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Storage of Olives (*Olea europaea* L.) under CO_2 Atmosphere: Liquid Chromatography−Mass Spectrometry Characterization of Indices Related to Changes in Polyphenolic Metabolism

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Olives (*Olea europaea* cv. Chondrolia) were stored under a CO_2 atmosphere immediately after harvesting for a period of 12 days. Samples obtained at 24-h intervals were analyzed by HPLC to identify components that may reflect changes in the biochemical behavior of the tissue. Four substances were shown to undergo significant fluctuations during storage, while their evolution was found to be different in olives stored under CO_2 from those stored under regular atmospheric conditions (control). On the basis of data provided by liquid chromatography−electrospray ionization mass spectrometry, these substances were tentatively identified as hydroxytyrosol glucoside, demethylated ligstroside aglycone, ibotalactone A methyl ester, and verbascoside. The data are discussed in relation to the effect of postharvest treatments of olives for purposes of manipulating their polyphenolic content and plausible development of novel debittering processes.

KEYWORDS: Antioxidants; liquid chromatography−mass spectrometry; hydroxycinnamates; secoiridoids; functional ingredients; *Olea europaea*; olives; postharvest treatments; polyphenols

INTRODUCTION

A growing number of epidemiological investigations show a protective effect of vegetables and fruits against degenerative diseases, such as cardiovascular disorders and cancer. The hypothesis that lies behind health claims is that plant food commodities contain a wide spectrum of polyphenolic antioxidants, which are believed to exert beneficial biological effects (*1*, *2*). Olives (*Olea europaea*) and olive oil are largely produced and consumed in the countries of the Mediterranean basin. Both products contain considerable amounts of polyphenolic phytochemicals, including simple phenols (tyrosol, hydroxycinnamates, etc.), secoiridoid derivatives (oleuropein), lignans, and various flavonoids (*3*). Several of these components have been demonstrated to possess functional effects in vitro and in vivo (*4*), and thus they are considered to be bioactive constituents.

Currently, there is an increasing awareness with regard to the enhancement of food plants in biologically significant phenolics with the view to ameliorate their functional properties, and in this context, several techniques have been proposed, including appropriate plant breeding (*5*), genetic modification (*6*), and postharvest irradiation (*7*). Postharvest gaseous treatment is also a promising means of modifying the polyphenolic composition (*8*), but studies on several commercially important food plants have not been carried out. The detailed examination of such treatments and their impact on fruit and vegetable quality is nevertheless highly essential, because different plant tissues may behave in a variable manner upon exposure to modified atmospheres, in relation to alterations observed in the profile of various polyphenol classes. In particular, storage of intact clusters of red grapes under CO_2 atmosphere was shown to give rise to increased anthocyanin biosynthesis (*9*), but this finding

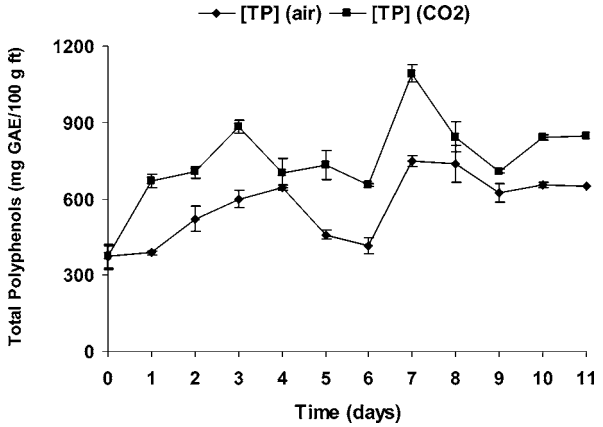

Figure 1. Changes in the total polyphenol content during postharvest storage of olives under CO_2 and regular atmospheric conditions (air).

