24 h, a considerable decrease in L* and hue angle parameters was observed, indicating a severe browning of the olive surface. The reddish parameter (a*) did not change drastically but the yellowish one (b*) presented a large decrease. At time zero, the colour parameters were slightly higher in pasteurized olives with respect to the others, except for the parameter a* which showed a significantly lower value. This result was associated with the pheophytinization reaction occurring in the chlorophyll compounds during food heat treatments. It must be noticed that the colour parameters of the pasteurized olives slightly modified only the b* value during the experiment and, consequently, chroma parameter showed a low decrease. Therefore, this browning effect on olives must be associated with enzymatic reactions.

Moreover, the phenolic compounds in the olive skin were determined (Table 2). A significant decrease in the *o*-diphenols hydroxytyrosol, verbascoside and HyEDA concentrations in non-pasteurized fruits was detected after 24 h exposure to air, and was

not observed for the glucoside oleuropein, which is the main polyphenol in olives and has also an *o*-diphenol structure. As expected, the concentration of phenolic compounds remained almost constant during the exposure of pasteurized olives to air for 24 h due to the absence of oxidative enzymes in these fruits. The pasteurized olives showed higher amounts of oleuropein than non-pasteurized and the former did not contain the *o*-diphenols HyEDA, these phenomena have been previously reported by other authors (Medina et al., 2009).

4. Conclusion

As processing of Natural green olives does not include any alkaline treatment, the main difference found in the chloroplastic pigment composition, regarding Spanish-style table olives, was the absence of chlorophyll derivatives with a chlorin or rhodin-type structure. However, this result could not explain the different colour shown by olives processed by one or another style since all the chlorophyll pigments formed were Mg-free derivatives (mostly pheophytins in both cases), which have similar colourations, ranging from grey to green brownish. The results of the present study indicate that the browning observed in Natural green olives was mainly due to compounds of a polyphenolic nature, resulting from an enzymatic oxidation by the PPO of the *o*-diphenolic compounds present in the fresh fruits. In the case of Spanish-style table olives, the colour was due to chloroplastic pigments because the PPO enzyme was probably inactivated by the initial alkaline treatment of the process which prevented the oxidation of the *o*-diphenolic compounds in the fruits.

Acknowledgements

This work was supported by the "Comisión Interministerial de Ciencia y Tecnología (CICYT-EU Spanish Government) under projects AGL-2009-07512 and AGL 2012-39714/ALI, and Junta de Andalucía through financial support to groups AGR 148-2011 and AGR-125, partially financed by European regional development funds. ER thanks MINECO for her FPI fellowship. We thank Sergio Alcañiz for technical assistance. BGR and LGG are members of the IBERCAROT Network, funded by CYTED (ref. 112RT0445).

References

Aparicio-Ruiz, R., Riedl, K. M., & Schwartz, S. J. (2011). Identification and quantification of metallo-chlorophyll complexes in bright green table olives by high-performance liquid chromatography-mass spectrometry quadrupole/time-of-flight. *Journal of Agricultural and Food Chemistry, 59,* 11100-11108.

Ben-Shalom, N., Kahn, V., Harel, E., & Mayer, A. M. (1977). Catechol oxidase from green olives: properties and partial purification. *Phytochemistry, 16,* 1153–1158.

Brenes, M., García, P., & Garrido, A. (1988). Regeneration of Spanish style green table olive brines by ultrafiltration. *Journal of Food Science, 53,* 1733-1736.

Brenes, M., Hidalgo, F. J., García, A., Rios, J. J., García, P., Zamora, R., & Garrido, A. (2000). Pinoresinol and 1-acetoxypinoresinol, two new phenolic compounds identified in olive oil. *Journal of the American Chemical Society, 77,* 715–720.

Cilliers, J. J. L., & Singleton, V. L. (1989). Nonenzymic autoxidative phenolic browning reactions in a caffeic acid model system. *Journal of Agricultural and Food Chemistry, 37,* 890-896.

