Monitoring endogenous enzymes during olive fruit ripening and storage: Correlation with virgin olive oil phenolic profiles

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ABSTRACT:

The ability of olive endogenous enzymes activities β-glucosidase, polyphenol oxidase (PPO) and peroxidase (POX), to determine the phenolic profile of virgin olive oil was investigated. Olives used for oil production were stored for one month at 20 °C and 4 °C and their phenolic content and enzymatic activities were compared to those of ripening olive fruits. Phenolic and volatile profiles of the corresponding oils were also analyzed. Oils obtained from fruits stored at 4°C show similar characteristics to that of freshly harvested fruits. However, the oils obtained from fruits stored at 20 °C presented the lowest phenolic content. Concerning the enzymatic activities, results show that the β-glucosidase enzyme is the key enzyme responsible for the determination of virgin olive oil phenolic profile as the decrease in this enzyme activity after 3 weeks of storage at 20 °C was parallel to a dramatic decrease in the phenolic content of the oils.

KEYWORDS: Virgin olive oil; olive storage; β-glucosidase; peroxidase; polyphenoloxidase; phenolic compounds; volatile compounds; oil stability.

Highlights

- Similar endogenous enzymatic activities pattern were observed between 4 ºC stored fruits and fresh harvested ones.
- Contribution of POX, PPO and β-glucosidase activities in shaping the oil phenolics profile.
- β-glucosidase was the key enzyme directly related to the phenolic profile.
- Similar phenolic profiles of the oils extracted from fruits stored at 4 ºC and fresh harvested ones were established.
- C6 volatile compounds increased during ripening whilst a decrease was noted in oils extracted from fruits stored at 20 °C.

1. Introduction

Virgin olive oil (VOO) is exclusively extracted from olive fruits by means of mechanical techniques (Clodoveo, 2012). Olive constitutes a complex medium in which several endogenous enzymes such as pectinases, lipases, lipoxygenases, hydroperoxide lyases, beta-glucosidases, peroxidases, and polyphenol oxidases operate (Clodoveo, Hachicha Hbaieb, Kotti, Mugnozza, & Gargouri, 2014). These enzymes were liberated when fruit tissues were disrupted due to mechanical damage during harvest, pathogenic micro-organism attacks occurring during storage or extraction process. VOO sensory properties are essentially related to both its content of phenolic compounds (Fregapane & Salvador, 2013) responsible for oil stability and protection against autoxidation, and also its richness in volatile compounds responsible for fruity and green notes (Angerosa, 2002). VOO phenolic content depends on endogenous factors, such as phenolic glycosides amount and enzymatic activities in olive fruit tissues and technological factors during olive oil extraction (Romero-Segura, García -Rodriguez, Sánchez-Ortiz, Sanz, & Pérez, 2012). The most important classes of endogenous enzymes are β-glucosidase which hydrolyze phenolic glycosides and oxidoreductase enzymes which are responsible for phenolic compounds oxidation. Moreover, the level of oxidoreductases activities depends, to a large extent, on the olive variety, maturation degree, location and altitude (Clodoveo et al., 2014; Gutiérrez, Jímenez, Ruíz, & Albi, 1999).

The main phenolic glycosides identified in olive fruits from different cultivars and maturation stages are oleuropein, ligstroside, demethyloleuropein, verbascoside, elenolic acid glucoside, luteolin-7-glucoside, apigenin-7-glucoside, rutin and quercetin-3-rutinoside (Gómez-Rico, Fregapane, & Salvador, 2008). These phenolic glycosides are hydrolysed during extraction process by an endogenous β-glucosidase (E.C.3.21.1.21) generating secoiridoid compounds which constitute the most important phenolic fraction of VOO

(Romero-Segura, Sanz, & Pérez, 2009). Secoiridoid compounds are depicted by the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol or tyrosol (3,4-DHPEA-EDA or p-HPEA-EDA), and the aldehydic forms of the oleuropein aglycone (3,4-DHPEA-EA) and the ligstroside aglycone (p-HPEA-EA) (García-Rodríguez, Romero-Segura, Sanz, Sánchez-Ortiz & Pérez, 2011).

Moreover, endogenous oxidoreductases in particular, polyphenol oxidase (PPO) and peroxidase (POX) promote the oxidation of phenolic compounds during the milling and kneading steps in olive oil extraction process (Servili, Taticchi, Esposto, Urbani, Selvaggini, & Montedoro, 2008). On the one hand, peroxidases (EC 1.11.1.7) are glycoproteins catalyzing the oxidation of phenolic compounds using either hydrogen peroxide (H_2O_2) or organic peroxides as the oxidizing agent, thus producing free radical, highly reactive and easily polymerizable intermediates (Gajhede, 2001). While the phenol oxidation by the POX activity is limited by the H_2O_2 availability, the auto-oxidation of the phenolic compounds in the damaged tissue increases the H_2O_2 concentration which can be used by the POX and thus contribute to phenolic compounds oxidation (Takahama & Oniki, 2000). On the other hand, (PPO) (EC 1.14.18.1) is a copper-containing enzyme broadly distributed in nature, which plays an important role in many plant metabolic processes (Waliszewski, Mârquez, & Pardio, 2009). Otherwise, it contributes to the enzymatic browning in many plants and vegetables damaged by improper handling, resulting in bruising, compression or indentations (Zawitowski, Bilideris, & Eskin, 1991).

PPO catalyzes two distinct reactions the o-hydroxylation of monophenols to odiphenols (monophenolase activity) and oxidation of the o-diphenols to o-quinones (diphenolase activity).

VOO from Arbequina is highly appreciated by the consumers thanks to its excellent sensorial quality (Yousfi, Weiland, & García, 2012). However, this quality depends on the

maturity of the fruit. In fact, Morello, Romero, and Motiva (2004) showed a clear reduction of sensory positive attributes and VOO oxidative stability due to the reduction of contents on photosynthetic pigments (chlorophylls and carotenoids) and phenolic compounds when the fruits are overripe. Thus, it is recommended that Arbequina fruit should be harvested at an early maturation stage and for a short period (Romero, Diaz, &Tous, 2002). However, this situation will make the milling industries face an extreme concentration of production, which might exceed their processing capacity, and oblige them to store the fruit for longer periods varying from weeks to months.

