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**Biochemical aspects of olive freezing-damage: impact on the phenolic
and volatile profiles of virgin olive oil**

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Running title header: Freezing and virgin olive oil flavor

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Abstract

In this study, the biochemical changes induced by severe freezing of olives of the Picual and Arbequina cultivars were evaluated, paying particular attention to the phenolic and volatile profiles of the oils obtained from them. The content of the main phenolic glycosides, oleuropein and demethyloleuropein, and the activity levels of β -glucosidase and polyphenol oxidase in the olive tissue were severely reduced by freezing. On the contrary, unusually high levels of hydrolytic secoiridoid derivatives were found in freeze-damaged fruits. In both cultivars, the oils obtained from freeze-damaged fruits contained significantly lower levels of secoiridoid compounds, with a 21% and 42% decrease in Picual and Arbequina, respectively. The volatile composition of the oils obtained from Arbequina freeze-damaged fruits were also more strongly affected than that of Picual oils, with a higher reduction in the content of C6-aldehydes, 85% and 65% respectively. The changes observed in the main volatile components were closely related to the changes provoked by freezing in the activity level of the enzymes of the lipoxygenase pathway.

Key words: *Olea europaea* L.; quality; lipoxygenase; phenolic compounds; freezing.

Chemical compounds studied in this article

Oleuropein (PubMed CID 5281544); Demethyloleuropein (PubMed CID 6450302)
Tyrosol (PubChem CID: 10393); Hydroxytyrosol (PubChem CID: 82755); (Z)-hex-3-enal (PubChem CID: 643139); (E)-hex-2-enal (PubChem CID: 5281168); hexanal (PubChem CID: 6184); hexan-1-ol (PubChem CID: 8103); (Z)-hex-3-enol (PubChem CID: 5281167); (E)-hex-2-enol (PubChem CID: 54670067); hexyl acetate (PubChem CID: 8908); (E)-hex-2-en-1-yl acetate (PubChem CID: 5363374).

1. Introduction

Virgin olive oil (VOO) is extracted exclusively from fresh undamaged olive fruit by mechanical or other physical means, and under conditions that do not alter the oil, particularly thermal conditions. Due to this peculiar extraction process, VOO retains a significant amount of biologically active metabolites (e.g., phenolic compounds, tocopherols, sterols and pigments) which enhance its nutritional value (Visioli & Bernardini, 2013), also contributing to its extraordinary flavor.

Phenolic and volatile compounds undoubtedly exert the main influence on the sensory quality of VOO. The biosynthesis of these flavor related compounds takes place mostly during the milling of olive fruits, being the biosynthetic activity strongly limited during the malaxation process (Clodoveo, Hachicha-Hbaieb, Kotti, Mugnozza, & Gargouri 2014). In this sense, despite the fact that processing technology may severely affect VOO quality (Cevik, Ozkan & Kiralam, 2016) it is quite clear that the composition and biochemical status of the olive fruit are the most important variables defining the quality of the oil. Oleuropein, ligstroside and demethyloleuropein are the main phenolic glycosides in olive fruit, and experimental evidences point to β -glucosidase, polyphenol oxidase (PPO) and peroxidase (POX) as the main enzymes involved in the biosynthesis and degradation of phenolic compounds during the extraction of VOO (Romero-Segura, García-Rodríguez, Sanz, & Pérez, 2011). Thus, the hydrolytic derivatives identified as the dialdehydic forms of decarboxymethyloleuropein and ligstroside aglycones (3,4-DHPEA-EDA and *p*-HPEA-EDA, respectively), and the aldehyde forms of oleuropein and ligstroside aglycones (3,4-DHPEA-EA and *p*-HPEA-EA, respectively), are the most abundant phenolic components in most VOOs. In a similar way, it is well established that the biosynthesis

of VOO aroma occurs through the lipoxygenase (LOX) pathway, comprising mainly the actuation of LOX, hydroperoxide lyase (HPL), alcohol dehydrogenase (ADH), and alcohol acyltransferase (AAT) enzymes, which gives rise to the straight-chain six-carbons (C6) aldehydes, alcohols, and their respective esters identified as the key volatile components of VOO (Olías, Perez, Rios & Sanz., 1993).

Agronomic factors may induce some changes to the biochemical pathways that determine the phenolic and volatile composition of VOOs (Romero, & Motilva, 2010; Romero, Saavedra, Tapia, Sepúlveda, & Aparicio, 2016). Thus, severe freezing damage due to an abrupt drop in temperature can induce intracellular freezing in the fruit, affecting the protoplasmic structure, while only indirect damage occurs when ice forms outside of the cells (i.e.: extracellular freezing) (Ruiz-Baena, Lorite-Torres, Gavilan-Zafra, Navarro-Garcia, & Estevez- Gualda, 2009). However, there is little information as to the metabolic changes induced in the olive fruit damaged by freezing, particularly in reference to markers of aroma in VOO (Inarejos-García, Santacatterina, Salvador, Fregapane, & Sanchez-Alonso, 2010; Zhu,H., Wang, & Shoemaker, 2014). Likewise, the phenolic profile of the oils obtained from freeze-damaged olives has not been well characterized. In addition to contributing to a deeper understanding of the biochemistry of plant foods, the biochemical characterization of damaged olive fruits could be a useful complement to the chemical determination of VOO defects and to the sensory evaluation of such defects.

