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Novel Endotherapy-Based Applications of Ozonated Water to Bobal Grapevines: Effect on Grape Quality

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Abstract: Ozonated water has recently been incorporated in the management of grapevine diseases, which in turn can alter the fruit quality. When wood-inhabiting pathogens are involved, trunk injection or "endotherapy" represents a promising application method. Thus, the present study aimed to evaluate the effect on grape quality of ozonated water applied to *Vitis vinifera* L. cv. Bobal grapevines through endotherapy (E) or its combination with spraying (E + S). Grape quality at harvest was evaluated through several enological and chromatic parameters, the phenolic maturity, the varietal aroma potential index (IPAv) and the phenolic and volatile composition. The E treatment improved the chromatic characteristics and favored the accumulation of phenolic compounds. Conversely, E + S had a detrimental effect on the color and phenolic content and, although their synthesis was enhanced, the extractability of anthocyanins was negatively affected. In terms of aroma, both treatments reduced the content of glycosylated precursors but increased certain free volatiles. The application of ozonated water to grapevines, even when injected into the trunk, results in changes in fruit quality and a possible impact on wine attributes. Our findings and those in the literature support that, with the appropriate dose, frequency and method of application, ozonated water could be used with a twofold objective: disease management and grape quality improvement.

Keywords: ozone; ozonated water; endotherapy; spraying; Bobal; grape quality; phenolics; volatiles

1. Introduction

Grapevines are one of the most extensively grown fruit crops in the world and also one of the leaders in pesticide consumption. On average, 35% of the pesticides used worldwide are attributed to viticulture [1]. This can be largely explained by the fact that grapevines host the highest number of pathogens of any woody species [2]. Fungal pathogens are particularly abundant, especially those causing the grapevine trunk diseases (GTDs), which are currently considered one of the most relevant threats for viticulture [3]. They include a number of different diseases (esca, eutypa dieback, botryosphaeria dieback, etc.) caused by a complex range of pathogenic fungi that attack the perennial organs, eventually causing the death of grapevines [4]. Since the ban of sodium arsenite at the beginning of the 21st century, there are no curative methods to fight against GTDs, so merely preventive measures are frequently used [5]. The development of an effective treatment against the agents causing these diseases has become a major necessity in a scenario where efforts are being made to promote a sustainable use of pesticides and the search for environmentally friendly alternatives.

Recent studies have revealed that ozone (O_3) in aqueous solution is a promising candidate for limiting grapevine infection by *Phaeoacremonium aleophilum*, a fungus associated with esca [6]. In the last few years, in fact, spraying grapevines with ozonated water for disease control is becoming more frequent among winegrowers [7,8]. The reason lies mainly in the high oxidizing power of ozone and its spontaneous decomposition to oxygen without forming hazardous residues. The decomposition of aqueous ozone also leads to the production of reactive oxygen species (ROS), which are more reactive but less selective than molecular ozone [9]. The hydroxyl radical (•OH), for example, is an important transient species that propagates additional radicals, all of them contributing to the overall oxidizing power of ozone [10]. In water, the triatomic form of oxygen is even more unstable and decays faster than in the gaseous phase [11]. However, molecular ozone or its decomposition products require short contact times to inactivate a wide range of microorganisms by reacting with numerous cellular constituents, such as intracellular enzymes, nucleic material, components of their cell envelope, spore coats, or viral capsids [12].

These properties make ozone, in either gaseous or aqueous form, a widely used sanitizer in the food industry, especially to preserve and extend the shelf life of harvested fruits and vegetables [13]. In addition to inhibit or prevent decay, postharvest ozone exposure has been shown to induce a metabolic response in fresh commodities—for example, affecting either positively or negatively the phenolic composition and the aroma profile of grapes and resulting wines, depending on many factors, such as the ozone dose, the exposure duration and the variety [14–20]. Before harvest, aqueous ozone has been demonstrated to improve several key performance metrics when applied via drip irrigation to hydroponic tomato [21], to reduce the severity of powdery mildew when sprayed on cucumber leaves [22], and to modify the quality of grapes and wines when sprayed on grapevines for phytosanitary purposes [7,8,23]. However, most of the research has been done on the physiological changes induced in growing plants exposed to ozone as a pollutant. The major entry pathways to the plant tissues are openings or breaks in the cuticle, including stomata, lenticels and physical breaks or cracks [24]. Once in the apoplast, ozone and derived ROS react with molecules in the extracellular fluid as well as components of the cell wall and plasma membrane [24]. As ozone and its breakdown products are very reactive, they are unlikely to penetrate the cytoplasm; however, the disruption of the normal membrane function [24] and the activation of a signal transduction [25] may alter the metabolism of the whole cell. The accumulation of ROS induces plant defense mechanisms that resemble those elicited by pathogens [25]. These defensive responses include the production of phenolic compounds and volatile organic compounds because of their roles as antioxidants [25–30].

Foliar spraying is the most common way of applying protection products to plants. However, this method entails off-target losses due to drifts that reduce the actual dose received by the plant. In vineyards, Bonicelli et al. showed that spray applications led to 30–40% of air losses, and 10–40% of ground depositions depending on the stage of development of the grapevines or the canopy density [31]. Alternatively, plant protection products can be directly applied to the vascular system through a delivery method called “endotherapy” or “trunk injection”. This approach reduces the environmental impact and user exposure and, at the same time, promotes an efficient use of agrochemicals since the root or cuticle barriers are avoided and the whole dose is delivered in the sap flux [32]. Endotherapy was first investigated by Leonardo Da Vinci in the 15th century when he managed to poison apples by introducing arsenic into the trunk of an apple tree [32]. In woody plants, xylem sap moves from the roots to the leaves through the vessels due to a decreasing gradient of water potential. This movement follows the Bernoulli’s principle on fluid dynamics, so the introduction of an object (e.g., a drill) into the trunk causes a temporary reduction in the cross section of the vessels that leads to a Venturi effect—i.e., the speed of the sap increases while its pressure decreases [33]. Under such circumstances, and when the natural speed of sap is substantial, liquids from an external source are passively absorbed by the tree, although the uptake can also be forced by applying a low external pressure [33].

Nowadays, endotherapy is a method particularly used to apply pesticides to large trees and near urban areas [32]. On grapevines, this delivery method has been little studied, although some

trials injecting fungicides, plant-defense elicitors and different chemicals have been conducted against esca [34–38], eutypa dieback [36] or downy mildew [39]. In the particular case of ozonated water, endotherapy represents an interesting alternative application method to grapevines for the following reasons: (i) spray drift is prevented; (ii) in the case of grapevines affected by GTDs, the active substance is directly applied to the infected parts of the plant since the fungal pathogens causing these diseases colonize the woody tissues [4]; (iii) the low persistence of ozone may be counterbalanced by the shorter time to reach the target pathogens.

