Chapter

THE EFFECT OF EXOGENOUS ABSCISIC ACID SPRAY ON PHENOLICS AND SCAVENGING FREE RADICAL ACTIVITY OF OLIVES DURING STORAGE

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ABSTRACT

The olive tree (*Oleo europaea*) is largely popular for the production of both oil and table olives, which are positively considered in nutrition studies. Apart from fatty acids, olives also contain some minor components such as phenolics which are regarded as highly beneficial to the health because of their antioxidant properties. Phenolics occurring in olive pulp are also present in processing by-products. However, phenolics decrease drastically during the storage in such a way that postharvest techniques guaranteeing oil quality are essential when oil processing is delayed. In the present study we investigated abscisic acid (ABA) spray treatment as a postharvest procedure to minimize losses of phenolics during olive storage. With this purpose, we evaluated the effect of ABA on olive pulps and stones on days 7, 15 and 30 after the treatment. The total phenolic contents were measured in ABA treated olives by the Folin-Ciocalteu method whereas the 1,1-diphenyl-2-picrilhydrazyl free radical (DPPH) scavenging assay was used to evaluate the scavenging free radical capacity The values obtained were compared with those of the pulps and the stones obtained from untreated olives. The results

indicate that ABA treated olive pulps had higher total phenolic content and DPPH scavenging capacity as compared with untreated samples. A similar effect was also observed for the stones, although the increases in both total phenolic content and scavenging free radical activity were not as pronounced as in the pulps. For both olive pulps and stones, exogenous ABA spray was more effective after prolonged storage periods. Concerning phenolics, we focused our study on oleuropein and hydroxytyrosol for their abundance in olive fruit and their pharmacological properties. The effect of ABA on contents of oleuropein and hyrdroytyrosol was evaluated by HPLC-UV. The identification was carried out on the basis of retention times and UV spectra while calibration curves were performed for their quantification. Oleuropein and hydroxytyrosol exerted significantly ($p < 0.05$) higher contents in olive pulps and stones after the ABA treatment. In line with the overall results on phenolics and scavenging free radical activity, the increments were more marked for the pulps than for the stones and particularly after longer storage periods. These findings indicate that the postharvest ABA spray treatment might be a promising approach to avoid the natural losses of the phenolic content and antioxidant properties of olive fruits during the storage. The postharvest method proposed allows olive pulps and stones with improved antioxidant properties to be obtained. Since the stones are a residue from oil processing, ABA treated olive stones are proposed as a high added value olive by-product. These results can be interesting to the olive oil industry, in particular when delay in oil processing occurs and olives have to be stored.

Keywords: olives, oleuropein, hydroxytyrosol, phenolics, abscisic acid, stones, pulps, by-products, postharvest treatment

1. INTRODUCTION

Nowadays the awareness of the relationship between the diet and the health has led to a growing search for functional foods. In this context, it has been demonstrated that the chemical elicitation of plant foods is a powerful strategy to produce functional foods by means of the increase of health promoting constituent contents (Baenas, García-Vigueras & Moreno, 2014). Various elicitors have been reported to stimulate the production of bioactive compounds in plant foods. In particular, certain phytohormones have demonstrated to be particularly effective since, besides regulating all aspects of the plant growth and development, they are also involved in the bioformation of secondary metabolites. Among them, the use of abscisic acid

(ABA) increases the accumulation of tanshinones and phenolic acids (Yang, Ma, Liang, Wei, Liang, Liu & Liu, 2012), anthocyanins (Cui, Liang, Liu, Liu & Zhu, 2012) and other phenolics (Cantín, Fidelibus & Crisosto, 2007; Huang, Cai, Ye, Hu, Li & Zhang, 2016) in derived plant foods.

We have largely investigated the effects of certain phytohormones as chemical elicitors on the phenolic content. The focus of our studies has been oriented to induce the production of anthocyanins, phenolic acids and flavonols, among other phenolic compounds, in berries through the treatment with phytohormones other than ABA (de la Peña Moreno, Monagas, Blanch, Bartolomé & Ruiz del Castillo, 2010; Ruiz del Castillo, Flores & Blanch, 2010; Flores & Ruiz del Castillo, 2014; Flores, Blanch & Ruiz del Castillo, 2015). Lately, we have also started evaluating the effects of ABA on the phenolic acid content in olive fruits.