Therefore, the aim of this paper was to evaluate the influence of fruit freezing on the biochemical events related to the synthesis of VOO flavor. For this purpose, the biochemical status of severely freeze-damaged olive fruits was studied paying particular attention to the key biochemical factors involved in phenolic and volatile biosynthesis.

2. Materials and methods

2.1 Plant material.

Olive fruits (*Olea europaea* cv. Picual and Arbequina) were cultivated at the experimental fields of Instituto de la Grasa and collected at an average maturity index of 2.5 (turning stage). Olive fruits (6 kg) were immediately transported to the lab, randomly distributed and placed in perforated plastic boxes. To evaluate the biochemical changes induced by intracellular ice crystal formation caused by a rapid freezing (Levitt, 1980), fresh harvested olives were subjected to a simulated fast freezing process in the lab. Fruits (3 kg) were placed in a laboratory freezer (-18 °C) for three days and then subjected to a gradual thawing process (24 h at 5 °C plus 5 h at room temperature) prior to fruit analysis and oil extraction. Control fruits (3 kg) were kept 24 h at 5 °C and 5 h at room temperature.

2.2 Chemicals.

Reagents to assess enzyme activity, for enzyme extraction and other measurements were supplied by Sigma-Aldrich (St. Louis, MO, USA). Phenolic compounds were purchased from Extrasynthese (Genay, France).

2.3 Olive oil extraction.

Olive oil was extracted using an Abencor analyzer (Comercial Abengoa, S.A., Seville, Spain) that simulates the industrial process of VOO production on a laboratory scale (Martínez, Muñoz, Alba & Lanzón, 1975). As previously mentioned control and freeze-damaged fruits were processed after 5 h conditioning to room temperature. Two

batches of 1 kg olive fruits were processed per each treatment. Abencor processing parameters have been precisely described in a previous study (Sánchez-Ortiz et al., 2012b).

2.4 Enzyme activities

The activity levels of different enzymes related to the synthesis of phenolic and volatile compounds that characterize VOO were measured three times in each of the two extracts prepared for each of them as described below. All enzyme activities were expressed as nano katal per gram of fruit ((*nk*at/g FW)

LOX activity was extracted from fresh olive pulp as described previously (Luaces, Pérez, García, & Sanz, 2005). LOX activity was determined at 25 °C, using linolenic acid as the substrate, and monitoring the increase in absorbance at 234 nm ($\epsilon = 2.5 \times 10^4 \text{ mol}^{-1} \text{ L cm}^{-1}$) due to the conjugated double bonds formed by hydroperoxidation of this acid.

HPL activity was extracted from fresh olive pulp as described elsewhere (Sánchez-Ortiz et al., 2013). HPL activity was determined spectrophotometrically at 25 °C, using 13-hydroperoxylinolenic acid as the substrate and quantifying its degradation by measuring the decrease in absorbance at 234 nm as described for LOX.

ADH was extracted from acetone powders prepared from fresh olive pulp as described by Sánchez-Ortiz et al. (2012a) and its activity was measured by monitoring the reduced nicotinamide adenine dinucleotide (NADH) oxidation at 338 nm using acetaldehyde as the co-substrate ($\epsilon = 6160 \text{ mol}^{-1} \text{ L cm}^{-1}$).

AAT enzyme extracts were prepared from 0.1 g of acetone powders obtained from fresh olive seeds, diluted in 3 mL of extracting buffer (50 mmol/L Tris-HCl, pH 8.0, 7

mmol/L DTT, 100 g/L glycerol and 2 g/L Triton X-100). AAT activity was assayed as described previously (Olías, Pérez, & Sanz, 2002) with minor modifications, using 5,5'-dithiobis nitrobenzoic acid (DTNB) as the substrate.

POX activity was extracted from olive seeds (0.5 g) and determined spectrophotometrically by following guaiacol oxidation at 470 nm ($\epsilon = 26600 \text{ mol}^{-1} \text{ L cm}^{-1}$) through the method described elsewhere (García-Rodríguez, Romero-Segura, Sanz, Sánchez-Ortiz, & Pérez, 2011).

PPO extracts were prepared from acetone powders as described by García-Rodríguez et al. (2011). PPO activity was determined by constantly monitoring the increase in absorbance at 400 nm due to the oxidation of *tert*-butylcatechol (TBC).