Since previous experiments using ozonated water to treat grapevines by spraying have revealed an impact on the quality of grapes and resulting wines, our hypothesis is that this active substance would also trigger the response of the fruit when it is delivered directly through the vascular system of the plant or in combination with additional spraying applications. Thus, the aim of this study was to evaluate the effect of ozonated water, applied to Bobal grapevines through endotherapy or its combination with spraying on the enological, chromatic, phenolic and aromatic quality of grapes. As previously stated, spray treatments with ozonated water are increasingly used in the vineyard as a complementary tool in disease management but, as far as we know, this is the first attempt to use endotherapy as the delivery method and to assess the possible impact on grape quality.

2. Materials and Methods

2.1. Grapevines

Field trials were carried out during the year 2017 in an unirrigated vineyard of *Vitis vinifera* L. cv. Bobal located in Casas de Haro (Cuenca, Castilla-La Mancha, Spain) at an altitude of 730 m, latitude 39°18′ N and longitude 2°13′ W. The grapevines, which were planted in 1999, were grown on a vertical trellis system (2.8 × 2.8 m) with a row distribution north to south. The local climate is cold and semi-arid with an annual average temperature of 14.4 °C, a minimum of −10.3 °C (January) and a maximum of 40.9 °C (July). The annual precipitation was around 195 mm. A visual evaluation of the health status of the plot, especially with regard to GTDs, was carried out before, during and after the experiments. Moreover, other diseases and pests, such as the grapevine moth, downy mildew, powdery mildew, botrytis, the leafhopper *Empoasca vitis* and the spider mite *Tetranychus urticae* Koch, were controlled weekly.

2.2. Ozonated Water

A specific prototype intended for use in agriculture (Nutricontrol, S.L., Cartagena, Spain) was employed to generate the ozonated water in the vineyard immediately prior to the treatments. The equipment consisted of an ozone generator from atmospheric oxygen and a 500 L thermally insulated tank of water. Water from a well located in the vineyard with an approximate temperature of 15 °C was used to prepare the aqueous ozone. Ozone gas was bubbled into the water according to the manufacturer's instructions. The prototype also contained an electrode to measure the oxidation-reduction potential (ORP) of the water in millivolts (mV), and a controller to automatically maintain the desired ORP. During water ozonation, the increase in dissolved ozone and consequent ROS makes the ORP of water rise because they are strong oxidizers. Thus, ORP provides a real-time indicator of the oxidizing activity of the water and, indirectly, of the dissolved ozone concentration. The ORP of the ozonated water was 875 ± 25 mV in all applications.

2.3. Grapevine Treatments

Two different ozonated water treatments were applied to the grapevines by means of different application strategies: endotherapy (E)—i.e., direct injection of the ozonated water into the trunk—as well as the combination of endotherapy and foliar spraying (E + S). In the case of endotherapy, preliminary tests were carried out to establish the most appropriate injection procedure in grapevines (data not shown). A 3 mm diameter hole was drilled above the graft union of the trunk to a depth of

1 cm, which was immediately sealed with a cannula containing a connector to the ozonated water tank and a hermetic closure (Figure 1). A volume of ozonated water of approximately 0.5% of the trunk volume was injected under a maximum pressure of 1 bar into each grapevine in each application. A sufficient injection volume was selected so that the ozonated water was distributed through the xylem and reached the different parts of the plant. Ozonated water was applied through endotherapy four times before harvest for both E and E + S treatments, in particular before flowering and fruit setting (15 weeks before harvest), after the fruit set (10 weeks before harvest), at veraison (5 weeks before harvest) and during the ripening period (3 weeks before harvest). Each application timing corresponded, respectively, to the growth stages 15, 32, 35 and 37, according to the Modified E-L system [40]. In the case of E + S treatment, spraying was done eight times, specifically 2 days before and after each endotherapy application. Approximately 300 mL of ozonated water was sprayed per plant to cover the entire canopy. Each treatment was performed in quadruplicate over a total number of 15 randomly selected plants, and another 15 plants were used as a control (without treatment, C), leaving 3 of each group as a backup. The plants under study were visually healthy in order to minimize the effect of pests or diseases on the quality of grapes. A row with untreated plants between the treatments and the control was left to avoid the drift effect. The plants under study were located in a homogeneous portion of the plot and the outer rows were avoided. The treatments were carried out early in the morning, when the environmental temperature was around 20 °C. When the optimal technological maturity of the controls was achieved (most suitable °Baumé/titratable acidity ratio), grapes were manually collected and frozen at −18 °C until further analysis.

Figure 1. Procedure for the application of ozonated water through endotherapy: (**a**) Drilling of the trunk; (**b**) Insertion of the cannula; (**c**) Placement of the connector to the ozonated water tank.

2.4. Analytical Methods

2.4.1. Sample Preparation

Grapes were defrosted and manually destemmed prior to the analyses. All the analyses described below were done in duplicate.

2.4.2. Grape Enological Parameters

Grapes classical parameters, such as the weight of 100 berries (g), °Baumé (°Bé), pH and titratable acidity (TA, g/L of tartaric acid), were determined according to the Official Methods established by the European Union [41].

2.4.3. Chromatic Parameters

The chromatic parameters of grapes were determined by measuring the absorbances at 420, 520 and 620 nm, and then calculated as follows: color intensity (CI) = $A_{420} + A_{520} + A_{620}$ and tonality (T) = A_{420}/A_{520} [42]. Grapes were first crushed and filtered through a strainer. The resulting must was centrifuged at 4000 rpm for 5 min and filtered through a PVDF Durapore filter of 0.45 µm (Millipore, Bedford, MA, USA). Lambda 25 UV–Vis equipment (Perkin Elmer, Norwalk, CT, USA) and 0.1 cm path length cells were used for the spectrophotometric determinations, by referring the absorbances to 1 cm path length cells to calculate CI and T.

2.4.4. Phenolic Maturity

The phenolic maturity of the grapes was determined according to the method described by Saint-Cricq de Gaulejac et al. [43]. Grapes were macerated for 4 h at pH 1.0 and 3.4, the latter being more relevant to the musts of the area under study than the pH 3.2 proposed in the original method. The total phenol index (TPI) of the grape solution at pH 3.4 and the anthocyanin content (A) of both solutions were obtained by measuring the absorbances at 280 nm [44] and 520 nm [45], respectively. The aforementioned UV–Vis equipment and 1 cm path length cells were used for the spectrophotometric determinations. The anthocyanin extractability index (%AEI) and the seed maturity index (%SMI) were calculated with the following equations: $\%AEI = [(A_{pH1.0} - A_{pH3.4})/A_{pH1.0}] \times 100$; $\%SMI = [(TPI_{pH3.4} - A_{pH3.4} \times 40/1000)/TPI_{pH3.4}] \times 100$.