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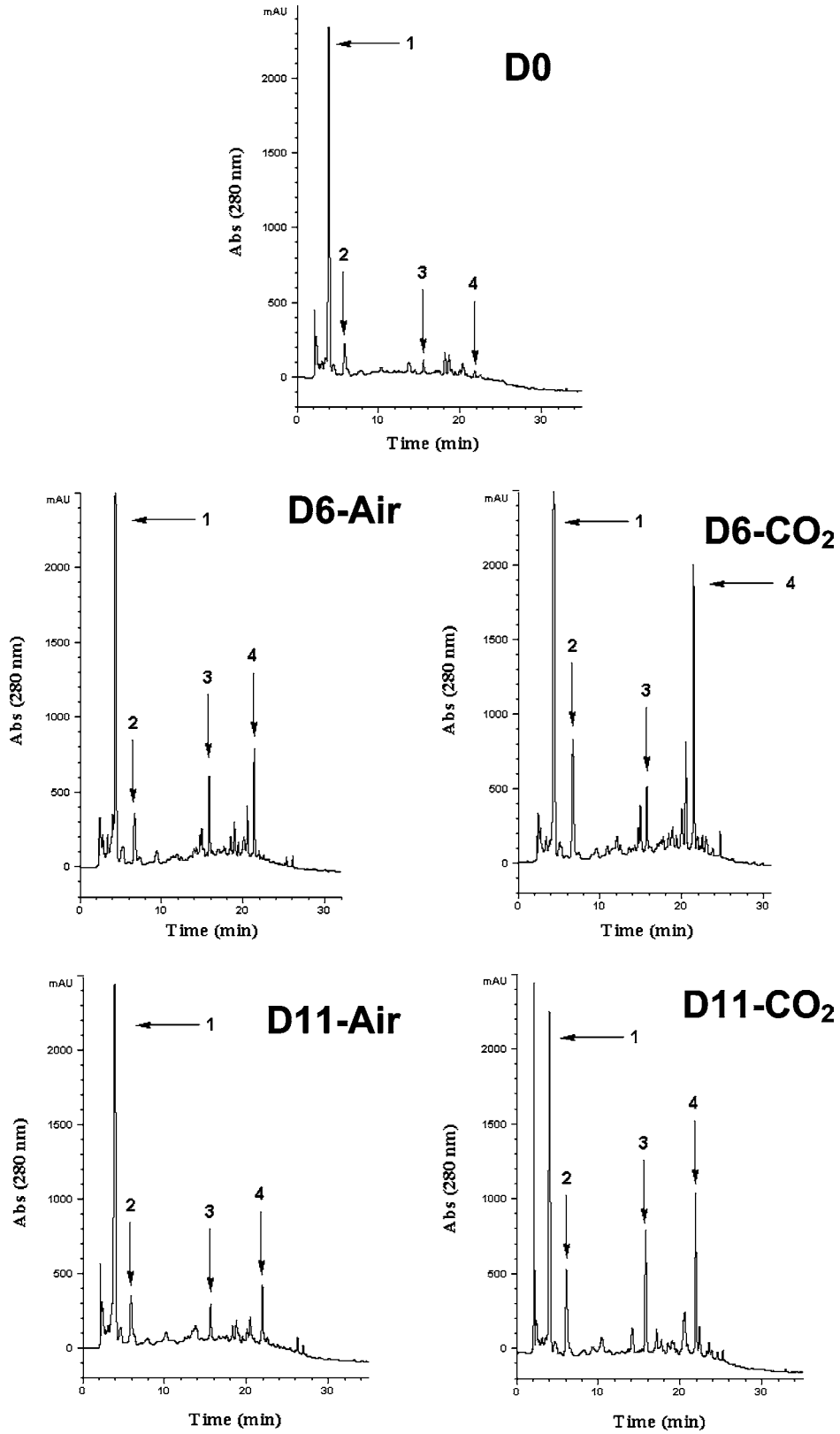

Figure 2. Characteristic chromatograms showing representative changes in the four major peaks detected (1, 2, 3, and 4), upon storage under CO_2 and regular atmospheric conditions (air). D0, D6, and D11 correspond to samples obtained at the beginning, after 6, and after 11 days of storage. Monitoring was performed at 280 nm.

contrasted with studies on strawberries, where storage under CO_2-enriched atmosphere was demonstrated to promote either anthocyanin degradation or decreased synthesis compared with samples stored under air (*10, 11*). In apples, ultralow oxygen or CO_2-enriched storage showed that only minimal changes may occur in phenolics and flavonoids (*12, 13*), which is in accordance with investigations on packaging of Swiss chard (*Beta vulgaris*) under CO_2-containing modified atmospheres that had no effect on flavonoid content (*14*).

In the case of olives, storage under atmospheres containing 1−5% CO_2 resulted in increased polyphenol retention (*15*), most probably because of polyphenoloxidase (PPO) inhibition (*16*).

