

Research Article

Postharvest Ozone Fumigation of Grapes (cv Sangiovese) Differently Affects Volatile Organic Compounds and Polyphenol Profiles of Berries and Wine

Margherita Modesti ^{1,2}, Stefano Brizzolara,¹ Roberto Forniti,² Brunella Ceccantoni,² Andrea Bellincontro,² Cesare Catelli,³ Fabio Mencarelli,⁴ and Pietro Tonutti¹

¹Crop Science Research Center, Scuola Superiore Sant'Anna, Piazza Martiri della Libertà 33, Pisa, Italy

²DIBAF, University of Tuscia, Via DeLellis, Viterbo, Italy

³P.C. di Pompeo Catelli S.R.L., Via Roma 81, Uggiate Trevano, Como 22029, Italy

⁴Department of Agriculture Food and Environment, University of Pisa, Via del Borghetto 80, Pisa 56124, Italy

Correspondence should be addressed to Margherita Modesti; margherita.modesti@unitus.it

Received 8 October 2022; Revised 17 December 2022; Accepted 11 January 2023; Published 1 February 2023

Academic Editor: Keren Bindon

Copyright © 2023 Margherita Modesti et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Consumers are more and more oriented towards the purchase of safer food and beverages, which is pushing the wine sector to find alternatives to the use of sulfur dioxide. Ozone (O₃) is already applied in the wine industry to produce sulfur dioxide-free wines through the patented method Purovino®. The aim of this two-year study was that of evaluating whether the postharvest treatment of grapes with ozone affects volatile organic compounds (VOCs) and polyphenol profile in berries, and in turn, wine composition. Grape bunches (*Vitis vinifera* L.) of cv Sangiovese were fumigated overnight with gaseous ozone (max 20 g·h⁻¹ with 6% w·w⁻¹ of ozone) in a cold room at 4°C (±0.5). After treatment, grapes were processed into wine. In grapes, ozone treatments increased total polyphenol and flavonoid content and upregulated specific genes (*phenylalanine ammonia lyase*, *VvPAL*, *flavanol synthase 1*, and *VvFLS1*) involved in polyphenol biosynthesis. Wine obtained from ozone-treated grapes had higher flavanol content than the control. Fumigation only slightly affected the different VOC classes of grapes and wine, including aroma compounds derived from the lipoxygenase (LOX) pathway. Although a season-dependent effect was observed, results showed that postharvest ozone treatments applied to avoid the use of sulfur dioxide introduced limited but, in general, positive modifications to grape and wine composition. This information provides assurance to winemakers that the maintenance of wine quality and typicity will be guaranteed when using ozone treatments.

1. Introduction

In winemaking, sulfur dioxide (SO₂) is the most used preservative. It has antioxidant and antimicrobial properties, which stabilise wine over time. However, it is well known that SO₂ can reduce the aromatic bouquet of the wines [1, 2]. Aromatic composition is one of the most important quality traits of wine grapes, since it plays a key role in determining the sensory properties of the resulting wine. In addition to these quality-related problems, SO₂ is known to have some drawbacks for human health. Nowadays, the number of consumers asking for safer and healthier food and beverages

is growing, pushing the wine sector to reduce the use of SO₂. Different alternative methods and treatments, being considered as a complement to SO₂ in low-sulfite winemaking, have been proposed [3]. Recently, the use of ozone (O₃) has received special attention due to its strong antioxidant properties and instability in wine, in that it degrades spontaneously. As a result, it is effective in inactivating the microbial population, attacking cellular constituents of bacteria, fungi, and yeasts, and it also rapidly reconverts to molecular oxygen thereby leaving no byproduct in the wine. A patented method called Purovino® [4, 5] is an SO₂ replacement based on the use of ozone, that is being employed

in an increasing g number of wineries as a sanitising agent for both facilities and harvested grapes [1, 6, 7]. Purovino® is a treatment applied in three steps: first to presterilise the container used to hold grapes with O₃-enriched water, then to apply O₃-enriched water to the grapes directly at two sequential concentrations, at a controlled temperature. In addition to controlling microbial growth, due to its high oxidative potential, O₃ may induce a significant shift in fruit metabolism that may positively or negatively affect the composition of the treated product. In biological tissues, O₃ is rapidly converted to reactive oxygen species (ROS), which leads to a cyclic endogenous production of ROS and consequently, to an imbalance in the cellular redox status and oxidative stress [8]. Biological tissues respond to the induced oxidative stress by triggering the production of different antioxidant compounds such as isoprene, monoterpenes, C₆ volatiles, and polyphenols. For instance, when applied during the storage of different fruits, including table grapes, O₃ enables prolonged shelf life, inhibits the growth of grey mold [9, 10], and boosts the biosynthesis of antioxidant and aromatic compounds. An increase in ascorbic acid content was reported in kiwifruit stored for 3 months with 300 ppb of O₃ [11]. A higher amount of ascorbic acid was also found in strawberries treated with 300–350 ppb of O₃ [12] and in papaya (exposed to 1.5–5 ppm) [13], and in each case was associated with an observed increase in polyphenol content. The observed increase in polyphenols has been found to be associated with higher antioxidant activity during post-harvest storage under O₃-enriched atmosphere [11, 12]. Concerned aromatic composition, aroma was found to be reduced in tomatoes (exposed to 4 ppm of O₃ for 30 min every 3 h) [14] and strawberries (treated with 0.35 and 1.5 ppm for 3 days) [15]. On the other hand, in cantaloupe treated with 10,000 ppm of O₃ for 30 min [16], and table grapes continuously exposed to 100 ppb of O₃ for 60 days [17], changes in volatiles composition were not observed. Considering wine grapes, specific changes in the aromatic profile have been reported in response to O₃ treatments [6, 18]. The formation of sugar oxidation-derived compounds with sweet, breadly, caramelly, and buttery flavour (e.g., furaldehyde, hydroxy methyl furfural, and 3-hydroxy-2,3-dihydro maltol) have been described in O₃-treated grapes [6]. Moreover, different publications report an increase of LOX-derived compounds and terpenoids content in grapes and wine after O₃ treatment [6, 18–21]. Furthermore, short-term (between 12 and 48 h) exposure to O₃ has proven to be effective in promoting the content of flavanols, specifically of catechins [6] and total stilbenes, mainly *trans*-resveratrol and *trans*-piceatannol, in wine grapes [22]. The authors in [1] reported an increase of polyphenols and anthocyanins in red wines derived from O₃-treated grapes. Therefore, the postharvest application of O₃ as a sanitising agent must be carefully evaluated in terms of changes in berry metabolism and composition, as this has obvious consequences on composition and sensory properties on the resulting wine. To date, only a few studies are available reporting the effect of O₃ treatment on important wine traits, and among them are many contradictory results. This is mainly because O₃-induced changes appear to differ

in relation to seasonality and cultivar, thus making it difficult to clearly identify specific responses to O₃ treatment. Considering this, the current study addresses this knowledge gap by presenting the results of a two-year experiment to determine the compositional and metabolic changes induced in wine grapes (*Vitis vinifera* cv Sangiovese) following postharvest O₃ treatment.

2. Materials and Methods

2.1. Fruit Samples and Experimental Design. The experiments were carried out in two different seasons, 2018 and 2019. Bunches of red wine grapes (*Vitis vinifera* L. cv Sangiovese) were hand harvested (approximately 100 kg each year) in the vineyards of Rossi's farm located in Podere Poggio d'Elci, Scansano (GR), Tuscany, Italy (42°63'704.2" N latitude, 11°36'294.9" E longitude) on September 26th in 2018 and on October 1st in 2019. Grapevines were planted on hilly terrain. The planting layouts, training systems, and the pruning systems were those established by the disciplinary of production for the Appellation of Controlled and Guaranteed Origin (DOCG) "Morellino di Scansano" (i.e., planting density not less than 4000 vines per hectare with a maximum yield of 9 tonnes per hectare and a bilateral cordon with a vertical shoot-positioned trellis system). The harvest was performed by collecting alternating bunches (collecting the apical bunch from the first shoot, the basal bunch from the second shoot, etc.) from 9 grapevines (from 3 adjacent panels). Grapes were harvested at an average total soluble solid of about 22 (±1). Brix and sorted to ensure homogeneous colour of the berries and an absence of injuries. A 50 kg parcel of harvested grapes were treated with gaseous O₃ according to the Purovino® method as reported by the authors in [1]: ozone gas (max 20 g·h⁻¹ with 6% w.w⁻¹ of ozone) at the maximum flow rate of 150 NL·h⁻¹ (NL = normal liter) (Ozone generator A series, P.C. di Pompeo Catelli SRL, Uggiate-Trevano, Italy) in a 9 m³ cold room for 12 hours at 4 (±0.5)°C and 70% RH. As a control, 50 kg of grapes were not treated with O₃ but kept in a cold room with the same thermohydrimetric conditions and the same treatment duration. The treatments were carried out at the postharvest laboratory of Tuscia University. On completion of O₃ treatment, grapes were used for small-scale winemaking.