2.4.5. Varietal Aroma Potential Index (IPAv)

The varietal aroma potential index (IPAv) of grapes was analyzed using a commercially available kit (Teknokroma S.A., Barcelona, Spain). The method is based on the one described by Salinas et al. [46], but modified to enable the spectrophotometric determination of the glycosyl glucose (G-G) released by acid hydrolysis from the glycosylated aroma precursors.

2.4.6. Determination of Low Molecular Weight Phenolic Compounds by HPLC-DAD

The analysis of grape phenolic composition was based on the method described by Pardo-García et al. [47]. Before the analysis, grapes were crushed and filtered through a strainer. The resulting must was centrifuged at 4000 rpm for 5 min and passed through a PVDF Durapore filter of 0.22 µm (Millipore, Bedford, MA, USA). A volume of 20 µL of the filtrate was injected into an Agilent 1200 High Performance Liquid Chromatograph (HPLC, Palo Alto, CA, USA) equipped with a Diode Array Detector (DAD, Agilent G1315D, Palo Alto, CA, USA), coupled to an Agilent ChemStation (version B.03.01) data-processing station. Separation was performed on a reversed-phase Zorbax-Eclipse XDB-C18 (4.6 mm × 150 mm, 5 µm particle sizes) and a precolumn of the same material at 30 °C.

The standards employed to identify and quantify the phenolic compounds were gallic acid, syringic acid, *t*-caffeic acid, *t*-*p*-coumaric acid, quercetin and malvidin 3-O-glucoside (Sigma-Aldrich, Steinheim, Germany). The identification of phenolic compounds was carried out by comparison with their corresponding UV–Vis spectra and the retention time of their pure standards. The identification of compounds for which no standard was available was carried out as previously described by Pardo-García et al. [47]. The respective absorption maxima in the UV–Vis region and retention times were used as additional means of identification. Compounds were identified and quantified at different wavelengths: gallic acid and syringic acid at 280 nm; *t*-caftaric acid at 324 nm; *t*-*p*-coutaric acid at 308 nm; flavonols at 365 nm and anthocyanins at 520 nm. Quantification was based on calibration curves of the respective standards at five different concentrations ($R^2 > 0.98$). Acids *t*-caftaric and *t*-*p*-coutaric were quantified as *t*-caffeic acid and *t*-*p*-coumaric acid, respectively. Flavonols were quantified as quercetin equivalents and the anthocyanins as malvidin 3-O-glucoside equivalents.

2.4.7. Determination of Volatile Compounds by HS-SBSE-GC-MS

The grape volatile compounds were determined according to the method described by Campayo et al. [7]. First, they were extracted by Headspace Stir Bar Sorptive Extraction (HS-SBSE). Volatiles were later analyzed using an automated thermal desorption unit (TDU, Gerstel, Mülheim and der Ruhr, Germany) mounted on an Agilent 7890A gas chromatograph system (GC) coupled to a quadrupole Agilent 5975C electron ionization mass spectrometric detector (MS, Agilent Technologies, Palo Alto, CA, USA). The GC system was equipped with a fused silica capillary column (BP21 stationary phase, 30 m length, 0.25 mm i.d., and 0.25 µm film thickness) (SGE, Ringwood, Australia) and the carrier gas was helium with a constant column pressure of 20.75 psi.

MS data acquisition was carried out at positive scan mode, although to avoid matrix interferences, the MS quantification was performed in the single ion-monitoring mode using the characteristic *m/z* values of the volatiles. The identification of the compounds was performed using the NIST library and confirmed by comparison with the mass spectra and retention time of their pure standards (Sigma-Aldrich, Steinheim, Germany). The standards employed to identify and quantify volatiles were (the numbers in brackets indicates the *m/z* used for quantification): benzaldehyde (106), *β*-damascenone (121), geraniol (69), geranyl acetone (43), guaiacol (109), 1-hexanol (56), *cis*-3-hexen-1-ol (67), *trans*-2-hexen-1-ol (57), *trans*-2-hexenal (41), linalool (71), nonanal (57), 1-octen-3-ol (57) and syringol (154). Internal standard 3-methyl-1-pentanol was used. Quantification was based on calibration curves of the respective standards at five different concentrations (R^2 = 0.95–0.97).

2.5. Statistical Analysis

The statistical analysis of the data was performed using SPSS statistics software package version 23.0 for Windows (SPSS, Chicago, IL, USA). Data were processed using the one-way analysis of variance (ANOVA). Differences between means were compared using the Tukey post hoc test at 95% confidence interval (α = 0.05).

3. Results and Discussion

It is well known that tropospheric ozone triggers a series of physiological and metabolic changes in plants [26]. The effect of ozone used as a postharvest treatment on a wide range of fruits and vegetables, including table and wine grapes, has also been extensively studied [13]. As a phytosanitary treatment, however, the direct application of aqueous ozone to the plant, and specifically to the grapevine, is a recent practice whose effect on the quality of the fruit has just begun being assessed [7]. In this study, the effect on grape quality of the ozonated water applied to Bobal grapevines through endotherapy (E) or its combination with foliar spraying (E + S) was evaluated.

3.1. Effect on Enological Parameters

The enological parameters of control (C) and treated (E and E + S) grapes are shown in Figure 2. The weight of the berries and the °Bé showed no significant differences between control and treated grapes, but both treatments lowered their pH, especially the E treatment, which also increased the TA. As a consequence, the °Bé/TA ratio of E grapes at harvest was slightly lower than the ratio of the control, suggesting a delay in the ripening process as a result of this treatment. Similarly, a slight delay in the technological maturity was observed when spraying ozonated water in a vineyard of the Vermentino cultivar [8]. However, the combination treatment carried out in this study did not significantly modify the °Bé/TA ratio with respect to the untreated samples.