Olive fruits contain a great amount of healthful compounds. Apart from the known richness of monounsaturated fatty acids, olive fruits possess some minor components with biological properties. Although there are more than two hundred minor components in olives, particular attention has been focused on those with antioxidant activity. In this regard, nutraceutical properties have been attributed to oleuropein and its derivatives, belonging to the secoiridoid polyphenols family, as well as to the main alcohol 3,4-dihydroxyphenyl ethanol, also known as hydroxytyrosol (Bendini et al., 2007). Their activities have been investigated as pure compounds or as components of extracts, showing important pharmacological effects (Omar, 2010; Cicerale, Lucas & Keas, 2010). Oleuropein is abundant in unprocessed olive leaves and fruit, while higher concentration of hydroxytyrosol may be found in other tissues owing to chemical and enzymatic reactions occurring during the fruit maturation (Morello, Motilva, Tovar & Romero, 2004). Such compounds are released from the olive fruit to the oil during the extraction process. However, it has been described that phenolic content decrease drastically during the olive storage, which is necessary when a delay in oil processing occurs (Benito, Oria & Sánchez-Gimeno, 2009; Gómez-Alonso, Salvador & Fregatane, 2007). In these cases, a methodology allowing the increase of the concentration of phenolic compounds in the collected fruit will help to obtain oils of higher quality and to reduce the loss of nutritional value of oils obtained from stored olives.

The olive pomace obtained from olive fruit waste contains seed husk and a small amount of seeds, pulp, and peel, which can be separated by common industrial methods. Part of the phenolic compounds such as oleuropien and hydroxytyrosol contained in the olive fruit also exist in processing by-

products. In particular, the olive stone is a source rich in valuable components (Rodríguez, Lama, Rodríguez, Jiménez, Guillén, & Fernández-Bolaños, 2008). However, while many studies have been conducted regarding phenolics in olive oil (Schwartz, Ollilainen, Piironen & Lampi, 2008), much less attention has been paid to the study of bioactive compounds in olive processing by-products (Visioli et al., 1999).

Recently, we have started working on a postharvest technique based on the application of exogenous ABA spray over olives.. Preliminary results obtained from these first studies suggest that ABA might induce accumulation of phenolic acids in olive pulps during their storage (data accepted for publication).

The objective is now to evaluate the effect of exogenous ABA spray on the total phenolic content, the ability to scavenge free radicals, and oleuropein and hydroxytyrosol contents in olive fruits during the storage. This study includes not only olive pulps but also stones. Since the stones are obtained as a waste from oil processing, the final aim was to propose a postharvest technique to obtain olive fruits enriched in antioxidant content and simultaneously a high added value product. This technique can be particularly practical from an industrial standpoint.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

HPLC-grade MeOH were supplied by VWR Inc. (Bridgeport, PA, USA), whereas acetic acid was purchased from Fisher Scientific (UK). Ultrapure water was collected from a purification system (Millipore Milford, MA, USA). For the elicitation, ABA was obtained from Across Organics (New Jersey, USA). Sodium carbonate and Folin-Ciocalteu reagent were supplied by Merck (Darmstadt, Germany). Oleuropein and hydroxytyrosol standards and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were acquired from Sigma-Aldrich (Steinheim, Germany). Olive fruits *(Manzanilla* cultivar) were randomly hand-picked from the trees in November 2014 in Cáceres (Spain). Only undamaged fruits without any kind of infection of physical injury were selected for the experiments. All fruits were picked at optimum ripening stages for the production of olive oil, according to their skin colour. After harvesting, the olive fruits were immediately kept in cool bags for a couple of days up to treatment, as explained below.

2.2. ABA Treatment

A 200-g weight of olives were placed in a glass container and treated with 45 mg of ABA (0.4 mg g^{-1} olive fruit) in 0.05% Tween-20 mixture, which was prepared by mixing 35 µl Tween-20 in 75 ml distillated water. These conditions were selected in an earlier study (data submitted for publication). ABA was applied as a spray to runoff over the olives. Simultaneously, other 200 g of olives were also placed in other glass container and sprayed with 0.05% Tween-20 mixtures alone (i.e, without ABA) to be used as a control. Both control and treated containers were stored at 4°C for 30 days. To study the evolution of the samples during the storage, analyses were carried out on days 7, 15 and 30 after treatment. Part of the samples was extracted for the total phenolic content and DPPH assay. The rest was tested for the oleuropein and hydroxytyrosol contents. The whole procedure was performed with two different batches of olives and all analyses were carried out in duplicates within each batch.

