1 **Influence of short-term postharvest ozone treatments in nitrogen or air**

2 **atmosphere on the metabolic response of white wine grapes**

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11 **Abstract**

12 Grapes were exposed to ozone gas in air (GO) or in nitrogen atmosphere (GNO) for
13 12 hours at 10°C and then kept in normal atmosphere for one day at room
14 temperature. Grapes kept at 10°C, for 12 hours without ozone treatment plus one day
15 at room temperature, were considered as the control sample (GC). Low temperature
16 induced a great change of ripening features only in GC with reduction of weight, loss
17 of sugars (about 20%) and increase in titratable acidity but, above all, 15% of increase
18 in malondialdehyde (MDA). The use of ozone in air or in nitrogen neither altered the
19 fruit quality attributes nor modified MDA concentration. Ozone provoked a high
20 decrease of hydroxycinnamic acids, compared to the harvest grapes, overall in GNO
21 samples (caftaric acid: -38.8%; coutaric acid: -26.1%). Cold storage treatment without
22 ozone enhanced antiradical capacity. Three main flavonols, named hyperoside
23 (quercetin-3-O-galactoside), rutin (quercetin-3-O-rutinoside) and kaempferol-3-O-
24 glucoside were found in all grape extracts analysed and the total flavonol content
25 increased significantly ($p < 0.008$) in all the samples starting from 1.79 mg/g dry
26 matter of grapes at harvest (GH sample) up to 2.13 mg/g of GNO one. Both cold
27 storage and ozone in air treatments significantly increased catechin content compared
28 to that in the grapes at harvest (+54.6% and +35.7% for GC and GO, respectively; $p <$
29 0.008). The interrelationships between the parameters analysed and the different
30 postharvest treatments, as well as the relationships among variables, were investigated
31 by principal component analysis. Component loadings showed significant groupings
32 for concentrations of hydroxycinnamic acid derivates and flavonols, which appeared
33 as good candidates to be further considered as biomarkers of the physiological status
34 of grapes. Similarly, the component scores grouped according to the different

35 postharvest treatments, highlighting hyperoside as an useful indicator of the ozone
36 effect on the grape's physiology.
37 **Keywords**
38 Ozone, wine grape, quality, antiradical capacity, principal component analysis
39

40 **Introduction**

41 After detachment from the plant and until cell death occurs, the fruits remain
42 metabolically active and react to internal and external stimuli and stresses, resulting in
43 compositional changes. Postharvest strategies are generally aimed at reducing
44 metabolic activity and at maintaining the physico-chemical properties of fruit at
45 harvest. However, for certain food products, postharvest controlled stresses are
46 applied to induce desired physical/chemical changes and/or to positively affect their
47 phytochemical content (Schreiner and Huyskens-Keil 2006). Recently, an innovative
48 postharvest technology, Purovino®, based on the treatment of wine grapes with ozone
49 was presented (Mencarelli et al. 2011). Authors confirmed ozone as an efficient and
50 safe bactericidal and fungicidal agent which, when used to treat the grapes before the
51 winemaking, allows to avoid SO_2 use. This aspect is very interesting, as it is well
52 known the detrimental effect of SO_2 on human health. Ozone has been widely used on
53 table grapes with the aim of reducing decay and controlling fungi development or
54 maintaining fruit quality (Romanazzi et al. 2012; Feliziani et al. 2014). However,
55 almost no information exists about the role of this gas on the quality of wine grapes.
56 Ozone, the triatomic form of oxygen (O_3), is an unstable compound that decomposes
57 either spontaneously, producing hydroxyl radicals and other free radical species, or in
58 contact with oxidizable surfaces. It has been suggested that in plants, O_3 enters the
59 mesophyll cells via the stomata, while in harvested fruits through lenticels, cuts or
60 cracks in the cuticle (Forney 2003). Here it is converted into the so-called reactive
61 oxygen species (ROS) such as superoxide anion, hydroxyl radicals, and H_2O_2
62 (Mehlhorn et al. 1990), participating in the oxidative burst mechanism.