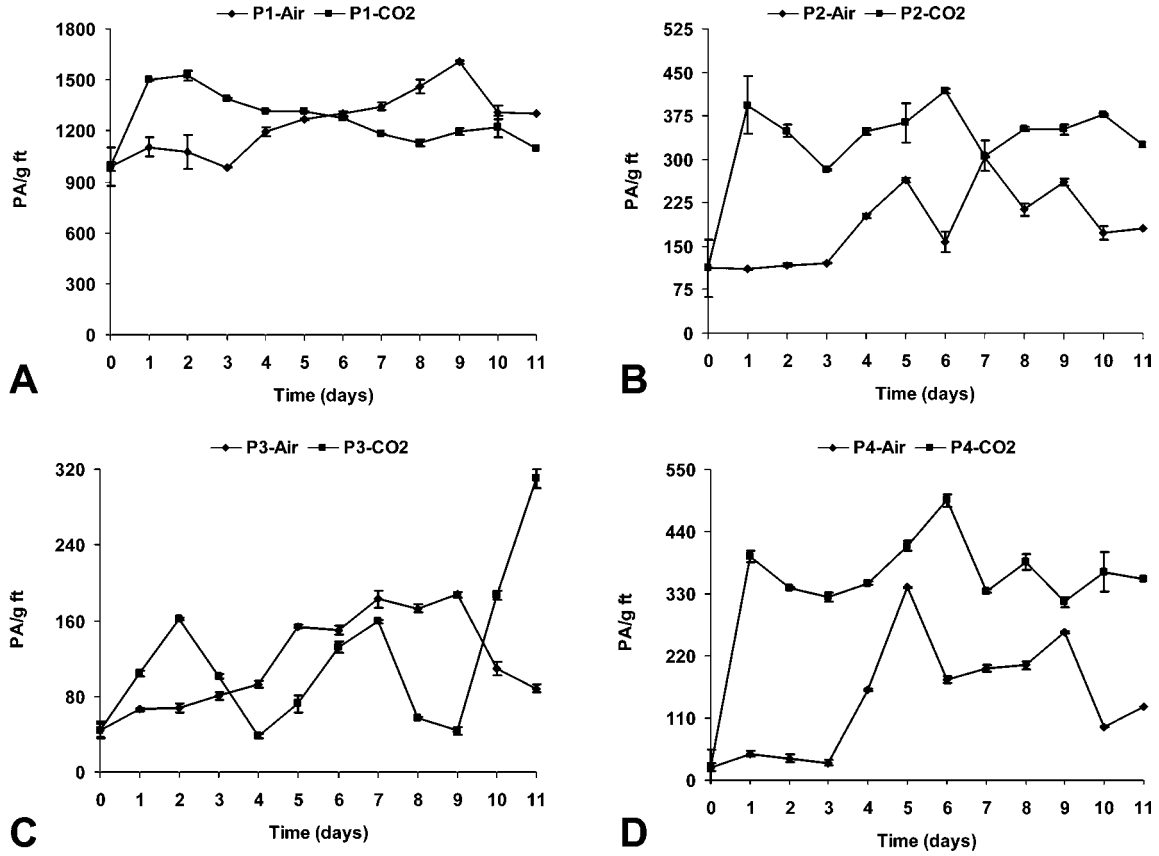

Figure 3. Evolution pattern of the major peaks during storage under CO_2 and regular atmospheric conditions (air). Panels **A**, **B**, **C**, and **D** show the evolution of the corresponding peaks 1–4 (**Figure 2**). Each value represents the mean of triplicate determination (±SD).

Also, atmospheres containing 2 kPa CO_2 were shown to induce faster color development than atmospheres containing lower CO_2 levels (*17*), evidence that gas composition may be critical in affecting polyphenolic metabolism. To the best of our knowledge, postharvest gaseous treatments including extreme CO_2 levels for purposes of manipulating polyphenolic composition in olives have never been performed, and therefore the responses of this fruit upon exposure to CO_2 atmosphere have never been investigated. This study was undertaken to examine the effect of storage of olives under an atmosphere composed entirely of CO_2 on certain secondary metabolites as a first step toward identifying possible activation of specific biochemical routes.

MATERIALS AND METHODS

Chemicals. All solvents used for HPLC and LC-MS were of HPLC grade. Folin−Ciocalteu phenol reagent was from Merck (Darmstadt, Germany). Gallic acid, caffeic acid, and *p*-coumaric acid were from Sigma Chemical Co. (St. Louis, MO).