Several studies have been carried out to elucidate the influence of fruits storage on oil quality. Storage of 'Picual' olives at 5 °C kept the value of free acidity, peroxide value, ultraviolet absorbance, and sensory quality of the resulting oil within the limit admitted for extra quality until a 45-day-fruit storage (García, Gutiérrez, Barrera, & Albi, 1996a). Moreover, García, Gutiérrez, Castellano, Perdiguero, Morilla, and Albi (1996b) and Canet and García (1999) demonstrated that olive refrigeration (Olea europaea cv. Villalonga and Blanqueta) at 5 °C on an industrial scale delayed deterioration of the physical, chemical, and sensorial parameters of oil quality, without changes in the initial oil quality during the 30 day storage. Clodoveo, Debora, Tommaso and Giancarlo (2007) studied the effect of different temperatures and storage atmospheres on Coratina olive oil quality. Results showed that oils retained their initial chemical qualities during 30 days of olives storage at 5 °C under a flow of humidified air or a flow of 3% O2 + 5% CO2. However, the oils lose their qualities after 15 days of olive storage at room temperature.

Given that phenolic content is a key parameter to guarantee the VOO nutritional and organoleptic properties, so as to obtain a product with a high economic value, suitable for commercialization in international markets (Lazzez et al., 2011), it is important to under-

stand the evolution of the enzymatic activities related to phenolic metabolism during olive fruits storage.

Nevertheless, according to our knowledge no studies have been carried out to investigate those endogenous enzymatic activities during fruits storage and their impact on extracted VOO quality.

The main purpose of the present study is to follow the evolution of olive (β glucosidase, POX and PPO) during fruit storage at two different temperatures and to assess their possible role in shaping the phenolic profile of VOO.

2. Materials and methods

2.1.Chemicals

Reagents for enzymatic activity extraction and measurements were supplied by Sigma-Aldrich (St. Louis, MO) except for phenolic compounds (oleuropein, and verbascoside) purchased from Extrasynthese (Genay, France).

2.2. Plant material

Olives (*Olea europaea*) of the Arbequina variety, cultivated at the experimental fields of Institute de la Grasa (Seville, Spain), were harvested (10 kg) in plastic containers at the green mature stage October 2012, 24 weeks after flowering (24WAF). Two different storage conditions were tested: refrigeration at 4 °C and ambient temperature (20 ± 2 °C) during one month (24 to 28 WAF). Every 7 days, samples of 1 kg were taken from each storage condition and used for olive oil extraction while about 250g were used for the enzymatic activities determination. The same experimental procedure was carried out with fresh harvested fruits.

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 at laboratory scale (Mar-

tínez, Muñoz, Alba, & Lanzón, 1975). Milling of olive fruits (1 kg) was performed using a stainless steel hammer mill operating at 3000 rpm provided with a 5 mm sieve. Malaxation was carried out for 30 min with the Abencor thermo beater operated at 30 °C. Centrifugation of the kneaded paste was performed in a basket centrifuge at 3500 rpm for 1 min. After centrifugation, the oils were decanted and paper filtered. Then, the oils were transferred into dark glass bottles, and stored in the dark at -20 °C until analysis.

2.4.Enzyme extraction

Acetone powders were prepared from mesocarp of fresh harvested olive fruits, following the method described by García-Rodríguez et al. (2011).

2.4.1. POX extraction

POX enzyme extracts were prepared as described previously by García-Rodríguez et al. (2011). 0.5 g of olive seeds was homogenized in 5 mL of buffer by means of an Ultraturrax homogenizer (5×1min) and then centrifuged at 15,000 *g* for 20 min at 4 °C. The clear supernatant was used as crude extract.

2.4.2. PPO extraction

PPO enzyme extracts were prepared from acetone powder, following the method described by García-Rodríguez et al. (2011).

2.4.3. β-glucosidase extraction

β-glucosidase enzyme extracts were prepared also from acetone powder as reported by Romero-Segura et al. (2009).

2.5. Enzyme activity assay

2.5.1. POX activity assay

Peroxidase activity in the extracts was determined spectrophotometrically at 470 nm (ε = 26600 M^{-1} cm^{-1}), using the method described by García-Rodríguez et al. (2011). One unit

of POX activity corresponds to the amount of enzyme oxidizing 1 μmol of guaiacol per min.

2.5.2. PPO activity assay

PPO activity was determined by constantly monitoring the increase in absorbance at 400 nm related to the oxidation of tert-butylcatechol (TBC) (García-Rodríguez et al., 2011). One unit of PPO activity was defined as the amount of enzyme forming 1 μmol of TBC-quinone per min.

2.5.3. β-glucosidase activity assay

β-Glucosidase activity was determined spectrophotometrically by following the hydrolysis of the synthetic glucoside *p*-nitrophenyl-β,D-glucopiranoside (pNPG) at 405 nm (ε = 552.8 M^{-1} cm^{-1}), according to Romero-Segura et al. (2012). One unit of β-glucosidase activity was defined as the amount of enzyme able to liberate 1 μmol of p-nitrophenol per min.

2.6. Extraction of fruit phenolic compounds

Fruit phenolics were extracted according to a previously developed protocol (García-Rodríguez et al., 2011). Representative fruits samples (1.65 g) were kept at 4 °C during 24h with dimethyl sulphoxide (10 mL). The extracts obtained were filtered through 0.22 μm before HPLC analysis.

2.7. Extraction of VOO phenolic compounds

VOO phenolics were isolated by SPE on a diol-bonded phase cartridge (Supelco, Bellefonte, PA) following a previously described procedure (Mateos et al., 2001). A solution of *p*-hydroxyphenyl-acetic acid (4.64 x 10^{-2} mg/mL) and *o*-coumaric acid (9.6 x 10^{-3} mg/mL) in methanol was used as internal standard in this extraction procedure. 0.5 mL of standard solution was added to each oil sample (2.5 g).

