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Comparison of Ozone and UV-C Treatments on the Postharvest Stilbenoid Monomer, Dimer, and Trimer Induction in Var. 'Superior' White Table Grapes

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Postharvest treatment of seedless white table grapes (var. 'Superior') with different gas ozone concentrations (3.88 and 1.67 g/h) for 1, 3, and 5 h induced an increase in stilbenoid biosynthesis [*trans*-resveratrol, piceatannol, and viniferinas (resveratrol dehydrodimers and dehydrotrimers)] during storage at 22 °C and 95% relative humidity. The maximal resveratrol concentration was reached after 2 days of storage, and this amount was similar to that induced by optimized UV-C treatments (1 min, 510 W, 40 cm). Although similar resveratrol concentrations accumulated in grapes after both UV-C and O_3 treatments (maximum ozone production and time), the ozone treatment was more efficient in inducing viniferins accumulation in grape berries. A sequence in the biosynthesis of stilbenoids was observed, starting with the resveratrol monomer, continuing with the resveratrol dehydrodimers ϵ-viniferin and δ-viniferin, and ending with four different resveratrol dehydrotrimers. These trimers were different from α-viniferin, a trimer previously reported to be induced in grapes after biotic and abiotic stresses. Two α-viniferin isomers were also detected in the ozone-treated grapes, although at very low concentrations that prevented their quantification.

KEYWORDS: Table grapes; postharvest; ozone; resveratrol; stilbenoids; viniferins; dehydrodimers; dehydrotrimers; UV-C

INTRODUCTION

Stilbenes, in general, and resveratrol (3,5,4′-trihydroxystilbene), in particular, are bioactive phenolic compounds occurring in grapes (*1*) and wine (*2*). These are the main food products contributing to the human intake of stilbenoids. The induction of stilbene biosynthesis in response to biotic and abiotic stresses, such as pathogenic attack (*3*, *4*), as well as preharvest (*5*, *6*) and postharvest UV-C irradiation (*7*−*9*), has been previously described.

trans-Resveratrol has been reported to have a number of health-beneficial effects related to its antioxidant (*10*), antimutagenic (*11*), anti-inflammatory (*12*), antiestrogenic (*13*), and antiarrhythmic and cardioprotective (*14*) properties. These activities, together with the lack of toxicity after oral administration of high doses to rats (*15*), have led to the proposition of this molecule as a cancer chemopreventive agent (*16*−*19*).

The resveratrol, and other stilbenoids, content of different food products (grapes, peanuts, and wines mainly) is rather small. Postharvest irradiation with UV-C light has been proposed as a valuable method to increase the resveratrol content of table grapes (*7*, *8*, *20*), wine grapes (*21*), and red wines (*22*) by inducing stilbenoid biosynthesis.

It was also shown that resveratrol biosynthesis was elicited by ozone treatments of harvested grapes in a similar way to UV-C irradiation (*3*) and the 3-fold increase of stilbenoids in 'Napoleon' grapes by ozone gas treatments (*23*). The ozone-induced expression of genes involved in phytoalexin biosynthesis has been reported to occur via ethylene-dependent and -independent signaling pathways (*24*).

The aim of the present work was the evaluation of stilbenoid induction by ozone gas in harvested seedless 'Superior' white table grapes and comparison of this with the induction capacity of UV-C treatments that have been previously optimized.

MATERIALS AND METHODS

Grapes. White table seedless grapes of the variety 'Superior' were harvested at commercial maturity during June and July of both 2003 and 2004, in Murcia (Spain), transported to the laboratory, and stored at 0 °C until being treated with ozone and UV-C irradiation (not later than 1 week after harvest).

Ozone Gas. Extra-dry compressed air (0.7 Pa) was let through a water-cooled corona discharge generator (model 1A, Steriline, Ozono Electrónica Ibérica, Granada, Spain) to produce ozone. Gaseous ozone concentration was measured with an ozone gas analyzer (model H1-SPT, IN USA Inc., Needham, MA). Ozone gas was supplied in air

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constant flow of 0.16 Nm^3/h, with productions (P) of 3.88 g/h and 1.67 g/h produced by the ozone generator in a 50 L methacrylate chamber at 22 °C and 98% relative humidity (RH). The values of ozone obtained were constant throughout the experiments, and the gas mixture expelled from the chamber was destroyed by a catalytic destroyer (OIE, Granada, Spain). The chamber temperature and relative humidity were determined and controlled with a data logger model Tinytag TGU-1500 (Gemini Dataloggers Ltd., Sussex, U.K.). All of the experiments with ozone were made in the pilot plant of CEBAS-CSIC in accordance with strict safety and protection rules.

Gaseous Treatment. Five hundred grams of grape berries was treated for 1, 3, and 5 h in air for the control treatment and with ozone concentrations in the gas carrier (air) of 11300 and 4800 ppm (v/v) of O_3 for the production rates of 3.88 and 1.67 g/h, respectively (22 °C, 98% RH, at atmospheric pressure). These ozone concentrations were chosen because lower concentrations did not provoke significant stilbenes induction according to preliminary experiments. All samples were then stored at 22 °C and 95% RH in air for up to 5 days. Three replicates were evaluated initially and after 1, 2, 3, 4, and 5 days of storage.