Plant Material and Postharvest Treatments. Green, unripe olives (*Olea europaea* cv. Chondrolia) were collected on the 20th of September 2004 from an olive tree plantation located inside the T. E. I. of Athens. To obtain a uniform amount of fruits, collection was from three adjacent trees and from different parts of each tree, so as to minimize the effect of watering, sun exposure, and differences related to different maturation stages. After collection, fruits were pooled and randomly divided into two lots, each being approximately 1 kg. One lot was immediately placed under CO_2 atmosphere, in a glass jar. The other lot was spread out on a plastic tray to ensure full contact of all fruits with air. Sampling was performed on a 24-h interval basis, over a period of 12 days. All treatments were carried out at 23 ± 2 °C at 90−95% relative humidity.

Extraction Procedure. Olives (approximately 30 g) were manually destoned. The tissue was ground with sea sand and a small portion of extraction solvent (1% HCl in MeOH) with a pestle and a mortar, and then placed in a round-bottom flask with 50 mL of 1% HCl in MeOH. The flask was attached to a rotary evaporator, and extraction was performed for 15 min at maximum spin without vacuum, at 40 °C. The extract was filtered through a paper filter, and this procedure was repeated twice. The extracts were pooled and concentrated until all MeOH was removed. The aqueous residue was then extracted with petroleum ether (4 × 30 mL) to remove pigments and fats, and solvent residue was removed under vacuum. The remaining aqueous solution was made up to 25 mL with MeOH and filtered through 0.45 μm syringe filters.

Determinations. *Moisture Content*. Olives stored under air showed considerable moisture loss (>8%) from the fifth day of storage. For this reason, moisture losses were determined and taken into account for further determinations. Moisture content of olives was estimated after drying in an oven at 105 °C until constant weight.

Total Polyphenols. Measurements were carried out according to a previously published protocol (*15*), employing the Folin−Ciocalteu methodology. Gallic acid was used as the reference standard, and results were expressed as mg gallic acid equivalents (GAE) per 100 g of fresh tissue.

HPLC Analysis. A HP 1050 liquid chromatograph equipped with a HP 1100 diode array detector and controlled by HP ChemStation was used. The column was a Waters Spherisorb ODS2, 250 mm × 4.0 mm i.d., 5 μm, protected by a guard column packed with the same material and maintained at 25 °C. Chromatograms were performed at a flow rate of 1 mL/min, and injections were made with a 20-μL fixed loop. Eluent A was 2.5% aqueous acetic acid, and eluent B was MeOH. The elution program was as follows: 90% A for 5 min, then 50% A in 20 min, 90% A in another 10 min, which remained constant for further 5 min. Monitoring of chromatograms was accomplished at 280 nm.

Liquid Chromatography−Mass Spectrometry. A Finnigan MAT spectra system P4000 pump was used coupled with a UV6000LP diode array detector and a Finnigan AQA mass spectrometer. Analyses were carried out on a Superspher RP-18, 125 mm × 2 mm i.d., 4 μm, column (Macherey-Nagel, Germany), protected by a guard column packed with the same material, and maintained at 40 °C. Samples were analyzed