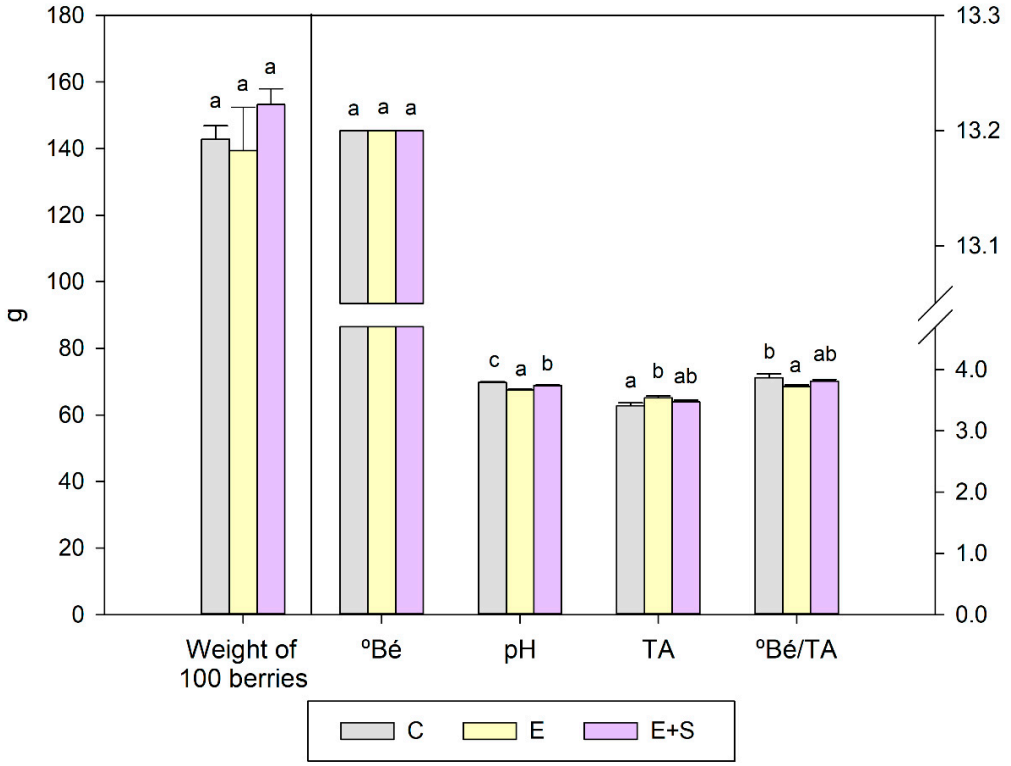

Figure 2. Enological parameters determined in Bobal grapes on harvest day. C: control grapevines (untreated); E: grapevines treated by endotherapy; E + S: grapevines treated by endotherapy and spraying. Left *y*-axis: weight of 100 berries (g). Right *y*-axis: °Bé (Baumé degrees); pH; TA (titratable acidity expressed as g/L of tartaric acid); °Bé/TA. All data are expressed as mean ± SD ($n = 4$). For each parameter, different letters indicate significant differences according to the Tukey test ($p < 0.05$).

3.2. Effect on Chromatic Parameters

The chromatic parameters—i.e., the color intensity (CI), tonality (T) and the absorbances from which they are calculated—are shown in Figure 3. These parameters can be used to predict the color quality of wines, which is more appreciated when CI is higher and T is lower. This is the case of the grapes subjected to the E treatment, which, compared to the control showed 16%, 32% and 52% higher absorbances at 420, 520 and 620 nm, respectively. The E + S treatment, while increasing the absorbance at 620 nm compared to the control, decreased the absorbances at 420 and 520 nm by 30% and 47%, respectively, resulting in grapes with lower CI and higher T. Therefore, the ozonated water applied exclusively by endotherapy had a positive effect on the chromatic characteristics of grapes, which showed a greater presence of yellow, red and blue tones, whereas the treatment that involved endotherapy and spraying was detrimental, presenting these grapes with more blue but less yellow and red shades than the control. This suggests that the impact of aqueous ozone on the color quality of grapes depended on factors, such as the dose, the application frequency or even the application method itself, as the E + S grapevines were subjected to more frequent applications by means of additional sprays, which resulted in a higher dose received. Previous studies have shown that acute (400 ppb, 6 h) and chronic (90 ppb, 8 h/day, 28 days) ozone exposure do not induce identical mechanisms of ozone damage within soybean leaves [48]. A different behavior was also observed when different intensities of ozonated water treatments were applied by spraying to Bobal grapevines, although in this case, other variables, such as the season and its climatic conditions, the characteristics of the plot or the training system, could play a role in the different response [7].

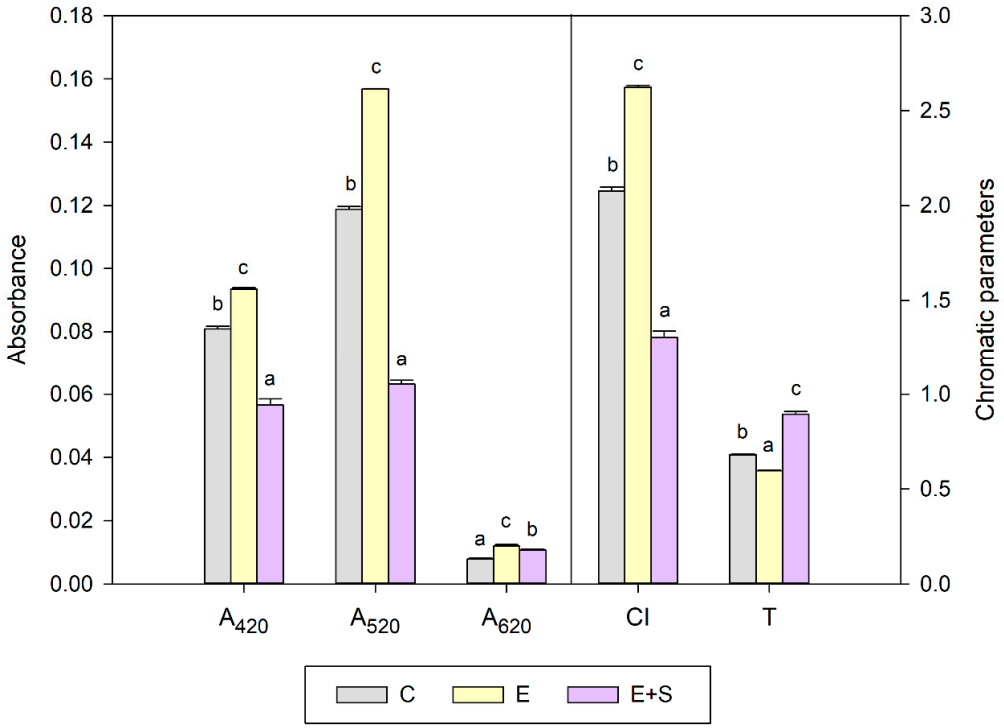

Figure 3. Chromatic parameters determined in Bobal grapes on harvest day. C: control grapevines (untreated); E: grapevines treated by endotherapy; E + S: grapevines treated by endotherapy and spraying. Left *y*-axis: A_{420} (absorbance at 420 nm); A_{520} (absorbance at 520 nm); A_{620} (absorbance at 620 nm). Right *y*-axis: CI (colour intensity); T (tonality). All data are expressed as mean ± SD (n = 4). For each parameter, different letters indicate significant differences according to the Tukey test ($p < 0.05$).