561 Gallardo-Guerrero, L., Gandul-Rojas, B., Moreno-Baquero, J. M., López-López, A.,
562 Bautista-Gallego, J., & Garrido-Fernández, A. (2013). Pigment,
563 physicochemical, and microbiological changes related to the freshness of
564 cracked table olives. *Journal of Agricultural and Food Chemistry, 61*, 3737-
565 3747.
566 Gandul-Rojas, B., Roca, M., & Gallardo-Guerrero, L. (2012). Detection of the colour
567 adulteration of green table olives with copper chlorophyllin complexes (E-141ii
568 colourant). *LWT Food Science and Technology, 46*, 311-318.
569 García, A., Romero, C., Medina, E., García, P., de Castro, A., & Brenes, M. (2008).
570 Debittering of olives by polyphenol oxidation. *Journal of Agricultural and Food*
571 *Chemistry, 56*, 11862–11867.
572 Garrido-Fernández, A., Fernández-Díez, M. J., & Adams, M. R. (1997). *Table olives:*
573 *Production and processing*. London: Chapman and Hall.
574 Goupy, P., Fleuriet, A., Amiot, M. J., & Macheix, J. J. (1991). Enzymatic browning,
575 oleuropein content, and diphenol oxidase activity in olive cultivars (*Olea*
576 *europaea* L.). *Journal of Agricultural and Food Chemistry, 39*, 92-95.
577 Hornero-Méndez, D., Gallardo-Guerrero, L., Jarén-Galán, M., & Mínguez-Mosquera,
578 M. I. (2002). Differences in the activity of superoxide dismutase, polyphenol
579 oxidase and Cu-Zn content in the fruits of Gordal and Manzanilla olive varieties.
580 *Zeitschrift fur Naturforschung, 57*, 113-120.
581 Hynninen, P. V. (1991). Chemistry of chlorophylls: modifications. In H. Scheer (Ed.),
582 *Chlorophylls*, (pp. 145-209). Boca Raton, FL: CRC Press.
583 McLaren, K. (1980). Food colourimetry. In J. Walford (Ed.), *Developments in food*
584 *colours, Vol. I*, (pp. 27-45). London: Applied Science Publishers.

585 Medina, E., Brenes, M., Romero, C., García, A., & de Castro, A. (2007). Main
586 antimicrobial compounds in table olives. *Journal of Agricultural and Food*
587 *Chemistry, 55*, 9817–9823.
588 Medina, E., García, A., Romero, C., de Castro, A., & Brenes, M. (2009). Study of the
589 anti-lactic bacteria compounds in table olives. *International Journal of Food*
590 *Science and Technology, 44*, 1286-1291.
591 Medina, E., Romero, C., de Castro, A., Brenes, M., & García, A. (2008). Inhibitors of
592 lactic acid fermentation in Spanish-style green olive brines of the Manzanilla
593 variety. *Food Chemistry, 110*, 932–937.
594 Mínguez-Mosquera, M. I., & Gallardo-Guerrero, L. (1995). Anomalous transformation
595 of chloroplastic pigments in Gordal variety olives during processing for table
596 olives. *Journal of Food Protection, 58*, 1241-1248.
597 Mínguez-Mosquera, M. I., & Gandul-Rojas, B. (1994). Mechanism and kinetics of
598 carotenoid degradation during the processing of green table olives. *Journal of*
599 *Agricultural and Food Chemistry, 42*, 1551-1554.
600 Mínguez-Mosquera, M. I., & Gandul-Rojas, B. (1995). High-performance liquid
601 chromatographic study of alkaline treatment of chlorophyll. *Journal of*
602 *Chromatography, 690*, 161-176.
603 Mínguez-Mosquera, M. I., & Garrido-Fernández, J. (1989). Chlorophyll and carotenoid
604 presence in olive fruit *(Olea europaea*). *Journal of Agricultural and Food Chemistry,*
605 *37*, 1-7.
606 Mínguez-Mosquera, M. I., Gandul-Rojas, B., & Mínguez-Mosquera, J. (1994).
607 Mechanism and kinetics of the degradation of chlorophylls during the processing
608 of green table olives. *Journal of Agricultural and Food Chemistry, 42*, 1089-
609 1095.