2.8. Analysis of fruit and VOO phenolic compounds

Phenolic extracts were then analyzed by HPLC in a Beckman Coulter liquid chromatographic system equipped with a System Gold 168 detector, a solvent module 126 and a Mediterranea Sea 18 column (4.0 mmi.d. x 250 mm, particle size 5 μm) (Teknokroma, Barcelona, Spain) following a previously described methodology (Luaces et al., 2007). Quantification and identification of phenolic compounds was performed as described by García-Rodríguez et al. (2011).

2.9. Stability of VOO

Oxidative stability was expressed as the oxidation induction time (h), measured with the Rancimat apparatus Model CH 8970 (Metrohm AG, Herison, Switzerland) using an oil sample of 2.5 g, warmed to 100ºC, and a purified air flow rate of 20 l/h. Three determinations per oil were carried out.

2.10. Extraction and analysis of VOO volatile compounds

Olive oil samples were conditioned to room temperature and then placed in a vial heater at 40 °C. After 10 min of equilibrium time, volatile compounds from headspace were adsorbed on a solid-phase microextraction (SPME) fiber DVB/Carboxen/PDMS 50/30 μm (Supelco Co., Bellefonte, PA). Sampling time was 50 min at 40 °C. Desorption of volatile compounds trapped in the SPME fiber was performed directly into the gas chromatograph (GC) injector.

Volatiles were analyzed using a HP-6890 GC equipped with a DB-Wax capillary column (60 m × 0.25 mm inner diameter; film thickness, 0.25 μm; J&W Scientific, Folsom, CA). Quantification and identification of volatile compounds was carried out following the method described by Sánchez-Ortiz, Pérez, and Sanz. (2013). Volatile compounds were clustered into different classes according to the polyunsaturated fatty acid and the LOX pathway branch origin. Quantitative data for every volatile class was done as previously detailed by Sánchez-Ortiz et al. (2013).

2.11 Statistical analysis

Data were statistically evaluated using Statgraphics Plus 5.1 (Manugistic Inc., Rockville, MD). Analysis of variance (ANOVA) was applied, and comparison of means was done by the Student-Newman-Keuls/Duncan test at a significance level of 0.05.

Moreover, the correlation between the amounts of total phenolics, o-diphenolics and secoiridoid compounds and the oxidative stability of the oils was assessed by simple regression applying the best fitting model (linear). The degree of correlation was expressed by regression coefficient (r).

3. Results and discussion

POX, PPO and β-glucosidase activities were extracted and assayed in crude protein extracts obtained from green mature Arbequina fruits stored during 4 weeks at two different temperatures (4 and 20 °C ± 2 °C) in order to determine if endogenous enzymatic activities levels could be correlated to the oils phenolic profiles.

3.1. Monitoring the olive endogenous enzymatic activities (POX, PPO, and β-glucosidase) during fruit ripening and storage

The evolution of POX activity in Arbequina olive seeds during ripening and fruit storage at 4 and 20 °C is illustrated in (Figure 1). Concerning the POX activity during fruit ripening from 24 to 28 WAF, the initial value was found to be low at early fruit maturation stages (9.4 ± 0.2 U/g FW at 24 WAF). Then results demonstrate an increment on POX activity during the ripening with a maximum activity level equal to 13.93 ± 0.4 U/g FW at 28 WAF. These activities confirmed those reported by García-Rodríguez et al. (2011).

A gradual increase in seeds POX activities was also observed during storage, except in the second week of fruits storage at 4 °C when the POX activity passed from 9.4 U/g FW to 8.77 ± 0.3 U/g FW. Moreover, during the first week of storage, POX activities were almost constant for both storage temperatures and were respectively. 11.36 ± 0.3 and 10.5

± 0.2 U/g FW at 4 and 20 °C. On the contrary, during the second and third week of storage, POX activities were higher in olives stored at 20 °C compared to those stored at 4 °C. Maximum activity level was observed on the last week of storage, reaching 15 ± 0.6 U/g FW and 14 ± 0.5 U/g FW for Arbequina fruits stored at 20 and 4 °C, respectively.

Results show that POX activities in olives seeds during storage and ripening have almost the same behavior with slightly higher activities in fruits stored at ambient temperature.

The evolution of PPO activity in olive fruit mesocarp during ripening and storage at two different temperatures was shown in (Figure 1). Results show that PPO activities decrease during fruit ripening at both storage temperatures which was more marked when storage was carried out at 20 °C. In fact, after one month of storage at 20 °C, PPO activity passed from 287.1 ± 10.4 to 147.7 ± 24 U/g FW corresponding to 49% reduction in respect to the initial value. However, when fruits were stored at 4°C, the diminution was only 24% (218.4 ± 15.7 U/g FW) and followed similar pattern to that found in fresh harvested fruits. In this sense, the PPO activity values found along Arbequina fruit ripening, were in good agreement with previous studies on PPO activity in the fruits of Gordal and Manzanilla olive varieties which had reported that olive PPO decreases sharply during the first month of olive fruit development and then at a slower rate during fruit maturation and ripening (Hornero- Méndez, Gallardo-Guerrero, Jarén-Galán, & Mínguez-Mosquera, 2002).

As previously observed for POX activity, PPO activity in olive fruits stored at 4 °C followed the same trend that in fresh fruit, and was significantly different from those stored at 20 ºC.

The olive β-glucosidase activity during ripening and storage was determined and results were shown in (Figure 1). The activities found for both storage temperatures (4 and 20 °C) were almost equal on the first week of storage (31.2 ± 1.8 and 33 ± 2.1 U/g FW respectively). However, their behavior was very different during the last three weeks. In

fact, at 20 °C, the β–glucosidase activity fell considerably from 33 to 0.6 ± 0.2 U/g FW. While, at 4 °C, it continued to increase until the third week of storage (57.2 ± 6.3 U/g FW) and then decreased slightly at the last week of storage (50.6 ± 6.2 U/g FW).