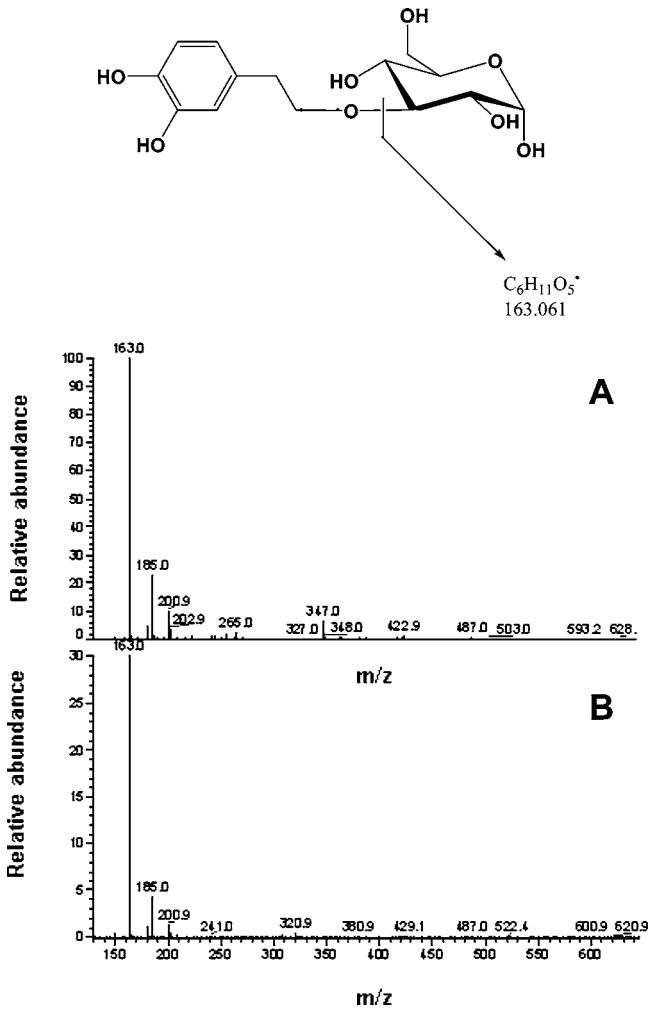

Figure 4. Mass spectrum of peak 1 obtained employing electrospray ionization operated in positive mode. Spectra **A** and **B** were obtained with 20 and 80 eV collision energy, respectively.

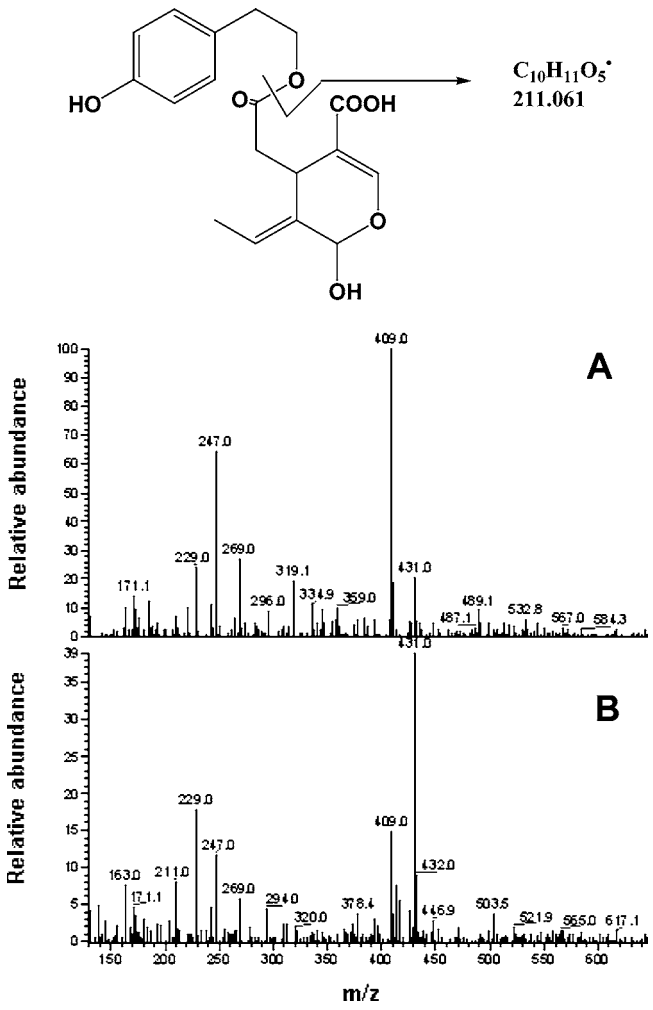

Figure 5. Mass spectrum of peak 2. Spectra **A** and **B** were obtained with 20 and 80 eV collision energy, respectively.

employing electrospray ionization (ESI) in the positive ion mode with acquisition set at 1 eV, capillary voltage at 3.50 kV, source voltage at 45 V, detector voltage at 650 V, and probe temperature of 250 °C. All other conditions were as for HPLC analyses, except that flow rate was set at 0.33 mL/min.

Statistics. Chromatographic analyses were carried out in duplicate. All other analyses were performed at least in triplicate. Values reported are means ± standard deviation (SD). For all statistics, Microsoft Excel was employed.