UV-C Treatment. Grape berries were UV-treated as previously described (*20*). Briefly, the standard irradiation parameters used along the present study were irradiation power of 510 W, irradiation distance of 40 cm, and irradiation time of 60 s. Both irradiated and control (nontreated) grape berries were stored at 22 °C in perforated plastic bags at a relative humidity of 90−95% to avoid water loss and shriveling during storage. Irradiation experiments were repeated three times.

Extraction of Phenolic Compounds. Five hundred grams each of control and UV-C- and ozone-treated grapes (three replicates) was peeled with the help of a sharp knife and the skins were collected. Three grams of these skins was homogenized in Ultraturrax T-25 equipment (Janke and Kunkel, Ika-Labortechnick, Germany) at 24000 rpm for 1 min after the addition of 4 mL/g HPLC grade methanol + 3% formic acid. The extracts were centrifuged at 5000*g* for 5 min in a Centromix centrifuge (Selecta, Barcelona, Spain), filtered through 0.45 μm, and HPLC analyzed.

HPLC Analysis of Phenolics. The HPLC analyses were performed on an L-6200 liquid chromatograph (Merck-Hitachi, Darmstadt, Germany) equipped with a Shimadzu SPD-M6A UV diode array detector, and a Licrochart RP-18 column (Merck, Darmstadt, Germany) (25 × 0.4 cm, 5 μm particle size), using as solvents water + 5% formic acid (solvent A) and HPLC grade methanol (solvent B) at a flow rate of 1 mL/min. Elution was performed with a gradient starting with 2% B to reach 32% B at 30 min, 40% B at 40 min, and 95% B at 50 min and then maintained isocratic for 5 min. Chromatograms were recorded at 320 nm. The different phenolic compounds were identified by their UV spectra recorded with a diode array detector, by their MS spectra recorded with an ion trap MS-MS, and by chromatographic comparisons with resveratrol (Sigma, St. Louis, MO). The different stilbenoids were quantified at 320 nm using resveratrol as standard (*20*), and the contents are expressed as milligrams of stilbenoid per 100 g of fresh berries.

HPLC-MS-MS. Chromatographic separation was carried out as detailed above. The HPLC system equipped with DAD and mass ion trap detectors in series consisted of a HPLC binary pump (G1312A), an autosampler (G1313A), a degasser (G1322A), and a photodiode array detector (G1315B) controlled by software (v. A08.03) from Agilent Technologies (Waldbronn, Germany). The mass detector was an ion-trap mass spectrometer (G2445A, Agilent Technologies) equipped with an electrospray ionization (ESI) system and controlled by software (v. 4.0.25). The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. Mass scan (MS) and daughter (MS-MS) spectra were meassured from *m*/*z* 100 to 1500. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, and the collision energy was set at 50%. Mass spectrometry data were acquired in the negative ionization mode.

Graphs and Data Analysis. Graphs of the experimental data were carried out by using the Sigma Plot 6.0 program for Windows. Mean values from (at least) three measurements ± standard deviation (SD) of stilbene contents in both control and treated grapes are shown.