63 Unless efficiently metabolized, ROS may alter plant metabolism by structurally
64 modifying proteins and enhancing their susceptibility to "proteolytic degradation"

65 (Pell and Dann 1991). Plants metabolize ROS by activating the endogenous
66 antioxidant defence system (Foyer et al. 1994), which consists of low molecular
67 weight antioxidants, as well as several antioxidant enzymes (Sandermann et al. 1998).
68 Besides, plants respond to biotic and abiotic stresses also through a chemical defence
69 response, synthetizing specific metabolites, some of them recognized as healthy
70 compounds (i.e. stylbens and polyphenols). In this sense, O_3 can be considered a
71 postharvest elicitor that may promote the levels of healthy phytochemicals in fruit and
72 vegetables. In *Vitis vinifera* L., the most frequently observed chemical defence
73 response to biotic and abiotic stresses is the accumulation of phytoalexins, a restricted
74 group of phenolic compounds belonging to the family of stilbenes, which derive
75 primarily from trans-resveratrol (3,5,40-trihydroxystilbene). In this regard, the
76 accumulation of phenolic compounds (i.e. flavonols, anthocyanins, etc.), induced by
77 O_3 elicitation mechanism was reported, in both table grapes (Artés-Hernandez et al.
78 2003) and wine grapes (Mencarelli et al. 2011). Phenolic compounds are marked by a
79 broad spectrum of health-promoting functions as antioxidants, blood pressure or
80 blood sugar influencing substances, or agents with anticarcinogenic, immunity-
81 supporting, antibacterial, antifungal, antiviral, cholesterol-lowering, antithrombotic or
82 anti-inflammatory effects. In this regard, literature data show the influence of grape
83 variety, gas concentration and time of exposure on the response to ozone treatment,
84 which for some genotypes can lead to a depletion of some of these important
85 metabolites (Artés-Hernandez et al. 2003; Artés-Hernandez et al. 2007; De Sanctis
86 2013). Moreover, when ozone is used in combination with air during the fumigation
87 process, the increased oxygen concentration, caused by the ozone decomposition,
88 around and within the commodity, even though of a minimal entity, may result in an
89 alteration in the balance of redox system of the cells. Besides, when ozone is used in

90 an inert atmosphere, even if it decomposes into O_2, the observed effect can be
91 ascribed totally to a direct or an indirect effect (by means of its decomposition
92 products) of ozone itself. Starting from these considerations, the present study aimed
93 at investigating the effects exerted by short-term postharvest ozone treatments on the
94 metabolic response of wine grapes. White wine grapes (var. Grechetto) were exposed
95 to two different ozone-enriched atmospheres in cold storage: ozone in air (GO) and
96 ozone in pure nitrogen (GNO). Quality traits, phenolic profiles and the antiradical
97 capacity of grapes were studied together with an index of lipid peroxidation.
98 Moreover, the interrelationships between the parameters analysed and the different
99 postharvest treatments, as well as the relationships among variables, were investigated
100 by means of the principal component analysis (PCA).
101
102 **Materials and Methods**
103
104 Chemicals
105
106 All used reagents were of analytical spectrophotometric grade (Carlo Erba,
107 Rome, Italy). Folin-Ciocalteu reagent, gallic acid, catechin, 2,2-diphenyl-1-
108 picrylhydrazyl radical (DPPH•), 2,2'-azinobis-(3-ethylbenzothiazolin-6-sulfonic acid)
109 (ABTS), potassium persulfate, and vanillin were purchased from Sigma-Aldrich
110 (Milan, Italy). Standards used for identification and quantification purposes with
111 HPLC were purchased from Extrasynthese (Genay, France) and Sigma-Aldrich
112 (Milan, Italy). Organic solvents used for chromatography were of HPLC ultragradient
113 grade (Sigma Aldrich, Milan, Italy), while the water employed was previously
114 purified in a Milli-Q system (Millipore, Milan, Italy). 0.45-μm pore size membrane