RESULTS AND DISCUSSION

As a first approach for the investigation of possible changes that may be induced in olive polyphenolic metabolism under CO_2 atmosphere, the total polyphenol content was monitored throughout treatments using the Folin−Ciocalteu reagent. In **Figure 1**, it can be seen that olives stored under CO_2 produced larger amounts of phenolics, while the content of total phenols remained higher in olives under CO_2 compared with those stored under air during the whole period of storage. Additional examination included more detailed insight into the polyphenolic composition employing HPLC. The chromatogram at 280 nm corresponding to the sample just after harvesting indicated that the predominant constituent was a peak accompanied by a minor one, eluted at approximately 4 and 6 min, respectively (**Figure 2**, D0). Their UV spectra were very similar with corresponding λ_{max} at 276 and 278 nm. The analysis of samples obtained after 6 days, however, showed a pronounced increase in peaks 1 and 2, as well as in another two peaks, assigned as 3 and 4, with corresponding λ_{max} at 328 and 312 nm. Comparison of their UV spectra with original standards suggested that peak 3 was a caffeic acid derivative (λ_{max} 325), whereas peak 4 was a *p*-coumaric acid conjugate (λ_{max} 309).

The evolution of these four peaks during the examination period was not affected in the same fashion, and the storage atmosphere was proven to play a crucial role in this regard (**Figure 3**). Peak 1 exhibited a declining trend after an initial increase under CO_2, but its content under air showed a constant increasing tendency. Peaks 2 and 4 appeared to be affected in the same manner, giving very large increases in the first 24 h and maintaining higher levels under CO_2. On the other hand, peak 3 showed intense fluctuations under CO_2 without a constant trend, but its content increased significantly at the end of the treatment. For the sample stored under air, peak 3 increased constantly up to the ninth day, but declined considerably thereafter.

To obtain some additional information on the nature of these components, liquid chromatochraphy−electrospray ionization mass spectrometry was carried out. Peak 1 gave two ions of *m*/*z* 185 and 163, whereas no molecular ion was observed, even under mild ionization conditions (**Figure 4**). The ion with *m*/*z* 163 is a characteristic fragment of glucose elimination, while the ion with *m*/*z* 185 was ascribed to an adduct of the hydroxytyrosyl fragment with methanol $[M - 163 + H + 32]^+$. Thus this compound was identified as the glucoside of hydroxytyrosol, in accordance with previous observations (*16*).

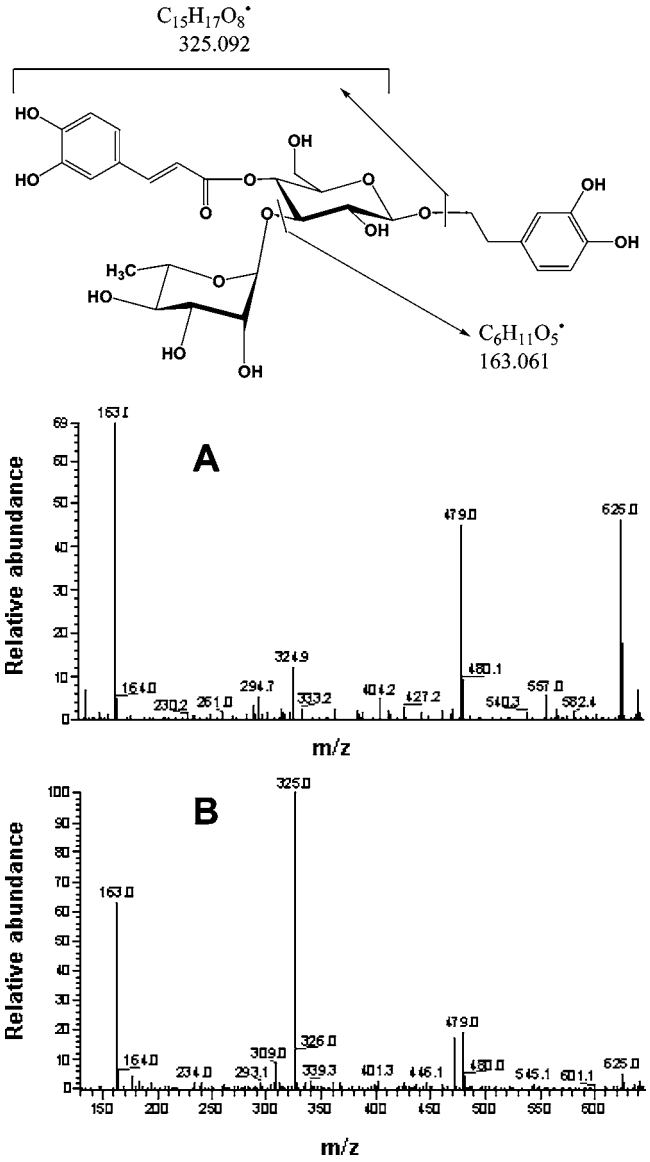

Figure 6. Mass spectrum of peak 3. Spectra **A** and **B** were obtained with 20 and 80 eV collision energy, respectively.