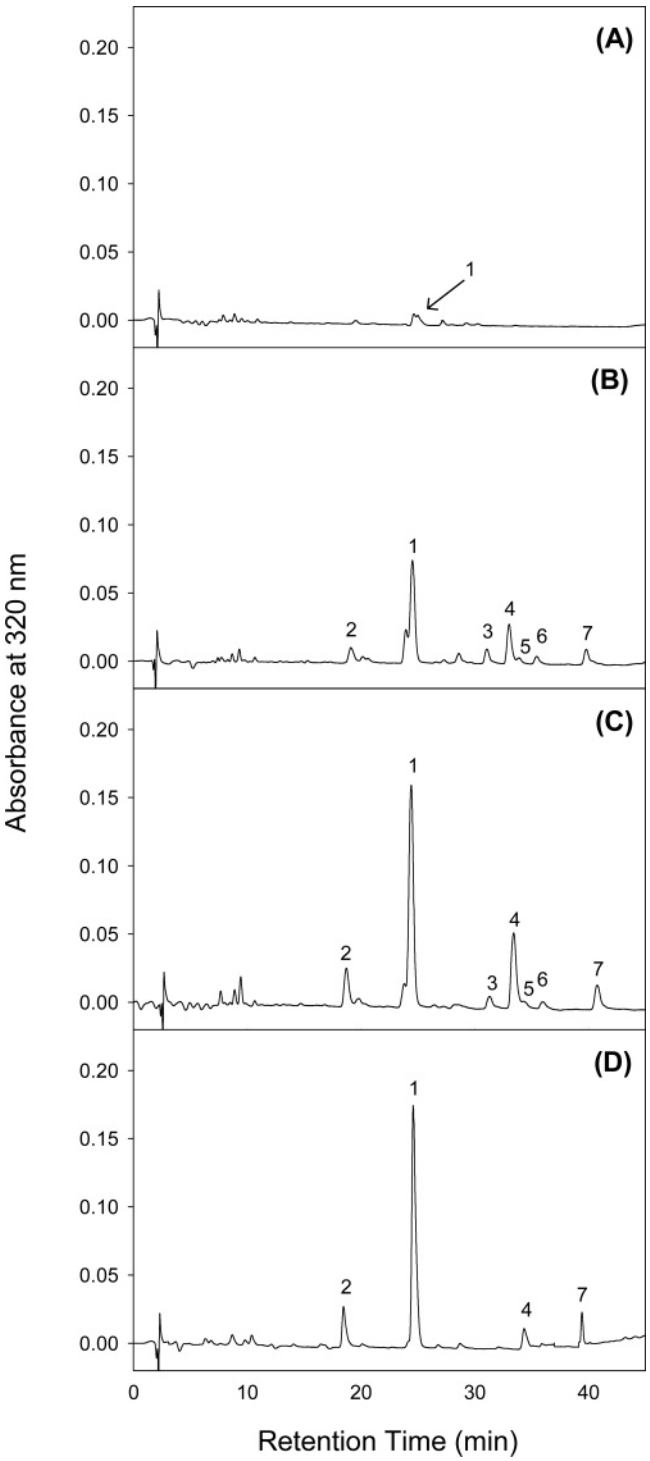

Figure 1. HPLC chromatograms of grape skin extracts at 320 nm: (**A**) control (nontreated) grapes; (**B**) O_3 production = 1.67 g/h for 5 h and after 2 days of storage; (**C**) O_3 production = 3.88 g/h for 5 h and after 2 days of storage; (**D**) UV-C treatment after 2 days of storage. Peaks: (1) *trans*-resveratrol; (2) piceatannol; (3) resveratrol dehydrotrimer t_1; (4) ϵ-viniferin; (5) resveratrol dehydrotrimer t_3; (6) resveratrol dehydrotrimer t_4; (7) δ-viniferin.

RESULTS AND DISCUSSION

Identification of Stilbenoids Induced by Ozone Gas. White grapes were treated with ozone at two different production rates for 5 h at room temperature. The treated and control grapes were stored for 2 days at 22 °C, and the phenolic composition of their skins was evaluated by HPLC coupled with a diode array detector and an ion trap MS spectrometer. A significant

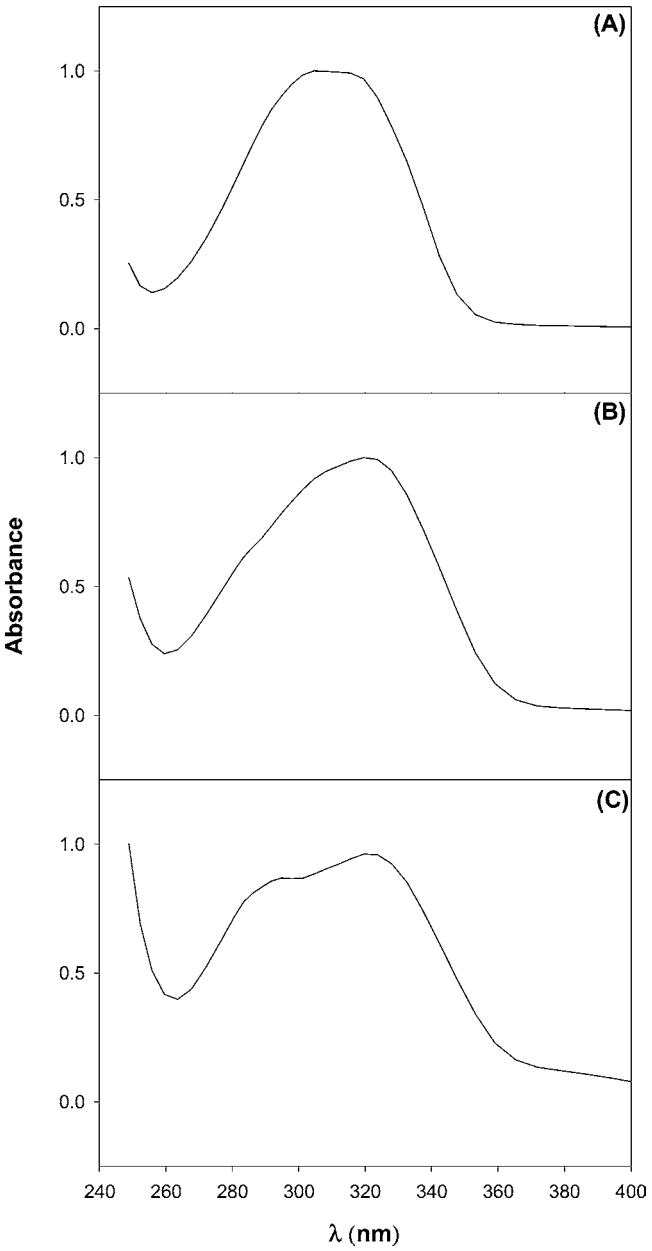

Figure 2. spectra of different induced stilbenes: (**A**) *trans*-resveratrol; (**B**) ϵ-viniferin; (**C**) resveratrol dehydrotrimers.

increase in the peak corresponding to *trans*-resveratrol was observed as a consequence of the ozone treatment (**Figure 1**). In addition, other chromatographic peaks were produced de novo after the treatment. A first peak was identified as piceatannol by its UV spectrum and MS analysis. Several more lipophilic stilbenoids were also detected in the chromatograms (**Figure 1**). These compounds were chemically similar to those induced after UV-C irradiation of grapes (*20*). A previous study reported slight increases in *trans*-resveratrol biosynthesis after ozone treatment of harvested grapes (*23*). The UV spectra of the induced compounds are shown in **Figure 2**. All UV spectra showed maxima around 320−330 nm, similar to those of *trans*-resveratrol, suggesting that all compounds should have at least one *trans*-stilbene grouping. Their MS analyses confirmed the occurrence of two types of compounds in addition of *trans*-resveratrol (3,5,4′-trihydroxystilbene) and *trans*-piceatannol (3,5,3′,4′-tetrahydroxystilbene) (MS negative form: resveratrol, *m*/*z* 227; piceatannol, *m*/*z* 243; two compounds with *m*/*z* 453; four compounds with *m*/*z* 679) (**Figure 3**). The UV spectrum

