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Oleuropein Hydrolysis in Natural Green Olives: Importance of the Endogenous Enzymes

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ABSTRACT

The bitter taste of olives is mainly caused by the phenolic compound named oleuropein and the mechanism of its hydrolysis during the processing of natural green olives was studied. First, a rapid chemical hydrolysis of oleuropein takes place at a high temperature of 40 ºC and at a low pH value of 2.8, but the chemical hydrolysis of the bitter compound is slow at the common range of pH for these olives (3.8–4.2). However, decarboxymethyl elenolic acid linked to hydroxytyrosol and hydroxytyrosol have been found in a high concentration during the elaboration of natural green olives. When olives were heated at 90 ºC for 10 minutes before brining, these compounds are not formed. Hence, the debittering process in natural green olives is due to the activity of β-glucosidase and esterase during the first months of storage and then a slow chemical hydrolysis of oleuropein happens throughout storage time.

Keywords: natural table olives; oleuropein; enzymatic hydrolysis; chemical hydrolysis

1. Introduction

Like many fruits and vegetables, olives contain a significant amount of phenolic compounds distributed in the skin, flesh and seed. Oleuropein is the major polyphenol in fresh olives and, due to its bitter taste; it must be completely or partially removed or transformed to make this fruit edible. This compound consists of a molecule of elenolic acid linked to the orthodiphenol hydroxytyrosol by an ester bond and to a molecule of glucose by a glycosidic bond (Panizzi, Scarpati, & Oriente, 1960).

There are very few studies on the presence of endogenous β-glucosidase enzymes in olives and scarce information on esterase enzymes (Briante et al., 2002; Ramírez, Medina, Brenes, & Romero, 2014), although they could be involved in the hydrolysis of oleuropein. The affinity of the β-glucosidase enzyme for oleuropein has been observed in numerous olive varieties from Italy, Tunisia and Spain (Briante et al., 2002; Jemai, Bouaziz, & Sayadi, 2009; Gutiérrez-Rosales, Romero, Casanovas, Motilva, & Mínguez-Mosquera, 2010, 2012). Moreover, researchers have demonstrated in model solutions that oleuropein is hydrolysed by the action of β-glucosidase, forming glucose and the corresponding aglycone (Walter, Fleming, & Etchells, 1973; Capasso, Evidente, Visca, Gianfreda, Maremonti, & Greco, 1997; Romero-Segura, Sanz, & Pérez, 2009; De Leonardis, Macciola, Cuomo, & López, 2015). Recently, the enzymatic hydrolysis of oleuropein by an olive leaf protein extract has been proposed (De Leonardis, Testa, Macciola, Lombardi, & Iorizzo, 2016). It has also been reported that oleuropein is converted into the decarboxymethyl dialdehydic form of oleuropein aglycone, HyEDA (Figure 1) by the action of the endogenous β-glucosidase enzyme during the elaboration of olive oil (Montedoro, Baldioli, Selvaggini, Begliomini, Taticchi, & Servili, 2002; Romero-Segura, García-Rodríguez, Sánchez-Ortiz, Sanz, &

Pérez, 2012). In addition, endogenous esterase can hydrolyse the oleuropein ester bond, forming hydroxytyrosol and a derived glycosylate (Amiot, Fleuriet, & Macheix, 1989).

Among table olive elaborations, the most popular ones are the Spanish-style green and the California-style black olives. In both processes, oleuropein is chemically hydrolysed by treating the fruits with diluted sodium hydroxide solution; this alkaline solution produces the breakage of the ester bond oleuropein with the consequent formation of hydroxytyrosol and elenolic acid glucoside, both non-bitter compounds (Brenes & de Castro, 1998).

On the other hand, there are other trade preparations that involve the direct brining of olives, either green or purple fruits, which are called natural olives because the fruits are not subjected to any treatment with sodium hydroxide (Medina, Gori, Servili, de Castro, Romero, & Brenes, 2010). The harvested fruits are currently put into an acidified brine (Romeo, Piscopo, & Poiana, 2010). Supposedly, the olives lose their bitterness slowly due to the diffusion of oleuropein from the pulp to the brine (Romero, Brenes, García, García, & Garrido, 2004; Arroyo-López, Romero, Durán-Quintana, López-López, García-García, & Garrido-Fernández, 2005). In addition, the bitter compound can be chemically hydrolysed by the acidic conditions of the solution, giving rise to glucose, elenolic acid and hydroxytyrosol (Figure 1), none of which are bitter.