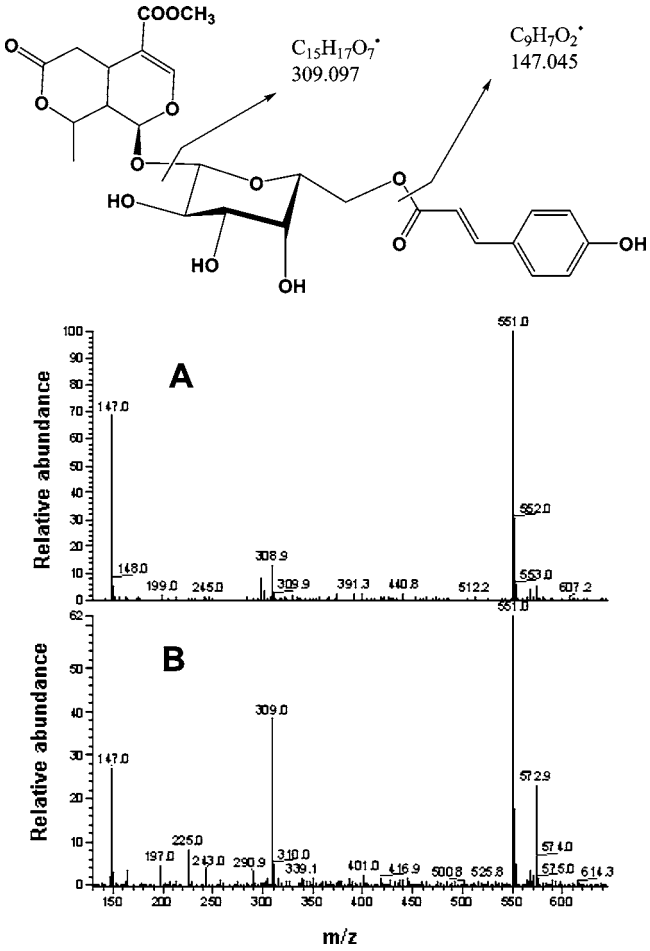

Figure 7. Mass spectrum of peak 4. Spectra **A** and **B** were obtained with 20 and 80 eV collision energy, respectively.

Peak 2 gave a predominant ion at *m*/*z* 409, but the ion with *m*/*z* 431 was also observed, especially under more drastic ionization conditions (**Figure 5**). The ion with *m*/*z* 229, along with *m*/*z* 247, indicated the formation of a fragment ion giving adducts with one and two molecules of water, respectively. These data are consistent with the structure of the demethylated form of ligstroside aglycone (**Figure 5**). In this case, the principal peak is an adduct of the compound with acetic acid $[M + 60 + H]^+$. Upon cleavage at the ester bond, which is thermodynamically the weakest bond of the molecule, there is the formation of the ion *m*/*z* 211, which is better manifested upon enhanced ionization (**Figure 5B**). Under these conditions the adduct of this ion with water is more pronounced, but milder ionization (**Figure 5A**) yields the adduct with two water molecules (*m*/*z* 247) and the predominant *m*/*z* 431, which is ascribed to an adduct with acetic acid and Na^+ $[M + 60 + 23]^+$. Peak 3 exhibited a molecular ion at *m*/*z* 625, and the fragmentation pattern suggested the detachment of the rhamnosyl group (*m*/*z* 163). This was confirmed by the ion at *m*/*z* 479, which was ascribed to an adduct of $[M - 163]^+$ with water. Furthermore, the cleavege of a hydroxytyrosyl was hypothesized by the existence of *m*/*z* 325 $[M - 137]^+$ (**Figure 6**). On the basis of these results, peak 3 was assigned to verbascoside. This is in agreement with ion collision-induced dissociation fragmentation of this component (*17*). Peak 4 showed a molecular ion at *m*/*z* 551, while ions with *m*/*z* 309 and 147 were observed, mainly under stronger ionization conditions, where an adduct with Na^+ (*m*/*z* 573) was also seen. As illustrated in **Figure 7**, the ion with *m*/*z* 147 clearly indicates the elimination of a coumaroyl unit, while the ion with *m*/*z* 309 may represent a secoiridoid residue. Therefore, this peak was tentatively assigned as the coumaroyl derivative of the oleoside methyl ester, ibotalactone A methyl ester.