2.3. Sample Preparation

In all cases, the olive stone was carefully removed from the flesh manually by using a scalpel. Then, the stones were frozen in liquid nitrogen and freeze-dried to inhibit enzymatic activities. The dried stones were finally ground to a fine powder using a knife mill (Grindomix GM 300; Retsch, Dusseldorf, Germany) prior to analysis. The pulp, however, was immediately analyzed without any further pretreatment. The separation of the stone from the pulp did not imply any significant pulp modification.

2.4. Total Phenolic Content and DPPH Assay

2.4.1. Extraction

Phenolic compounds from control and ABA treated olive pulps and stones were extracted by following the analytical procedure described elsewhere

(Shin et al., 2008). In brief, 10-g weight of sample was homogenized for 3 min with 10 mL of 80% acetone at 4°C using a coffee grinder. The resulting mixture was filtered through No. 1 Whatman paper and the acetone was evaporated off by using a rotary evaporator at 45°C. The dry extracts were then split in two different batches: the first one, which was meant to determine the total phenolic content, was brought to 10 mL with deionized water and then divided into several 1 mL aliquots. The second batch, which was meant for DPPH assay, was brought to 5 mL MeOH.

2.4.2. Determination of Total Phenolic Content

The determination of total phenolic content was carried out by following the method described elsewhere (Singleton & Rossi, 1965). Basically, the method is based on the oxidation of the hydroxyl groups of phenols in basic media by the Folin-Ciocalteu reagent. A 0.1-ml volume of the extract, 0.5 mL of Folin-Ciocalteu reagent and 10 mL of sodium carbonate solution (75 g L^{-1}) were mixed, and the volume was made up to 25 mL with distilled water. After 1 h, the absorbance was measured at 750 nm against a blank prepared in the same way but without adding the reagent. Gallic acid was used as the standard to prepare the calibration curve. The results were expressed as milligrams of gallic acid equivalents per kg of olive fruit. Analyses were performed in duplicate.

2.4.3. 1,1-Diphenyl-2-picrylhydrazyl Free Radical (DPPH) Scavenging Assay*

The ability of the extracts to scavenge DPPH* radicals was performed according to a slight modification of the method developed by Smith, Reeves, Dage & Schnettler (1987). Each extract was further diluted to final concentrations of 15.6, 62.5, 125, 250, and 500 µg/mL before being transferred to a 96-well microtiter plate. Each extract solution before adding DPPH was used as a blank. Each well contained 50µL aliquot of the sample and 150 µL of DPPH (400 µM). Decrease of absorbance, with respect to DPPH solution measured immediately, was monitored at 517 nm after 30 min of incubation at 37°C. The percentage inhibition of the DPPH by each dilution of samples was calculated considering the percentage of the steady DPPH in solution after the reaction. The results were expressed as the concentration of extract that gives rise to a 50% reduction in the DPPH. The experiments were performed in quadruplicate.

2.5. Oleuropein and Hydroxytyrosol Contents

2.5.1. Extraction

The isolation of oleuorpein and hydroxytyrosol from olive pulp and olive stone powder was performed on the basis of the method elsewhere published (Vinha et al., 2005) with slight modifications. First, a 60 ml-volume of 80:20 (v/v) methanol:water was added to a 5 g-weight of sample. Then, the mixture was homogenized by using and Ultraturrax (IKA, Sigma-Aldrich, Madrid, Spain) and subsequently centrifuged at 1500 rpm for 10 min at room temperature. The supernatant was filtered through filter paper. An additional 60 ml of methanol:water was added to the extract, which was re-extracted. After that, 30 ml of hexane was added to the resulting extract to eliminate the remaining oil. Once discharged the hexane layer, the combined methanolic extracts were collected, filtered through Whatman No. 1 filter paper and analysed by HPLC as detailed below. Extractions of each single sample including controls and samples treated with ABA spray were accomplished in duplicate.