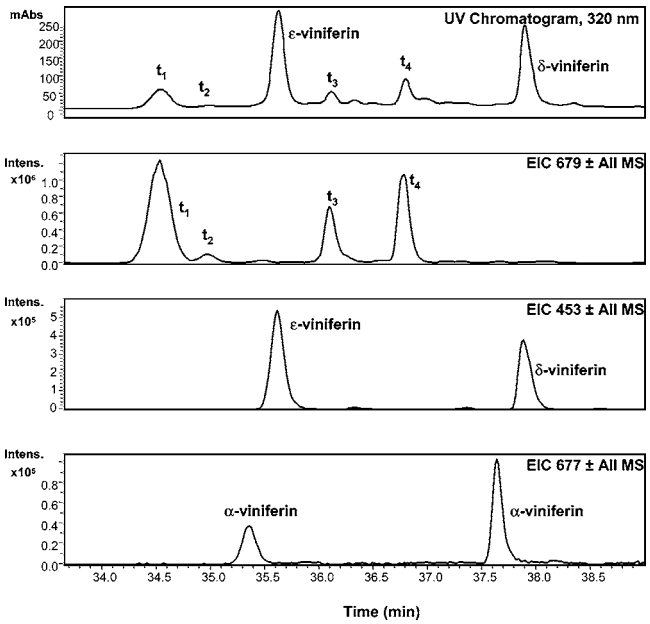

Figure 3. HPLC-MS-MS analysis of ozone-induced stilbenoids: resveratrol dehydrodimers (*m*/*z*$^-$ 453); resveratrol dehydrotrimers (*m*/*z*$^-$ 679); α-viniferins (*m*/*z*$^-$ 677).

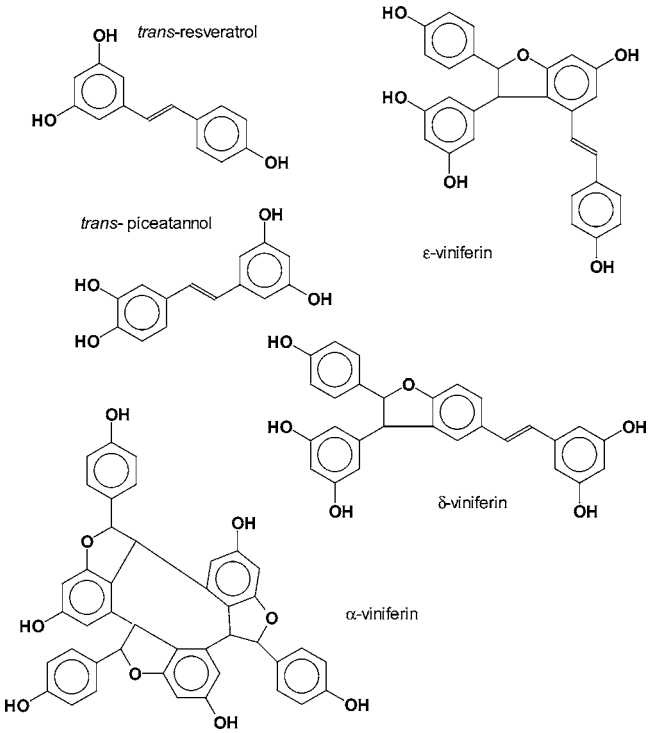

Figure 4. Structures of *trans*-resveratrol, piceatannol, ϵ-viniferin, δ-viniferin, and α-viniferin.

and MS spectra (including MS-MS fragments) clearly indicated that the compounds at *m*/*z* 453 were the resveratrol dehydrodimers ϵ-viniferin [t_R 34.6 min; HPLC-MS-MS fragments at *m*/*z* 435 (M − H − H_2O), 411 (M − H − 42), and 359 (M − H − 94)] and δ-viniferin [t_R 39.7 min; HPLC-MS-MS fragments at *m*/*z* 435 (main fragment), 411, 369, and 359] (**Figure 4**) in agreement with previous reports for the dehydrodimers synthesized by stressed grapevine leaves (*25*). The four isomers at *m*/*z* 679 did not coincide with the previously reported resveratrol trimer α-viniferin (*26*), as this last compound has a molecular weight of 678 and therefore should have a [M − H] ion at *m*/*z* 677, showing that the trimers mainly