It is assumed that the endogenous enzymes of olive are degraded or inactivated during the treatment with sodium hydroxide, due to the high pH of the solution (Pandey, & Ramachandran, 2008). In contrast, in the case of natural olives, the penetration of sodium chloride and acetic acid into the pulp of the fruit causes a breakdown of the tissue and, consequently, the endogenous hydrolase enzymes (β-glucosidase and esterase) could act on the oleuropein molecule. Jemai et al. (2009) have proposed the possibility of synergism between esterases and β-glucosidases in the fresh fruit on the

tree. Firstly, β-glucosidase enzyme breaks the bond between glucose and the rest of the oleuropein molecule; then the esterase enzyme hydrolyses the aglycone so that hydroxytyrosol and elenolic acid are formed. The formation of a high concentration of HyEDA in the brine of 17 olive varieties processed as natural olives in brine has been reported (Medina, García, Romero, de Castro, & Brenes, 2009). Although researchers have proposed many theories for the debittering of olives not treated with sodium hydroxide, such as simple diffusion of the glucoside to the surrounding brine, chemical hydrolysis or microbial action, until now this phenomenon remains unsolved.

The aim of this work was to elucidate the mechanisms by which natural green olives lose their bitterness. In particular, the roles of the endogenous hydrolase enzymes and the chemical conditions of the medium in the loss of bitterness in natural green olives have been investigated.

2. Materials and methods

2.1. Hydrolysis of oleuropein in model system

2.1.1. Experiment A

Aliquots of 2 mL of a solution containing 6% sodium chloride, 0.2% acetic acid and 5 mM commercial oleuropein (Sigma-Aldrich, St Louis, MO) were stored under a nitrogen atmosphere at different temperatures (10, 22 and 40 ºC). The phenolic composition was measured at 1.5, 3 and 5 months. The experiment was carried out in duplicate.

2.1.2. Experiment B

Sterile brine from olives with an oleuropein concentration of 4.7 mM, 3.4% sodium chloride and 0.3% acetic acid was heated at 90 ºC for 30 minutes. Aliquots of 2

mL were stored under a nitrogen atmosphere at different pHs (3.5, 3.8, 3.9 and 4.3 units) at room temperature. The phenolic composition was measured at 1 and 2 months. The experiment was carried out in duplicate.

2.2. Hydrolysis of oleuropein in olive elaboration on a laboratory scale

2.2.1. Experiment A

Fruits of the Hojiblanca variety (*O. europaea* L.) from the 2011–2012 season, at the ripening stage corresponding to a green-yellow colour on the surface, were supplied by local farmers. The olives (190 g) were put into a bottle of 250 mL capacity and covered with a 5% sodium chloride and 0.5% acetic acid solution (control, C).

To eliminate interference from the activity of microorganisms, the olives were also elaborated in aseptic conditions (S) in accordance with Medina, Brenes, Romero, García, and De Castro (2007). The fruits were selected to remove those with blemishes, cuts, and insect damage. After washing thoroughly with tap water to remove impurities, the olives were placed in a sodium hypochlorite solution (50 mg/L active chlorine) at 35 ºC for 2 minutes and then they were washed with sterilised water twice to remove chlorine. Subsequently, 190 g of fruits were put in autoclaved bottles (250 mL capacity) and covered with a 5% sodium chloride and 0.5% acetic acid sterile solution. These manipulations were carried out in a laminar flow cabinet.

To eliminate interference from the activity of the endogenous enzymes and microorganisms, the olives were heated at 90 ºC for 10 minutes and elaborated in aseptic conditions as explained above (sample S-P).