115 filters from Pall (Pall Corporation, Michigan, USA) were used for filtration of both
116 mobile phases and samples.
117
118 Plant material and experimental design
119 Samples of Grechetto grapes (*Vitis vinifera*, L; vintage 2012) were collected
120 from a 14-year-old experimental vineyard of the CRA- Research Unit for Enology,
121 located in Velletri (Rome) in the Lazio region (Italy) (41°40’30” N latitude,
122 12°50’42” E longitude) at 355 m a.s.l. Grapes were harvested at technological
123 maturity (sample name: GH) between 20 and 21°Brix and with a value of titratable
124 acidity around 1 g tartaric acid/L. Then, bunches were carefully selected, placed in
125 perforated plastic boxes, in a cold room (T = 10 ± 1°C) at 90(±2)% relative humidity
126 and removed soon after the end of the treatments (12 hours). The following
127 atmosphere treatments were applied (exposure time: 12 hours; three replicates for
128 each treatment): (i) air continuous flow (GC); (ii) a continuous flow of 1.5 g/h of
129 ozone in air (GO) ; (iii) a continuous flow of 1.5 g/h of ozone in 100% nitrogen flow
130 (GNO). The specific ozone flow value was chosen in accord with previous treatment
131 on wine grapes. A generator of ozone (PC Eng., Uggiate Trevano, Como, Italy) was
132 employed to produce gaseous ozone, while nitrogen was generated by a 100%
133 nitrogen tank (Rivoira, Terni, Italy). After the treatment samples were kept in normal
134 atmosphere for one day at room temperature (20±2°C). The treatments were carried
135 out in three stainless steel containers (volume = 1 m^3), tightly closed for the treatment
136 time, and placed in three different cold rooms. Initially and at the end of the
137 experiment, selected berries (300 from each box of each treatment, sampled by
138 different bunches) of a similar size and from the central part of the bunch, were

139 detached, and stored at −20 °C for the phytochemical extraction, while fresh berries
140 were used for the proximate analysis.
141
142 Quality chemical analysis
143
144 Berries were weighed, washed, deprived of seeds, homogenized, and the
145 homogenate samples were analysed for total soluble solid (TSS) content using a
146 digital refractometer (Refracto 30 PX, Mettler Toledo, Milan, Italy); data are given as
147 °Brix. The method for analysis of titratable acidity (TA) was based on titration of the
148 acids present in the berries juice with sodium hydroxide (0.1 N). Data are given as g
149 tartaric acid/L, since this is the dominant organic acid in grapes. The pH value was
150 measured using a digital pH-meter (785 DMP, Methrom, Milan, Italy). The dry matter
151 (DM) of the berries was measured drying them in an oven at 105°C until reaching a
152 constant weight. Total browning was evaluated by measuring the absorbance at 420
153 nm (A420) and normalized to the total polyphenol content (TPC) according to Boselli
154 et al. (2010). Each analysis was replicated three times.
155
156 Extraction of grape's bioactive compounds
157
158 Grapes were extracted to determine their phytochemical content (e.g. total flavans:
159 FLC, total polyphenols: TPC) and antiradical capacity (AC). Berries (15 g) were
160 deprived of seeds, placed in 50-mL tubes and homogenized at high speed with 15 mL
161 of a hydro alcoholic solution (methanol: water = 80:20, v/v) acidified with 0.1% HCl
162 (v/v) using an Ultra-Turrax mixer (Ultra-Turrax, Model T25; Jancke & Kunkel, IKA-
163 Works, Germany). Each homogenization (three replicates) lasted 15 s with intervals

164 of 30 s. The homogenization was carried out in an ice bath. Then, the mixture
165 underwent to an ultrasound-assisted extraction for 30 min. The resulting extracts were
166 then centrifuged at 6792 *g*, for 15 min at 4°C. Pellets were extracted once again in the
167 same manner. Then, the supernatants were collected together and immediately
168 analyzed.

169 Bioactive compound content and antiradical capacity of grapes

170

171 Bioactive compound content of extracts were determined according to Carbone
172 et al. (2011). Data were expressed as mg gallic acid equivalents (GAE)/g (DM) and
173 mg catechin equivalents (CAE)/g (DM) for total polyphenol content (TPC) and
174 flavan-3-ol content (FLC), respectively. The radical scavenging power of the
175 analysed samples was assessed by measuring their ability to scavenge synthetic
176 radicals (e.g. DPPH• and $ABTS^{\bullet+}$). The DPPH• quenching capacity was estimated
177 spectrophotometrically according to Carbone et al. (2011) and data were expressed in
178 terms of EC_{50} according to Sánchez-Moreno et al. (1998). $ABTS^{\bullet+}$ stock solution
179 (daily prepared) was produced by reacting a 7.0 mM aqueous solution of ABTS with
180 aqueous 2.45 mM potassium persulfate (final concentration). The mixture was
181 allowed to stand in the dark at room temperature for 12 h before use. Then, the
182 working solution was prepared by dissolving the ABTS radicalized solution above in
183 ethanol (1:90, v/v) to reach an absorbance of 0.700 ± 0.002 at 734 nm at room
184 temperature. Extracts (20 μL) were added to 980 μL of ABTS radical solution. The
185 mixture was incubated in darkness in a 37 °C water bath for 10 min, and the
186 absorption at 734 nm was measured by a UV-Vis spectrophotometer (Evolution 300,
187 THERMO Scientific, Italy) in reference to a blank. EC_{50} values were calculated based
188 on dose-response curves.