610 Mínguez-Mosquera, M. I., Gandul-Rojas, B., Montaño-Asquerino, A., & Garrido-
611 Fernández, J. (1991). Determination of chlorophylls and carotenoids by high-
612 performance liquid chromatography during olive lactic fermentation. *Journal of*
613 *Chromatography, 585,* 259-266.
614 Montaño, A., Rejano, L., & Sánchez, A H. (1986). Proceso rápido de elaboración de
615 aceitunas verdes aderezadas. *Grasas y Aceites, 37,* 141-147.
616 Montaño, A., Sánchez, A. H., & de Castro, A. (1993). Controlled fermentation of
617 Spanish-type green olives. *Journal of Food Science, 58,* 842-844.
618 Ortega-García, F., & Peragón, J. (2009). Phenylalanine ammonia-lyase, polyphenol
619 oxidase, and phenol concentration in fruits of *Olea europaea* L. cv. Picual,
620 Verdial, Arbequina, and Frantoio during ripening. *Journal of Agricultural and*
621 *Food Chemistry, 57,* 10331-10340.
622 Rejano, L., Montaño, A., Casado, F. J., Sánchez, A. H., & de Castro A. (2010). Table
623 olives: varieties and variations. In: V. R. Preedy, & R. R. Watson (Eds), *Olives*
624 *and olive oil in health and disease prevention* (pp. 5-15). Oxford: Academic
625 Press.
626 Roca, M., Gallardo-Guerrero, L., Mínguez-Mosquera, M. I., & Gandul-Rojas, B.
627 (2010). Control of olive oil adulteration with copper-chlorophyll derivatives.
628 *Journal of Agricultural and Food Chemistry, 58,* 51-56.
629 Sánchez, A. H., de Castro, A., Rejano, L., & Montaño, A. (2000). Comparative study on
630 chemical changes in olive juice and brine during green olive fermentation.
631 *Journal of Agricultural and Food Chemistry, 48,* 5975-5980.
632 Sánchez, A. H., Romero, C., Ramírez, E., & Brenes, M. (2013). Storage of
633 mechanically harvested Manzanilla olives under controlled atmospheres.
634 *Postharvest Biology and Technology, 81,* 60-65.

635 Schiedt, H., & Liaaen-Jensen, S. (1995). Isolation and analysis. In G. Britton, S. Liaaen-
636 Jensen, & H. Pfander (Eds.), *Carotenoids. Volume 1A: Isolation and analysis* (pp.
637 81-108). Basel, Switzerland: Birkhäuser Verlag.
638 Sciancalepore, V., & Longone, V. (1984). Polyphenol oxidase activity and browning in
639 green olives. *Journal of Agricultural and Food Chemistry*, *32*, 320-321.
640 Segovia-Bravo, K., Jarén-Galán, M., García-García, P., & Garrido-Fernández, A.
641 (2007). Characterization of polyphenol oxidase from the Manzanilla cultivar
642 (*Olea europea pomiformis*) and prevention of browning reactions in bruised
643 olive fruits. *Journal of Agricultural and Food Chemistry*, *55*, 6515–6520.
644 Segovia-Bravo, K., Jarén-Galán, M., García-García, P., & Garrido-Fernández, A.
645 (2009). Browning reactions in olives: mechanism and polyphenols involved.
646 *Food Chemistry*, *114*, 1380-1385.
647 Zawistowski, J., Biliaderis, C. G., & Eskin, N. A. M. (1991). Polyphenol oxidase. In
648 D.S. Robinson, & N. A. M. Eskin (Eds.), *Oxidative enzymes in foods* (pp 217-
649 273). London: Elsevier Applied Science.
650

651 **Figure Captions**

652 **Fig. 1.** Changes in concentrations (μmol kg^{-1}) of chlorophyll and carotenoid pigments
653 during the processing of Manzanilla and Hojiblanca olive varieties as Spanish-style or
654 Natural green table olives. (FF: Fresh fruit). (A) chlorophyll compounds with Mg in
655 their structure (solid line for series *a* and dotted line for series *b*): chlorophyll (□),
656 allomerized derivatives (AD) (Δ), Mg-15^2-Me-phytol-chlorin e_6 (or -rhodin g_7) ester
657 (○); (B) Mg-free chlorophyll derivatives (solid line for series *a* and dotted line for series
658 *b*): pheophytin (□), AD (Δ), 15^2-Me-phytol-chlorin e_6 (or -rhodin g_7) ester (○),
659 pyropheophytin (◇), pheophorbide (✳), pyropheophorbide (+); C) carotenoid pigments:
660 lutein (■), β-carotene (●), violaxanthin (▲), neoxanthin (◆), antheraxanthin (✳),
661 luteoxanthin (Δ, with dotted line), auroxanthin (Δ, with solid line), neochrome (◇),
662 mutatoxanthin (+).