Finally, all the bottles were sealed and stored at room temperature for 1, 2, 4 and 6 months. After this time, the bottles were opened and checked for microbial growth by visual appearance and plate counts. Microorganisms were not detected in any aseptic

brine. The phenolic composition was analysed both in olive pulp and brines. The experiment was carried out in duplicate.

2.2.2. Experiment B

Fruits of the Manzanilla variety (*O. europaea* L.) from the 2012–2013 season, at the ripening stage corresponding to a green-yellow colour on the surface, were supplied by local farmers. Olives (190 g) were put into bottles of 250 mL capacity and covered with a 5% sodium chloride and 0.5% acetic acid solution under aseptic conditions. Some fruits were not heated (S), others were heated at 60 ºC for 10 minutes (S-P60) and, finally, the rest were heated at 90 ºC for 30 minutes (S-P90).

Bottles were stored at room temperature for 15 and 60 days. After this time, they were opened and checked for microbial growth by visual appearance and plate counts. It must be said that microorganisms were not detected in any aseptic brine. The phenolic composition was also analysed in both olive pulp and brine. The experiment was carried out in duplicate.

2.3. Analysis of phenolic compounds

The extraction of phenolic compounds from the olive pulp was based on the methodology proposed elsewhere (Kumral, Korukluoglu, Romero, De Castro, Ruiz-Barba, & Brenes, 2013). Approximately 10 g of olive pulp were mixed in an Ultra-Turrax homogeniser with 30 mL of dimethyl sulfoxide (DMSO). After 30 minutes resting contact, the mixture was centrifuged at 6000 *g* for 5 min (22 ºC), and 0.25 mL of the supernatant were diluted with 0.5 mL of DMSO plus 0.25 mL of 0.2 mM syringic acid in DMSO (internal standard). The analysis of phenolic compounds in brine was carried out by mixing 0.25 mL of brine, 0.25 mL of internal standard (2 mM syringic

acid in water), and 0.5 mL of deionised water. The analyses were performed in duplicate.

All samples were filtered through a 0.22-μm pore size nylon filter and an aliquot (20 μL) was injected into the HPLC. The chromatographic system consisted of a Waters 717 plus autosampler, a Waters 600 E pump, a Waters column heater module, and a Waters 996 photodiode array detector operated with Empower software (Waters Corporation. Milford, MA.). A 25 cm × 4.6 mm i.d., 5 μm, Spherisorb ODS-2 (Waters) column, a flow rate of 1 mL/min and a temperature of 35 ºC were used in all experiments. Separation was achieved by gradient elution using (**A**) water (pH 2.5 adjusted with 0.15% phosphoric acid) and (**B**) methanol. The initial composition was 90% **A** and 10% **B**. The concentration of **B** was increased to 30% over 10 min and was maintained for 20 minutes. Subsequently, **B** was raised to 40% over 10 min, maintained for 5 min, and then increased to 50%. Finally, **B** was increased to 60, 70, and 100% in 5-min periods. Initial conditions were reached in 10 min. Chromatograms were recorded at 280 nm (Medina et al., 2007).

The evaluation of each compound was performed using a regression curve with the corresponding standard. Hydroxytyrosol, oleuropein and verbascoside were purchased from Extrasynthèse S.A. (Lyon Nord, Genay, France), and tyrosol from Sigma Chemical Co. (St. Louis, MO). Hydroxytyrosol-1-glucoside was quantified using the response factors of hydroxytyrosol. Salidroside and ligustroside were quantified using the response factors of tyrosol. Hydroxytyrosol-4-glucoside was obtained using an HPLC preparative system (Romero, Brenes, García, & Garrido, 2002). HyEDA was obtained by HPLC preparative system as described elsewhere (Brenes et al., 2000).

2.4. Statistical analysis

Data were expressed as mean values ± standard error. Statistical software Statistica for Windows version 7.0 (StatSoft, Tulsa, OK) was used for data processing.