3.3. *Effect on Phenolic Maturity*

Plants, and also postharvest fruits, respond to ozone synthesizing antioxidant substances, such as phenolic compounds [25–27,49,50], although published results, which are discussed below, are inconsistent. The parameters associated with the phenolic maturity of grapes are included in Figure 4a. In comparison with the control, the TPI was increased with the E treatment and decreased with the combined one, which is in line with the higher CI found in E grapes and the lower CI of E + S grapes (Figure 3). Anthocyanins are the phenolic compounds responsible for the color of red grapes. Their extraction from the berry skin is maximized at pH 1.0, while during winemaking the pH tends to be closer to 3.4. As shown in Figure 4a, both E and E + S treatments significantly increased the total anthocyanin content ($A_{pH1.0}$) of grapes, especially the E treatment. Nevertheless, the easily extractable anthocyanins ($A_{pH3.4}$) were only increased in the E-treated grapes, their content being approximately 60% higher than in the control samples. The stress caused by ozone has been demonstrated to stimulate the production of phenolic compounds in plants through the activation of the phenylalanine ammonia-lyase (PAL), the first enzyme in the general phenylpropanoid pathway and, consequently, key in the biosynthesis of phenolic compounds [27,51]. Accordingly, increased phenolic content has been found in postharvest fruits following ozone exposure [14,49,50]. However, long-term ozone treatments can have the opposite effect, decreasing the concentration of phenolic substances and anthocyanins in postharvest grapes through a supposed oxidant activity [14]. This decrease, in addition to the direct oxidation by ozone or its intermediary products, may be due to the increased activities of polyphenol oxidase and peroxidase, which has been described in soybean leaves after exposure to this gas [51]. We hypothesize that PAL induction may prevail over these other enzymes at low ozone doses, whereas with higher doses the oxidation of phenolic compounds may be greater than their synthesis.

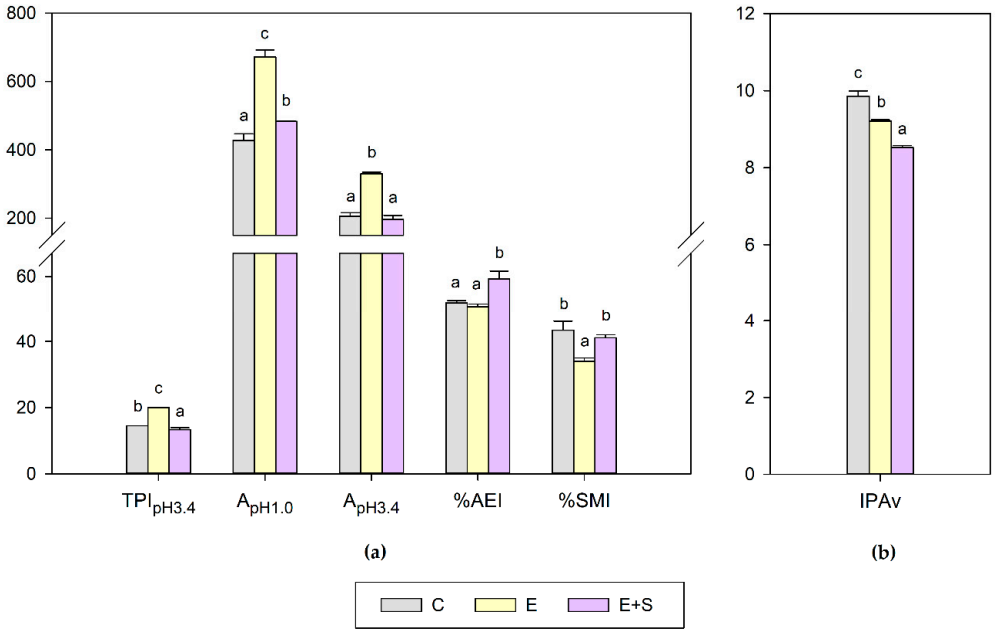

Figure 4. Phenolic (**a**) and aromatic (**b**) maturity determined in Bobal grapes on harvest day. C: control grapevines (untreated); E: grapevines treated by endotherapy; E + S: grapevines treated by endotherapy and spraying. (**a**): TPI (total phenol index); A (total anthocyanins, mg/L); %AEI (anthocyanin extractability index); %SMI (seed maturity index). (**b**): IPAv (varietal aroma potential index). All data are expressed as mean ± SD (n = 4). For each parameter, different letters indicate significant differences according to the Tukey test ($p < 0.05$).

Grapes with high anthocyanin content do not necessarily produce wines rich in these compounds, but their extractability must be also considered. The impact of the treatments on the extractability of anthocyanins is presented in Figure 4a. Lower values of the anthocyanin extractability index (%AEI) correspond to smaller differences between $A_{pH1.0}$ and $A_{pH3.4}$ and therefore greater ease of extraction. The E treatment did not modify the %AEI of grapes, which means that the higher content of anthocyanins found in these grapes is due to an increased biosynthesis and not to a higher extractability. In contrast, the more intensive treatment increased this index in grapes and therefore the difficulty to extract their anthocyanins. Ozone applied at postharvest is known to affect the cell wall composition and mechanical properties of the berry skin, which are closely related to the release of anthocyanins and other phenolic compounds. In table and wine grapes, the continuous exposure to ozone gas (30 µL/L, 24 h) led to an increase in the skin hardness and consequently slow extraction kinetics of phenolic compounds [52]. Indeed, the genes involved in cell wall stiffening were up regulated in *Arabidopsis* plants exposed to ozone (150 ppb, 8 h/day, 2 days) [53]. Similarly, short-term gaseous ozone treatment (10 µL/L, 10 min) decreased pectin solubilization and the activity of pectin methylesterase (PME) in tomatoes, which resulted in the reduced disassembly of pectic polysaccharides and a delay in fruit softening [50]. However, the effect of ozone on the extractability of phenolic compounds strongly depends on the grape variety and the exposure time [14,54,55]. For example, higher activities of PME and polygalacturonase (PG) were detected in Pignola wine grapes after ozone gas shock treatment (1.5 g/h, 18 h), which could explain the higher polyphenol content due to a higher extractability [14]. Regarding our results, it is logical that the endotherapy treatment did not modify anthocyanin extractability since, in this case, the ozonated water did not come into direct contact with the surface of the berries. Nevertheless, when combining this treatment with spraying over the aerial part of the plant, anthocyanin extractability was negatively affected as observed in a previous experiment consisting of three preharvest spraying applications of ozonated water [7]. This reduced extractability

is probably related to lower activities of the pectin-degrading enzymes and consequent skin hardening, as reported in previously commented literature.