2.2. Grape Compositional Analysis. Berry samples (30 berries per replicate from different bunches, three replicates per treatment) were collected at harvest and at the end of the respective O₃ or control treatments for chemical analyses. The methods used were the same reported in [23]. Briefly, grapes samples were manually pressed and the obtained must was centrifugated (9302 rfc, 5 min, 18°C), filtered with syringe filters (0.22 μm pore size, 33 mm diameter, Sigma-Aldrich, Italy), and used for the following analyses: pH, using a pH meter (GLP21, Crison Instruments S.L.U., Spain); total soluble solids (TSSs), employing an optical refractometer and expressed in g/L of sugars; titratable acidity (TA), by titrating 7.5 mL of filtered must with 0.1 N

sodium hydroxide, expressed in g/L tartaric acid equivalent. Total polyphenol content (TPC) was measured using the Folin Ciocalteu method [24]. Polyphenol content have been determined by interpolating the obtained data with those obtained from the calibration curve (prepared with gallic acid solutions at concentrations 10–800 mg/L in methanol). Content was expressed as mg of gallic acid equivalents (GAE) $\times 100 \text{ g}^{-1}$ fresh weight. Total flavonoid content (TFC) was measured using the colorimetric method of [25] and expressed as mg of flavonoids $\times \text{kg}^{-1}$ fresh weight, determined against a catechin calibration curve.

Analysis of free and bound volatiles at the end of each treatments 15 berries were randomly sampled in 10 different tubes, representing 10 replicates. Grapes were analysed as reported in [23]. Grapes were homogenised with 1 M NaCl buffer solution (1 : 1 ratio in weight) by using an UltraTurrax (Mod. T25, IKA) and immediately frozen in liquid nitrogen and stored at -80°C for VOCs analysis. The prehomogenized samples were thawed at 15°C for 15 minutes and 10 g was transferred to a 20 mL glass crimp vial for headspace analysis (Cat. No. SU860049, Sigma-Aldrich, Italy) and sealed with silicone septa for SPME (Cat. No. 27362, Sigma-Aldrich, Italy). For wine analyses, 5 different bottles representing 5 replicates were used for the analyses. A 6 g sample of wine was mixed with 1 g of sodium chloride in a 20 mL glass crimp vial for headspace analysis and sealed. Grapes samples were incubated under agitation for 45 min at 40°C . Wine samples were incubated under agitation for 30 minutes at 40°C . VOCs were sampled at the same temperature after a further 45 and 30 min, respectively, for grapes and wine, using an SPME fibre (50/30 μm , DVB/CAR/PDMS, 1 cm long; Supelco, Bellefonte, PA, USA). VOCs were desorbed from the fibre for 5 min into the GC injector set at 250°C (splitless mode). The employed GC-MS settings were those reported in [26] with minor modifications. A Clarus 680 Gas Chromatograph equipped with a split/splitless injector (PerkinElmer®, Waltham, Massachusetts) was used for the analysis. Volatiles were separated on a fused silica capillary column (DBWax, 60 m, 0.32 mm ID, 0.25 μm film thickness; Restek, Bellefonte, PA), using helium as carrier gas with a flow rate of $1 \text{ mL}\cdot\text{min}^{-1}$. Compounds were identified using a mass spectrometer (Clarus 500 Mass spectrometer, PerkinElmer®, Waltham, Massachusetts) coupled to the GC. In 2019, glycosylated volatile compounds content was analysed in grapes. Glycosylated volatile compounds were extracted from 10 mL of homogenate from 15 berries using Sep-Pak C₁₈ cartridge (Waters Corporation, Milford, MA, USA) with 10 mL of methanol. The liquid was then evaporated to dryness using a rotavapor set at 37°C and dissolved in 5 mL of 0.2 M citrate-phosphate buffer at pH 5. The hydrolysis was carried out using 50 mg of glycosidase enzymes (Cytolase® M 102, Ferrari SRL, Verona, Italy) incubated for 20 hours at 40°C . The following day, the extract was mixed with an equal volume of deionized water and 2 g of sodium chloride, using 20 $\mu\text{g}/\text{L}$ of 1-heptanol was used as internal standard. A GC Agilent 7890 B and Agilent 7010, with split/splitless injector (PAL RSI 85) set at 230°C was used for glycoside quantification. The GC oven heating

program was $40^\circ\text{C} \times 1 \text{ min}$, $10^\circ\text{C}/\text{min}$ up to 60°C , and $4^\circ\text{C}/\text{min}$ up to 230°C . The source temperature was set at 230°C . Volatiles were separated on a JeW DB WAX polyethylene glycol column (30 m, 0.25 mm ID, 0.25 μm film). Helium was used as a carrier gas with a flow rate of $1 \text{ mL}\cdot\text{min}^{-1}$. Quantification of glycosides was carried out using a calibration curve specific to each of the different compounds, and expressed as μg per 1000 berries.

2.3. Gene Expression Analysis. 30 berries were randomly collected in triplicate from O₃-treated and control bunches. Seeds were discarded, and grapes were immediately frozen in liquid nitrogen and stored at -80°C . Frozen berries were ground to powder with liquid nitrogen and 100 mg of ground tissue were used for total RNA extraction, using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Italy), including DNA digestion with On-Column DNase I Digestion Set (Sigma-Aldrich, Italy). RNA concentration, purity, and integrity were determined with a Nanodrop 2000 spectrophotometer (Thermo Scientific, Italy), and on a 1% (weight/volume) agarose gel. Reverse transcription of the RNA templates to cDNA was carried out using 50 ng of RNA using ReadyScript™ cDNA Synthesis Mix (Sigma-Aldrich, Italy). The PCR conditions were set according to manufacturer's protocol. Gene-specific primers of *lip-oxygenase A* (VvLOXA), *hydroperoxides lyase* (VvHPL), *alcohol dehydrogenase 1* (VvADH1), *phenylalanine ammonia lyase* (VvPAL), *stilbene synthase* (VvSTS), and *flavanol synthase 1* (VvFLS1) were designed with an NCBI primer designing tool, based on the mRNA sequences of the target genes from the *Vitis vinifera* genome present in GenBank [23]. The primer couples were then inserted to the NCBI Basic Local Alignment Search Tool (BLAST) to verify specific amplification. Primers were synthesized by Sigma-Aldrich (Milano, Italy). Before sample analysis, the amplification efficiency of each couple of primer were determined using the [27] method and a range of acceptable efficiencies have been determined (90–110%) for further analysis. The forward and reverse sequences, GenBank Accession, as well as primer efficiencies are given in Table S1. For samples analysis, RT-qPCR was performed using the SYBR Green PCR Master Mix (Life Technologies™), with a final reaction volume of 10 μL , running on the CFX Connect Real-Time PCR System (BioRad®). The RT-qPCR cycle was set as follows: initial denaturation at 95°C for 2 min, followed by 40 cycles of amplification with denaturation at 95°C for 15 s, and annealing and elongation at specific temperature suited for each couple of primers (Table S1) for 1 min. Following 40 cycles, a melt cycle was performed at 95°C for 15 s, 60°C for 1 min, 95°C for 15 s, and 60°C for 15 s, to detect possible primer dimers or nonspecific amplification in cDNA samples [23]. PCR reactions were run in three biological and two technical replicates, and a negative control was performed in all qPCR runs for each couple of primers. Results were processed with the comparative Ct method [27] employing Actin 7 (VvACT7) as references gene. The relative quantification of each gene tested was calculated using the $2^{-\Delta\Delta\text{Ct}}$ method.

2.4. Winemaking. Approximately, 50 kg of bunches per treatment were destemmed and crushed. The must pH was then adjusted to 3.5 with the addition of tartaric acid, before addition of 100 mg/L of diammonium phosphate and yeast inoculation (20 g/hL) with rehydrated commercial *Saccharomyces cerevisiae* (Premium Supertuscan, Vason, Verona, Italy). The must obtained was divided into 3 25 Lt containers and fermented at $24 \pm 1^\circ\text{C}$. For the first 5 days, the mass was punched down twice a day. Between day 5 and until the end of sugar fermentation (0.5–0.6 g/L), 10% of the whole mass was punched down once a day. The fermentation lasted about 16 days in 2018 and 14 days in 2019. Once at dryness, the treated and control wines were racked from gross lees directly into barrels. For wine made from ozone-treated, the winemaking tanks and facilities (i.e., pumps and press) were previously washed with ozonated water and saturated with ozone gas until filled with wine, according to the Purovino method. In the control vinification, SO_2 (as 8% solution of potassium metabisulphite) was added at the beginning of fermentation (5 g/hL) and after fermentation (3 g/hL).