β -Glucosidase activity was extracted from acetone powders and determined spectrophotometrically by following the hydrolysis of the synthetic glucoside *p*-nitrophenyl- β ,D-glucopyranoside (pNPG) at 405 nm (Romero-Segura, García-Rodríguez, Sánchez Ortiz, Sanz, & Pérez, 2012).

2.5 Extraction and analysis of fruit and VOO phenolic compounds.

Fruit phenolic compounds were extracted according to a previously developed protocol (García-Rodríguez et al., 2011). Representative fruits samples (1 g) were kept at 4 °C for 24 h in dimethyl sulphoxide (DMSO, 6 mL) and the extracts were filtered through a 0.45 μm mesh nylon before HPLC analysis. VOO phenolics were isolated by solid phase extraction (SPE) on a diol-bonded phase cartridge (Supelco, Bellefonte, PA, USA) A solution containing *p*-hydroxyphenyl-acetic acid ($4.64 \times 10^{-2} \text{ mg/mL}$) and *o*-coumaric acid ($9.60 \times 10^{-3} \text{ mg/mL}$) in methanol provided internal standards to this extraction procedure, and 0.5 mL of the standard solution was added to each oil sample (2.5 g). Phenolic extracts were analyzed by HPLC on a Beckman Coulter liquid

chromatography system (Beckman coulter, CA, USA) equipped with a System Gold 168 photodiode array detector, a solvent module 126 and a Waters column heater module (Waters, Milford, USA) on a Superspher RP 18 column (4.6 mm i.d. x 250 mm, particle size 4 μm : Dr Maisch GmbH, Germany). Elution was performed at a flow rate of 1.0 mL min⁻¹, and a temperature of 35 °C, using phosphoric acid 5 g/L (solvent A) and methanol/acetonitrile (50:50 mL:mL) (solvent B) as the mobile phases. The quantification of phenols (except ferulic acid) and lignans was carried out at 280 nm using *p*-hydroxyphenyl-acetic acid as internal standard. The quantification of flavones and ferulic acid was done at 335 nm using *o*-coumaric acid as internal standard. Response factors were calculated for each phenolic compound and the tentative identification of compounds by their UV-Vis spectra was confirmed by HPLC/ESI-qTOF-HRMS as described previously (García-Rodríguez et al., 2011). Two extracts per sample were analyzed in duplicate.

2.6 Extraction and analysis of VOO volatile compounds.

Olive oil samples (0.5 g) were placed in a vial heater at 40 °C and after a 10 min equilibration, volatile compounds from the headspace were extracted by a solid-phase microextraction (SPME) fiber DVB/Carboxen/PDMS 50/30 μm (Supelco Co., Bellefonte, PA, USA). Four vials per sample were analyzed. The volatile compounds were analyzed using a HP-6890 GC equipped with a DB-Wax capillary column (60 m $\times$ 0.25 mm inner diameter; film thickness, 0.25 μm ; J&W Scientific, Folsom, CA, USA), and they were identified on a HRGC-MS Fisons Series 8000 (Fisons, Loughborough, UK) following the method described by Sánchez-Ortiz et al. (2013). Quantification was performed using individual calibration curves for each identified compound. Volatile compounds were clustered into different classes according to the polyunsaturated fatty

acid and the LOX pathway branch origin. Quantitative data for each volatile class is the sum of the content of the corresponding individual compounds (Sánchez-Ortiz et al., 2013).

2.7 Statistical analysis.

The data were analyzed using Statgraphics Plus 5.1 (Manugistic Inc., Rockville, MD), applying an analysis of variance (ANOVA) and comparing the means with the Student-Newman-Keuls/Duncan test. Significance was accepted at a level of 0.05.

3. Results and discussion

3.1 Effect of freezing on the biochemical factors involved in VOO phenolic synthesis.

The phenolic composition of the fresh undamaged Picual and Arbequina fruits analyzed was consistent with the typical phenolic profile of these two cultivars, whereas the phenolic composition of the freeze-damaged olives was characterized by a strong reduction in the levels of the phenolic glycosides, oleuropein and demethyloleuropein, and significant high levels of the corresponding hydrolyzed products, 3,4-DHPEA-EA and 3,4-DHPEA-EDA in Picual and Arbequina cultivars, respectively (Table 1). Although ripening may increase tissue disorganization and cause a certain degree of glucoside hydrolysis in the fruit, it is quite unusual to find significant amounts of these two secoiridoid derivatives, typical of VOO, in intact olive fruits. It is commonly accepted that only when cell integrity is disrupted during milling enzymes and substrates come into contact, and the biosynthesis/degradation of phenolic compounds is promoted. In this sense, the phenolic composition of freeze-damaged fruits it is similar to that found in olive pastes during the industrial process to obtain the oil, albeit with even higher levels of secoiridoid derivatives (Romero-Segura et al., 2012).