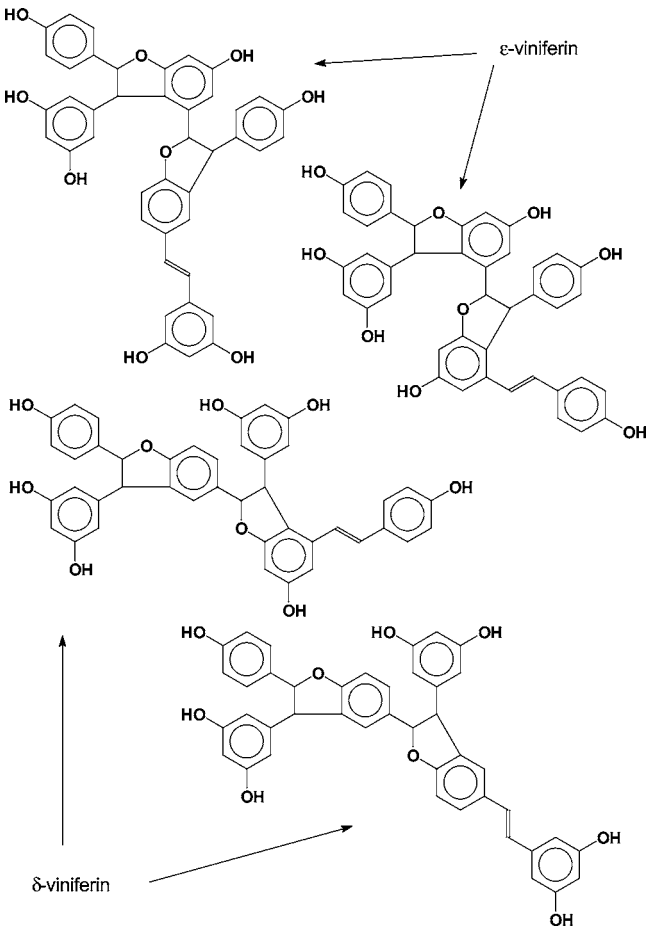

Figure 5. Proposed structures for the resveratrol dehydrotrimers induced by ozone treatments.

induced after ozone treatment (t_1-t_4; **Figure 3**) have two more hydrogen atoms than α-viniferin. Traces of two isomers of α-viniferin were also tentatively detected in the HPLC-MS analysis (M − H at *m*/*z* 677, **Figure 3**), although they were below the limit of detection in the HPLC-UV analysis. Another relevant difference between α-viniferin and the trimers mainly induced by ozone is their UV spectra. The spectrum of α-viniferin should not have a maximum at wavelengths around 325 nm as is the case of resveratrol, ϵ-viniferin and δ-viniferin, and the trimers t_1-t_4, as there is no *trans*-stilbene grouping in the α-viniferin structure (**Figure 4**). The main ozone-induced trimers t_1-t_4 detected here show UV spectra with maxima around 325 nm, indicating that at least one *trans*-stilbene grouping should be present in the molecules. This feature together with the occurrence of two more hydrogen atoms than in the molecule of α-vinferin suggests that these trimers should be resveratrol dehydrotrimers with structures similar to those of the dehydrodimers ϵ-viniferin and δ-viniferin. The proposed structures for these four isomers are shown in **Figure 5**. The MS-MS analyses in the negative form of these compounds show fragments at *m*/*z* 661 (M − H − H_2O), 637 (M − H − 42), and 585 (M − H − 94), with losses similar to those found for the resveratrol dehydrodimers ϵ- and δ-viniferins, supporting similar structural features. No effect of ozone on the phenolic constituents of grape flesh was observed (data not shown), in agreement with previous reports on the effect of UV-C irradiation on the phenolic composition of grapes that only produced an increase in the stilbenoid content of the skin tissue (*8*).

Effect of Time and Ozone Concentration on *trans*-Resveratrol Biosynthesis. The effect of treatment time and ozone concentration on the resveratrol induction was evaluated and compared with the increase in biosynthesis produced by UV-C irradiation under the specific conditions previously optimized (*8*, *20*). Treatments with higher ozone production rates and for longer time did not induce necessarily higher resveratrol concentrations. The treatment that clearly produced a high resveratrol concentration was 3.88 g/h of O_3 for 5 h (**Figure 6**). The maximum resveratrol concentration for each ozone treatment slightly fluctuated after several storage days (**Figure 6**). This fluctuation could be partially due to the transformation