2.5.2. HPLC analysis

A Konik-Tech model 560 (Barcelona, Spain) liquid chromatograph fitted with a manual injection valve (model 7725i, Konik-Tech, Barcelona, Spain) and having a 20-µl sample loop was used for the analyses. The separation was accomplished on a ODS reverse phase (C18) column (250 nm × 4.6 mm i.d., 5-µm particle size, ACE, Madrid, Spain). A mixture of water/acetic acid (95/5, v/v) and methanol were used as solvents A and B, respectively and the flow rate was 1 ml/min. A linear gradient was programmed as follows: initial composition 95/5% A/B, 85/15 A/B at 3 min, 80/20 A/B at 13 min, 75/25 A/B at 25 min, 70/30 A/B at 35 min, 65/35 A/B at 40 min, 60/40 A/B at 45 min, 55/45 A/B at 47 min, 53/47 A/B at 50 min, 52/48 A/B at 60 min, 50/50 A/B at 64 min, 50/50 A/B at 70 min, 95/5 A/B at 75 min. Chromatograms were recorded at 280 nm. Blanks between consecutive runs were performed to assure the washing of the equipment. Three HPLC runs were performed for each single extract. Stock solutions of the standard compounds were prepared in 70% (v/v) methanol to final concentration of 1 mg/mL. Each stock solution was further diluted to obtain six concentrations of the standard. Calibration curves of the standards were established on six data points, and each standard dilution was injected in triplicate. Peak areas for the extracts and standards were integrated by use of Konikrom Plus (KNK-725-240).

2.6. Statistical Analysis

The results are given as mean values ± standard deviation (SD). Student´s *t*-test was used for comparison between two means and a one-way analysis of variance (ANOVA) was used for comparison of more than two means (Runyon & Haber, 1984). A difference was considered statistically significant when $p \leq 0.05$.

3. RESULTS

3.1. Total Phenolic Content and DPPH Assay

3.1.1. Extraction

Table 1 depicts the weights of the extracts determined gravimetically and obtained from ABA treated olive stones and pulps after 7, 15 and 30-day storage. Data corresponding to the controls are also included as a reference. By comparing the weight of the extracts of the controls throughout the storage process, no significant changes ($p > 0.05$) were observed either for the stones or the pulps. In contrast, ABA treated extracts exhibited significantly ($p < 0.05$) higher weights as compared with the controls for both the stones and the pulps. This increase suggests accumulation of phenolics as a result of the postharvest exposition of olives to ABA spray. For this reason, the total phenolics as well as the oleuropein and hydroxytyrosol contents were determined in both olive stones and pulps.

3.1.2. Total Phenolic Content and DPPH Assay

Figure 1 represents the total phenolic content, expressed as mg gallic acid kg^{-1} in stone (a) and pulp (b) of untreated-control and ABA treated olive fruits after 7, 15 and 30 storage days. Total phenolic values in Figure 1 are comparable to those reported in the literature for the whole olive fruit (Arslan & Özcan, 2011). It is worth noting that the olive stone extracts exerted total phenolic content in the same range as the pulp extracts. This is probably related to the high content of some phenolic compounds (ie, tyrosol, hydroxytyrosol, oleuropein….) present in olive stones (Fernández-Bolaños, Felizón, Brenes, Guillén & Heridia, 1998). Considering that large amounts of

stones are produced as an olive oil waste, the high content in phenolics reflects its potential use as a natural source of bioactive compounds.