To investigate the effect of freezing on the phenolic biosynthesis, the three main enzymes involved in the formation/transformation of phenolic compounds in olive were assessed: β -glucosidase, PPO and POX (Table 2). β -glucosidase activity in Picual (270 *nkat/g* FW) and Arbequina (41.6 *nkat/g* FW) control fruits was consistent with the levels previously reported for this enzyme (Romero-Segura et al., 2012). However, no β -glucosidase activity was detected in the olive fruits of either cultivar after freezing. PPO activity behaved similarly and it could not be detected in freeze-damaged fruits (control Picual and Arbequina fruits displayed 4446 and 1741 *nkat/g* FW, respectively). POX was the only enzyme that was not affected by the freezing of Picual (2995 *nkat/g* FW) or Arbequina (1202 *nkat/g* FW) olive fruits. It should be noted that olive POX is mainly located in the seed and for physiological reasons, the seed tissue is quite resistant to adverse environmental conditions. Curiously, very similar results were obtained when these three enzymatic activities were analyzed during olive paste kneading. A dramatic decrease in β -glucosidase and PPO activities has been described during the milling step, with almost no activity detected at the beginning of the kneading step as opposed to significant POX activity during and at the end of kneading (García-Rodríguez, Romero-Segura, Sanz, & Pérez, 2015). Taking into account the proven inactivating role of oxidized phenolics during the isolation of plant enzymes (Loomis & Battaile, 1966), the increasing content of oxidized phenolics during olive paste kneading has been associated to the reduction of some enzyme activities during the oil extraction process (Clodoveo et al., 2014; Sánchez-Ortiz et al., 2012b). Similarly, the accumulation of oxidized phenolic components in the tissue of freeze-damaged fruit might explain the inactivation of β -glucosidase and PPO enzymatic activities. Therefore, it appears that rapid freezing of the olive fruits promotes the formation of intracellular ice crystals that cause mechanical damage in the protoplasmic

structure, releasing enzymes and substrates that were previously compartmentalized. Hence, the intracellular status of freeze-damaged olive fruit could be compared with that of mechanical ground olives, since most of the reactions that usually occur during milling have already been initiated in the cells of freeze-damaged fruits and similarly the activity of these endogenous enzymes is being reduced as a consequence of the parallel increase of potential inhibitors.

The increase of oxidative and hydrolytic reactions after olive freezing damage may be inferred from the high peroxide and free acidity values found in oils obtained from freeze damaged fruits from different olive cultivars (Morello et al., 2003; Inarejos-García et al., 2010). Valuable information was obtained on the competitive hydrolytic and oxidative enzymatic reactions taking place within the freeze-damaged tissue (Table 1). Firstly, it is important to note that olive β -glucosidase possesses very high substrate and product specificity (Romero-Segura et al., 2012), with maximal specificity towards oleuropein, followed by demethyloleuropein, but no activity on verbascoside, a disaccharide caffeoyl ester. This is consistent with the stronger reduction in oleuropein and demethyloleuropein contents compared to that of verbascoside (Table 1); and more importantly, with the high specificity of the hydrolytic products formed in freeze-damaged fruits: DHPEA-EA and 3,4-DHPEA-EDA, respectively (Romero-Segura et al., 2012). Thus, significant amounts of 3,4-DHPEA-EA and 3,4-DHPEA-EDA were synthesized in Picual and Arbequina freeze damaged fruits (461 and 626 $\mu\text{mol}/100\text{g}$ fruit) in which very low levels of oleuropein and dimethyloleuropein were found in comparison with the levels detected in control fruits (1672 and 1806 $\mu\text{mol}/100\text{ g}$, respectively). A very recent publication (Ramirez, Medina, García, Brenes & Romero, 2017) found very similar oleuropein degradation rates in frozen Manzanilla and Hojiblanca olives stored at freezing temperatures. As olive PPO and POX can oxidize

the main phenolic glycosides present in the fruit and their hydrolysis products (García-Rodríguez et al., 2011), it is clear that the final contents of oleuropein, demethyloleuropein, 3,4-DHPEA-EA and 3,4-DHPEA-EDA depend not only on the β -glucosidase activity but on the oxidative activities. By contrast, verbascoside is highly susceptible to oxidative degradation by olive PPO (García-Rodríguez et al., 2011) but, as mentioned above, it is not a suitable substrate for β -glucosidase so that its reduction could not be associated to hydrolytic activity but only to oxidative degradation. The decrease in the verbascoside content of freeze-damaged fruits is significantly lower (33 and 23 $\mu\text{mol}/100\text{ g}$ in Picual and Arbequina, respectively) than the decrease observed in the contents of oleuropein and demethyloleuropein. These data suggest that the oxidative degradation of phenolic compounds induced by freezing-damage seems to not reach the phenolic oxidation rates observed during the mechanical process to obtain the VOO (Clodoveo et al., 2014; García-Rodríguez et al., 2011). Thus, while it seems clear that rapid freezing promotes cellular disruption, triggering hydrolytic and oxidative enzymatic reactions, oxygen availability could be a limiting factor for oxidation phenomena in freeze-damaged fruits. In this sense, a strong correlation has been observed between oxygen availability and phenolic oxidation during the kneading of the olive paste (Servili, et al., 2008).