Regarding, the β–glucosidase ripening evolution, the maximum activity was attained at the 27 WAF (66.2 ± 4.5 U/g FW) and then decreased slowly to reach 54.3 ± 3.5 U/g FW at the 28 WAF corresponding to the green-brown stage. The activity was high in green fruit, when the oleuropein amount was highest and low in last stage when the oleuropein concentration declined. These results were in accordance with those reported by Bitonti, Chiappetta, Innocenti, Muzzalupo, and Uccella (2000) concerning the evolution of oleuropein concentration at different ripening stages. In fact, in green fruits the debittering, due to the hydrolysis of oleuropein, does not occur because enzyme and substrate are in the separate cell compartments although the respective concentrations in tissues are very high. In green-brown fruits the debittering is provoked by the damage of cell structures, due to the senescence of tissues or pathogens injury or mechanical damage that brings in contact β–glucosidase and oleuropein, despite lower enzyme activity. Moreover, Mazzuca, Spadafora, and Innocenti (2006) showed that the β–glucosidase activity trend during ripening appeared to be linked to oleuropein degradation and the release of glucose and aglycones molecules, with the consequent physiological debittering of fruit tissues.

For these reasons, the clear decline on β–glucosidase activity from the first week of olive storage at ambient temperature could be related to the accelerated ripening and to the damage of cell structures happening during olives storage at high temperature (García et al., 1996b). However, at 4 °C, the decrease on β–glucosidase activity was lagged by 2 weeks compared to that determined in the ambient temperature storage. This may be related to the fact that olive refrigeration delayed olive ripening and tissue softening (García et al., 1996a; Yousfi et al., 2012).

In conclusion, monitoring the olive endogenous enzymatic activities (POX, PPO, and β-glucosidase) during fruit ripening and storage showed a similar pattern between 4 ºC stored fruits and fresh harvested ones.

3.2. Phenolic compounds

Given the importance of the phenolic fraction, the change in the phenolic compounds over time in fruits and oils could be an essential quality control parameter of VOO.

To investigate whether the observed enzymatic activities changes correlate with fruits and oils phenolic profile, the monitoring of fruits and oils phenolic compounds in different experimental conditions (fresh harvested fruits and stored ones) was studied.

3.2.1. Behavior of phenolic compounds in fruits

The phenolic composition of fresh harvested and stored olive fruits is shown in Table 1. As illustrated, significant differences on phenolic composition were noted between samples. Moreover, the most representative complex phenols identified in olive fruits were oleuropein, dimethyloleuropein, hydroxityrosol and ligstroside. The content of oleuropein decreased significantly during ripening and vanished after 4 weeks of storage at 20 °C. In fact, the levels of oleuropein decreased from 7382.3 μg/g to 1840.5 μg/g (25% of its initial content) during ripening. However, in fruits stored at 4 °C, oleuropein content increased during the two first weeks up to 10768.3 μg/g

Dimethyloleuropein, is a phenolic glycoside characteristic of the Arbequina variety, that is considered by some authors as a degradation product of the oleuropein (Gomez-Rico et al., 2008). Dimethyloleuropein doubled its content during ripening. In fact, it passes from 6027.7 μg/g to 11399.8 μg/g from 24 to 28 WAF. In the same way, the content of this compound also increased significantly during olive storage at 20 °C and the same pattern was observed for hydroxityrosol.

Oleuropein degradation could explain hydroxityrosol and dimethyloleuropein concentration increment.

The concentration of ligstoside decreases along ripening and storage particularly at 20 °C.

3.2.2. Phenolic compounds in the oils

Changes in oil phenolic profiles obtained from fresh harvested fruits and from olive fruits stored at different temperatures are shown in Table 2. Olive oils samples did not show significant qualitative differences in their phenolic fraction profiles. However, significant quantitative differences were observed for many of the phenolic compounds.

The most representative phenolic compounds were 3,4-DHPEA-EDA and p-HPEA-EDA. In fact, the concentration of these two compounds in the VOO obtained from fruits harvested at 24 WAF was 1.0951 ± 0.0545 and 0.452 ± 0.0258 μmol/g olive oil, respectively. The secoiridoid compound p-HPEA-EA was not detected in this oil and other simple phenols, such as vanillic, cinnamic acid and *p*-coumaric acid, were detected in very low amounts. Considerable changes were observed in oil phenolic composition obtained through fruit ripening. The content of total phenolics, o-diphenolics and secoiridoids derivatives in the oils decreased about 20-25% from fruits harvested at 24 WAF to those of 28 WAF.

The total content of phenolic compounds, orthodiphenols and secoiridoids derivatives in the oils extracted also decreased as the fruit storage period progressed. Moreover, the negative effect of storage time on oil phenolic compounds was more noticeable when storage was carried out at 20 °C than at 4 °C. In fact, when fruits were kept at 20 °C, the reduction rate of these compounds was 90.31, 95.78 and 91.73% respectively whereas, at 4 °C, it was 22.5, 25.93 and 26.17 % respectively.

In addition, secoiridoids compounds, 3,4-DHPEA-EDA and 3,4-DHPEA-EA, were the most affected phenolic compounds along the total storage period. Indeed, oil loses 28.81

and 97.07% of these compounds when fruits were stored at 4 °C and 20 °C respectively. However, the loss of p-HPEA-EDA was 18.85 and 77.54 % at 4 and 20 °C. In the same way, the reduction rate of 3,4-DHPEA-EDA, and p-HPEA-EDA was respectively 27.41, and 13.59% during olive ripening. However, only 3.36% of 3,4-DHPEA-EA was reduced during fruit ripening.

Concerning the phenolic alcohols, the tyrosol concentration varied between the analyzed samples. In fact, it maintained its concentration during ripening and storage at 4°C and decreased only after 3 weeks when fruits were kept at 20 °C. Tyrosol concentration may be associated to the ligstroside aglycone and derivatives hydrolysis which contains tyrosol as an aromatic alcoholic fragment.