663

664 **Fig. 2.** (A) Mechanism of degradation of the chlorophyll and carotenoid pigments
665 during Spanish-style or Natural green table olive processing. Pigments included into a
666 rectangle with dashed line are exclusive of the Spanish-style, and the rest, included into
667 a rectangle with continuous line are common for both processing types. Pigments in:
668 transparent rectangles are of green colourations, dark grey rectangles are of grey-
669 brownish colourations, and light grey rectangles are of yellow colourations. (B)
670 Structural comparison between chlorophylls *a* and *b*, their Mg-free derivatives, and their
671 derivatives with an open isocyclic ring (V) (chlorin or rhodin-type structure,
672 respectively. (C) Structural comparison between carotenoids with 5,6-epoxy groups and
673 5,8-furanoid groups.

674

675 **Fig. 3.** Changes in the percentage distribution (with respect to total chlorophyll
676 pigments), of the chlorophyll compounds with Mg in their structure, i.e., bright green
677 pigments (black bars) and Mg-free chlorophyll derivatives, i.e., pigments with brownish
678 and grey tones (dashed bars) during the processing of Manzanilla and Hojiblanca olive
679 varieties as Spanish-style or Natural green table olives. (FF: Fresh fruit).
680
681 **Fig. 4.** Concentration of total *o*-diphenols of the different solutions generated during the
682 processing of Spanish-style and Natural green olives of the Manzanilla (A) and
683 Hojiblanca (B) varieties. Bars mean the standard deviation of two samples. The *o*-
684 diphenol compounds analyzed were hydroxytyrosol, hydroxytyrosol-1-glucoside,
685 caffeic acid, verbascoside, dialdehydic form of decarboxymethyl elenolic acid linked to
686 hydroxytyrosol, oleuropein and caffeoyl ester of secologanoside.
687

688 **Table 1.** Physicochemical parameters of olive brines and fruits (Manzanilla and
689 Hojiblanca varieties) elaborated as Spanish-style and Natural green olives for 6 months
690 of storage at room temperature.

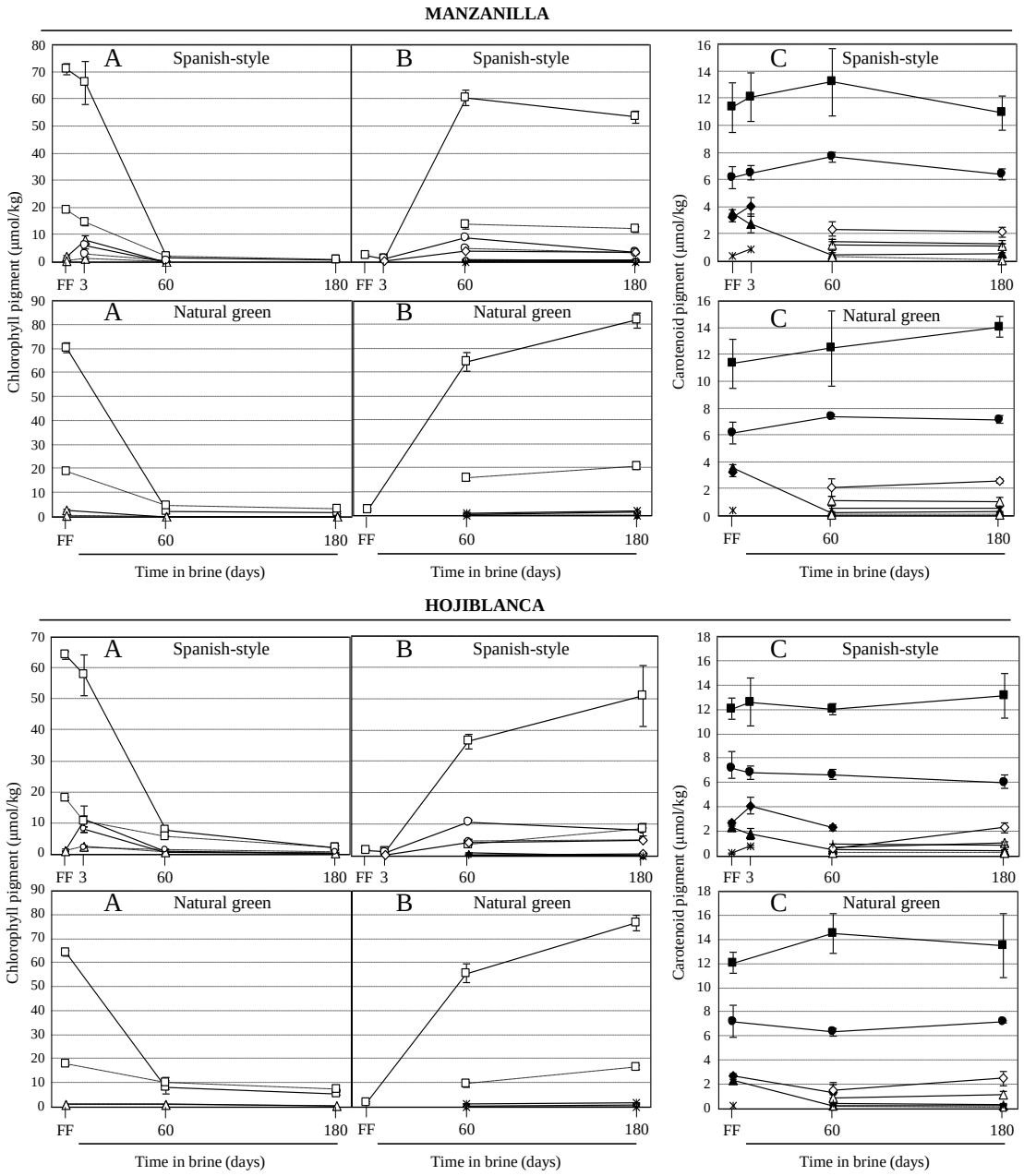
	Manzanilla olives		Hojiblanca olives	
	Spanish-style	Natural green	Spanish-style	Natural green
Chemical parameters of olive brines [a,b]				
Salt (%)	6.8 ± 0.1 a	5.0 ± 0.2 b	6.2 ± 0.3 a	5.5 ± 0.2 b
pH	3.8 ± 0.0 a	3.7 ± 0.1 a	4.8 ± 0.1 b	4.4 ± 0.0 c
Free acidity (%) [c]	1.1 ± 0.0 a	1.4 ± 0.1 b	0.5 ± 0.1 c	0.6 ± 0.1 c
Color parameters of olive fruits [b,d]				
L*	55.20 ± 0.65 a	48.64 ± 0.99 b	53.88 ± 0.83 c	53.01 ± 0.87 c
a*	3.78 ± 0.03 a	5.79 ± 0.16 b	2.18 ± 0.29 c	4.43 ± 0.39 d
b*	36.68 ± 1.25 a	29.92 ± 0.79 b	32.20 ± 0.96 c	32.44 ± 0.79 c
h	84.86 ± 1.43 a	79.03 ± 0.48 b	86.14 ± 0.40 c	82.23 ± 0.56 d
C	36.87 ± 1.25 a	30.47 ± 0.76 b	32.28 ± 0.97 c	32.74 ± 0.82 c