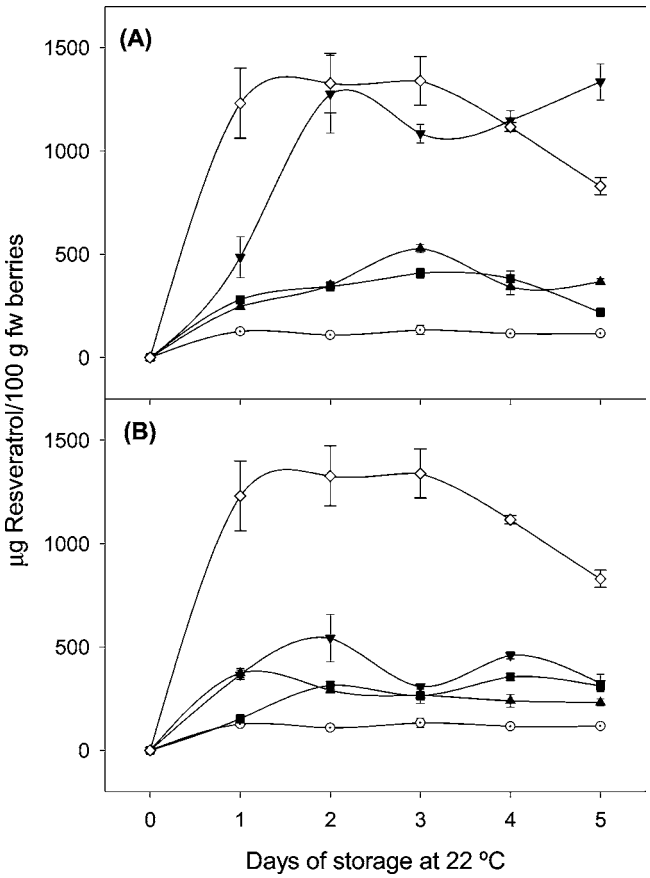

Figure 6. Induction kinetics of *trans*-resveratrol after UV-C irradiation and O_3 treatments: (**A**) UV-C and O_3 production = 3.88 g/h; (**B**) UV-C and O_3 production = 1.67 g/h: (⊙) control (nontreated) grapes; (◇) UV-C-irradiated grapes; (■) O_3 1 h treatment; (▲) O_3 3 h; (▼) O_3 5 h.

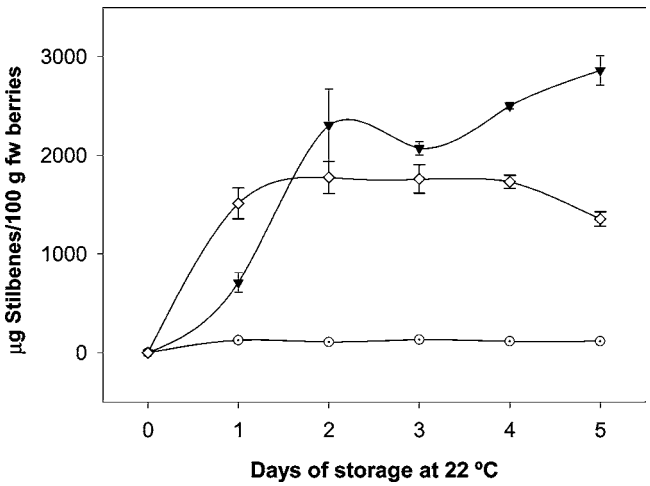

Figure 7. Induction kinetics of total stilbenes after UV-C irradiation and O_3 treatments (3.88 g/h; 5 h): (⊙) control (nontreated) grapes; (◇) UV-C-irradiated grapes; (▼) O_3-treated grapes.

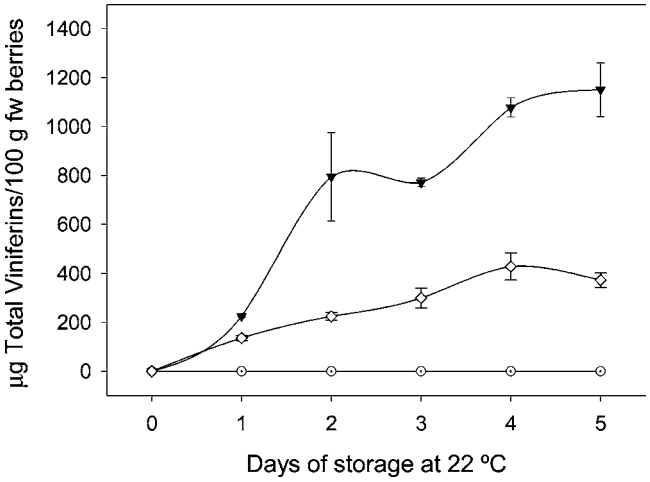

Figure 8. Induction kinetics of total viniferins (dehydrodimers and dehydrotrimers) after UV-C irradiation and O_3 treatment (3.88 g/h; 5 h): (⊙) control (nontreated) grapes; (◇) UV-C-irradiated grapes; (▼) O_3-treated grapes.

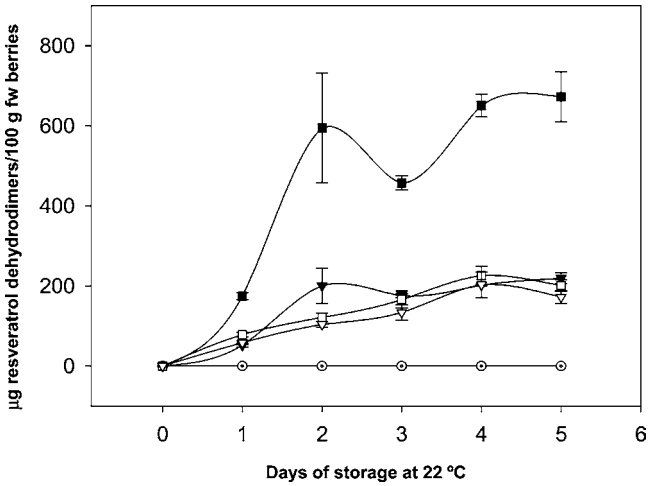

Figure 9. Induction kinetics of different resveratrol dehydrodimers after UV-C irradiation and O_3 treatment for 5 h and production = 3.88 g/h: (⊙) control (nontreated) grapes; (■) O_3, ϵ-viniferin; (▼) O_3, δ-viniferin; (□) UV-C, ϵ-viniferin; (▽) UV-C, δ-viniferin.

of *trans*-resveratrol produced by stilbene synthase after abiotic stress into dehydrodimers and dehydrotrimers (viniferins + trimers). This oxidative dimerization or trimerization of *trans*-resveratrol has been suggested to be carried out by grape basic peroxidases (*27*). The results show that UV-C was generally much more efficient in inducing resveratrol than ozone. Ozone treatments, at the maximum production and treatment time (5 h), induced resveratrol concentrations similar to those induced by optimized UV-C irradiation (**Figure 6**). With regard to total stilbenoids accumulated in the grape skin [resveratrol + piceatannol + viniferins (dimers and trimers)], the ozone treatment led to higher stilbenoid levels than the UV-C treatment (**Figure 7**), showing that ozone at the highest concentration and longest treatment time induced in addition the accumulation of stilbenoid derivatives different from resveratrol in a more efficient manner than did UV-C.