However, hydroxytyrosol showed a decrease at the last ripening stage and after 4 weeks of storage at 20 °C. It was noted that tyrosol concentration was higher than hydroxytyrosol. This result was similar to those reported by several authors (Tsimidou, Papadopoulos, & Boskou, 1992; Caponio, Allogio, & Gomes, 1999).

Regarding flavonoids, for all samples the concentration of luteolin was higher than that of apigenin. Moreover, the flavonoids contents of the oils extracted from olives stored at 4 °C were higher than those at 20 °C.

In oils extracted from stored olives at 4 °C, both luteolin and apigenin contents registered a clear rise during all the storage period. However, in those extracted from olives kept at 20 °C, flavonoids contents increased until 2 weeks of storage and then decreased sharply during the last two weeks of fruit storage.

The significant reduction of phenolic compounds found in the oils obtained from olives stored at 20 °C can be related to the fact that high storage temperatures affect the physiological status of the olive fruits and accelerate the processes of fruit ripening. Moreover, the olives suffer softening and become very sensitive to mechanical damage and to the ac-

tion of pathogenic microorganisms which are able to metabolize a wide variety of aromatic compounds, such as phenol and its derivatives (Watanabe, Hino, Onodera, Kajie, & Takahashi, 1996). However, olive refrigeration at 4 °C delayed deterioration of the fruit in terms of physical, chemical, and sensorial quality so that VOOs obtained from cold stored fruits retain good organoleptic and nutritional properties. In addition, low temperatures may have a bacteriostatic action by controling microbial development (Clodoveo et al., 2007).

In conclusion, our results revealed a very similar phenolic profile of the oils extracted from fruits stored at 4 ºC and fresh harvested ones. Whereas, the oil extracted from fruits kept at 20 °C has a very poor quality marked by its low phenolic content.

Moreover, our data showed that the β-glucosidase was the key enzyme directly related to the oil phenolic profile since the sharp decrease in β-glucosidase activity after 3 weeks of storage at 20 ºC was parallel to a dramatic decrease of phenolic compounds in the corresponding oils even if the fruit was rich in phenolic compounds.

3.3. Oil stability

The oil stability evaluates the time (hours) of their resistance to oxidation and plays a key role in the assessment of the olive oil quality.

Oxidative stability, measured by Rancimat method, of the oils extracted during fruit ripening and storage was shown in Table 3. Results showed that the oil stability was clearly affected by fruit ripening and storage conditions. In fact the oil stability decreased with olive maturation degree and storage. Moreover, this reduction was more important when fruits were stored at 20 °C. Indeed, 60.62% of oil stability was lost when olives were kept at 20 °C during 4 weeks, whereas only 20 and 15.93% was lost respectively during refrigeration and ripening. García and Yousfi (2006) demonstrated that storage at low temperature delayed olive fruit ripening and that VOO stability decreased with olive maturation.

The results obtained were in good agreement with those found in a previous study on Picual fruits stored at 5 °C, 8 °C and ambient temperature during 60 days, in which the oil stability decreased 35, 70 and 93% respectively.

Numerous studies have reported a correlation between phenolic compounds content and oil oxidative stability. As described elsewhere, the contribution of phenolic compounds to oil stability was estimated at approximately 30%, of fatty acids at 27% and of carotenoids at 6% (Apparicio, Roda, Albi, & Gutiérrez, 1999).

Figure 2 showed the relation between the oil phenolics, o-diphenolics and secoridoids content, with their corresponding oxidative stabilities. Data shown by this figure evidenced a positive correlation between the oxidative stability measured by Rancimat and total phenolics, o-diphenolics and secoridoid compounds analysed by HPLC. As illustrated, the maximum correlation (r=0.985) was found between total phenols in oils extracted from fruits stored at higher temperature and the resistance to oxidation. This result was due to the lowest amounts of phenolics compounds in oils obtained from stored olive fruits at high temperature and confirms the strict dependence between phenolic concentration and oil stability.

3.4. Volatile compounds

Given that the aroma is a key quality parameter for VOO, the volatile profiles of the different oils obtained were studied (Table 4). C6 aldehydes and alcohols and their corresponding esters, are the most important compounds in the VOO aroma, from either a quantitative or a qualitative point of view (Kotti, Gargouri, Chiavaro, & Bendini, 2011).

Moreover, Kalua, Allen, Bedgood Jr, Bishop, Prenzler, &Robards (2007) reported that storage of the fruit decreased the aldehyde and ester content that is responsible for the positive aroma.

During olive fruit ripening an increase of C6 compounds was observed, from 22645 to 29770 ng/g oil. The contents of C6 compounds, in oils extracted from fruits stored at 20 °C decreased through storage. Whereas oils extracted from stored fruits at 4 °C showed a progressive increase in these compounds until 2 weeks. These compounds are responsible for the positive aroma perceptions in olive oils.

The trend of C5 compounds including also aldehydes and alcohols compounds was similar to that of C6 compounds with a difference in the case of cold storage consisting in a decrease of C5 compounds content after 1 week of storage at 4 °C.

The esters content was also significantly affected by temperature and storage period. In fact, the content of these compounds was higher when fruits were stored at 20 °C than at 4 °C. Moreover, their concentrations reached highest level after 2 weeks of storage at both temperatures. However, these values were lower than the concentration measured in fresh fruits at the same period.

4. Conclusion

This study demonstrated that storage conditions affects VOO flavor quality. However, olive refrigeration at 4 °C retarded deterioration in the oil quality parameters by delaying alteration of the olive fruits. In this sense, for most quality parameters, a very similar pattern was observed between fruits stored at 4 °C and freshly harvested fruits. Thus, the prolonged storage of fruits caused a decrease in corresponding oil phenolic content. This negative effect was more noticeable when fruits were kept at ambient temperature (20 ± 2 °C) than at 4 °C.

A positive correlation was obtained between the decrease of total phenolics, *o*-diphenolics and secoiridoid compounds and the loss of oxidative stability measured by Rancimat method.