691 [a] Each value is the mean ± standard deviation of two samples. [b] Different letters in the
692 same mean value row indicates significant differences according to a Duncan's
693 multiple-range test ($p < 0.05$). [c] Expressed as lactic acid. [d] Each value is the mean ±
694 standard deviation of four samples.
695

Table 2. Changes in color parameters and polyphenol compounds (mmol kg^{-1}) of olive skin exposed to air for 24 hours. Harvested fruits were pasteurized or non-pasteurized before placed in sterile acidified brine and stored for 1 month under aseptic conditions.[a,b]

	Non-pasteurized olives		Pasteurized olives	
	0h	24h	0h	24h
Color parameters				
L*	50.44 ± 0.33 a	33.53 ± 1.26 b	52.29 ± 0.43 c	51.63 ± 0.58 c
a*	5.49 ± 0.22 a	5.18 ± 0.09 b	1.25 ± 0.18 c	1.24 ± 0.15 c
b*	34.72 ± 0.95 a	9.08 ± 0.97 b	38.29 ± 0.88 c	34.92 ± 0.92 a
h	81.02 ± 0.32 a	60.14 ± 2.34 b	88.11 ± 0.25 c	87.97 ± 0.21 c
C	35.15 ± 0.95 a	10.46 ± 0.88 b	38.31 ± 0.89 c	34.94 ± 0.92 a
Polyphenol compounds				
Hydroxytyrosol	4.73 ± 0.26 a	3.26 ± 0.30 b	0.73 ± 0.47 c	0.70 ± 0.08 c
Verbascoside	3.50 ± 0.55 a	1.17 ± 0.24 b	2.82 ± 0.72 a	2.51 ± 0.90 a
HyEDA[c]	3.07 ± 0.27 a	1.49 ± 0.31 b	-	-
Oleuropein	17.63 ± 1.74 a	16.91 ± 3.95 a	49.63 ± 6.94 b	64.38 ± 4.24 b

[a] Each value is the mean ± standard deviation of four samples. [b] Different letters in the same mean value row indicate significant differences according to a Duncan's multiple-range test ($p < 0.05$). [c] HyEDA is the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol.