2.5. Wine Polyphenol Analysis. For polyphenol analysis, 10 mL of wine sample was filtered with syringe filters (0.22 μm pore size, 33 mm diameter, Sigma-Aldrich, Italy) and then diluted 1:20 in MilliQ-Water. A 1 mL aliquot of diluted wine has been transferred into a 2 mL vial (amber glass, crew cap) for analysis. Wine samples were subjected to a targeted quantitative analysis of selected known polyphenols by UHPLC-MS/MS using a Sciex 5500 QTrap + mass spectrometer (AB Sciex LLC, Framingham, MA, USA), equipped with a Turbo V ion-spray source and coupled to an ExionLC AC System custom made by Shimadzu (Shimadzu Corporation, Kyoto, Japan), which includes ExionLC Controller, ExionLC Degasser, ExionLC Tray, 2 ExionLC AC Pumps, and an ExionLC AC Autosampler.

Chromatographic separation was performed using a Phenomenex Kinetex Biphenyl 2×100 5 μm column (Phenomenex, Torrance, CA, USA). The elution was carried out in gradient mode using acetonitrile containing 0.1% formic acid (solvent A) and water containing 0.1% formic acid (solvent B). The gradient elution was programmed as follows: 0.0 min, A 5%; 0.0–10.0 min, A 5–95%; 10.0–12.0 min, A 95%; followed by 4 min equilibration time (A 5%). Other chromatographic conditions were as follows: flow rate 300 $\mu\text{L}\cdot\text{min}^{-1}$, injection volume 20 μL , and column oven temperature 40°C . MS/MS experiments were performed in Electrospray negative ion mode using nitrogen as collision gas, with the operation source parameters: source type, Turbospray; nebulizer gas (GS1) 70 (arbitrary units); turbo gas (GS2) 50 (arbitrary units); curtain gas (CUR) 10 (arbitrary units); temperature (TEM) 500°C ; ionspray voltage (IS) -4500 V; entrance potential (EP) 10 V. Compound parameters, declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) were adjusted for the specific selected reaction monitoring (SRM) transition for each component. SRM transitions and the corresponding compound parameters are reported in Table S2. A set of 40 polyphenols were investigated but only

the following 21 compounds were identified and quantified in the tested samples: 4-coumaric acid, transferulic acid, resveratrol, naringenin, phloretin, apigenin, luteolin, catechin, epicatechin, quercetin, chlorogenic acid, rosmarinic acid, piceid, phloridzin, kampferol-3-O-glucoside, quercetin-3-O-glucoside, quercetagenin 7-O-glucoside, cynarin, kampferol-3-O-rutinoside, rutin, and quercetin-3,4-O-diglucoside. For each molecule, a 10-point calibration curve (1–512 ppb) was prepared and used for quantification. The standard used were unique for each compounds (Sigma-Aldrich, Milan, Italy).

Data were normalised according to the matrix effect and percentage recovery. The matrix effect was calculated as the peak area of the sample after extraction/peak area of the standard, while recovery was calculated as the peak area of the sample before extraction/peak area of the sample after extraction. Each sample was replicated three times. Qualitative confirmation was obtained taking advantage of one of the features of the Qtrap instrument. Information-dependent acquisition (IDA) was programmed so that the SRM transitions reported in Table S2 were used as a survey scan, switching the third quadrupole to act as a linear ion trap (LIT), performing an enhanced product ions (EPI) scan, affording the complete MS-MS product ions spectrum (MRM >> enhanced product experiment). A comparison with a custom-built MS-MS product ions spectra library allowed for qualitative confirmation.

2.6. Data Analysis. All data were statistically analysed through the Shapiro–Wilk and Bartlett test to verify normality and homogeneity of variances. Once these prerequisites were established, data were compared by an unpaired *T* Test (with $p \leq 0.05$) or one-way ANOVA test (for grape compositional analyses only) and Tukey's HSD posthoc test (p value ≤ 0.05). The statistical tests were performed using GraphPad Prism 7.01, separately for each year. For the VOCs data, chromatograms were treated as described in [21]. Briefly, each chromatogram was run on AMDIS software (National Institute of Standards, Gaithersburg, MD, USA) and each peak was identified by comparing the spectra with those of the National Institute for Standards and Technology (NIST 98, Version 2.0, USA) data bank. Only compounds with 80% identity or more were selected. Selected peaks were then quantified using TurboMass software (TurboMass R, Version 5.4.2 PerkinElmer Inc., USA, 2008), by integration of peak areas. The area of each peak was normalised based on the sum of the areas to eliminate variations due to fibre decay [28]. VOCs data were compared using an unpaired *T* Test and Tukey's HSD posthoc test (p value ≤ 0.05), separately for each year. The data were then auto scaled by mean-centering and division by the standard deviation of each variable. A partial least squares discriminant analysis (PLS-DA) was performed using the measured VOC levels as predictor variables, while using the treatment as the response variable. Variable importance in projection (VIP) scores (>1) were used for variable selection to create the reported PLS-DA models and tables. Features, which were common in two or more years,

or that resulted in a statistically significant difference between control and treated grapes (p value ≤ 0.05) in one or more year, are presented in the table as fold change values by normalisation based on their level in control grapes and transformation in logarithmic scale as following: $\log_2(\text{FC}) = \log_2(\text{ozone/control})$. For wine VOC data, the data from both years were finally assembled in a single dataset and a further PLS-DA was performed using the measured VOC level in both years. For wine polyphenol data, data were auto scaled by mean-centering and division by the standard deviation of each variable. PLS-DAs were performed using the measured polyphenols as predictor variables while using the treatment as the response variable, separately for each year. The data from both years were assembled in a single dataset, and then a principal component analysis (PCA) was performed. All multivariate analyses were performed using MetaboAnalyst online tool [29].

3. Results

3.1. Grape Basic Composition and Polyphenol Profile. Various compositional parameters were analysed in grapes at harvest time and after the O_3 treatments. Briefly, in both years O_3 treatment maintained sugars at a level comparable with that of grapes at harvest time (time 0), while in the control grape sugar content tended to increase and acidity decreased slightly (Table S3). The observed differences in terms of sugar and acidity due to O_3 treatment were generally very small, and were therefore not expected to perceptibly impact winemaking outcomes.

Postharvest O_3 fumigation increased TPC in grapes in both years (Table 1). 2019 season were also analysed for total flavonoid concentration, specific, and total anthocyanin content (TAC). TFC slightly, but significantly, increased in O_3 -treated grapes (1902 vs 2219 g/kg of FW in control and ozonated grapes, respectively), while no difference in terms of TAC and specific anthocyanins (delphinidin, malvidin, peonidin, cyanidin, and petunidin) were detected between control and O_3 -treated grapes (data not shown).

In both years, the relative expression level of three genes involved in polyphenol biosynthesis, specifically *PAL*, *STS*, and *FLSI*, were analysed. In 2018, none of the selected genes were significantly affected by the O_3 treatment, even though *FLS* gene expression showed an increasing trend in O_3 -treated grapes (data not shown). In 2019, *STS* expression confirmed to be unaffected by the O_3 treatment, while *PAL* and *FLS* showed significantly higher expression in O_3 -treated grapes than the control samples (Figure 1). In O_3 -treated grape samples, *PAL* gene expression was approximately 40 times greater than that observed in the control treatment.

3.2. VOC Profile of Grape Berries. The changes in VOC profile induced by postharvest O_3 treatment were analysed in grapes collected during the two seasons. Only compounds with 80% identity or more were considered. In 2018, 32 compounds were identified in total (Table S4) and among them, 4 compounds (i.e., hexanal, 1-hexanol, 2-hexenol, and

TABLE 1: Total polyphenol content (TPC) expressed in mg of gallic acid equivalent/100 g of fresh weight in Sangiovese grapes after postharvest ozone treatment and in control grapes. Values are the means of three replicates \pm standard deviation.

Year		TPC (mg GAE/100 g fw)
2018	Time 0	365.5 \pm 24.9 ^b
	Control	332.0 \pm 30.4 ^b
	Ozone	504.8 \pm 25.2 ^a
2019	Time 0	565.5 \pm 36.54 ^b
	Control	601.3 \pm 21.6 ^b
	Ozone	760.2 \pm 24.9 ^a

Means followed by different letters are statistically different ($p \leq 0.05$) based on one-way ANOVA performed separately for each year.

nonanal) were identified as significantly different between control and O_3 -treated grapes. Only nonanal increased after O_3 exposure (Figure S1), while the other compounds decreased. In 2019, on a total of 18 volatile compounds identified (Table S4), only acetic acid was statistically different between the treatments, being reduced following the O_3 treatment (Figure S2). Besides the univariate statistical approach, PLS-DA models were created to further investigate differences induced by the treatments in the two years, separately for each season. In order to identify common response in grape VOCs from different vintages, compounds with a VIP score higher than 1, which resulted common in the two years, or compounds statistically significant in one year, were used to create Table 2. The data are presented as $\log_2(\text{FC})$ ozone/control. Two compounds, namely, nonanal and 2-hexenol, were differently affected after O_3 exposure in the two years of the study. Specifically, in 2018 a statistically significant increase of nonanal was observed in O_3 -treated grapes, while in 2019 the content decreased, albeit nonsignificantly, after O_3 exposure. On the other hand, 2-hexanol decreased or increased in response to O_3 treatment in 2018 and 2019, respectively. Moreover, and in accordance with the univariate statistical analysis, in 2018, a significant reduction of 1-hexanol and hexanal was observed, while in 2019 O_3 significantly reduced acetic acid content.