189

190 Chromatographic analysis of phenolic compounds using photodiode array detection
191 (DAD)

192

193 Polyphenols were separated and identified by an analytical high performance
194 liquid chromatography (HPLC) system (Agilent 1100 series, Agilent, Italy) equipped
195 with a photodiode array detector (DAD; Agilent Technologies, Italy). DAD was
196 simultaneously set at 280 nm (benzoic acids and flavan-3-ols), 320 nm
197 (hydroxycinnamic acids) and 370 nm (flavonols). Besides, UV-Vis spectra were
198 recorded over the range 200-700 nm. The separation protocol was slightly adapted
199 from a previous one (Zhang et al. 2012). Briefly, the separation was done on a
200 Zorbax SB C18 column (Agilent, 4.6 x 250 mm; 5 mm particle size, set at 30°C), and
201 used in combination with the following binary gradient: 10-12% B for 16 min, 12-
202 38% for 9 min, 38-70% B for 7 min, 70-85% B for 8 min and 85-10% B for 10 min.
203 The time of post-run for reconditioning was 5 min. The following mobile phase was
204 used (flow rate: 0.7 mL/min): solvent A: water with 0.1% (v/v) formic acid (FA);
205 solvent B: 30% acetonitrile, 10% methanol, 59.90% water and 0.1% FA. The
206 injection volume was 20 μL and samples were membrane-filtered (Millipore PTFE
207 0.45 mm, Milan, Italy) before HPLC analysis. The different phenolic compounds
208 were identified by their retention time, spectral data as compared to individual
209 standards and according to literature data and by the method of standard addition to
210 the samples. Analytical data were evaluated using a software-management system of
211 chromatographic data (Chemstation 32.1, Agilent Technologies). 10-point calibration
212 curves based on external standard solutions (0 – 100 ppm) were obtained for
213 quantification.

214

215 Analysis of lipid peroxidation

216

217 Lipid peroxidation was determined by measuring the content of
218 malondialdehyde (MDA) as described by Du and Bramlage (1992) with some
219 modifications. Three milliliters of 0.67% thiobarbituric acid (TBA) in 10%
220 trichloroacetic acid (TCA) was added to one milliliter of the crude extract sample
221 solution in a test tube. The solution was heated at 95°C for 20 min, immediately
222 cooled and then centrifuged at 6792 *g* for 10 min, and the absorbance of supernatant
223 read at 532 nm. The value of non-specific absorption at 600 nm was subtracted. The
224 results were recorded as thiobarbituric acid reactive substances (TBARS), which
225 represent MDA equivalents. The amount of MDA was calculated from the extinction
226 coefficient 155 mM/cm and expressed as nmoles/g DM.

227

228 Statistical analysis

229

230 Statistical analysis was performed with SPSS 20.0 software (SPSS, Inc.,
231 Chicago, Illinois). Data were reported as means ± standard error of the mean (SE,
232 where not specified differently) of three independent experiments with three
233 replicates. An exploratory data analysis was made to check the data normal
234 distribution (Shapiro-Wilkinson test) and the equality of variances (Levene's test).
235 To reduce the impact of any detected outliers in the data, we replaced them with the
236 mean plus two standard deviations (Field, 2009). Data violated ANOVA assumptions,
237 even after their mathematical transformation. In light of the obtained results, we
238 analysed not-normal distributed data through the Kruskal-Wallis non parametric test,