3.2 Effect of freezing on the phenolic profile of VOO.

The phenolic contents in the oils obtained from freeze-damaged fruits were lower than those obtained from fresh undamaged olive fruits in both cultivars (Table 3). This decrease, more notable in Arbequina than in Picual oils, was mainly due to a reduced content of secoiridoid compounds. Thus, the most relevant alteration in the oils obtained from freeze-damaged Picual fruits was the low 3,4-DHPEA-EA content while in

Arbequina oils, 3,4-DHPEA-EDA underwent the greatest reduction. A lower content of secoiridoids was previously reported in oils extracted from freeze-damaged Arbequina fruits (Morello, Motilva, Ramo & Romero, 2003) coupled to a significant increase in the vanillin and vanillic acid contents. Similar results, marked by a low secoiridoid and high vanillin and vanillic acid contents were also obtained in oils from Picual trees grown in areas of Australia with recurrent freezing problems (Guillaume, Ravetti, & Gwyn, 2010). As shown in Table 2, the levels of vanillic acid were also significantly higher in the oils obtained from freeze-damaged Picual fruits.

Despite the aforementioned decrease in secoiridoids, the oils obtained from freeze-damaged fruits retained significant levels of phenolic components as reported for other oils with non-oxidative defects (Aparicio-Ruiz et al., 2016). As previously mentioned, the metabolic changes induced in freeze-damaged fruits could differ depending on the type and severity of the freezing-stress (Ruiz-Baena et al., 2009). Thus, Romero et al. (2016) found very high levels of apigenin and luteolin in oils from different olive cultivars affected by freezing. However, we found only significant differences in the luteolin content of Arbequina oils obtained from freeze-damaged fruits.

3.3 Effect of olive freezing on the biochemical factors involved in volatile biosynthesis.

The LOX activity in fresh undamaged olives was significantly higher in Arbequina (49 *nkat/g* FW) than in Picual fruits (9.6 *nkat/g* FW) (Table 2), as it was reported in a previous study (Sánchez-Ortiz et al., 2012b). This result is also in good agreement with the higher level of total volatiles found in the Arbequina oils (Table 4). However, freezing of olive fruit caused a dramatic decrease in LOX activity in both cultivars, with no detectable activity in freeze-damaged Picual fruits and very weak activity in freeze-

damaged Arbequina fruits (3.2 *nkat/g* FW). By contrast, the HPL activity level in fresh Picual fruits (11.2 *nkat/g* FW) was slightly higher than in Arbequina fruits (8.0 *nkat/g* FW). The HPL activity in freeze-damaged fruits from both cultivars differed significantly, falling to 6.4 *nkat/g* FW in Picual fruits while no detectable activity was found in Arbequina fruits.

The ADH enzyme also seems to be strongly affected by fruit freezing, with 20.8 and 9.6 *nkat/g* FW ADH activity in fresh-undamaged Picual and Arbequina fruits, respectively, and no detectable activity in freeze-damaged fruits. According to previous studies, the ADH activity is severely limited during the extraction process of VOO due to the presence of specific inhibitors in the olive tissue (Sánchez-Ortiz et al., 2012a), which might be also acting in freeze-damaged fruits. The last enzyme of the LOX pathway, AAT, showed a different pattern to those enzymes mentioned above. Both cultivars had similar AAT activity as previously suggested (Sánchez-Ortiz et al., 2012a). AAT activity was not significantly affected by freezing in Picual fruits while a significant decrease (less than 20%) was observed in Arbequina freeze-damaged fruits (Table 2). It is worth mentioning that, like olive POX, AAT is mainly located in the seed, a tissue localization that could protect the enzyme from inactivation.

3.4 Effect of freezing on the volatile profile of VOO

The changes induced by freezing-damage in the four enzymes from the LOX pathway seem to be related to the volatile composition of the oils analyzed in this study. Table 4 shows the volatile compounds clustered into different classes according to the polyunsaturated fatty acid and the LOX pathway branch origin. Thus, the main effects of freezing in the volatile profile of Picual oils are a moderate decrease in the content of C6 LnA compounds, a dramatic decrease in C5 LnA compounds (from 9523 to 427

ng/g oil) and a doubling of the amount of LOX esters (from 2282 to 4923 ng/g oil). Quantitative data for C6 LnA is the sum of the content of aldehydes and alcohols that are affected in different ways by the freezing process. The extremely significant decrease (65%) observed in aldehydes C6 LnA correlates with the inactivation of LOX and the moderate decrease in HPL activity. Likewise, the total inactivation of LOX could explain the extremely low levels of C5 compounds detected in freeze-damaged Picual fruits (Sánchez-Ortiz et al., 2012a). However, the apparent inactivation of ADH activity does not fit with the increase in C6 LnA alcohols. Conversely, the high content of these C6 LnA alcohols and the significant levels of AAT found in freeze-damaged olives agrees well with the higher content of LOX esters found in these oils.