In 2019, the content of bound volatiles was also analysed. Of a total of 29 glycosides *s* identified (Table S4), only the two oxides of linalool (C and D forms) were significantly affected by the O_3 treatment (Figures 2(a) and 2(b)). Both oxides showed were higher in concentration in O_3 -treated grapes, which was not surprising considering that they are formed via the oxidation of linalool.

Most of the compounds affected by O_3 exposure derive from the degradation of polyunsaturated fatty acids (PUFAs), which occurs in the LOX-HPL pathway [30], or non-enzymatically through the direct interaction between O_3 and the fatty acid double bond [31]. With this in consideration, a focused gene expression analysis was performed analysing the expression levels of *LOX*, *HPL*, and *ADH*. In 2018, none of the selected genes were affected by the treatment showing an expression level very similar to that of control grapes (data not shown). On the other hand, in 2019, out of the three analysed genes, *HPL* expression was significantly higher (doubled) in

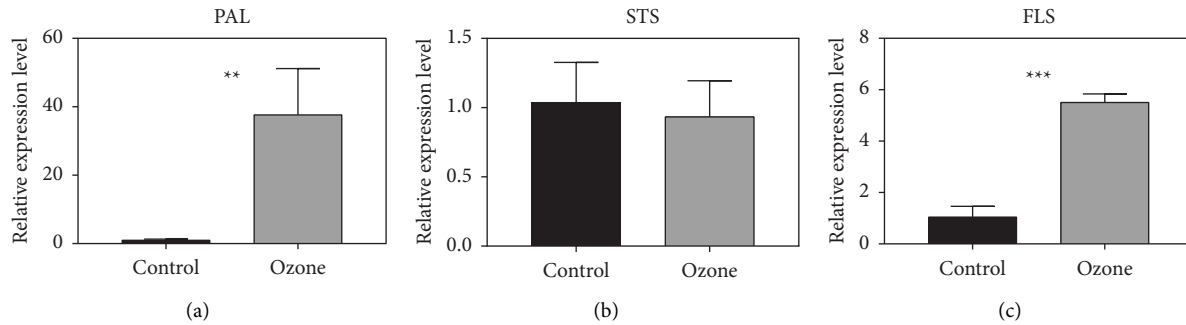


FIGURE 1: Relative expression level of (a) phenylalanine ammonia-lyase (PAL), (b) stilbene synthase (STS), and (c) flavanol synthase (FLS), analysed by RT-qPCR in control (black bars) and O₃-treated grapes (grey bars) from the 2019 season. The mean value of three biological replicates is reported with error bars representing standard deviation. Asterisks indicate significant differences between sample values (** $p = 0.0093$; *** $p = 0.0001$) based on an unpaired T test.

TABLE 2: Free volatiles identified as variables of importance in projection (VIP) (scores > 1) in the partial least squares discriminant analysis (PLS-DA) performed on the measured level of free VOCs (used as predictor variables) and treatments (as response variables). Only the compounds identified as VIPs for the two years, or compounds statistically significant in one of the two years, were selected. Data are presented as fold change values by normalisation to their level in control grapes and transformation in logarithmic scale as follows: \log_2 (FC) = \log_2 (ozone/control). A value followed by an asterisk means that in that year, the compound was significantly different between ozone-treated and control grapes ($p \leq 0.05$).

Log ₂ (FC)	2018	2019
1-Hexanol	-0.25*	—
Hexanal	-0.07*	—
Nonanal	0.04*	-0.006
Acetic acid	—	-1.14*
2-Hexenol	-0.27*	0.06

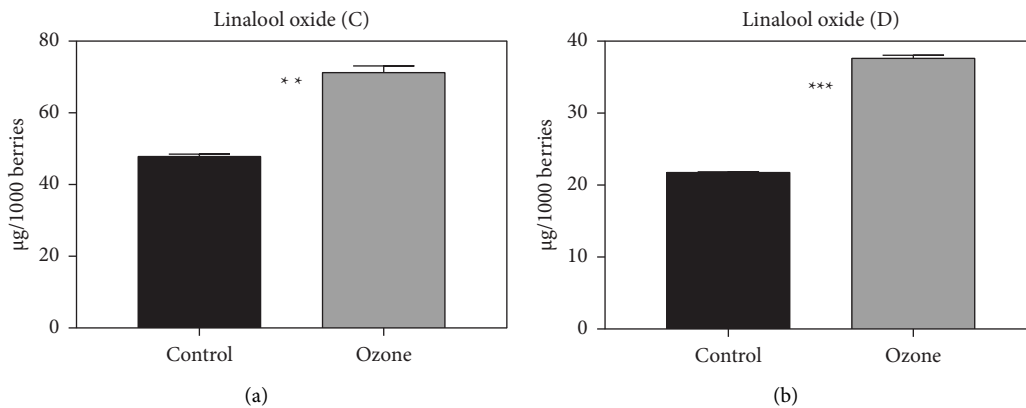


FIGURE 2: (a) Glycosides of linalool oxide C. (b) Glycosides of linalool oxide D. Data are expressed as μg per 1000 berries after postharvest ozone treatment (grey bars) and in control grapes (black bars) in the 2019 season. The mean value of three biological replicates is reported with error bars representing standard deviation. Asterisks indicate differences between sample values (** $p = 0.0033$; *** $p = 0.0003$) based on unpaired T test.

O₃-treated grapes (Figure 3(b)). The expression of *LOX* (Figure 3(a)) and *ADH* (Figure 3(c)) did not show any differences between control and O₃-exposed grapes.

The increase in the expression of *HPL* after O₃ treatment may indicate that an accumulation of C₆ aldehydes may have occurred. However, contrary to what might be expected, C₆ aldehydes were lower in the treated grapes (Table 2). Since

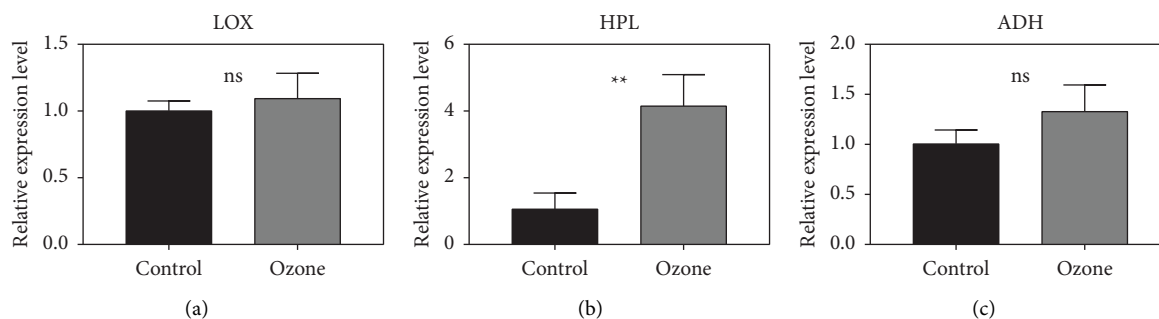


FIGURE 3: Relative expression levels of (a) lipoxygenase (LOX), (b) hydroperoxide lyase (HPL), and (c) alcohol dehydrogenase (ADH), analysed by RT-qPCR showing the results for control (black bars) and ozone-treated grapes (grey bars) in the 2019 trial. The mean value of three biological replicates is reported with the error bars representing the standard deviation. Asterisks indicate differences between sample values (** $p = 0.0071$) based on an unpaired T test.

this class of compounds was influenced by the O_3 treatment, they could be considered useful indicators of O_3 exposure in grapes.

3.3. The Profile of Wine VOCs. To have a more thorough understanding of the effect of O_3 application on grapes, wines made in the 2018 and 2019 seasons were analysed for their VOC profile. In 2018, a total of 60 compounds were identified in wines using the SPME-GC-MS approach. By means of univariate statistical analysis, 2 compounds were significantly different in wines prepared from control and O_3 -treated grapes. Specifically, pentanoic and propanoic acid increases or decreases after O_3 treatment, respectively (Figure S3). On the other hand, in 2019, 38 volatiles were identified using the same approach. Based on the T test results, none of the 38 compounds showed a significant difference between the control and O_3 -treated wines. In addition to the univariate statistical approach, PLS-DA models were created to further investigate differences and better visualise the results. To identified common response on volatile profile, VIP lists (scores > 1) of the PLS-DA was performed on the measured VOCs drawn from the VIP lists (scores > 1) for the two years of the study. Hexanoic acid, 1-butanol, and β -linalool were identified as VIPs in both years (data not shown). Only 1-butanol, which was observed to increase in O_3 -treated wines, showed the same trend in both years. Given the lack of duplicated response, the VOCs identified as significant for both vintages were integrated into a multiseason dataset and a further PLS-DA model was created. For the first two components, the model explained the 74.6% of the variability. Samples belonging to different clusters appear segregated but slightly overlapped (Figure 4(a)), thus suggesting that the volatile profiles of control and O_3 -treated wines were quite similar. Figure 4(b) shows the 15 compounds with VIPs scores ≥ 1 that contributed the most to the observed sample clustering effect. Out of 15 compounds, 4 belonged to the class of higher alcohols, for which 1-butanol and 3-methyl-1-propanol increased in the O_3 -treated wines relative to the control, while the other higher alcohols were lower in the treated samples. Among the other selected VIPs, 5 compounds belonged to the class of volatile fatty acids. Additionally, 2

compounds directly formed in the LOX-HPL pathway, 1-nonanol, and nonanoic acid, were present in the VIP list. In contrast with what observed in grapes, all these compounds, with the exception of 1-nonanol and propanoic acid, tended to increase in wine made from O_3 -treated grapes. Lastly, organic acids produced during fermentation by yeast metabolism (diethyl acetal, acetic acid, and butanedioic acid) were also listed among the VIPs. Specifically, after O_3 treatment, acetic acid decreased, which was in agreement with the observations for grapes in the 2019 season. On the other hand, diethyl acetal and butanedioic acid increased in O_3 -treated wines when compared to the controls. However, considering the overlap of the different wines in the PLS-DA model and the lack of significant differences in both years, O_3 treatment of Sangiovese grapes does not seem to remarkably affect the volatile profile of the resultant wine.