3. Results and discussion

3.1. Hydrolysis of oleuropein in model systems

Figure 2 shows that oleuropein hydrolysis is temperature dependent. A complete loss of this phenol can be observed after 1.5 months storage at 40 ºC, whereas half of the oleuropein concentration still remained after 5 months storage at the lowest temperature (10 °C). In addition, hydroxytyrosol, a product of the acid hydrolysis of oleuropein (Figure 1), steadily increased its concentration with time at all temperatures tested. A decrease in the concentration of this substance was also detected after 1.5 months in the solutions incubated at 40 ºC, which suggests that the degradation or oxidation of hydroxytyrosol occurred at this high temperature. Anyway, this compound was detected after 5 months storage at all of the temperatures tested.

These data led us to believe that the hydrolysis of oleuropein in acid brines is rapid; thereby the debittering of natural olives could be exclusively due to the chemical conditions. However, numerous researchers have demonstrated that the oleuropein concentration remains high after two months storage of the olives (Brenes, García, Durán, & Garrido, 1993; Ramírez, Gandul-Rojas, Romero, Brenes, & Gallardo-Guerrero, 2015) and the chemical hydrolysis of oleuropein is a slow process (Gikas, Papadoloulos, & Tsarbopoulos, 2006). In a normal fermentation of natural olives in brine, the pH of the brine ranges between 3.8–4.3 units, and the pH of the model solution was 2.8. This lower pH can explain the rapid oleuropein hydrolysis observed.

Therefore, a new experiment was planned to check the influence of the medium pH on the hydrolysis of oleuropein. A brine from olives stored aseptically that had been previously heated to inactivate olive enzymes was chosen as a model solution. The initial chemical conditions of this brine were 3.4% sodium chloride, 0.3% acetic acid and a pH value of 3.9. Then, the pH was adjusted to different values (3.5, 3.8 and 4.3), and the model brine solution was stored at room temperature.

Initially, the phenolic compounds present in this brine were hydroxytyrosol, hydroxytyrosol-1-glucoside, hydroxytyrosol-4-glucoside, salidroside, tyrosol, verbascoside, HyEDA, oleuropein and ligustroside. As expected, a decrease and increase in oleuropein and hydroxytyrosol were obtained, respectively, with time (Figure 3). However, the most interesting result was the low hydrolysis rate reached at pH 3.9–4.3, which is within the common range found for natural green olives. These data are in agreement with those reported by other researchers (Paiva-Martins & Gordon, 2005), and they are much different from those reflected in Figure 2.

It must be noticed that the hydroxytyrosol concentration increased by 1.35 mmol/L at pH 3.9, while the oleuropein concentration only decreased 0.86 mmol/L (Figue 3), which can be explained by the transformation of hydroxytyrosol derivatives, such as hydroxytyrosol-1-glucoside, verbascoside and HyEDA (Table1). A similar behaviour was observed for tyrosol derivatives; while the concentration of salidroside and ligustroside decreased, the tyrosol concentration increased after two months storage of the brine at room temperature (Table 1).

In conclusion, the chemical hydrolysis of oleuropein is a slow process that will take many months, or even years, at room temperature and pH values close to 4.0 units. Therefore another explanation must be found for the debittering process of natural olives in brine.

3.2. Hydrolysis of oleuropein in olive elaboration on a laboratory scale

To study the participation of endogenous enzymes in the hydrolysis of oleuropein during natural green olive processing, an experiment with Hojiblanca olives was carried out for six months at room temperature. The fruits were directly placed in bottles with brine of 5 % sodium chloride and 0.5 % acetic acid. Some of the olives were treated aseptically in sterile bottles before brining to avoid interference from microorganisms naturally present in olives, and other olives were also heat treated before brining in sterile conditions to inactivate the endogenous enzymes of the fruits as well as to eliminate microorganisms.

Figure 4 shows the evolution of the main phenols present in the bottles (mass balance of flesh and brine concentration) of Hojiblanca olives stored for six months. After one month, the oleuropein completely disappeared in both the control olives (C) and those treated aseptically (S), while this substance remained stable for six months in olives which were heat treated and preserved aseptically (S-P).