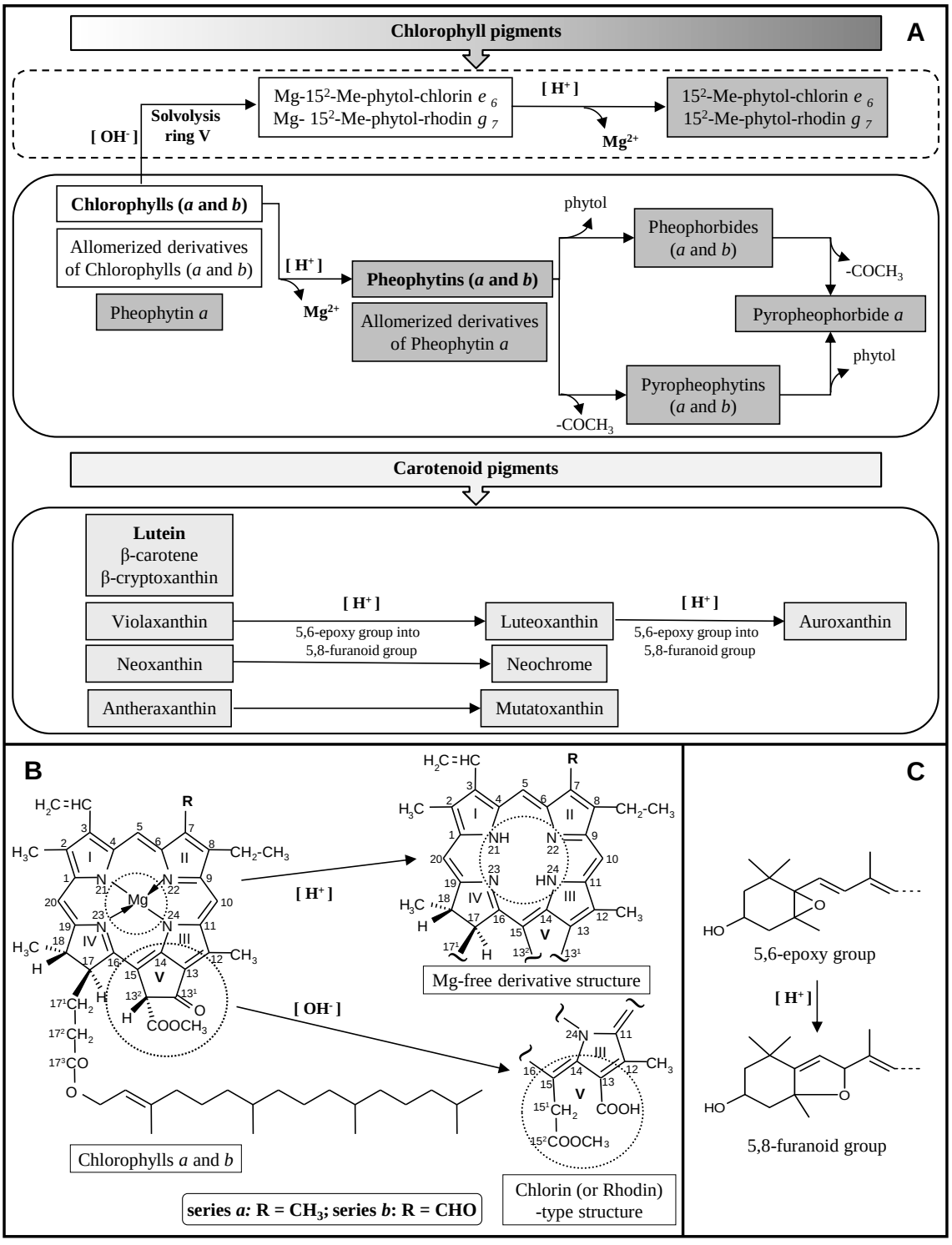
706

707

708

709 **Figure 1**

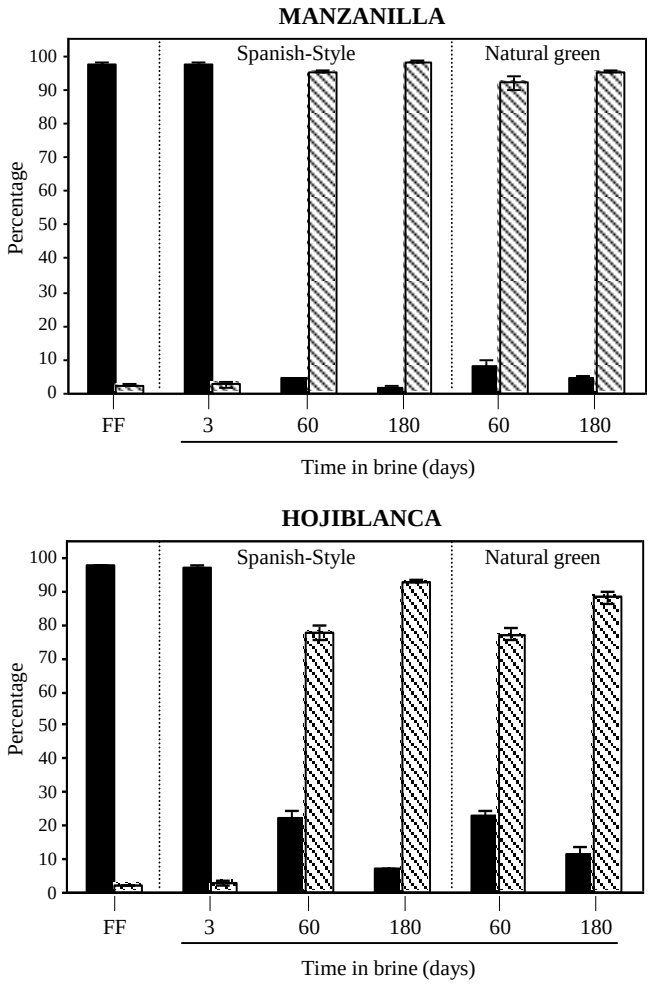
710



711

712

713 **Figure 2**

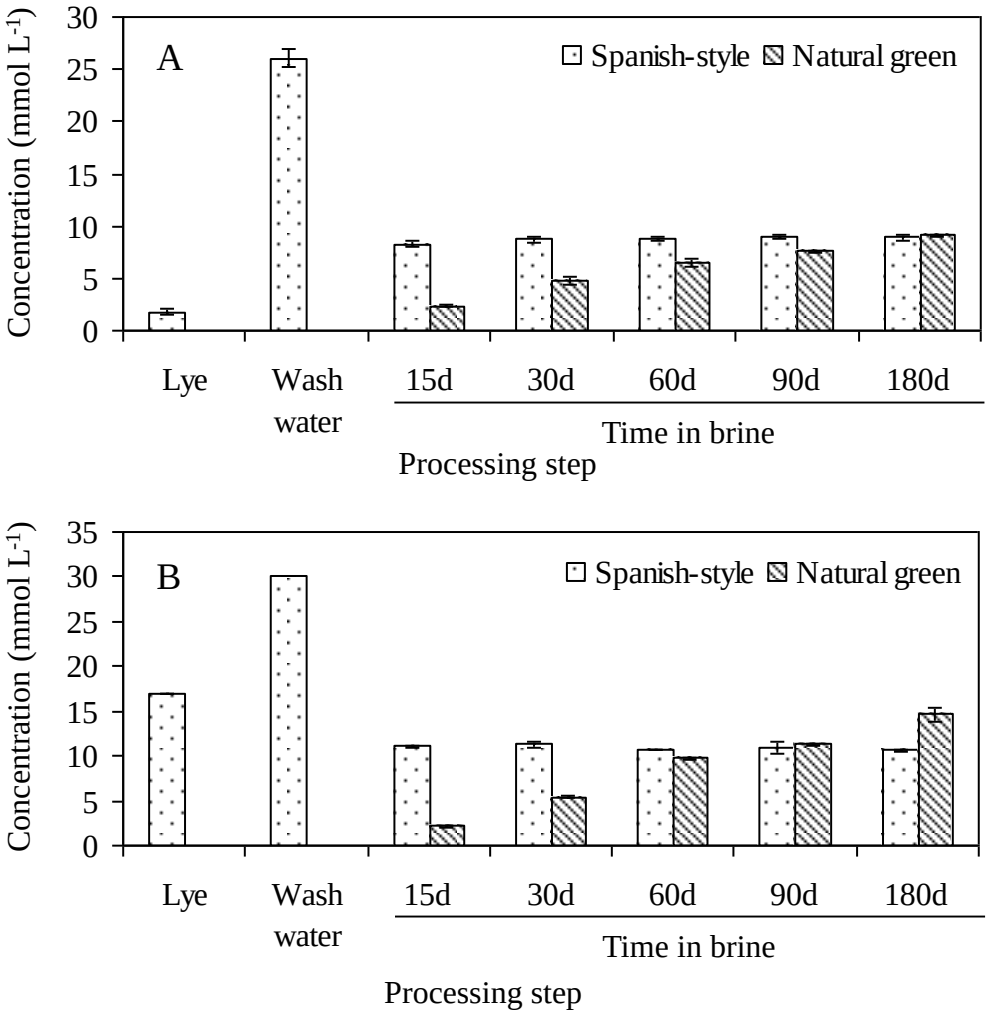