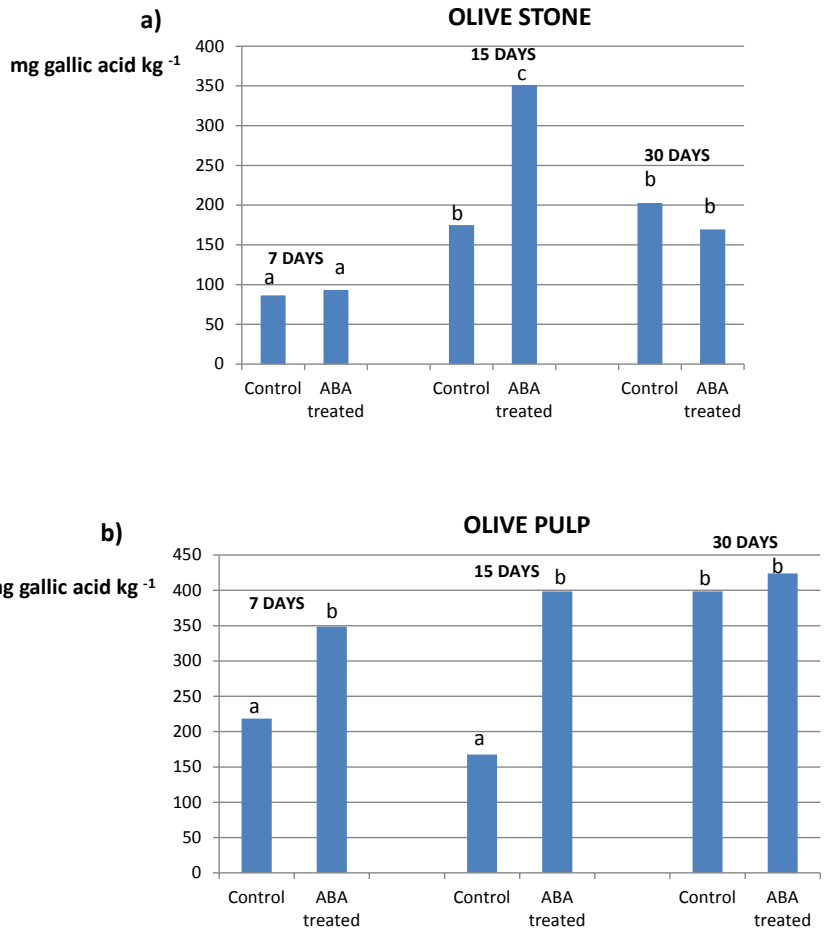

Figure 1. Total polyphenols (mg gallic acid kg-1) in stone (a) and pulp (b) of untreated-control and ABA treated olive fruits after 7,15 and 30 storage days.

Table 1. Weights (expressed as mg ± standard deviation) of dry extract obtained from untreated-control and ABA treated olive stones and pulps. Measurements performed on storage days 7, 15 and 30

OLIVE SAMPLES	WEIGHT (mg)		
	7 DAY STORAGE	15 DAY STORAGE	30 DAY STORAGE

	CONTROL	ABA TREATED	CONTROL	ABA TREATED	CONTROL	ABA TREATED
STONE	0.375 ± 0.022a	2.856 ± 0.048b	0.415 ± 0.030a	3.423 ± 0.071b	0.450 ± 0.084a	0.775 ± 0.015a
PULP	0.410 ± 0.013a	3.420 ± 0.052b	1.225 ± 0.065a	2.400 ± 0.033b	1.202 ± 0.029a	3.352 ± 0.041b

Different letters in the same column indicate significant ($p < 0.05$) differences between samples.

Following the evolution of control samples over the storage, it is interesting that the total phenolic content for both stones (Figure 1a) and pulps (Figure 1b) did not decrease with the storage. In fact, the total phenolic content was significantly ($p < 0.05$) higher in the stones obtained from olives stored for 15 and 30 days and in the pulps from olives stored for 30 days. It is worthy to highlight the particularly high total phenolic content of control olive pulp after 30-day storage (400 mg gallic acid kg^{-1}, Figure 1b). These results are in disagreement with data previously described in olives.

In this respect, we have found in an earlier study that the phenolic acid content in olive pulps decreased after a prolonged storage (article in press). Similarly, bibliographic reports have described drops in the total phenolic content of olive fruits after the storage (Benito et al., 2009). Also, it has been published the reduction of total phenolic compounds ranging from 43% to 73% in olive oil obtained from stored olive fruits with respect to the oil obtained from unstored olives (Gómez-Alonso et al., 2007). The decrease in the total phenolics in olives with the storage is attributed to the oxidative stress and the consequent formation of oxidized phenols (Armarforte, Mancebo-Campos & Bendini, 2007). The discrepancy found in the present work with respect to bibliographic works is possibly due to the selective extraction of certain phenolic compounds which are particularly stable during the storage. In fact, the influence of the extraction method on the phenolic total amount in olive oil is largely known (Gómez-Caravaca et al., 2008). To get insight into this possibility the most important phenolic constituents in olives (i.e., oleuropein and hydroxytyrosol) were further evaluated.

Concerning the ABA effect on olives, the total phenolic content increased significantly ($p < 0.05$) in both stones and pulps as a consequence of the treatment. As seen in Figure 1, the increase in the stones was exclusively observed in olives stored for 15 days (Figure 1a); however it is interesting to note that this increase was particularly remarkable (i.e., 350 mg gallic acid kg^{-1} in ABA treated stones *vs* 175 mg gallic acid kg^{-1} in control samples). Actually, it is worth pointing out that the value measured in stones (i.e., 350 mg gallic acid kg^{-1}) is close to that measured in ABA treated pulps (i.e., 400 mg gallic

acid kg^{-1}). This fact reflects, as previously mentioned, the potential value of the stone obtained from ABA treated olives. For the olive pulps, the ABA treatment resulted in a significant ($p < 0.05$) increment of the total phenolic content after 7- and 15-day storage (Figure 1b). In particular, pulps obtained from olives stored for 15 days exhibited an increase of the total phenolic content from 170 mg gallic acid kg^{-1} to 395 mg gallic acid kg^{-1} with the ABA treatment. Surprisingly the olive storage for 30 days did not provide a significant ($p < 0.05$) increase of the total phenolic content either in stones or in pulps. Therefore, the ABA treatment was effective in increasing the total phenolic content at the beginning of the storage but it was unsuccessful after a 30 day storage.