Results evidenced the contribution of POX, PPO and β-glucosidase activities in determining the oil phenolics profile. However, the experimental data obtained point to olive β-glucosidase as the key enzyme to determine the phenolic profile of the VOO.

Appendix:

ABBREVIATIONS USED

VOO = Virgin Olive Oil

POX = Peroxidase

PPO = Polyphenoloxidase

*p*NPG = p-nitrophenyl- β,D-glucopiranoside

TBC = Tert-butylcatechol

SDS =Sodium Dodecyl Sulfate

PMSF= Phenyl Methyl Sulfonyl Fluoride

WAF = Weeks After Flowering

EDTA = Ethylen Diamine Tetraacetic Acid

DTT = Dithiothreitol

3,4-DHPEA-EDA = di-aldehydic form of the oleuropein aglycone

p-HPEA-EDA = di-aldehydic form of the ligstroside aglycone

3,4-DHPEA-EA = aldehydic form of the oleuropein aglycone

p-HPEA-EA = aldehydic form of the ligstroside aglycone

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FIGURE CAPTIONS

Figure 1. Evolution of olive endogenous enzymatic activities (U/g FW, means of three replicates) during fruit ripening (a) and along the storage of Arbequina fruits at 4°C (b) and at 20°C (c)

Figure 2. Correlation between the amounts of total phenolics, o-diphenolics and secoridoids compounds with the oxidative stability of the oils extracted from fresh harvested Arbequina fruits (a) and fruits stored at 4°C (b) and at 20°C (c). The correlation was assessed by simple regression applying the best fitting model (linear). The degree of correlation was expressed by regression coefficient (r).

Table 1. Concentration of phenolic compounds in fresh harvested Arbequina fruits and fruits stored at different temperatures (4 and 20°C) during 4 weeks.

Table 2. Concentration of phenolic compounds in VOOs obtained from fresh harvested Arbequina fruits and fruits stored at different temperatures (4 and 20°C) during 4 weeks.

Table 3. Oxidative stability index (expressed as hours) of oil samples extracted from fresh harvested Arbequina fruits and fruits stored at different temperatures (4 and 20°C) during 4 weeks.

Table 4. Concentration of Volatile compounds in VOOs obtained from fresh harvested Arbequina fruits and fruits stored at different temperatures (4 and 20°C) during 4 weeks.

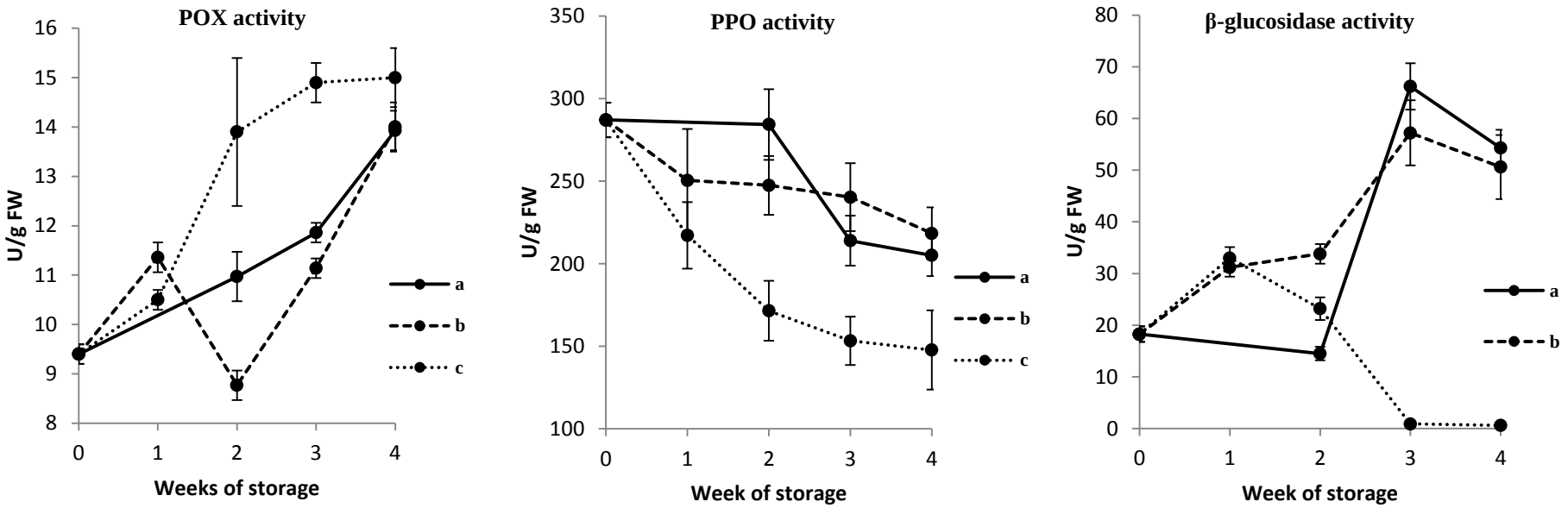

Figure 1. Evolution of olive endogenous enzymatic activities (U/g FW, means of three replicates) during fruit ripening (a) and along the storage of Arbequina fruits at 4°C (b) and at 20°C (c)