3.4. Wine Polyphenol Profile. Wines from the 2018 and 2019 trials were analysed using UHPLC-MS/MS, and a total of 21 polyphenols were identified. In 2018, by means of univariate statistical analysis, 6 compounds were found to be significantly affected by the O_3 treatment. Specifically, quercetin-7-O-glucoside, apigenin, rosmarinic acid, cyanin, luteolin, and kampferol-3-O-glucoside were all increased after O_3 treatment (Figure S4) relative to the control. On the other hand, as found for the VOC analysis, in 2019, none of the 21 polyphenols identified were significantly affected by the O_3 treatment (data not shown). PLS-DA models were then created to further investigate differences and to attempt to identify common responses in the polyphenol profile over the successive seasons (Figures S5 and S6 for 2018 and 2019, respectively). In 2018, the PLS-DA explained 94% of the variability within the multiseason dataset, and the samples from the respective treatments were clearly separated. In 2019, the PLS-DA model explained 91% of the variability in the polyphenol dataset. Separation of the samples by grape treatment was mainly explained by the first factor, showing a clear clustering of the samples, suggesting a strong effect of the O_3 treatment on the phenolic profile in both experimental years. The VIP lists (scores > 0.5) of the PLS-DAs were compared, showing that kampferol-3-O-glucoside, quercetin-7-O-glucoside, apigenin, luteolin, phloretin,

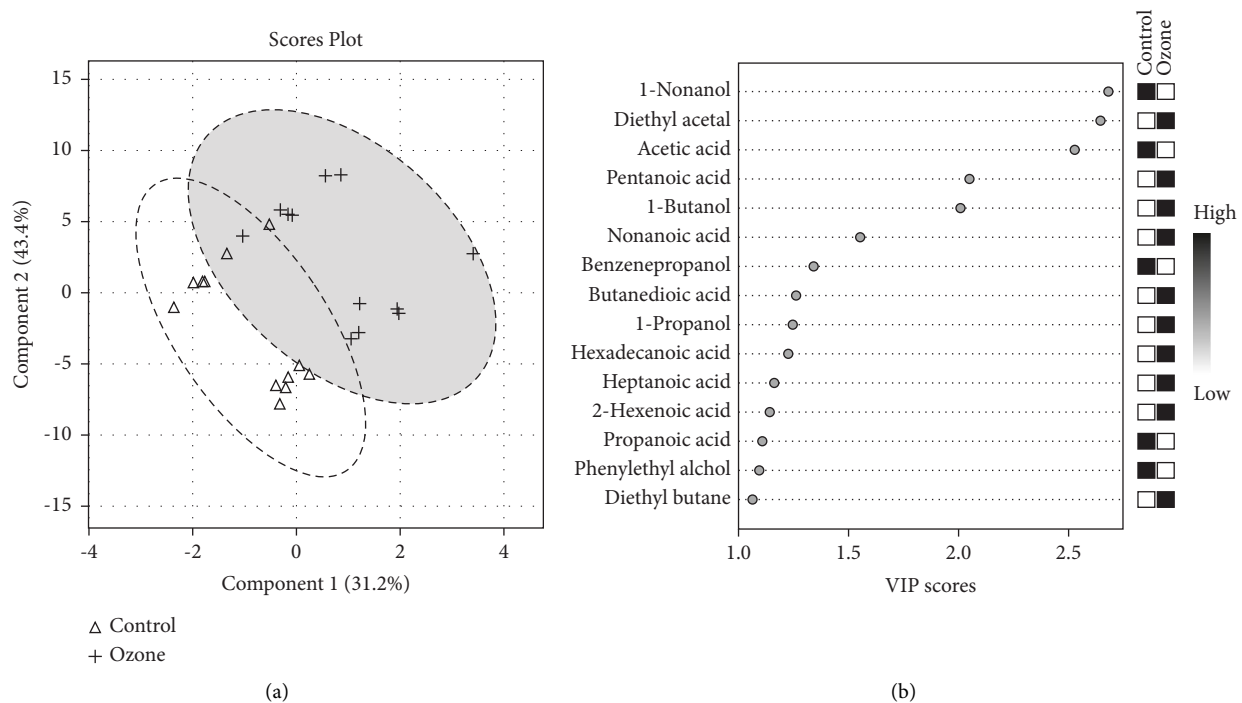


FIGURE 4: (a) PLS-DA performed on identified free VOCs in Sangiovese wines in the 2018 and 2019 vintages. The detected compounds were used as predictor variables while treatments (wine made from postharvest ozone-treated and control grapes) were used as response variables. The ellipses indicate 95% confidence intervals. (b) List of the highest 15 features, which contributed to sample clustering together with their VIP scores (>1) for the presented PLS-DA model. The boxes on the right indicate the relative concentrations of the corresponding metabolite in each group studied.

resveratrol, piceid, and quercetin-3,4-O-diglucoside, had high scores in both years. Of these, quercetin-7-O-glucoside, apigenin, and luteolin were increased by the O_3 treatments (Figures S5B and S6B) relative to the control treatments, while phloretin was decreased in both years. Lastly, the wine polyphenol data for both vintages were integrated and a PCA model was created (Figure 5). The PCA showed that the first two PCs described approximately 80% of the residual variance, where PC1 explained 57.7% and PC2, 21.8%, respectively (Figure 5(a)). Samples obtained from different seasons were well segregated in opposite quadrants, and clustering mainly occurred within the first PC. Discrimination in terms of treatment (control versus O_3) was mainly observed within PC2. However, as discussed previously, and as suggested by the PLS-DA, in 2019 the differences between samples was less evident in terms of their polyphenol profile. The loading plot of the PCA (Figure 5(b)) revealed that in 2018, O_3 treatment was correlated with increased rosmarinic acid, cynarin, and 4-coumaric acid. On the other hand, in 2019, the O_3 treatment was associated with increased luteolin, apigenin, kampferol-3-O-glucoside, piceid, and three forms of quercetin glycosides relative to the control.

4. Discussion

4.1. Effect of O_3 Treatment on Grapes and Wine Polyphenol Composition. The analysis of basic grape composition in response to O_3 treatment showed that it slightly reduced sugar content and increased acids and pH values. However,

the differences between treated and control grapes were generally very small. The ozone treatment lasted 12 hours, and this was probably insufficient to remarkably modify basic grape chemistry.

O_3 is one of the most powerful oxidative stressors, and one of the most known effects is its capacity to stimulate polyphenol biosynthesis, as a defence mechanism against induced oxidative stress [1, 14, 17]. Our data clearly confirmed that postharvest O_3 treatment increased total polyphenols concentration in wine grapes. Polyphenols are known to be important secondary metabolites with strong antioxidant activity, and the growing interest in O_3 treatment is related to its stress action on living tissue. Hence, in biological tissues O_3 is rapidly converted to ROS, which leads to an unbalanced cellular redox status and, consequently, to a rapid activation of the antioxidant system. The ROS scavenging system is needed to maintain normal level of ROS, and it results in the production of antioxidant compounds, such as polyphenols [32]. The increase of different phenolic fractions in table and wine grapes after O_3 treatment has, indeed, reported in previously OR reported elsewhere [1, 6, 7, 33], with the respective authors concluding that it is a defence response to an induced oxidative stress. Interestingly, a significant relationship between *PAL* expression and total polyphenol concentration has been observed, suggesting that O_3 treatment effectively induces an antioxidant response. Hence, under specific conditions, including stress responses, the phenylpropanoid pathway may activate with a consequent upregulation of polyphenol