The seed maturity index (%SMI), which represents the contribution of seed tannins in grapes, was decreased by the E treatment but unaffected by E + S (Figure 4a). This suggests that the grapes treated by endotherapy had a higher proportion of skin tannins, which are alleged to be less astringent because of their lower degree of galloylation [56]. This reduction in %SMI coincides with that observed in Bobal grapes that had received a single spraying application of ozonated water during the ripening period, while, as in E + S grapes, a higher exposure did not alter this index [7]. In Petit Verdot grapes, the postharvest ozone fumigation during 12 h did not modify seed tannin but significantly increased skin tannin [15], which could be the reason for the lower %SMI found in E grapes. Similar to anthocyanins, an increased content of tannins in the skins of E grapes could have its origin in the antioxidant defense response of the berries to the stress caused by ozone, with the subsequent induction of the synthesis of phenolic compounds [26].

3.4. Effect on Phenolic Compounds

The detailed phenolic compositions of control and treated grapes are shown in Table 1. The low molecular weight phenolic compounds identified have been grouped in phenolic acids, flavonols and anthocyanins. They were found in concentrations within normal ranges for Bobal musts [57]. Anthocyanins were the most abundant phenolic compounds in all the samples, followed by phenolic acids and flavonols. As shown in Table 1, the E treatment increased the overall content of flavonols and anthocyanins in grapes but did not affect the content of phenolic acids. On the contrary, the E + S treatment decreased the total content of phenolic acids, flavonols and anthocyanins. This is in accordance with the higher TPI, CI and $A_{pH3.4}$ found in E grapes and the lower TPI, CI and anthocyanin extractability found in E + S grapes (Figures 3 and 4a). According to the phenolic maturity results (Figure 4a), the lower content of anthocyanins, and possibly other phenolic compounds, found in E + S grapes is more likely to be due to a worse extractability than to a reduced biosynthesis or oxidation, as proposed in previously commented literature. However, the 4-h maceration of these grapes at pH 3.4 resulted in the extraction of an anthocyanin content similar to the control samples (Figure 4a). This disparity was probably due to the different extraction conditions used for the analysis of the individual phenolic compounds, since the maceration time with the skins was shorter and the pH of the grapes was slightly higher than 3.4 (Figure 2), hindering the extraction of anthocyanins, especially in grapes with higher %AEI.

With regard to phenolic acids, ozonated water modified the content of the hydroxybenzoic acids detected in grapes, decreasing, in both treatments, the amount of gallic acid compared to the control. The content of syringic acid, on the other hand, was favored with endotherapy but reduced with the combined treatment. Ozonated water had a lesser effect on the hydroxycinnamoyltartaric acids, since only the E + S treatment slightly reduced the *trans*-caftaric acid content, while the *trans-p*-coutaric acid was not modified. In table grapes, the content of hydroxycinnamic acid derivatives was preserved after the retail period by the continuous application of ozone (0.1 µL/L), while they were significantly decreased when shocks of 8 µL/L were applied [49]. Additionally, at postharvest, a high decrease in hydroxycinnamic acids was provoked in Grechetto wine grapes by a short-term ozone treatment (1.5 g/h, 12 h) [19], while the same gas flow over 16 h substantially increased these phenolic acids in Sauvignon blanc grapes [14]. The same contradictory results have been observed in terms of phenolic acids in Bobal grapes subjected to preharvest treatments with ozonated water [7].

Table 1. Phenolic compounds determined in Bobal grapes on harvest day.

Treatments	C	E	E + S
Phenolic Acids (mg/L)			
Gallic acid	0.55 ± 0.04 b	0.42 ± 0.01 a	0.38 ± 0.01 a
Syringic acid	1.51 ± 0.01 b	1.58 ± 0.02 c	1.45 ± 0.02 a
trans-Caftaric acid	0.18 ± 0.00 b	0.18 ± 0.00 b	0.16 ± 0.00 a
trans-p-Coutaric acid	0.19 ± 0.00 a	0.19 ± 0.00 a	0.19 ± 0.00 a
Σ Phenolic acids	2.42 ± 0.03 b	2.38 ± 0.01 b	2.17 ± 0.01 a
Flavonols (mg/L)			
Myricetin 3-O-glucuronide + glucoside [1]	0.79 ± 0.03 a	1.07 ± 0.05 b	0.71 ± 0.01 a
Quercetin 3-O-glucuronide + glucoside [1]	0.41 ± 0.01 a	0.37 ± 0.01 a	0.28 ± 0.06 a
Laricitrin 3-O-glucoside/galactoside [2]	nq [3]	0.08 ± 0.00	nq [3]
Syringetin 3-O-glucoside	0.15 ± 0.01 a	0.17 ± 0.02 a	0.13 ± 0.01 a
Σ Flavonols	1.35 ± 0.02 b	1.69 ± 0.04 c	1.12 ± 0.08 a
Anthocyanins (mg/L)			
Delphinidin 3-O-glucoside	1.99 ± 0.10 b	2.72 ± 0.13 c	1.48 ± 0.05 a
Cyanidin 3-O-glucoside	1.68 ± 0.03 c	1.38 ± 0.03 b	0.93 ± 0.01 a
Petunidin 3-O-glucoside	2.77 ± 0.10 b	3.77 ± 0.08 c	2.19 ± 0.08 a
Peonidin 3-O-glucoside	20.06 ± 0.58 c	18.52 ± 0.37 b	14.70 ± 0.16 a
Malvidin 3-O-glucoside	34.00 ± 1.24 a	47.21 ± 0.99 b	30.79 ± 0.12 a
Peonidin 3-O-(6′-acetyl)-glucoside	0.84 ± 0.03 b	0.88 ± 0.01 b	0.74 ± 0.02 a
Malvidin 3-O-(6′-acetyl)-glucoside	1.98 ± 0.07 a	2.71 ± 0.04 b	1.96 ± 0.03 a
Σ Anthocyanins	63.32 ± 2.13 b	77.18 ± 1.67 c	52.78 ± 0.35 a

C: control grapevines (untreated); E: grapevines treated by endotherapy; E + S: grapevines treated by endotherapy and spraying. All data are expressed as mean ± SD (n = 4). For each compound, different letters indicate significant differences according to the Tukey test ($p < 0.05$). [1] Coeluted compounds; [2] Can be glucoside or galactoside of laricitrin; [3] Not quantifiable.

As for flavonols, although significant differences were found in their total content between treated and control grapes, the increase in myricetin 3-O-glucuronide + glucoside caused by the E treatment was the only individually noticeable difference (Table 1). As in E grapes, an increase in the total flavonol content was observed in white wine grapes exposed for 12 h to ozone gas during postharvest [19], as well as in Bobal grapes that had received a single application of ozonated water during the ripening period [7]. In contrast, in the latter publication, a reduction in the content of these flavonoids was observed when ozonated water was applied three times, which agrees with the decreased total flavonol content found in the grapes subjected to the combined treatment.