Traditionally, optimal conditions for modified atmosphere (MA) storage have been selected on the basis of achieving maximum extension of postharvest life. However, postharvest storage under MA may have a prominent influence on several parameters related with plant food quality, including alterations in flavor, color, browning, and health-promoting properties (*8*, *18*). The postharvest storage of olives and its effects have been documented in relation to the quality of the oil produced (*19*−*21*). Nevertheless, postharvest treatments for optimization of storability of table olives and improvement of their quality have not been examined. The olive fruit contains a wide spectrum of secondary polyphenolic metabolites (*22*, *23*), which possess various functional effects (*4*, *24*). Therefore, their profile and content is of undisputed importance to the nutritional quality of olives.

In this study, the examination of storage of olives under a CO_2 atmosphere with respect to its impact on the secondary metabolism of phenolic compounds was attempted. The analyses

showed that CO_2 promotes increased polyphenol biosynthesis, as manifested by the determination of total polyphenol content (**Figure 1**). Further, more detailed investigations with HPLC-DAD of the polyphenolic composition revealed that initially there is one predominant component, but storage either under CO_2 or in air results in the prominent increase of at least another three metabolites (**Figure 2**). In accordance with the total polyphenol determination, increases in the content of these substances were more important under CO_2, but the pattern of biosynthesis and turnover should not be overlooked, because the profile might undergo significant changes within a few hours (**Figure 3**).

The liquid chromatography−mass spectrometry examination provided some valuable information about the nature of the components undergoing profound changes during storage. The most abundant constituent was identified as the glucoside of hydroxytyrosol, consistent with previous reports (*22*), accompanied by smaller amounts of the demethylated ligstroside aglycone. Storage gave rise to extensive production of two hydroxycinnamate derivatives, characterized as verbascoside and ibotalactone A methyl ester, which are conjugates of caffeic acid and *p*-coumaric acid, respectively. Ibotalactone A is known to occur in the *Ligustrum* genus, which belongs to the Oleacea family along with the olive tree (*25*). Although *p*-coumarate derivatives have been reported to occur in olive extracts (*22*), this is the first time that a structure is proposed. However, full structural characterization of this compound remains to be elucidated.

Rapid accumulation of phenolic metabolites in olives stored under CO_2 and maintenance of higher levels of them compared with those found in air-stored fruits, suggested that specific biochemical routes are stimulated, leading to the formation of these substances. In support of this assumption are studies on other plant tissues that are able to produce structurally and biosynthetically relevant phenolics. Elevated CO_2 was shown to stimulate subtle increases in the levels of verbascoside, *p*-coumaric acid, and caffeic acid in tissues of *Plantago maritima* (*26*), and suspension cultures of *Panax ginseng* roots accumulated high levels of phenolics, accompanied by a commensurate increase in the levels of the enzymes implicated in the relevant biosynthetic pathway, including phenylalanine ammonia lyase (PAL) (*27*). In this instance, higher levels of phenolics were associated with higher antioxidant activity. Likewise, storage of cherimoya fruit under elevated CO_2 (20%) resulted in enhanced phenolic content, but PAL activity was not influenced (*28*). Field-grown strawberries under elevated CO_2 produced increased levels of *p*-coumaric acid glucoside but also flavonol glycosides and anthocyanins, which were correlated with high antioxidant activity (*29*).

Several studies indicate that, in general, modified atmospheres containing high levels of CO_2 act in a stimulatory manner in relation to PAL activity but also to other enzymes of the phenylpropanoid pathway (*30*). In the study presented herein, it was demonstrated that an atmosphere entirely composed of CO_2 may cause very important alteration in the phenolic composition of freshly harvested olives, and this finding could be very critical with regard to manipulating the content of olives in potent bioactive phytochemicals. To the extent that these phenomena are confirmed for olives of different cultivars and maturation stages, the results may be directly applicable for the establishment of novel technologies that will aim at improving of the nutritional status of olives and presumably olive oil. Furthermore, the outcome of this study might be of value for the implementation of processes that would enable natural decomposition of bitter constituents, thus leading to olive treatment with minimal environmental impact.

ABBREVIATIONS

GAE, gallic acid equivalents; MA, modified atmosphere; PAL, phenylalanine ammonia lyase; TP, total polyphenols; SD, standard deviation.

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