Moreover, the mass balance found for HyEDA indicated that this phenol was formed during the first month of storage and then decreased or even disappeared after six months of storage in both control olives and those treated aseptically. On the contrary, this compound was not formed in heat treated olives. It is well known that HyEDA is formed *via* the action of β-glucosidase from the hydrolysis of oleuropein during the elaboration of olive oil (Montedoro et al., 2002). In our study, the fruits (S-P) were heated at 90 ºC for 10 min and it was enough to inhibit the endogenous glucosidase. It has been reported that the activity of β-glucosidase in fresh fruits is temperature dependent, with this activity being greatly reduced at 70 ºC and eliminated at 90 ºC (Ramírez et al., 2014). Our results confirm that the presence of the

antimicrobial substance HyEDA (Medina et al., 2007) in the brine of natural olives is a cause of the enzymatic degradation of oleuropein by endogenous enzymes.

Hydroxytyrosol is not currently present in fresh olives (Gutiérrez-Rosales et al., 2012; Ramírez et al., 2014) but is formed during the processing of the fruit by the action of hydrolase enzymes (Briante et al., 2002; Mazzei, Giorno, Piacentini, Mazzuca, & Drioli, 2009). Figure 4 shows that hydroxytyrosol was formed in a high concentration during the first month of storage and then its concentration remained stable throughout the study in both the control olives and those treated aseptically. This substance can come from oleuropein hydrolysis during the first months but then it can also be generated from HyEDA hydrolysis. It both cases, endogenous esterase enzymes are involved in the hydrolysis reactions. It must be highlighted that this compound was not formed in heat-treated olives (S-P), and was detected from the second month in a very small concentration. Undoubtedly, the heat treatment inactivated the endogenous esterase and the low concentration detected came from the chemical hydrolysis of the oleuropein, which is a very slow reaction, as indicated above.

A new experiment was carried out to confirm these data with olives of the Manzanilla variety. Two temperatures were tested, 60 and 90 ºC, and the heating time was 10 and 30 min, respectively. All the olives were aseptically prepared before brining.

The concentration of complex phenolic compounds, HyEDA, oleuropein and ligustroside, decreased in non-heated olives (S) with time, while that of simple phenols (hydroxytyrosol and tyrosol) increased (Figure 5). Again, these results were a consequence of the action of endogenous enzymes, because all the fruits had been aseptically treated and no hydrolase enzymes from microorganisms were available. It must be noticed that several researchers have reported the acceleration of olive debittering due to the growth of lactic acid bacteria during fermentation (Ciafardini,

Marsilio, Lanza, & Pozzi, 1994; Marsilio, Lanza, & Pozzi, 1996). It could be as a consequence of exogenous enzymatic production by these microorganisms or pH decrease in the brine that could favour chemical hydrolysis. However, our data indicate that chemical hydrolysis of oleuropein is a slow process.

The formation of HyEDA was not observed in heat-treated fruits at both temperatures. By contrast, hydroxytyrosol and tyrosol were detected in all the olives, even in those treated at 60 and 90 °C, thereby this heat treatment was insufficient for the complete inactivation of endogenous esterase, whose activity remains at high temperatures, as demonstrated by other authors (Ramirez et al., 2014).

4. Conclusion

The debittering process in natural green olives is mainly due to the activity of endogenous enzymes, esterase and β-glucosidase, on the oleuropein molecule during the first months of storage and, consequently, the formation of hydroxytyrosol and HyEDA, respectively is observed. By contrast, the chemical hydrolysis of oleuropein happens slowly at the current range of pH (3.8–4.2) of the fermented olives. These results open the possibility to optimise the natural debittering of table olives by modulating the enzymatic and chemical hydrolysis of oleuropein.

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Figure captions

Fig. 1. Structure of oleuropein and products obtained by its acid hydrolysis and by the action of hydrolase enzymes.

Fig. 2. Evolution of oleuropein and hydroxytyrosol in a model solution of commercial oleuropein stored for five months at different temperatures (10, 22 and 40 ºC). The chemical conditions were 6% sodium chloride and 0.2% acetic acid. Error bars indicate the standard error of duplicate analyses.