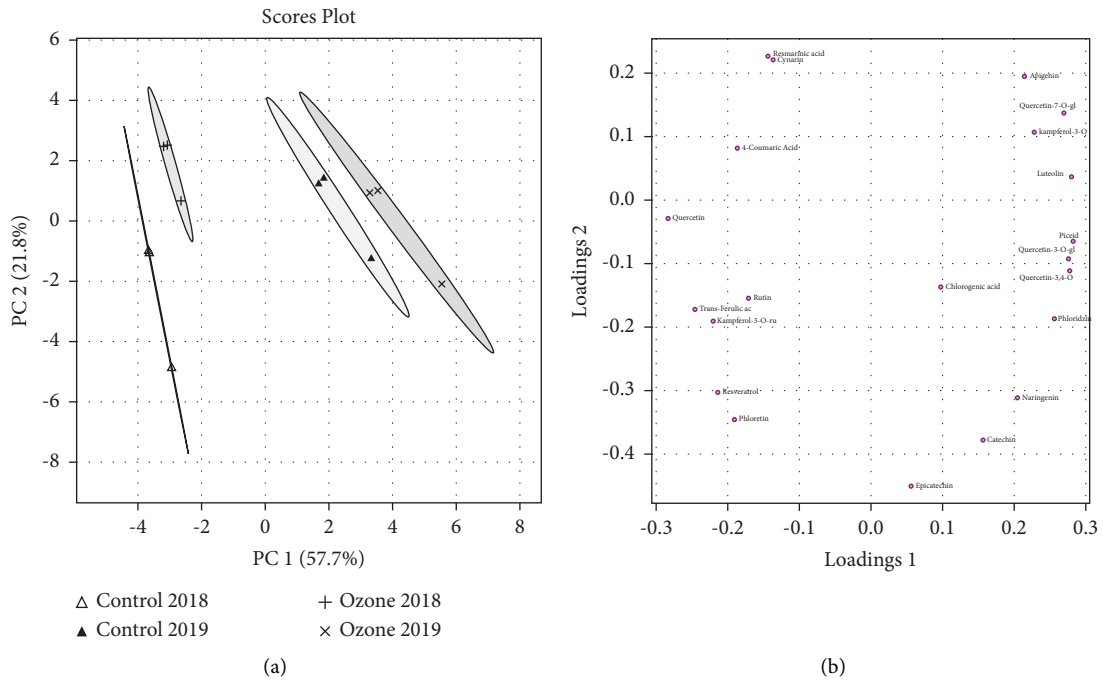


FIGURE 5: PCA performed on polyphenolic compounds detected in wines made from untreated (control 2018 and 2019) and ozonated (ozone 2018 and 2019) berries where (a) shows the scores plot of PC1 versus PC2 and (b) the loading plot.

biosynthesis, and thus accumulation. Additionally, *FLS* was found to rise in O_3 -treated grapes. *FLS* catalyses the key reaction of the phenylpropanoid pathway needed to synthesise flavanols. The accumulation of flavanols elicited by O_3 has already been reported in both table [17] and wine [6] grapes. Flavanols are known to be required as antioxidant defence mechanisms [34], and different stresses, including O_3 related stress in this instance, may induce the expression of *FLS* and result in a marked shift in flavanol biosynthesis in grape berries [6, 35, 36]. In order to study the response of grape polyphenols more in detail, flavonoid and anthocyanin concentration were also analysed. As mentioned earlier, total and specific anthocyanins did not show any response to O_3 treatment, while flavonoid concentration was elevated in treated grapes. Indeed, it seems that O_3 treatment not only stimulates the expression of the *PAL* gene and the subsequent accumulation of polyphenols but also induces specific routes within the phenylpropanoid pathway, namely, inducing *FLS1* expression and increasing flavanol concentration. Taken together, these results suggest that O_3 treatment may act as an abiotic elicitor.

Analysis of the wine polyphenol profile revealed that the O_3 treatment induced changes that were transferred from grape to wine during vinification. The effect of the O_3 treatment was greater in 2018 than in 2019. In 2018, some important compounds such as cynarin, luteolin, apigenin, rosmarinic acid, kampferol-3-O-glucoside, and quercetin-7-O-glucoside significantly increased in wines prepared from O_3 -treated grapes. Interestingly, out of 6 compounds identified to be enhanced by O_3 , three belong to the flavanol class of compounds (cynarin, kampferol-3-O-glucoside, and

quercetin-7-O-glucoside). Remarkably, this appears to confirm the hypothesis that O_3 treatment induces a specific pathway of polyphenol biosynthesis, stimulating the expression of *FLS* and, consequently, the production of flavanols. As a confirmation of this, the PCA model revealed that in both years of the study, O_3 treatment was well correlated with increase in flavanols (i.e., kampferol-3-O-glucoside, cynarin, and three form of quercetin glycosides). Other authors have also reported an increase of flavanols after O_3 exposure in both grapes [18] and wine, where the increase was more than double compared to the control wine [37]. In red wines, flavanols play an important role in stabilising the colour of young red wines, due in part to the copigmentation phenomenon with anthocyanins [38, 39]. Moreover, it has been suggested that flavanols play an important role in wine astringency and bitterness [39, 40]. Interestingly, the intake of flavanols has been associated with many health benefits due to their antioxidant potential and their role in reducing the risk of cardiovascular diseases [41]. Therefore, considering that ozonation induces the biosynthesis of phenolic substances in both grapes and wine, this could represent an advantage for the vinification process not only in terms of wine composition but also due to associated health-related benefits.

4.2. Implication of O_3 Treatment on the Grape and Wine Volatile Profile. Regarding the VOC profile of grapes, the first remarkable consideration was that most of the compounds identified as consistently responding to the O_3 treatment were derived from the LOX-HPL pathway. In particular, these were C_6 and C_9 aldehydes (2-nonanal,

hexanal, and nonanal) and C₆ alcohols (1-hexanol and 2-hexenol). These compounds belong to a group of molecules called “green leaf volatiles” (GLVs), which derive from fatty acid metabolism and are known to be important signalling molecules [42]. It is well known that abiotic stress, including oxidative stress, induces the accumulation of C₆ compounds [43, 44]. The biosynthesis of C₆ compounds is influenced by the presence of free polyunsaturated fatty acids (PUFAs), which are used as substrate, and which are released due to the membrane degradation and denaturation [43]. One of the known effects of O₃ mediated stress is the degradation of lipid membranes, and therefore, C₆ volatile accumulation can be increased by O₃ treatment, in particular given the release of PUFA precursor deriving from this process [19, 22, 45]. The degradation of PUFAs occurs in the LOX-HPL pathway [30], and three enzymes are mainly responsible for C₆ volatile biosynthesis: LOX, HPL, and ADH. Among them, in the present study, only the expression of *HPL* was significantly induced in O₃-treated grapes, showing more than double the expression level recorded for control samples. The increased expression of the *HPL* gene after O₃ treatment might suggest an accumulation of C₆ aldehydes in ozonated grapes. However, C₆ aldehydes were found to be lower after the O₃ treatment. As already known, the relationship between gene expression levels and accumulation of related compounds is rarely straight forward [22, 46]. Notably, and in accordance with our data, the expression profiles of the known *HPL* genes in *Vitis vinifera* very often do not match with the related accumulation of aldehydes [46]. Herein, the only LOX-derived volatile, which increase in O₃-treated grape, was the alcohol 2-hexenol, which, again, did not match the expression level of *ADH*, confirming that the expression gene genes within the LOX-HPL pathway did not necessarily correlate directly with the accumulation of related C₆ compounds, as stated by other authors [22, 46]. Another reasonable hypothesis is related to the capacity of O₃ to directly oxidise the double bond polyunsaturated fatty acid [31] acting therefore nonenzymatically.

In 2019, analysing the content of bound volatiles, two oxides of linalool (C and D forms) were higher in O₃-treated grapes. Some papers report an increase of terpenoids after O₃ exposure of grapes [1, 21, 22]. These authors suggest that these compounds are involved in an O₃-induced stress response, being known as antioxidant volatiles. It is also known that in grape terpenes are mainly present in glycosylated forms [47], especially in nonaromatic (or neutral) varieties such as Sangiovese. Glycoconjugate linalool oxides are released upon tissue disruption and associated stress responses [48]. Considering that the oxide forms are derived from linalool through chemical oxidation following tissue damage, the recorded increase of linalool as oxides is not surprising given the high oxidative capacity of O₃, as discussed earlier. Interestingly, previous studies reported that linalool oxide formation is a defence mechanism for linalool catabolism and cell detoxification in plants and fruits [49, 50]. Therefore, it could be proposed that linalool oxide formation after O₃ exposure could be a protection mechanism, involved in cellular detoxification.