In terms of anthocyanins, the aqueous ozone applied by endotherapy produced an increase in the content of the trisubstituted anthocyanins—i.e., delphinidin 3-O-glucoside, petunidin 3-O-glucoside and malvidin 3-O-glucoside—with respect to the untreated samples. However, the first two presented a lower content in E + S grapes. The amount of the disubstituted anthocyanins—i.e., cyanidin 3-O-glucoside and peonidin 3-O-glucoside—was reduced by both treatments, but to a greater extent in E + S grapes. The content of peonidin 3-O-(6′-acetyl)-glucoside was also decreased by the combined treatment, while the acetylated derivative of malvidin 3-O-glucoside was favored in the grapes treated exclusively by endotherapy. The postharvest ozone fumigation overnight (12 h) of Petit Verdot grapes increased anthocyanin content during maceration/fermentation [15]. However, Botondi et al. found that a shock ozone treatment (1.5 g/h, 18 h) in the postharvest dehydration of Pignola grapes did not alter the phenolic and anthocyanin content, while a long-term treatment significantly

reduced their concentrations through a supposed oxidant activity [14]. In fact, the degradation of anthocyanins in ozonated grape juice has been attributed to the strong oxidizing potential of ozone and intermediate radicals [58].

The results of the detailed phenolic composition indicate that the elicitor effect of ozonated water on phenolic compounds was dependent on the compound considered and the treatment type—i.e., dose, frequency and/or method of application, but in general the E treatment favored the accumulation of phenolics in Bobal grapes, while the E + S treatment caused a decline in their content, either due to a lower biosynthesis, their oxidation or, as has been demonstrated for anthocyanins in this work, a lower extractability.

3.5. Effect on the Varietal Aroma Potential Index (IPAv)

In order to determine whether the ozonated water treatments affected the glycosylated aroma precursors in grapes, the varietal aroma potential index (IPAv) was analyzed. This index is a global measure of glycosylated aroma precursors, such as alcohols, terpenes, phenols and C_{13}-norisoprenoids [46]. In grapes, most of the aroma compounds are found in the glycosylated or "bound" form. As shown in Figure 4b, both treatments reduced the IPAv, especially E + S, and therefore treated grapes would have less glycosylated aroma precursors to be released during winemaking. This reduction was already observed in Bobal grapes that had received three spraying applications of ozonated water prior to harvest [7]. On the contrary, a different pattern was found when ozone gas was applied during the dehydration of Sauvignon blanc and Moscato Bianco grapes, in which the volatile compounds in the glycosylated form were favored [16,17]. Additionally, in Moscato Bianco grapes, the content of bound compounds remained quite stable after short-term ozone exposure in both fresh and withered grapes [20]. However, in another study carried out on several aromatic and non-aromatic varieties, the impact of ozone applied during grape drying was variety-dependent, decreasing, in some cases, the content of bound volatile compounds [18]. It is important to note that these studies are performed with ozone gas in postharvest grapes, so it is reasonable that ozone applied in aqueous solution directly to the plant exerted a different effect on the glycosylated aroma composition. Ozone dissolved in water does not interact with plants or postharvest fruits in the same way as ozone in gaseous phase [22,59], and the metabolism of the grapes still attached to the plant is different from that of the grapes after harvest [60]. Furthermore, ozone in aqueous solution has been shown to lead to the extensive cleavage of glycosidic linkages in carbohydrates, either by direct ozone attack, hydroxyl radicals or acid hydrolysis [61], so it is likely to be able to break the glycosidic bond between the volatile fraction and the sugar moiety in aroma precursors. In addition, the lower pH found in treated grapes may favor the release of aroma precursors by acid hydrolysis [46].

3.6. Effect on Volatile Compounds

The impact on volatile compounds is another recognized effect of ozone. In plants exposed to this abiotic stressor, either a reduction [30,62] or a stimulation [28–30] in isoprene, terpenes and C_6 compounds was observed. The same applies to fruits detached from the plant—i.e., in postharvest, in which the ozone exposure led to an increased synthesis of volatile compounds [17], a significant decline [13,16,20], or no substantial impact on aroma [18]. The reason for the high variability found in the literature about the impact of ozone on the physiology and metabolism of plants or postharvest fruits has been attributed to the sensitivity of the species, the variety, the ripening stage, the environmental conditions, the application type, the dose and the exposure time [7,26,54,55,63]. Table 2 shows the free volatile compounds determined in the control and treated grapes, which have been classified into C_6 compounds, terpenoids (terpenes and C_{13}-norisoprenoids), volatile phenols and others. The treatments with ozonated water induced the accumulation of some individual volatiles, although no significant differences were found in the total amount of each group of compounds between treated and control grapes. The increase in certain free volatiles may be related to a greater synthesis and/or a greater

release of glycosylated aroma precursors, which is consistent with the lower IPAv found in the treated grapes (Figure 4b).

Table 2. Volatile compounds determined in Bobal grapes on harvest day.

Treatments	C	E	E + S
C_6 Compounds (μg/L)			
1-Hexanol	315.87 ± 9.93 ab	358.00 ± 15.16 b	295.23 ± 13.88 a
cis-3-Hexen-1-ol	14.32 ± 0.79 a	14.27 ± 0.41 a	13.61 ± 0.38 a
trans-2-Hexen-1-ol	58.91 ± 1.20 a	67.63 ± 2.38 a	55.96 ± 5.23 a
trans-2-Hexenal	14.45 ± 1.01 a	19.29 ± 0.02 ab	25.61 ± 3.04 b
Σ C6 compounds	403.55 ± 10.53 a	459.18 ± 17.98 a	390.40 ± 21.78 a
Terpenoids (μg/L)			
β-Damascenone	0.06 ± 0.01 a	0.16 ± 0.04 b	0.06 ± 0.01 a
Geraniol	2.95 ± 0.06 a	3.31 ± 0.29 a	2.99 ± 0.17 a
Geranyl acetone	0.29 ± 0.04 a	0.25 ± 0.03 a	0.31 ± 0.07 a
Linalool	0.40 ± 0.01 a	0.35 ± 0.04 a	0.61 ± 0.05 b
Σ Terpenoids	3.69 ± 0.11 a	4.08 ± 0.34 a	3.96 ± 0.30 a
Volatile phenols (μg/L)			
Benzaldehyde	8.03 ± 1.11 a	6.82 ± 0.70 a	22.14 ± 3.11 b
Guaiacol	7.94 ± 0.10 a	7.74 ± 0.33 a	12.97 ± 1.10 b
Syringol	80.04 ± 7.00 a	115.22 ± 3.56 a	116.27 ± 27.55 a
Σ Volatile phenols	96.02 ± 5.80 a	129.78 ± 4.59 a	151.38 ± 29.57 a
Others (μg/L)			
Nonanal	1.47 ± 0.11 a	1.58 ± 0.08 a	2.82 ± 0.29 b
1-octen-3-ol	1.22 ± 0.05 a	1.31 ± 0.11 a	1.15 ± 0.08 a

C: control grapevines (untreated); E: grapevines treated by endotherapy; E + S: grapevines treated by endotherapy and spraying. All data are expressed as mean ± SD (n = 4). For each compound, different letters indicate significant differences according to the Tukey test ($p < 0.05$).