Fig. 3. Evolution of oleuropein and hydroxytyrosol in a sterile brine stored at different pHs for two months at room temperature. The sterile brine was heat treated at 90 ºC for 30 min. The chemical conditions were 3.4% sodium chloride and 0% acetic acid. Error bars indicate the standard error of duplicate analyses.

Fig. 4. Differences between the final amount ($mmol_f$) and the initial amount ($mmol_i$) of oleuropein, decarboxymethyl elenolic acid linked to hydroxytyrosol (HyEDA) and hydroxytyrosol in the bottle of Hojiblanca olives with time. The fruits were subjected to three different treatments: not aseptic nor heat treated (C), aseptic but not heat treated (S) and aseptic and heat treated at 90 ºC for 10 min before brining (S-P). The total amount of each compound is calculated by the sum of the mmol present in the fresh pulp and in the brine. Error bars indicate the standard error of quadruplicate analyses.

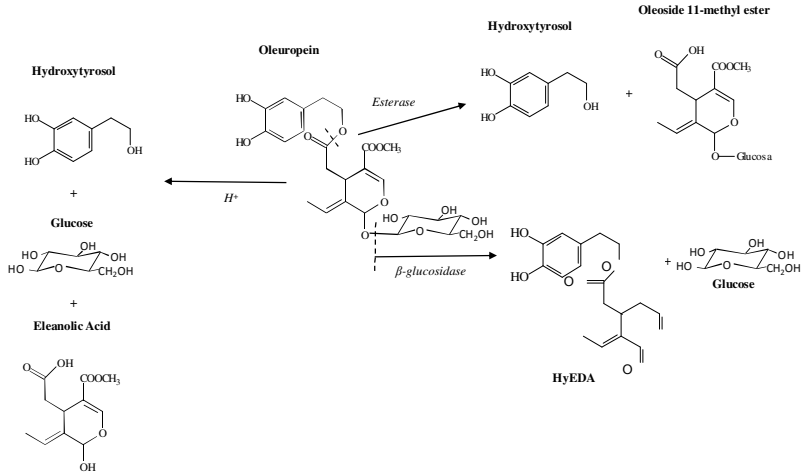
Fig. 5. Evolution of the total amount of hydroxytyrosol (Hy), decarboxymethyl elenolic acid linked to hydroxytyrosol (HyEDA), oleuropein (O), tyrosol (Ty) and ligstroside (L) concentration in the Manzanilla olive variety stored in a bottle of 250 mL for two

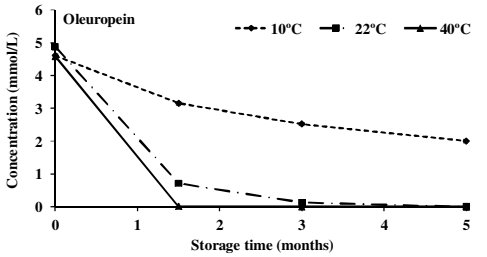
months. The fruits were subjected to three different treatments: aseptic but not heat treated (S), aseptic and heat treated at 60 ºC for 10 min before brining (S-P60) and aseptic and heat treated at 90 ºC for 30 mines before brining (S-P90). The total amount of each compound is calculated by the sum of the mmol present in the fresh pulp and in the brine. Error bars indicate the standard error of duplicate analyses.

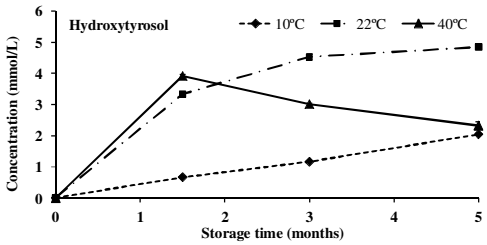
Table 1. Evolution of phenolic compounds concentration in a sterile brine stored at pH 3.9 for two months at room temperature. The sterile brine was heated at 90 ºC for 30 minutes. The chemical conditions were 3.4% sodium chloride and 0.3% acetic acid. The concentration is expressed as mmol/L. HyEDA: decarboxymethyl elenolic acid linked to hydroxytyrosol.