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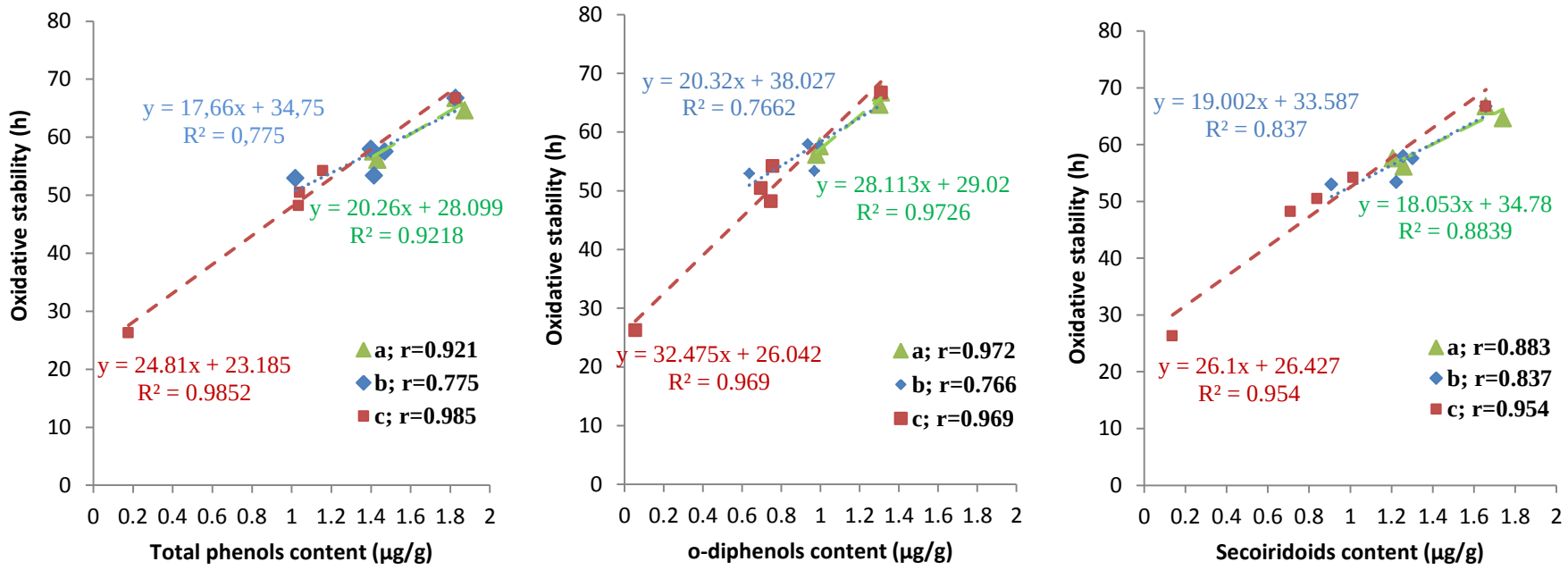

Figure 2. Correlation between the amounts of total phenolics, o-diphenolics and secoridoids compounds with the oxidative stability of the oils extracted from fresh harvested Arbequina fruits (a) and fruits stored at 4°C (b) and at 20°C (c). The correlation was assessed by simple regression applying the best fitting model (linear). The degree of correlation was expressed by regression coefficient (r).

Table 1. Phenolic contents (µmol/ g olive pulp) in Arbequina fruits ripen on the tree and stored at different temperatures (4 and 20°C) during 4 weeks.

Fruit Phenolics (µmol/g FW)		**On-tree ripening**			**Storage at 4ºC**				**Storage at 20ºC**			
	0 (24 WAF)	**2 week**	**3 weeks**	**4 weeks**	**1 week**	**2 weeks**	**3 weeks**	**4 weeks**	**1 week**	**2 weeks**	**3 weeks**	**4 weeks**
Hydroxytyrosol	2.5	1.9a	1.5a	2.2a	2.1	2.1a	2.0a	2.3a	3.1	4.4b	5.1b	6.7b
Demethyloleuropein	11.4	16.4b	14.8b	21.7b	9.3a	9.1a	12.4a	17.0a	17.7b	23.8c	34.9c	36.0c
Verbascoside	1.7	1.7	0.8a	1.4a	1.7	1.7	1.4b	1.5a	1.6	1.6	2.1c	2.4b
Luteolin-7-glucoside	0.5	0.7	0.5	0.8	0.7	0.9	0.6	0.8	0.8	0.6	0.8	0.6
Oleuropein	13.7	6.2a	2.7a	3.4b	16.6b	19.9c	15.3b	17.1c	8.4a	2.3b	1.6a	0a
Ligstroside	12.7	0.7b	0.5a	0a	1.1b	1.3c	0.8b	1.0c	0.7a	0.5a	0.6a	0.7b

*Mean values from three determinations.

**For each compound and week values with different letters are significantly different ($P \leq 0.05$).

Table 2: Concentration of phenolic compounds in VOOs obtained from fresh harvested Arbequina fruits and fruits stored at different temperatures (4 and 20°C) during 4 weeks