The changes observed in the volatile profile of Arbequina oils seem to be more severe than those analyzed in Picual oils (Table 4). Thus, the sharp decrease in the content of C6 LnA aldehydes and C5 LnA compounds in oils from Arbequina freeze-damaged fruits (88% and 95%, respectively) could only be explained by the almost total depletion of LOX and HPL activities in this cultivar as a consequence of fruit freezing (Table 2). Similarly, to what was described for Picual cultivar, the apparent inactivation of ADH in freeze-damaged Arbequina fruits does not correlate with the important increase of C6 alcohols detected in the corresponding oils. Despite this high availability of C6 alcohols to form LOX esters the concomitant decrease in AAT activity associated to freezing (Table 4) seems to be a limiting factor for ester biosynthesis that explains the slight reduction observed in the content of LOX esters (15%).

Besides the changes already described in connection with the main groups of volatile compounds, a detailed analysis of the content of individual volatile components (data not shown) in the aroma of the different oils was also carried out to identify possible markers of the freezing damage in harvested fruits. The compounds exhibiting the most

significant increases in the oils obtained from freeze-damaged Picual and Arbequina fruits were: 2-methyl butanal (an 8-fold increase in both cultivars), and 3-methyl butanal (10-fold and 6-fold increases in Picual and Arbequina, respectively). The defects associated to freezing damage of olives while on the tree have not been fully characterized, but some volatile compounds have been proposed to serve as markers for defective oils. Thus, good correlation was found between the wet wood defect, associated to freezing damage in the Cornicabra cultivar, and the of 2-methylbutanal and 3-methylbutanal content (Inarejos-García et al., 2010). More recently, limonene, 1-hexanol, octyl acetate, octanal, phenylethyl alcohol and pentanal were shown to be the most relevant volatile components in the aroma of VOO obtained from freeze-damaged Arbequina fruits (Zhu et al., 2014). Although the observed differences may not be relevant from a quantitative point of view when compared to the major quantitative alterations detected in C6 and C5 compounds from LnA, the possible use of some of these specific compounds as volatile markers of freezing in harvested olives should be explored in further studies.

4. Conclusion

The postharvest freezing of the olive fruits induces biochemical changes that give rise to a significant modification of the main compounds responsible for the sensorial properties of VOO. The content of the main phenolic glycosides and the levels of β -glucosidase and PPO activity in olive fruit are drastically reduced by freezing. As a result, oils obtained from freeze damaged fruits contain significantly lower levels of the major phenolic compounds in VOO, the secoiridoid compounds, as compared to freshly harvested fruits. Moreover, the volatile composition of the oils obtained from freeze-damaged fruits is also greatly affected. Most of the changes are closely related to the

changes caused by freezing in the activity level of the LOX pathway enzymes. They are characterized by a high reduction in the content of C6 aldehydes and C5 compounds, and an increase in the content of C6 alcohols and branched aldehydes, suggesting a profound repercussion on the VOO aroma.

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CAPTIONS

Table 1: Content of phenolic compounds ($\mu\text{mol}/100\text{ g}$ olive fruit) in freshly harvested (control) and freeze-damaged olive fruits from Picual and Arbequina cultivars.

Table 2: Enzyme activities (nkat/g FW) in freshly harvested (control) and freeze-damaged olive fruits from Picual and Arbequina cultivars.

Table 3: Content of phenolic compounds ($\mu\text{g}/\text{g}$ oil) in Virgin olive oils obtained from freshly harvested (control) and freeze-damaged olive fruits from Picual and Arbequina cultivars.

Table 4: Content of volatile compounds (ng/g oil) in Virgin olive oils obtained from freshly harvested (control) and freeze-damaged olive fruits from Picual and Arbequina cultivars.

Supporting Information captions

Table S1: Content of individual volatile compounds (ng/g oil) in Virgin olive oils obtained from freshly harvested (control) and freeze-damaged olive fruit from Picual and Arbequina cultivars.