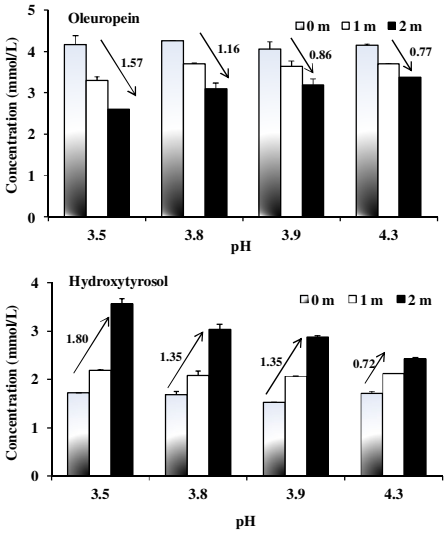
	storage time (months)		
	0	1	2
hydroxytyrosol-1-glucoside	0.73 ± 0.06[a]	0.70 ± 0.00	0.60 ± 0.01
hydroxytyrosol-4-glucoside	1.42 ± 0.01	1.43 ± 0.01	1.46 ± 0.03
salidroside	0.06 ± 0.00	0.05 ± 0.00	0.04 ± 0.00
tyrosol	0.26 ± 0.00	0.39 ± 0.01	0.39 ± 0.02
verbascoside	0.36 ± 0.01	0.36 ± 0.01	0.32 ± 0.00
HyEDA	0.91 ± 0.02	0.64 ± 0.02	0.38 ± 0.01
ligustroside	0.17 ± 0.01	0.13 ± 0.01	0.10 ± 0.01

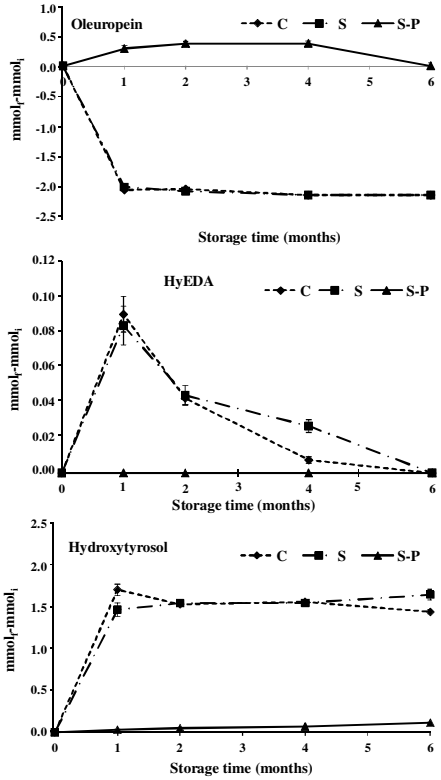
[a]Each value is the mean ± standard error of duplicate analyses

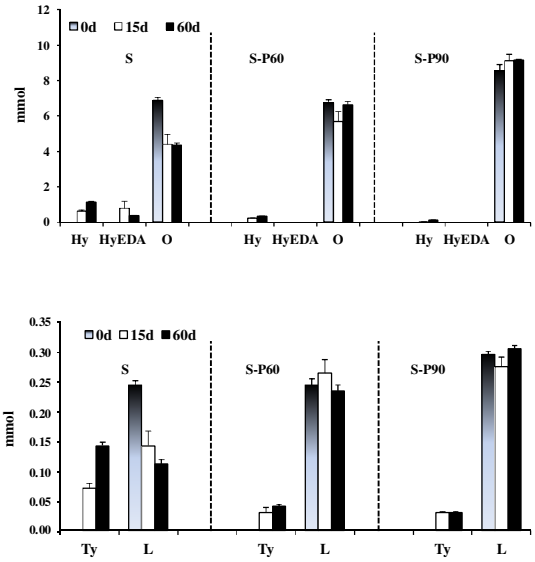












Highlights

- Endogenous enzymes hydrolyse oleuropein, a bitter compound of olives
- The enzymatic hydrolysis of oleuropein is rapid during the first months
- Chemical hydrolysis of oleuropein is slow over the common range of pH of natural olives
- A high concentration of HyEDA and hydroxytyrosol are found in natural green olives