		On-tree ripening			**Storage at 4ºC**				**Storage at 20ºC**			
Phenolics (μmol/g olive oil)	**0 (24 WAF)**	**2 weeks**	**3 weeks**	**4 weeks**	**1 week**	**2 weeks**	**3 weeks**	**4 weeks**	**1 week**	**2 weeks**	**3 weeks**	**4 weeks**
Hydroxytyrosol	0.0036*	0.0033	0.0033	0.0028	0.0033	0.00273	0.0045	0.0031	0.0026	0.0034	0.0030	0.0021
Tyrosol	0.0052	0.0054	0.0061	0.0046	0.0064	0.0074	0.0065	0.0073	0.0073	0.0079	0.0044	0.0032
Vanillic acid	0.0027	0.0016	0.0018	0.0008	0.0026	0.0023	0.0017	0.0015	0.0014	0.0008	0.0003	0.0002
p-coumaric acid	0.0029	0.0025	0.0013	0.0007	0.0034	0.0027	0.0018	0.0016	0.0013	0.0021	0.0014	0.0065
Hydroxytyrosol Ac.	0.0902	0.0529	0.1230	0.0943	0.0585	0.0317	0.0722	0.0972	0.0652	0.1177	0.2728	0.0154
3,4-DHPEA-EDA	1.0951	1.1381	0.8004	0.7949	0.7798	0.5195	0.8081	0.7470	0.6126	0.5112	0.4185	0.0311
OA-isomers	0.0400	0.0252	0.0038	0.0058	0.0270	0.0255	0.0365	0.0272	0.0165	0.0050	0.0051	0.0012
p-HPEA-EDA	0.4527	0.5057	0.3461	0.3912	0.3931	0.3157	0.3941	0.3674	0.3360	0.2771	0.2480	0.1017
Pinoresinol	0.0077	0.0084	0.0083	0.0084	0.0075	0.0036	0.0074	0.0084	0.0069	0.0075	0.0047	0.0020
Cinnamic acid	0.0012	0.0010	0.0009	0.0011	0.0004	0.0007	0.0011	0.0067	0.0006	0.0005	0.0007	0.0003
Acetoxipinoresinol	0.0427	0.0436	0.0475	0.0420	0.0495	0.0478	0.0577	0.0551	0.0462	0.0452	0.0269	0.0068
3,4-DHPEA-EA	0.0695	0.0711	0.0565	0.0671	0.0563	0.0476	0.0632	0.0820	0.0487	0.0449	0.0386	0.0003
p-HPEA-EA	0	0	0	0	0	0	0	0	0	0	0	0
Luteolin	0.0097	0.0103	0.0094	0.0140	0.0093	0.0094	0.0106	0.0123	0.0096	0.0131	0.0083	0.0023
Apigenin	0.0025	0.0031	0.0029	0.0049	0.0027	0.0026	0.0031	0.0038	0.0027	0.0032	0.0023	0.0007
Total phenolics**	**1.8264**	**1.8726b**	**1.4119b**	**1.4332b**	**1.4005b**	**1.0197a**	**1.4691b**	**1.4153b**	**1.8264**	**1.1581a**	**1.0403a**	**1.0355a**
Total *o*-phenolics	**1.3084**	**1.3011b**	**0.9965b**	**0.9792b**	**0.9345b**	**0.6366a**	**0.9952b**	**0.9690b**	**1.3084**	**0.7554a**	**0.6955a**	**0.7466a**
Secoiridoids	**1.6575**	**1.7402b**	**1.2070b**	**1.2592b**	**1.2563b**	**0.9084a**	**1.3020b**	**1.2237b**	**1.6575**	**1.0139a**	**0.8384a**	**0.7103a**

*) Average coefficient of variance was lower than 9%. (**) Total values with different letters within each week are significantly different ($P \leq 0.05$).

Table 3. Oxidative stability index (expressed as hours) of oil samples extracted from fresh harvested Arbequina fruits and fruits stored at different temperatures (4 and 20°C) during 4 weeks.

	Weeks				
	0 (24 WAF)	**1**	**2**	**3**	**4**
Fresh harvested fruits	66.7±1.5	-	64.6±2.3	57.5±0.3	56.1±0.7
Stored fruits at 4°C	66.7±1.5	57.9±0.3	52.9±0.6	57.5±0.3	53.4±0.6
Stored fruits at 20°C	66.7±1.5	54.2±0.3	50.4±0.6	48.2±0.4	26.2±0.3

Table 4. Concentration of Volatile compounds in VOOs obtained from fresh harvested Arbequina fruits and fruits stored at different temperatures (4 and 20°C) during 4 weeks.

	On-tree ripening				**Storage at 4ºC**				**Storage at 20ºC**			
Volatile class (ng/g olive oil)	**0 (24 WAF)**	**2 weeks**	**3 weeks**	**4 weeks**	**1 week**	**2 weeks**	**3 weeks**	**4 weeks**	**1 week**	**2 weeks**	**3 weeks**	**4 weeks**
C6/LnA aldehydes	21553±1422	25322±424	27208±222	26247±11	23813±1225	27687±195	23399±1298	23212±192	15865±441	15970±255	11557±160	9418±262
C6/LnA alcohols	723±79	1441±202	1701±42	1372±178	1228±140	1052±7	922±83	941±55	887±118	708±16	1284±247	874±12
Σ C6/LnA	22277±1342	26763±221	28910±179	27619±190	25042±1365	28739±187	24322±1381	24153±248	16752±323	16678±272	12842±408	10292±275
C6/LA aldehydes	353±121	499±132	629±24	903±13	561±149	807±36	494±28	682±10	714±11	987±56	763±38	1709±42
C6/LA alcohols	14.34±2	218±274	231±10	51±2	46±44	20±0	21±6	30±2	14±1	19±1	167±32	268±5
Σ C6/LA	367±124	717±407	860±34	954±10	608±104	827±37	515±22	713±13	729±10	1007±57	931±71	1978±36
C5/LnA carbonyls	497±54	584±48	582±49	448±15	351±32	259±59	246±16	259±20	412±97	414±24	334±48	214±5
C5/LnA alcohols	745±2	1166±288	1276±178	825±26	847±232	494±1	357±4	401±49	717±102	614±84	492±26	435±14
Σ C5/LnA	1242±51	1751±336	1859±228	1274±10	1199±264	754±57	603±12	661±70	1130±4	1029±109	826±74	650±20
C5/LA carbonyls	32±13	109±6	132±18	35±2	47±19	32±3	37±6	48±9	60±25	72±49	41±34	26±1
C5/LA alcohols	9±3	92±81	157±8	25±17	42±40	32±8	37±34	24±7	28±16	59±35	42±23	7±1
Σ C5/LA	42±10	201±87	289±10	60±15	89±58	65±5.	75±40	72±1	89±9	131±13	84±11	33±2
LOX esters	83±42	422±250	338±35	137±5	246±124	221±186	80±29	99±33	104±8	268±13	161±106	87±43
Non-LOX esters	70±28	339±12	425±1	183±4	50±11	176±103	144±10	133±56	176±58	329±17	197±68	159±35
Σ Esters	154±14	761±263	764±34	320±9	296±113	397±290	224±39.	232±89	280±49	597±31	358±174	246±7
Σ Volatile AA	120±43	236±54.6	340±57	158±77	132±36	79±8	77±14	72±37	98±8	183±17	113.85±26.07	131±81
Σ Terpenes	8989±353	8241±698	8190±269	5698±621	6636±205	4022±563	3814±252	3794±199	5293±298	6017±755	5281±465	4153±351
Total volatiles*	33194	38674b	41215c	36087c	34005b	34888c	29633b	29701b	24375a	25646a	20439a	17487a

* Total values with different letters within each week are significantly different ($P \leq 0.05$).

1
2
3
4