239 while significant mean differences were established using the Mann-Whitney test for
240 independent and non parametric procedures ($p < 0.008$ for Bonferroni's correction,
241 where not specified differently; Field, 2009). Spearman's correlation coefficient (r_S)
242 was used to determine the correlation among variables in the non-parametric analysis
243 ($p < 0.01$). Principal component analysis (PCA) was used to establish the
244 relationships among all the variables under study and to discriminate between
245 different postharvest treatments. It was performed using the data correlation matrix
246 and Varimax rotation between the samples.
247
248 **Results and Discussion**
249
250 Fruit quality attributes
251
252 In the present study, no alterations in the wine grape quality features due to
253 ozone treatments occurred (Table 1), in agreement with the findings reported by other
254 authors (Skog and Chu 2001; Salvador et al. 2006; Tzortzakis et al. 2007).
255 Particularly, no significant differences were found in dry matter, TSS, TA, and pH of
256 grapes treated with ozone, regardless of the atmosphere, air or nitrogen, compared to
257 the fresh samples. In contrast, GC samples showed a significant lower content of dry
258 matter and TSS, a lower value of pH and higher value of TA than those exposed to
259 ozone-enriched atmospheres (GO and GNO; Table 1). GC samples also showed an
260 unexpected higher weight loss (20% vs 10%) than the one of the other ozonated
261 samples. The behaviour of berries (decrease of sugars, increase in acidity and
262 peroxidation index) kept in cold storage without ozone treatment is surprising because
263 grape is considered tolerant to low temperature but the rapid thermal excursion from

264 the harvest temperature (30°C in our case) down to 10°C has likely induced a cold
265 stress. This phenomenon has been observed in grapes by Cirilli et al. (2012) during
266 postharvest dehydration, with a significant overexpression of genes dedicated to the
267 synthesis of polyphenols and it is something occurring even in the vineyard due to the
268 sensibility of polyphenolic fraction to temperature (Spayd et al. 2002; Cohen et al.
269 2012). Confirming a potential transient thermal stress, beyond the rapid decrease of
270 sugars content likely due to an immediate need of energetic substrate (increase of
271 respiration) for the thermal stress and water loss, GC samples showed a membrane
272 peroxidation index, determined by means of the malondialdehyde content,
273 significantly higher than the one of the other samples analysed (Table 1). Thus, the
274 thermal stress could be associated to an oxidative stress as reported by Wismer
275 (2003). In this regard, it has been shown that an oxidative stress can be induced in
276 harvested grapes as well as in other fruit during cold storage (Imahori et al. 2008;
277 Rosales et al. 2013). Yuan et al. (2014) pointed out that several antioxidant enzymes,
278 including ascorbate peroxidase (spots 5, 25 and 51), glutathione S-transferase (spot
279 35) and superoxide dismutase (spot 57) were up regulated after exposure to low
280 temperature. Furthermore, it has been shown that the lipid composition of the
281 membrane of the plant tissue, and consequently its physical properties, are influenced
282 by low temperature and by oxidation state of cell membrane (Dong et al. 2012).
283 Conversely to that observed for GC samples, ozone-treated berries, even though kept
284 at the same temperature of GC samples, did not show significant changes in the
285 chemical traits (Table 1). For these samples, it is assumed that exposure to ozone
286 could inhibit grape's respiration as reported for peach by Yang and Rao (2006) and
287 cucumber by Li et al. (2014). This effect was more pronounced for GNO samples,
288 where the replacement of oxygen by nitrogen in the atmosphere surrounding the berry

289 could have reduced significantly the respiration or even induced an anaerobic
290 respiration. Moreover, membrane lipid peroxidation was not affected by ozone, either
291 when the fumigation was done in air or nitrogen (Table 1). These findings are very
292 surprising because ozone is known for its strong oxidant action. In fact, it is a very
293 unstable molecule that decomposes either spontaneously, producing hydroxyl radicals
294 and other free radical species, or in contact with oxidizable substrates, such as cell
295 wall and plasma membranes, finding there suitable reactive sites (Forney 2003).
296 Chilling stress in plant cell is due mainly to a change of the ratio between unsaturated
297 to saturated fatty acids making the membrane more susceptible to ion leakage. It is
298 known that the effect of ozone on fatty acids is to produce ozonides, long chain
299 molecules, very unstable, which modify the fluidity of the lipid. Probably, the
300 modification of the lipid layers of the membrane due to the ozone treatments could
301 have induced cell resistance to cold stress as shown by less weight loss since it is
302 known that chilling injury favours water loss (Cohen et al. 1994). Further on, the
303 observed oxidation of phenolics in ozone-treated samples (see below) could have
304 prevented the peroxidation of membrane lipids (Robards et al. 1999).
305
306 Polyphenols, flavan-3-ols and antiradical capacity
307
308 Cold storage (GC samples) produced a statistically significant ($p < 0.008$)
309 increment of TPC if compared to those of grapes at harvest and ozonated samples
310 (Table 2). According to Toivonen (2003) and Wismer (2003), low temperature is a
311 causative agent of oxidative stress in postharvest of fruit and vegetables, and it
312 appears to enhance levels of antioxidants as observed in the present study. In
313 agreement with what has been discussed above about grape quality traits, the