Table 1

Phenolics	Picual		Arbequina	
	Control	Freeze damaged	Control	Freeze damaged
Hydroxytyrosol	*47.64 ± 0.05a	149 ± 169 b	74 ± 5 a	129 ± 44 b
Tyrosol	13 ± 6 a	249 ± 29 b	14 ± 1 a	13 ± 5 a
Demethyloleuropein	1.3 ± 0.4 a	2 ± 1 a	1806 ± 122 b	29 ± 23 a
3,4-DHPEA-EDA	1 ± 1 a	286 ± 45 b	2.1 ± 0.1 a	626 ± 75 b
Demethyligstroside	178 ± 18 b	0.95 ± 0.01 a	67 ± 4 b	4.6 ± 0.0 a
Oleuropein	1672 ± 215 b	5 ± 4 a	2.0 ± 0.1 b	4.5 ± 1.7 b
3,4-DHPEA-EA	112 ± 23 a	461 ± 5 b	0.83 ± 0.01 a	7.8 ± 1.3 b
Ligstroside	77 ± 58 b	7 ± 1 a	0.4 ± 0.1 a	0.27 ± 0.01 a
<i>p</i> -HPEA-EA	3.3 ± 0.2 a	10 ± 2 b	0.22 ± 0.01 a	0.3 ± 0.1 a
Verbascoside	59 ± 8 b	26 ± 8 a	94 ± 6 a	71 ± 7 a
Rutin	33 ± 9 b	13 ± 1 a	29 ± 2 b	21 ± 3 a
Luteolin-7-gluc	34 ± 9 b	5 ± 3 a	21 ± 1 b	9 ± 3 a
Apigenin-7-gluc	3 ± 1 b	0.7 ± 0.4 a	9 ± 1 b	4.1 ± 0.3 a
Luteolin	0.8 ± 0.2 a	19 ± 4 b	0.26 ± 0.01 a	10 ± 3 b
Apigenin	0.3 ± 0.1 a	0.5 ± 0.3 a	**ND	ND

* Mean ± standard deviation of four replicates. Different letters in the same olive cultivar designate statistically significant differences ($p < 0.05$) according to the Student-Newman-Keuls/Duncan test.

** Not detected.

Table 2

Enzyme activity	Picual		Arbequina	
	Control	Freeze damaged	Control	Freeze damaged
β -Glucosidase	*270 $\pm$ 3	**ND	41.6 $\pm$ 0.1	ND
PPO	4446 $\pm$ 138	ND	1741 $\pm$ 86	ND
POX	2774 $\pm$ 135 a	2995 $\pm$ 79 a	1153 $\pm$ 11 a	1202 $\pm$ 61 a
LOX	9.6 $\pm$ 0.3	ND	49 $\pm$ 3 b	3.2 $\pm$ 0.3 a
HPL	11.2 $\pm$ 0.2 b	6.4 $\pm$ 0.1 a	8.0 $\pm$ 0.3	ND
ADH	20.8 $\pm$ 0.1	ND	9.6 $\pm$ 0.3	ND
AAT	16 $\pm$ 1 a	17.4 $\pm$ 0.1 a	26 $\pm$ 1 b	20.6 $\pm$ 0.1 a

* Mean $\pm$ standard deviation of three replicates. Different letters in the same olive cultivar designate statistically significant differences ($p < 0.05$) according to the Student-Newman-Keuls/Duncan test.

** Not detected.

***PPO (polyphenol oxidase), POX (peroxidase), LOX (lipoxygenase), HPL (hydroperoxide lyase), ADH (alcohol dehydrogenase), AAT (alcohol acyltransferase).

Table 3

Phenolics	Picual		Arbequina	
	Control	Freeze damaged	Control	Freeze damaged
Hydroxytyrosol	* 0.92 ± 0.02 a	1.3 ± 0.4 b	0.97 ± 0.03 a	2 ± 1 b
Tyrosol	3.5 ± 0.3 a	3.8 ± 0.2 a	0.8 ± 0.2 a	1.7 ± 0.4 b
Vanillic acid	0.65 ± 0.01 a	1.0 ± 0.1 b	0.82 ± 0.01 a	0.1 ± 0.1 a
Vanillin	0.1 ± 0.1	**ND	ND	ND
p-coumaric acid	0.70 ± 0.01 a	0.5 ± 0.1 a	0.3 ± 0.1 a	0.2 ± 0.1
Hydroxytyrosol Ac.	2.47 ± 0.02 a	2.6 ± 0.1 a	46 ± 1 b	30 ± 5 a
3,4-DHPEA-EDA	106 ± 3 b	92 ± 17 a	502 ± 67 b	288 ± 76 a
p-HPEA-EDA	56 ± 1 a	63 ± 13 a	168 ± 17 b	90 ± 19 a
Pinoresinol	1 ± 1 a	0.8 ± 0.1 a	3.5 ± 0.2 a	2.8 ± 0.5 a
Cinnamic acid	1 ± 1 a	1.0 ± 0.1 a	0.5 ± 0.1 b	0.3 ± 0.1 a
Acetoxypinoresinol	18.7 ± 0.3 b	12 ± 1 a	15.5 ± 0.5 b	12 ± 1 a
3,4-DHPEA-EA	339 ± 3 b	215 ± 30 a	27.3 ± 0.1 a	22 ± 5 a
p-HPEA-EA	28.0 ± 0.4 a	47 ± 2 b	2 ± 2 a	3.14 ± 0.02 a
Ferulic acid	0.09 ± 0.01 a	0.11 ± 0.01 a	ND	ND
Luteolin	5.34 ± 0.01 a	4.93 ± 0.01 a	4.5 ± 0.3 a	5.3 ± 0.3 b
Apigenin	1.37 ± 0.05 a	1.31 ± 0.01 a	0.6 ± 0.1 a	0.6 ± 0.1 a
Total phenolics	565 ± 2 b	447 ± 3 a	773 ± 85 b	460 ± 108 a
Total o-phenolics	454 ± 1 b	316 ± 13 a	581 ± 66 b	348 ± 87 a