C_6 aldehydes and alcohols, characterized by their herbaceous aromas, were the most abundant free volatiles in all the samples (Table 2). These compounds are derived from the oxidative cleavage of polyunsaturated fatty acids. One of the effects attributed to ozone is the denaturation of the lipids in cellular membranes [64]. The accumulation of ROS also acts as a signal for the formation of free fatty acids, eventually originating in C_6 volatile compounds [28]. Therefore, the volatiles associated with lipid peroxidation are expected to be enhanced in plants exposed to this pollutant. Among C_6 compounds, the concentrations of 1-hexanol, *cis*-3-hexen-1-ol and *trans*-2-hexen-1-ol were not modified by either of the two treatments, whereas E + S grapes showed higher *trans*-2-hexenal content than the control samples. Accordingly, increased emissions of C_6 compounds, proportional to the ozone uptake, were observed in ozone-stressed leaves of tobacco plants [28]. In postharvest white wine grapes, Río Segade et al. found that ozone exposure during dehydration induced the biosynthesis of aldehydes, and, in particular, *trans*-2-hexenal, through the up-regulation of some genes involved in the lipoxygenase–hydroperoxide lyase (LOX–HPL) pathway [17]. However, significant differences between the control and treated grapes were only found at the greatest water loss—i.e., after a longer treatment [17]. In previous experiments, the content of *trans*-2-hexenal was decreased in Bobal grapes by a single spraying of ozonated water six weeks before harvest, while, as observed in E grapes, it remained similar to the control after three applications [7]. In general terms, although some changes were detected in individual C_6 compounds after the ozonated water treatments on Bobal grapevines,

their total concentration was not significantly favored. The reason for this could be the increase in certain terpenoids, which have been found to stabilize lipid membranes and reduce their denaturation caused by stresses [65].

Concerning terpenoids, both E and E + S increased the concentration of certain of these volatiles (Table 2). This particular group of compounds, associated with floral and fruity aroma, seems to play an important role in plant protection against abiotic stresses, including ozone-induced oxidative stress [66]. In particular, isoprene and monoterpenes have been demonstrated to reduce ozone damage and to quench ozone and ROS [67,68]. In response to ozone, it was hypothesized that the biosynthesis of terpenoids is enhanced when the dose surpasses a threshold that marks cellular damage, but photosynthesis is not yet heavily suppressed [68], a situation in which the two treatments under study must be found. In comparison with control grapes, the ozonated water applied through endotherapy increased the content of β-damascenone almost three-fold, while linalool was favored when this treatment was combined with spraying applications. Neither geraniol nor geranyl acetone was affected by the aqueous ozone. In agreement with these results, increased emissions of monoterpenes were detected in *Quercus ilex* (L.) leaves exposed to ozone [29]. Similarly, ozonated water spraying treatments carried out in Bobal and Vermentino vineyards increased the content of terpenoids at harvest [7,8]. During postharvest dehydration, linalool and other terpenes were also increased in grapes subjected to long-term ozone treatment [17]. In fact, these authors found that the methylerythritol phosphate (MEP) pathway, through which the biosynthesis of monoterpenes occurs in grapevine berries, was induced in the ozone-treated grapes.

Regarding volatile phenols, the E treatment did not modify their content, but E + S significantly increased the concentrations of benzaldehyde and guaiacol, which possess, respectively, almond-like and smoky odors. These benzenoids are derived from the phenylpropanoid metabolism, which is stimulated by ozone, as has been noted before. Moreover, guaiacol occurs in grapes mainly in the glycosylated form, so the lower IPAv found in the E + S grapes (Figure 4b) could explain the higher content of this volatile in the free form. Higher amounts of benzaldehyde were also found in postharvest grapes exposed to ozone for 16 to 48 h [16,20]. However, no significant differences in the content of volatile phenols were found in Bobal grapes when ozonated water was sprayed before harvest [7], probably due to an insufficient dose. As for nonanal and 1-octen-3-ol, the more intensive treatment favored the accumulation of the first in grapes, while no significant differences were found in the content of the latter between the grapes from the treated and control plants. Nonanal is an oxidation product of oleic acid, which has been detected in grape skin, pulp and seeds [69]. Therefore, the increased formation of this aldehyde could be related to the lipid peroxidation caused by ozone and derived products, as happens with C_6 compounds. Besides, the induced emission of saturated aldehydes, such as nonanal, has been reported when plants are exposed to ozone or attacked by pathogens or insects [70].

4. Conclusions

In the present study, two novel endotherapy-based application strategies of ozonated water to grapevines (E and E + S) have proven their feasibility for possible use in disease control, especially when wood-inhabiting pathogens are implicated. The exposure to this strong oxidant, even when injected into the trunk, triggers a physiological and metabolic response in the plant that results in changes in fruit quality. However, the effect is different, and in many cases opposite, depending on the treatment type. Both E and E + S treatments slightly decreased the pH, especially the former, which, in addition, increased the TA in comparison with the control grapes. The E treatment also improved the chromatic characteristics, favored the accumulation of phenolic compounds and anthocyanins, whose extractability was unaltered, and decreased the contribution of seed tannins compared to the untreated grapes. In contrast, the E + S treatment had a detrimental effect on the color, led to a reduction in phenolic compounds and, although their synthesis was enhanced (higher $A_{pH1.0}$), the extractability of anthocyanins was negatively affected. In terms of aroma, both treatments, and E + S to a greater

extent, reduced the content of glycosylated aroma precursors, indicated by a lower IPAv, but increased certain free volatile compounds, such as terpenoids. Therefore, the ozonated water used in the vineyard can not only help to control grapevine diseases, but also induces changes in grape quality and, consequently, could affect the wine attributes. In the particular case of Bobal grapevines, the ozonated water applied through endotherapy at four different phenological stages, ranging from before flowering to the ripening period, could improve the overall quality of grapes, but an intensification through additional spraying applications could overwhelm the antioxidant system of the plant and lead to the opposite effect, mainly in terms of color and phenolics. Future research to explore the influence of different ozonated water doses, application timing, years, and varieties would be appropriate to determine the optimal conditions for these types of treatments to be effective in disease management without altering, or even improving, the quality of grapes and wines.

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