Our results in olive fruits are in accordance with reports published about the ABA effect on the total phenolic content in grapes and lettuce (Sandhu, Gray, Lu & Gu, 2011; Li, Zhao, Shandu & Gu, 2010). The increase of the total phenolic content after the exposition to exogenous ABA can be explained by the promoting effect of ABA on the enzymes involved in the bioformation of phenolic compounds. In this respect, most phenolics are synthesized through phenylpropanoid pathway, which is initiated by phenylalanine ammonia-lyase (PAL) enzyme. Although the ABA effect on PAL has not been to our knowledge studied as yet, it is likely that PAL is being activated by ABA as a consequence of its hormonal activity.

In view of these results, it can be stated that the postharvest treatment of olives with ABA spray enabled the total phenolic content to be significantly increased in stones and pulps. The effectiveness of exogenous ABA depended to a great extent on the storage period of the olives.

Table 2 summarizes the results on DPPH scavenging activity of untreated and ABA treated olive stones and pulps after 7, 15 and 30 storage days.

As seen in Table 2, the DPPH scavenging values obtained in the control untreated pulps were higher than those measured in the stone samples. This agrees with the data reported elsewhere (Spizzirri et al., 2011). The IC_{50} values shown for the stones in Table 2 are comparable to those previously described in the literature (Spizzirri et al., 2011). By contrast, the free radical scavenging activity estimated in the pulps is slightly higher than that published in the literature for olive fruits (Arslan & Özcan, 2011). The phenolic profile and the antioxidant activity in olive fruit is known to vary with a number of factors, such as cultivar, location, olive ripening, storage conditions, etc. (Gómez-Rico, Fregapane & Salvador, 2008; Amiot, Fleuriet & Macheix, 1986). For this reason, small variations between samples are reasonable.

Table 2. DPPH scavenging activity expressed as IC_{50} (µg/ml) of olive fruit stones and pulps untreated and treated with ABA spray during storage. Values are expressed as means ± SD (n = 8)

OLIVE SAMPLE	IC_{50} (µg/ml)					
	7 DAY STORAGE		15 DAY STORAGE		30 DAY STORAGE	
	CONTROL	ABA TREATED	CONTROL	ABA TREATED	CONTROL	ABA TREATED
STONE	0.52 ± 0.08a	0.36 ± 0.05a	0.43 ± 0.07a	1.17 ± 0.06b	0.58 ± 0.05a	1.11 ± 0.09b
PULP	3.95 ± 0.14a	3.78 ± 0.09a	2.31 ± 0.11b	4.10 ± 0.089a	1.49 ± 0.11c	1.53 ± 0.09c

Values with different letters in the same row are significantly different ($p < 0.05$).

Table 3.Oleuropein contents (expressed as mg kg^{-1} weight ± SD) in olive fruit stones and pulps untreated and treated with ABA spray during storage. Data are presented as means of triplicated values

OLIVE SAMPLE	OLEUROPEIN CONTENT					
	7 DAY STORAGE		15 DAY STORAGE		30 DAY STORAGE	
	CONTROL	ABA TREATED	CONTROL	ABA TREATED	CONTROL	ABA TREATED
STONE	526.9 ± 3.3a	685.5 ± 3.6a	367.8 ± 4.3b	598.2 ± 2.6a	354.8 ± 3.1b	666.3 ± 3.6a
PULP	825.3 ± 3.6a	1356.9 ± 3.5b	613.3 ± 2.9c	806.9 ± 2.0a	416.7 ± 4.1d	606.9 ± 3.1c

Table 4.Hydroxytyrosol contents (expressed as mg kg^{-1} weight ± SD) in olive fruit stones and pulps untreated and treated with ABA spray during storage. Data are presented as means of triplicated values