* Mean $\pm$ standard deviation of four replicates. Different letters in the same olive cultivar designate statistically significant differences ($p < 0.05$) according to the Student-Newman-Keuls/Duncan test.

** Not detected.

Table 4

Volatiles	Picual		Arbequina	
	Control	Freeze damaged	Control	Freeze damaged
Σ aldehydes 6/LnA	* 8577 $\pm$ 360 b	3005 $\pm$ 624 a	33392 $\pm$ 1319 b	3940 $\pm$ 420 a
Σ alcohols C6/LnA	1259 $\pm$ 9 a	4381 $\pm$ 111 b	689 $\pm$ 78 a	1209 $\pm$ 192 b
Σ C6/LnA	9836 $\pm$ 351 b	7386 $\pm$ 513 a	34081 $\pm$ 1397 b	5149 $\pm$ 227 a
Σ aldehydes C6/LA	231 $\pm$ 16 a	809 $\pm$ 123 b	1504 $\pm$ 60 a	1972 $\pm$ 318 b
Σ alcohols C6/LA	242 $\pm$ 1 a	727 $\pm$ 19 b	125 $\pm$ 2 a	380 $\pm$ 19 b
Σ C6/LA	473 $\pm$ 18 a	1536 $\pm$ 104 b	1629 $\pm$ 58 a	2352 $\pm$ 336 b
Σ carbonyls C5/LnA	942 $\pm$ 28 b	59 $\pm$ 0 a	659 $\pm$ 7 b	33 $\pm$ 7 a
Σ alcohols C5/LnA	823 $\pm$ 2 b	183 $\pm$ 7 a	632 $\pm$ 47 b	132 $\pm$ 6 a
Σ DP	7758 $\pm$ 570 b	185 $\pm$ 5 a	5692 $\pm$ 232 b	141 $\pm$ 4 a
Σ C5/LnA	9523 $\pm$ 600 b	427 $\pm$ 2 a	6983 $\pm$ 273 b	307 $\pm$ 9 a
Σ carbonyls C5/LA	268 $\pm$ 14 a	202 $\pm$ 71 a	41 $\pm$ 0 a	51 $\pm$ 2 b
Σ alcohols C5/LA	20 $\pm$ 4 a	49 $\pm$ 21 a	25 $\pm$ 1 a	25 $\pm$ 7 a
Σ C5/LA	288 $\pm$ 17 a	251 $\pm$ 50 a	67 $\pm$ 1 a	76 $\pm$ 5 b
Σ LOX esters	2282 $\pm$ 80 a	4923 $\pm$ 144 b	542 $\pm$ 15 b	464 $\pm$ 43 a
Σ non LOX esters	78 $\pm$ 1 a	73 $\pm$ 12 a	100 $\pm$ 18 a	75 $\pm$ 35 a
Σ esters	2360 $\pm$ 79 a	4996 $\pm$ 157 b	642 $\pm$ 33 b	539 $\pm$ 12 a
Σ aldehydes AA	17 $\pm$ 1 a	157 $\pm$ 40 b	113 $\pm$ 2 a	843 $\pm$ 74 b
Σ alcohols AA	2 $\pm$ 0 a	7 $\pm$ 0 b	2 $\pm$ 1 a	1 $\pm$ 0 a
Σ volatiles AA	19 $\pm$ 1 a	164 $\pm$ 40 b	114 $\pm$ 1 a	845 $\pm$ 74 b
Σ terpenes	3049 $\pm$ 68 b	2479 $\pm$ 64 a	477 $\pm$ 77 b	354 $\pm$ 4 a
Total volatiles	25549 $\pm$ 533 b	17240 $\pm$ 473 a	43994 $\pm$ 983 b	9621 $\pm$ 152 a

* Mean $\pm$ standard deviation of four replicates. Different letters in the same olive cultivar designate statistically significant differences ($p < 0.05$) according to the Student-Newman-Keuls/Duncan test.