714

715

716 **Figure 3**

717



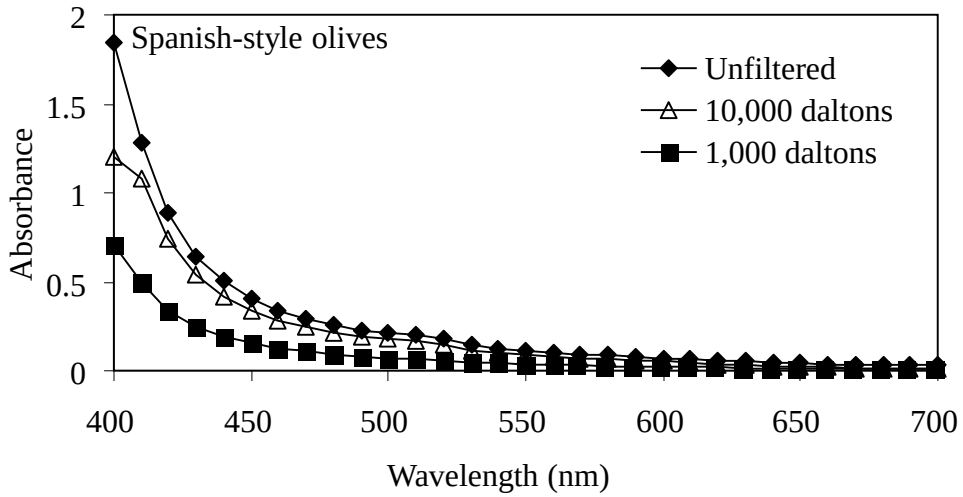
718

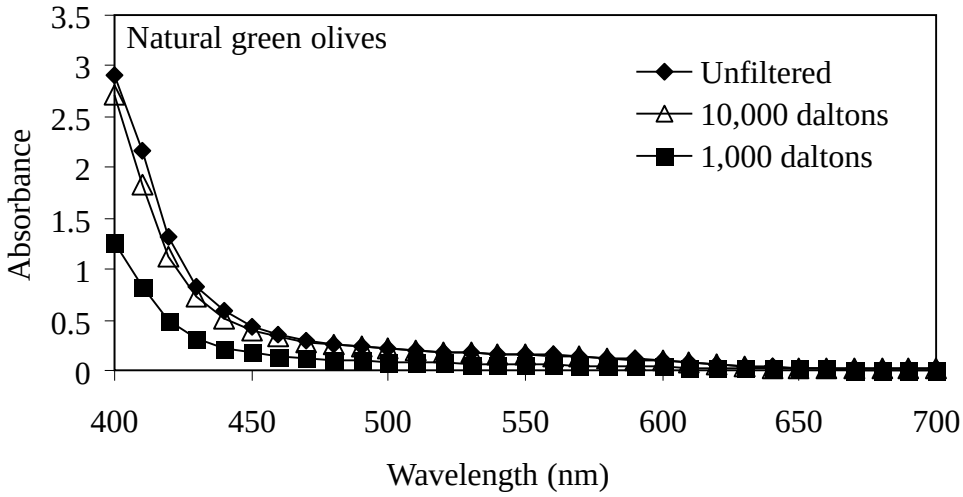
719

720

721 **Figure 4**

722





Supplementary data. Absorption spectra of the same original olive juice after having been filtered through different pore size membranes. Juices were of Hojiblanca olive variety processed for 6 months.

723