Effect of Ozone and UV-C Treatments on Viniferins Accumulation. The concentration of viniferins produced by the UV-C treatment was quite small when compared with those induced by ozone treatments, especially for longer treatment times (**Figure 8**). This was particularly relevant for the longest treatment time and the highest production rate, by which the

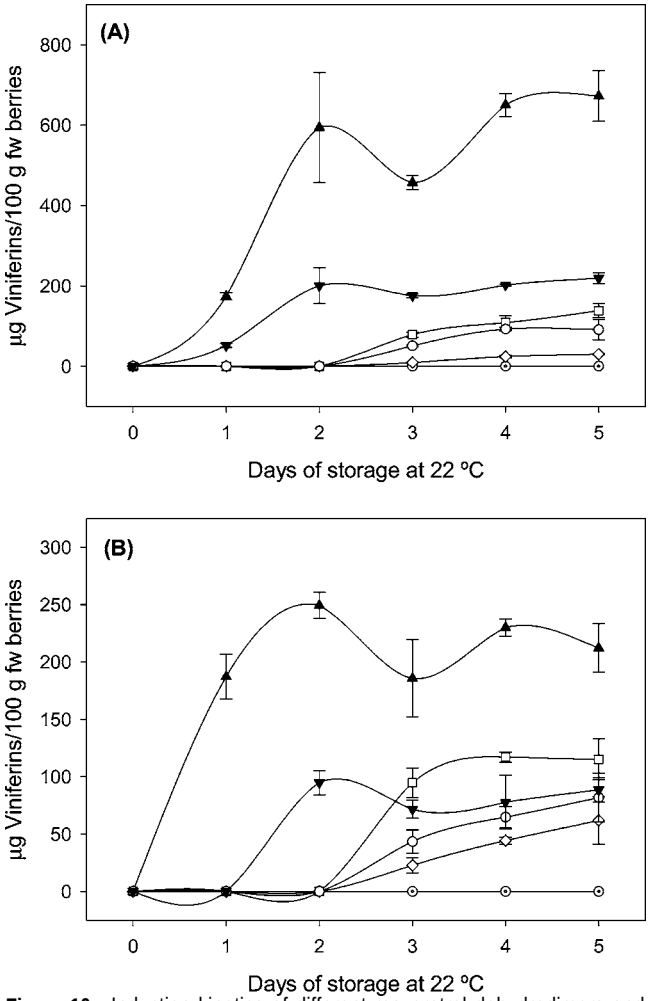

Figure 10. Induction kinetics of different resveratrol dehydrodimers and dehydrotrimers after O_3 treatment for 5 h and production = 3.88 g/h (**A**) and 1.67 g/h (**B**): (⊙) control (nontreated) grapes; (▲) ϵ-viniferin; (▼) δ-viniferin; (□) dehydrotrimer t_1; (◇) dehydrotrimer t_3; (○) dehydrotrimer t_4.

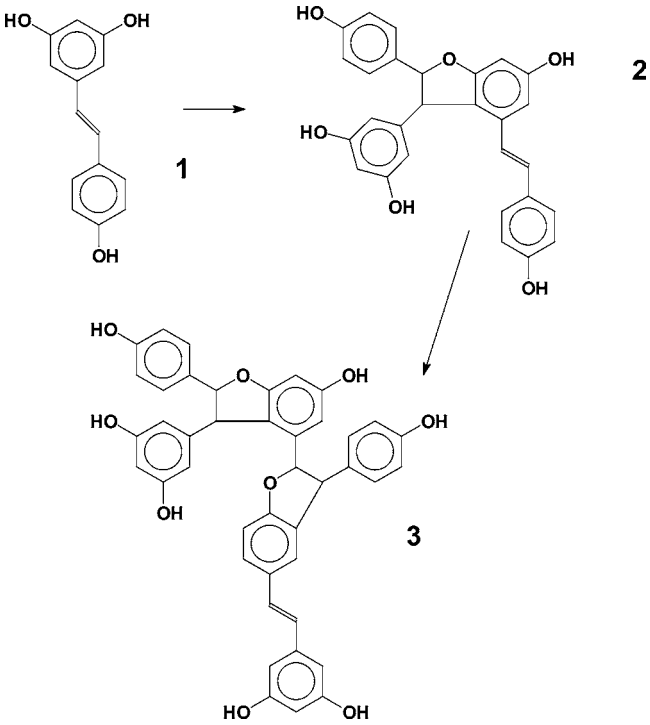

Figure 11. Sequential biosynthesis of stilbenoid derivatives: (**1**) *trans*-resveratrol; (**2**) resveratrol dehydrodimers; (**3**) resveratrol dehydrotrimers.