The analysis of wine aromatic profile revealed that, as observed for grapes, the O₃ treatment induced only limited changes in wine volatile composition. Only two compounds, pentanoic and propanoic acid, were significantly affected by O₃ treatment in 2018, and were unaffected in 2019. Furthermore, of 15 compounds considered due to their high VIP score (>1), only hexanoic acid, 1-butanol, and beta-linalool maintained high VIP scores in successive years. However, two out of three compounds showed opposite trends in each of the successive years of the study. Taken together, analysis of the VOC composition over successive years, 4 compounds were found to contribute most significantly to define the treatment and control samples, and these all belonged to the class of higher alcohols (i.e., 1-butanol, 1-propanol, 3-methyl, phenylethyl alcohol, and benzene propanol). Among them, 1-butanol and 3-methyl-1-propanol increased in wine prepared from the O₃-treated grapes. Higher alcohols are produced during fermentation from yeast metabolism and, if at low concentration, can positively contribute to the aromatic profile of wines, giving complexity and increasing fruity and spicy notes [51]. Other classes of volatiles, which were affected by the O₃ treatment, included the fatty acids, and C₆ volatiles derived from LOX-HPL pathway. However, contrary to what was observed in grapes, most of these compounds increased in wine prepared from O₃-treated grapes relative to the control treatment. The increase of volatile fatty acids and C₆ compounds in O₃ wines was not surprising. Indeed, as discussed earlier, the production of these volatile is stimulated by abiotic stress, primarily due to membrane degradation [43]. Finally, it was found that some organic acids associated with yeast metabolism, and thus produced during fermentation, were affected by the O₃ treatment. After grape O₃ treatment, acetic acid content decreased in the resultant wines, while on the other hand, diethyl acetal and butanedioic acid were found to increase. Yeast-derived volatile acids are generally associated with unpleasant pungent, acetic, and rotter aroma [52], but only when present at high concentrations. However, none of the mentioned classes showed a clear trend of accumulation or reduction in wines made from ozonated grapes, confirming a minimal effect of O₃ exposure on wine aroma.

5. Conclusions

In the wine industry, O₃ is becoming more and more popular, and in recent years is being used for the sanitation of both equipment and grapes. Purovino® is a patented method, which involves the use of O₃ at different stages of winemaking to produce wine without the addition of SO₂. In the present study, the effect of O₃ postharvest O₃ treatment, using the Purovino® method, on grape and wine composition were investigated in two different vintages. Our findings confirm the elicitor role played by O₃ since ozonation stimulated the accumulation of both polyphenols and flavonoids in grapes and were conferred to the resultant wine. Consistently, specific key genes involved in flavonoids biosynthesis were upregulated. Considering this, the treatment has good prospects to both produce wine without

chemicals and to increase important health-related compounds in grapes. The aromatic profile of wines, aside from minor effects on a subset of specific compounds, appeared to be only marginally affected by O₃ treatment. Considering that the treatment is generally used to prevent the use of sulfur dioxide, the finding that the treatment did not dramatically alter the quality of the treated grapes and the resultant wines, was in itself a positive result. We could then conclude that O₃ treatment may safely be used to produce sulfur dioxide-free wines, preserving the aromatic characteristics of the starting grapes and the resultant wines and, at the same time, potentially increasing wine health-related antioxidant properties by positively affecting wine polyphenolic composition. Finally, aspects of wine ageing and long-term protection must be taken into account. The safety and stability of wine produced without SO₂ will require careful monitoring during longer-term aging and storage, and warrants ongoing investigation.

Data Availability

The data presented in this study are available on request from the corresponding author (pending privacy and ethical considerations).

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors gratefully acknowledge Dr. Simone Baccelloni for winemaking process. The research was funded as part of a PhD program in Agrobiosciences at the Life Sciences Institute, in the Sant'Anna School of Advanced Study (Pisa, Italy).

Supplementary Materials

Table S1: gene name, forward and reverse sequence, GenBank accession number, reaction temperature, and primer efficiency (%) of the genes studied in the work. Table S2: SRM transitions and relative compound parameters for targeted polyphenol compounds. Table S3: total soluble solids (TSSs) expressed in g/L of sugar, titratable acidity (TA) expressed in g/L of tartaric acid equivalent, and pH in Sangiovese grapes at harvest (Time 0) and after postharvest ozone treatment and in control grapes. Table S4: free and glycosylated volatiles detected by GC-MS in grapes and wine. Figure S1: original and normalized concentration of 1-hexanol, 2-hexenol, hexanal, and nonanal identified in control and ozone-treated Sangiovese grapes from 2018 trial. Figure S2: original and normalized concentration of acetic acid, identified in control and ozone-treated Sangiovese grapes from 2019 trial. Figure S3: original and normalized concentration of pentanoic and propanoic acid, identified in wine made starting from control and ozone-treated Sangiovese grapes from 2018 trial. Figure S4: original and normalized concentration of cynarin, luteolin, apigenin, kampferol-3-O-glucoside, quercetin-7-O-glucoside, and

rosmarinic acid identified in wine made starting from control and ozone-treated Sangiovese grapes from 2018 trial. Figure S5: PLS-DA performed on identified polyphenols in Sangiovese wines from 2018 trial. Figure S6: PLS-DA performed on identified polyphenols in Sangiovese wines from 2019 trial. (*Supplementary Materials*)

References

- [1] A. Bellincontro, C. Catelli, R. Cotarella, and F. Mencarelli, "Postharvest ozone fumigation of petit verdot grapes to prevent the use of sulfites and to increase anthocyanin in wine," *Australian Journal of Grape and Wine Research*, vol. 23, no. 2, pp. 200–206, 2017.
- [2] S. Río Segade, M. A. Paissoni, S. Giacosa et al., "Winegrapes dehydration under ozone-enriched atmosphere: influence on berry skin phenols release, cell wall composition and mechanical properties," *Food Chemistry*, vol. 271, pp. 673–684, 2019.
- [3] M. T. Lisanti, G. Blaiotta, C. Nioi, and L. Moio, "Alternative methods to SO₂ for microbiological stabilization of wine," *Comprehensive Reviews in Food Science and Food Safety*, vol. 18, no. 2, pp. 455–479, 2019.
- [4] C. Catelli and F. Mencarelli, "Process for the treatment and the winemaking of grapes," U.S. Patent No. 11,332,703, 2022.
- [5] F. Mencarelli and C. Catelli, "Process for the treatment and the winemaking of grapes," Italy Patent No. PCT/IB2012/000036, 2012.
- [6] K. Carbone and F. Mencarelli, "Influence of short-term postharvest ozone treatments in nitrogen or air atmosphere on the metabolic response of white wine grapes," *Food and Bioprocess Technology*, vol. 8, pp. 1739–1749, 2015.
- [7] M. Modesti, M. Petriccione, R. Forniti, L. Zampella, M. Scortichini, and F. Mencarelli, "Methyl jasmonate and ozone affect the antioxidant system and the quality of wine grape during postharvest partial dehydration," *Food Research International*, vol. 112, pp. 369–377, 2018.
- [8] N. Tzortzakis, *Postharvest Management Approaches for Maintaining Quality of Fresh Produce*, M. W. Siddiqui, J. F. Ayala Zavala, and C.-A. Hwang, Eds., Springer International Publishing, Berlin, Germany, 2016.
- [9] E. Feliziani, G. Romanazzi, and J. L. Smilanick, "Application of low concentrations of ozone during the cold storage of table grapes," *Postharvest Biology and Technology*, vol. 93, pp. 38–48, 2014.
- [10] A. Duarte-Sierra, E. Aispuro-Hernández, I. Vargas-Arispuro, M. A. Islas-Osuna, G. A. González-Aguilar, and M. Á. Martínez-Téllez, "Quality and PR gene expression of table grapes treated with ozone and sulfur dioxide to control fungal decay," *Journal of the Science of Food and Agriculture*, vol. 96, no. 6, pp. 2018–2024, 2016.
- [11] I. S. Minas, G. Tanou, M. Belghazi et al., "Physiological and proteomic approaches to address the active role of ozone in kiwifruit post-harvest ripening," *Journal of Experimental Botany*, vol. 63, no. 7, pp. 2449–2464, 2012.
- [12] E. M. C. Alexandre, T. R. S. Brandão, and C. L. M. Silva, "Efficacy of non-thermal technologies and sanitizer solutions on microbial load reduction and quality retention of strawberries," *Journal of Food Engineering*, vol. 108, no. 3, pp. 417–426, 2012.
- [13] A. Ali, M. K. Ong, and C. F. Forney, "Effect of ozone preconditioning on quality and antioxidant capacity of papaya fruit during ambient storage," *Food Chemistry*, vol. 142, pp. 19–26, 2014.