OLIVE SAMPLE	HYDROXYTYROSOL CONTENT					
	7 DAY STORAGE		15 DAY STORAGE		30 DAY STORAGE	
	CONTROL	ABA TREATED	CONTROL	ABA TREATED	CONTROL	ABA TREATED
STONE	732.4 ± 5.6a	802.3 ± 4.1a	718.5 ± 2.8a	792.3 ± 3.5a	743.8 ± 3.7a	986.8 ± 3.8b
PULP	418.3 ± 2.9a	697.2 ± 2.1b	425.3 ± 3.4a	633.4 ± 3.3b	389.6 ± 1.9a	512.0 ± 2.2b

From Table 2, the results on the DPPH assay indicates that the ability to scavenge free radicals of control untreated stone samples was not significantly ($p > 0.05$) affected by the storage. On the contrary, the IC_{50} values measured in pulps declined significantly ($p < 0.05$) over the storage (i.e., from 3.95 µg/ml after a 7-day storage to 1.49 µg/ml after 30 days). Drops of the DPPH free

radical scavenging capacity with very prolonged storages, around 6 months, have also been reported for olive fruits and olive oil (Haouhay, Sanchez, Asehraou, Mir & De la Serrana, 2016). The decrease of free radical scavenging activity is directly related to the decline of antioxidant phenolic compounds.

Interestingly, a good correlation between the free radical scavenging ability and the total phenolic content could not be established. While the total phenolic content mostly increased with the storage (see Figure 1), the ability to scavenge free radicals did not show this trend either in stones or in pulps (see Table 2). This observation has also been occasionally found by other authors (Baiano, Gambacorta, Terracone, Previtali, Lamacchia & La Notte, 2009).

Considering the ABA treatment effect, the DPPH results indicate that in general terms the free radical scavenging activity was significantly ($p < 0.05$) higher in the ABA treated olives than in the controls. In particular, for stone samples the increment was particularly significant ($p > 0.05$) after the storage for 15 days (1.17 µg/ml *vs* 0.43µg/ml) and 30 days (1.11 µg/ml *vs* 0.58 µg/ml) whereas in pulp samples, the ABA effect was only observed after the storage of olives for 15 days (4.10 µg/ml in ABA treated samples *vs* 2.31 µg/ml in the controls). It is noticeable that the IC_{50} values obtained in ABA treated olive pulps stored for 15 days was comparable to those measured in untreated control pulps on day 7 of storage (4.10 µg/ml *vs* 3.95 µg/ml).

On the other hand it is observed that although, as above mentioned, a good correlation between the total phenolic content and the free radical scavenging activity was not found during the storage, exogenous ABA affects equally both the total phenolic content and the IC_{50} values. Both parameters enhanced after the exposition of the olives to ABA spray. Furthermore, this effect was observed in stone and pulp samples. Therefore, according to these results, the ABA treatment might be compensating the natural degradation of antioxidant phenolics during the extended olive storage.

3.3. Oleuropein and Hydroxytyrosolcontents

Tables 3 and 4 depict the oleuropein and hydroxytyrosol contents, respectively, expressed as mg kg^{-1} weight in untreated control and ABA treated olive fruit stones and pulps after 7, 15 and 30 storage days.

As observed in the tables, the oleuropein content was in general higher in pulps than in stones (Table 3) whereas the hydroxytyrosol content in control olives was higher in stones than in pulps (Table 4). This result agrees with the data on olive fruit composition published by other authors (Fernández-Bolaños et al., 1998). By comparing control samples, the oleuropein content fell with the storage in both stones and pulps, although the drop was more pronounced for pulps (see Table 3). As an example, the oleuropein content in olive pulps stored for 30 days was half that of measured on day 7 (416.7 *vs* 825.3 mg kg^{1}). For the stones, decline in the oleuropein content was only significant ($p < 0.05$) from the 15-day storage on. Unlike oleuropein, the hydroxytyrosol content was not affected by the storage either in stones or in pulps (see Table 4). This is in principle unexpected since oleuropein is a derivate of hydroxytyrosol. However, similar results have been found by other authors, which describe a linear increase of hydroxytyrosol in olive oil during 21 month storage at room temperature (Gómez-Alonso et al., 2007).

In view of these results, the evolution of the oleuropein content during the storage correlates well, in general, with the free radical scavenging activity and, in particular, for the pulp samples. This, in turn, implies that no correlation could be established between the total phenolics and the oleuropein content during the storage. On the contrary, a positive relation between the hydroxytyrosol content and the free radical scavenging ability was not established. Considering that both oleuropein and hydroxytyrosol are equally regarded as the most powerful radical scavengers in olives (Chimi, Cillard, Cillard, & Rahmani, 1991), it is deduced that the decrease in the free radical scavenging activity observed with the storage is partially due to the decline of the oleuropein content. In any case, care must be exercised in the interpretation of data relating to the antioxidant activity as the analytical technique influences the results.