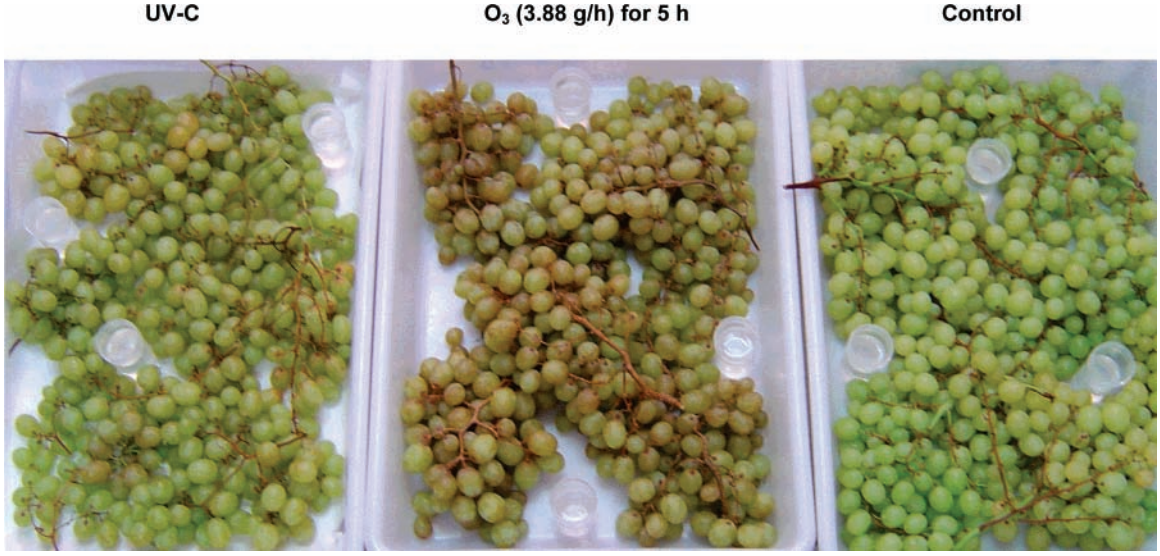

Figure 12. Var. 'Superior' grapes: nontreated (control) and submitted to ozone and UV-C treatments after 3 days of storage.

viniferin content accumulated was 3-fold than that induced by the UV-C treatment. The largest difference observed between ozone and UV-C treatments was due to the ϵ-viniferin accumulation, which was much more relevant in grapes treated by ozone (**Figure 9**).

When the induction rates of the biosynthesis of specific resveratrol dimers and trimers by the ozone treatments are considered, it is clear that resveratrol dehydrodimers (ϵ-viniferin and δ-viniferin) started to accumulate in the skin after 1 day of storage, reaching maximum values after 2 days of storage (**Figure 10**), whereas resveratrol dehydrotrimers (t_1-t_4) were present in nonquantifiable amounts during the first 2 days of storage, increasing their contents after 3 days of storage and reaching maximal values at day 4. This sequential induction in the biosynthesis of stilbenoid derivatives confirms that resveratrol (the monomer) is synthesized first upon abiotic stresses in grape skins, whereas the resveratrol dehydrodimers are produced in a second stage and the dehydrotrimers accumulate during a third sequential stage (**Figure 11**) in agreement with previous reports (*28*).

Although the application of ozone gas under some conditions (long treatment times and high ozone production rates) can lead to stilbenoid accumulation at levels similar to those found after UV-C irradiation, it could be concluded that UV-C is in general more efficient than ozone as a treatment to increase resveratrol content with shorter treatment times (1 min of irradiation + 1−2 days of storage at 22 °C to allow resveratrol biosynthesis) that are more compatible with industrial processing. Furthermore, UV-C light generally produces less damage to grape tissues than ozone gas (**Figure 12**). Ozone treatments diminished the sensory quality due to browning development in the skin after 48 h of storage. Although the use of ozone to increase stilbenoid content in grapes would not be appropriate to market fresh grapes, it could be used to obtain stilbenoid-enriched musts or grape extracts as previously proposed for UV-C treatments (*8*, *20*). However, it should be stressed that the white grape cultivar assayed (var. 'Superior') is particularly susceptible to browning (*29*).

When UV-C was used to induce resveratrol biosynthesis, the accumulation of resveratrol dehydrotrimers was less relevant than when ozone treatments were applied, this being the main phytochemical difference between both treatments. This might be due to a shorter treatment time for UV irradiation versus ozone treatment (minutes versus hours, respectively). In fact, dehydrotrimers upon UV-C treatment were detected (by UV spectra and ion mass) but not quantified due to their low content.

The biological activity of resveratrol is well documented (*10*−*18*), but studies on viniferin biological properties are scarce (*30*, *31*). Their activity, however, seems to be relevant, and the accumulation of viniferins might complement the biological effects of resveratrol. Nevertheless, nothing is known about viniferins bioavailability, but their chemical nature would suggest a lower bioavailability compared to that of resveratrol, possibly because the solubility of viniferins decreases as their molecular weight increases. If this parallels what is observed for procyanidins in which monomers are absorbed much better than dimers, and these better than trimers (*32*), viniferins bioavailaibility could be a critical point for studying the in vivo biological activity of these compounds.

Milder ozone treatments, such as those using ozonated water, which are effective in the control of microbial growth in fresh-cut commodities (*33*), did not induce stilbenoid accumulation in grapes (data not shown). This could be due to the low solubility of ozone in water (up to 5 ppm) and the short treatment times used (minutes).

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