- [14] E. Aguayo, V. H. Escalona, and F. Artés, "Effect of cyclic exposure to ozone gas on physicochemical, sensorial and microbial quality of whole and sliced tomatoes," *Postharvest Biology and Technology*, vol. 39, no. 2, pp. 169–177, 2006.
- [15] A. Nadas, M. Olmo, and J. M. García, "Growth of botrytis cinerea and strawberry quality in ozone-enriched atmospheres," *Journal of Food Science*, vol. 68, no. 5, pp. 1798–1802, 2003.
- [16] M. V. Selma, A. M. Ibáñez, M. Cantwell, and T. Suslow, "Reduction by gaseous ozone of Salmonella and microbial flora associated with fresh-cut cantaloupe," *Food Microbiology*, vol. 25, no. 4, pp. 558–565, 2008.
- [17] F. Artés-Hernández, E. Aguayo, F. Artés, and F. A. Tomás-Barberán, "Enriched ozone atmosphere enhances bioactive phenolics in seedless table grapes after prolonged shelf life," *Journal of the Science of Food and Agriculture*, vol. 87, no. 5, pp. 824–831, 2007.
- [18] R. Botondi, F. de Sanctis, N. Moscatelli, A. M. Vettraino, C. Catelli, and F. Mencarelli, "Ozone fumigation for safety and quality of wine grapes in postharvest dehydration," *Food Chemistry*, vol. 188, pp. 641–647, 2015.
- [19] S. Río Segade, M. Vilanova, S. Giacosa et al., "Ozone improves the aromatic fingerprint of white grapes," *Scientific Reports*, vol. 7, no. 1, Article ID 16301, 2017.
- [20] F. Mencarelli and A. Bellincontro, "Recent advances in postharvest technology of the wine grape to improve the wine aroma," *Journal of the Science of Food and Agriculture*, vol. 100, no. 14, pp. 5046–5055, 2020.
- [21] M. Modesti, S. Baccelloni, S. Brizzolara et al., "Effects of treatments with ozonated water in the vineyard (cv vermentino) on microbial population and fruit quality parameters," *BIO Web of Conferences*, vol. 13, Article ID 04011, 2019.
- [22] S. Río Segade, M. Vilanova, M. Pollon, S. Giacosa, F. Torchio, and L. Rolle, "Grape VOCs response to postharvest short-term ozone treatments," *Frontiers of Plant Science*, vol. 9, 2018.
- [23] M. Modesti, R. Shmuleviz, M. Macaluso et al., "Pre-processing cooling of harvested grapes induces changes in berry composition and metabolism, and affects quality and aroma traits of the resulting wine," *Frontiers in Nutrition*, vol. 8, Article ID 728510, 2021.
- [24] V. L. Singleton and J. A. Rossi, "Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents," *American Journal of Enology and Viticulture*, vol. 16, 1965.
- [25] J. Zhishen, T. Mengcheng, and W. Jianming, "The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals," *Food Chemistry*, vol. 64, no. 4, pp. 555–559, 1999.
- [26] G. Genova and G. Montanaro, *APPLICATION NOTE*, PerkinElmer, Inc. 940 Winter Street Waltham, MA 02451 USA, 2012.
- [27] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ CT method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.
- [28] M. J. Noonan, H. V. Tinnesand, and C. D. Buesching, "Normalizing gas-chromatography-mass spectrometry data: method choice can alter biological inference," *BioEssays*, vol. 40, no. 6, Article ID 1700210, 2018.
- [29] J. Chong, D. S. Wishart, and J. Xia, "Using MetaboAnalyst 4.0 for comprehensive and integrative metabolomics data analysis," *Current Protocols in Bioinformatics*, vol. 68, no. 1, 2019.
- [30] M. N. ul Hassan, Z. Zainal, and I. Ismail, "Green leaf volatiles: biosynthesis, biological functions and their applications in biotechnology," *Plant biotechnology journal*, vol. 13, no. 6, pp. 727–739, 2015.
- [31] J. N. Roehm, J. G. Hadley, and D. B. Menzel, "Oxidation of unsaturated fatty acids by ozone and nitrogen dioxide," *Archives of Environmental Health: An International Journal*, vol. 23, no. 2, pp. 142–148, 1971.
- [32] R. L. Heath, "Modification of the biochemical pathways of plants induced by ozone: what are the varied routes to change?" *Environmental Pollution*, vol. 155, no. 3, pp. 453–463, 2008.
- [33] M. A. Paissoni, S. Río Segade, S. Giacosa et al., "Impact of post-harvest ozone treatments on the skin phenolic extractability of red winegrapes cv barbera and nebbiolo (vitis vinifera L.)," *Food Research International*, vol. 98, pp. 68–78, 2017.
- [34] C. D. Foy, E. H. Lee, R. Rowland, T. E. Devine, and R. I. Buzzell, "Ozone tolerance related to flavonol glycoside genes in soybean," *Journal of Plant Nutrition*, vol. 18, no. 4, pp. 637–647, 1995.
- [35] L. Haselgrove, D. Botting, R. Heeswijck et al., "Canopy microclimate and berry composition: the effect of bunch exposure on the phenolic composition of vitis vinifera L cv. Shiraz grape berries," *Australian Journal of Grape and Wine Research*, vol. 6, no. 2, pp. 141–149, 2000.
- [36] S. E. Spayd, J. M. Tarara, D. L. Mee, and J. C. Ferguson, "Separation of sunlight and temperature effects on the composition of Vitis vinifera cv. Merlot berries," *American Journal of Enology and Viticulture*, vol. 53, no. 3, pp. 171–182, 2002.
- [37] A. Campayo, C. Cebrián-Tarancón, M. M. García-Martínez, M. R. Salinas, G. L. Alonso, and K. Serrano de la Hoz, "Preliminary studies on endotherapy based application of ozonated water to bobal grapevines: effect on wine quality," *Molecules*, vol. 27, no. 16, 2022.
- [38] M. R. Arcena, S. Y. Leong, M. Hochberg et al., "Evolution of volatile and phenolic compounds during bottle storage of merlot wines vinified using pulsed electric fields-treated grapes," *Foods*, vol. 9, no. 4, 2020.
- [39] R. Gutiérrez-Escobar, M. J. Aliaño-González, and E. Cantos-Villar, "Wine polyphenol content and its influence on wine quality and properties: a review," *Molecules*, vol. 26, no. 3, 2021.
- [40] R. Brouillard and J.-E. Dubois, "Mechanism of the structural transformations of anthocyanins in acidic media," *Journal of the American Chemical Society*, vol. 99, no. 5, pp. 1359–1364, 1977.
- [41] A. N. Panche, A. D. Diwan, and S. R. Chandra, "Flavonoids: an Overview," *Journal of Nutrition Sciences*, vol. 5, 2016.
- [42] K. Matsui, "Green leaf volatiles: hydroperoxide lyase pathway of Oxylipin metabolism," *Current Opinion in Plant Biology*, vol. 9, no. 3, pp. 274–280, 2006.
- [43] F. Loreto and J.-P. Schnitzler, "Abiotic stresses and induced BVOCs," *Trends in Plant Science*, vol. 15, no. 3, pp. 154–166, 2010.
- [44] P. Piombino, A. Genovese, L. Rustioni et al., "Free and glycosylated green leaf volatiles, lipoxygenase and alcohol dehydrogenase in defoliated nebbiolo grapes during postharvest dehydration," *Australian Journal of Grape and Wine Research*, vol. 28, no. 1, pp. 107–118, 2022.
- [45] E. J. Pell, C. D. Schlagnhauser, R. N. Artega, E. J. Schlagnhauser, C. D. Artega, and E. J. Pell, "Ozone-induced oxidative stress: mechanisms of action and reaction," *Physiologia Plantarum*, vol. 100, no. 2, pp. 264–273, 1997.

- [46] X. Zhu, J. Luo, Q. Li et al., "Low temperature storage reduces aroma-related volatiles production during shelf-life of Banana fruit mainly by regulating key genes involved in volatile Biosynthetic pathways," *Postharvest Biology and Technology*, vol. 146, pp. 68–78, 2018.
- [47] P. J. Williams, C. R. Strauss, B. Wilson, and R. A. Massy-Westropp, "Studies on the hydrolysis of vitis vinifera monoterpene precursor compounds and model monoterpene .Beta.-D glucosides rationalizing the monoterpene composition of grapes," *Journal of Agricultural and Food Chemistry*, vol. 30, no. 6, pp. 1219–1223, 1982.
- [48] R. A. Raguso and E. Pichersky, "New perspectives in pollination biology: floral fragrances. A day in the life of a linalool molecule: chemical communication in a plant-pollinator system, Part 1: linalool biosynthesis in flowering plants," *Plant Species Biology*, vol. 14, pp. 95–120, 1999.
- [49] J. C. R. Demyttenaere and H. M. Willems, "Bio-transformation of linalool to Furanoid and Pyranoid linalool oxides by *Aspergillus Niger*," *Phytochemistry*, vol. 47, no. 6, pp. 1029–1036, 1998.
- [50] G. Bock, I. Benda, and P. Schreier, "Biotransformation of linalool by *Botrytis Cinerea*," *Journal of Food Science*, vol. 51, no. 3, pp. 659–662, 1986.
- [51] A. de-la-Fuente-Blanco, M.-P. Sáenz-Navajas, and V. Ferreira, "On the effects of higher alcohols on red wine aroma," *Food Chemistry*, vol. 210, pp. 107–114, 2016.
- [52] C. Liu, Q. Li, C. Niu, F. Zheng, and Y. Zhao, "Simultaneous determination of Diethylacetal and Acetaldehyde during beer fermentation and storage process," *Journal of the Science of Food and Agriculture*, vol. 98, no. 12, pp. 4733–4741, 2018.