As far as the ABA treatment is concerned, both oleuropein and hydroxytyrosol exhibited significantly ($p < 0.05$) higher contents after the exposition of olive fruits to exogenous ABA spray. This increase was once again more pronounced in pulps than in stones. In fact, the ABA effect on olive pulp composition was apparent throughout the whole storage period. As an example, olives treated with ABA and stored for 30 days exerted oleuropein and hydroxytyrosol contents in pulps similar to those estimated in control samples stored just for 7 days (606.9 *vs* 825.3 mg kg^{-1} in Table 3 and 512.0 *vs* 418.3mg kg^{-1} in Table 4). Unlike pulp samples, the ABA effect on stone samples was not steady but it increased with the storage time. This way, the oleuropein and hyrdroxytyrosol contents were significantly ($p < 0.05$)

higher in ABA treated olive stones at the end of the storage period (666.3 mg kg^{-1} oleuropein and 986.8 mg kg^{-1} hydroxytyrosol after 30-day storage).

The mechanism by which ABA induces the increase of the oleuropein and hydroxytyrosol contents in olive fruits is not well understood. It is believed that ABA penetrates through the olive skin, accumulates inside and enhances the generation of oleuropein and hydroxytyrosol through the activation of the specific enzymes regulating their formation. In particular, although most phenolics are, as already commented, produced by the common phenylpropanoid pathway (Vogt, 2000), specific enzymes are involved in the synthesis of each phenolic compound in particular, which may respond differently to exogenous ABA. For oleuropein and hydroxytyrosol, endogenous enzymes esterase and β-glucosidasa affect the molecule during the first months of olive storage. Therefore ABA would be activating these endogenous enzymes which would induce the accumulation of oleuropein and hydroxytyrosol.

Due to their chemical properties, oleuropein and hydroxytyrosol, as most phenolics, act as natural antioxidants by inhibiting lipid oxidation (Teissedre, Frankel, Waterhouse, Peleg, & German, 1996). In particular, oleuropein and hydroxytyrosol exert their antioxidant power by scavenging initiating radicals. In this line, various researchers have demonstrated a positive linear relationship between oil stability and phenolic content (Gutfinger, 1981). Therefore, higher antioxidant phenolic content is recommendable to assure olive oil quality when olives have to necessarily be stored.

In addition, both oleuropein and hydroxytyrosol have been described to possess a number of pharmacological properties. Their occurrence in olive oil not only contributes to the oxidative stability of the oil but also to the health promoting characteristics associated with olive oil.

CONCLUSION

The extended storage of olive fruits brings about the reduction in the ability to scavenge free radicals as well as in the content of relevant antioxidant phenolics such as oleuropein. Oleuropein, together with hydroxytyrosol, not only exerts nutritional properties but also acts as a natural antioxidant of lipids. Therefore their reduction is directly linked to the oxidative instability of the oil and as a result to lower quality of olive oil.

The exogenous ABA spray treatment does not avoid phenolic losses over the olive storage but it results in an increase in the scavenging free radical

activity and enrichment in oleuropein and hydroxytyrosol in the starting olive fruits. Consequently, ABA treated olives stored for prolonged time exhibited scavenging free radical activity and contents of oleuropein and hydroxytyrosol similar to those encountered in olive fruits at the beginning of the storage. The postharvest ABA treatment compensates hence the storage effects on olive fruit composition. This is particularly interesting when delay in oil processing occurs and olive fruits have to be necessarily stored.

On the other hand, olive stones, which are obtained as by-products in oil processing, obtained from ABA treated olives exhibited high phenolic content and scavenging free radical activity. For this reason, it can be considered as a source of natural antioxidants to be used in the industry.

As a conclusion, the postharvest exposition of olive fruits to ABA spray is a promising technique to preserve the antioxidant content and the scavenging free radical ability in olive fruits during the storage. This method helps to minimize the detrimental storage effect which is particularly relevant when oil processing is delayed. Stones obtained from ABA treated olive fruits contain significant phenolic content and, hence, they are regarded as a potential source of natural antioxidants for industrial applications, such as food, cosmetic and pharmaceutical industries.

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