314 hypothesized transient thermal stress associated to an immediate need of energetic
315 substrate (increase respiration) induced by low temperature, may be responsible for
316 the increase in phenylpropanoid metabolism and thus in the accumulation of
317 phenolics in cold stored grapes (Saltveit 1999). Conversely, ozone-fumigated grapes
318 showed a great reduction of both TPC and FLC compared to cold storage (GO:
319 -25.8% and -21.1%, for TPC and FLC respectively), which was greater for grapes
320 exposed to ozone in pure nitrogen (-43.5% and -34.3%, for TPC and FLC
321 respectively). The significant higher decrease in the phytochemical content observed
322 for ozone-treated samples could be due to the strong redox potential (+2.07 eV) of
323 ozone (Torres et al. 2011). However, in case of anaerobic atmosphere (pure nitrogen),
324 we would have expected a lower oxidation of phytochemicals, due to the absence of
325 molecular oxygen (+1.23 eV), and in this sense the observed data are surprising.
326 Since the loss regards mainly the phenolic acids, we could suppose that the lack of
327 oxygen, reducing the ethylene biosynthesis, has halted the synthesis of phenolic acids
328 acting on phenylalanine ammonia-lyase (PAL), which is ethylene-dependent.
329 Summarising, while cold stress induces the synthesis of polyphenols, ozone plays an
330 oxidative role buffering this synthesis; however, if the ozone treatment is done in pure
331 nitrogen, beyond a slight oxidation, a reduction in the synthesis of phenolic acids is
332 observed. According to literature data, the effects exerted by ozone on the fruit quality
333 during storage are in some cases contradictory and strictly dependent on several
334 factors such as the ozone concentration, time of exposure, storage temperature and
335 finally the nature of regulatory mechanisms underlying their ripening process (i.e.
336 climacteric or not) (Horvitz and Cantalejo 2014). In particular with regard to table
337 grapes, the authors underlined that the effect of ozone on phenolics seems to be
338 related to the time of exposure and the class of phenolic compounds considered.

339 Regarding wine grapes, De Sanctis (2013) reported a positive effect of ozone
340 (concentration: 1.5 g/h, time of exposure: 16 hours) on the phenolic content of
341 Sauvignon blanc grapes. Moreover, the author pointed out a link between the phenolic
342 content and the degree of grape ripeness, while other authors highlighted the influence
343 of the degree of ripeness on susceptibility to oxidative stress (Toivonen 2003). The
344 antiradical potential of grapes was evaluated in the present study by means of two
345 different in vitro assays: the $DDPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging methods (Table 2). Cold
346 storage enhanced AC of grapes respect to that at harvest, followed by ozone in air
347 treatment, while no significant differences were recorded between GH and GNO
348 samples. In this case, the lower TPC of GNO samples compared to that of GH ones
349 indicate that probably non-phenolic compounds significantly contribute to their
350 radical scavenging capacity (Piljac-Žegarac and Samec 2011).

351 Non-parametric correlation analysis pointed out a good negative correlation
352 between TPC and AC (r_s = -0.80 and -0.74, for $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, respectively)
353 significant at 0.01 level (2-tailed). Interestingly, both TA and AC and TA and TPC
354 also correlated (r_s = -0.70 and -0.69, for $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, respectively and r_s =
355 0.62; $p < 0.05$, 2-tailed) but in these cases the correlations were not so good as in the
356 previous one. In the present study, cold storage of wine grapes produced an increment
357 of both TPC and TA levels, to which was also associated an increment of the
358 antiradical capacity of grapes (Table 1; $p < 0.008$). Literature data highlighted that
359 organic acids possess a biological activity both in reducing ROS (Van den Berg et al.
360 2003) and in enhancing polyphenols bioavailability (Yamashita et al. 2002), while Lo
361 Scalzo (2008) demonstrated that organic acids generally enhanced the scavenging
362 activity of ascorbic acid on $DPPH^{\bullet}$ at a steady rate.
363

364 Quantification of polyphenols by HPLC-DAD

365

366 Regarding specific polyphenols, our research focused on the phenolic
367 compounds typical of white wine grapes such as phenolic acids (mainly
368 hydroxycinnamic ones), flavanols (i.e. catechin and epicatechin) and flavonols (i.e.
369 quercitin glycosides).

370 Hydroxybenzoic (HBAs) and hydroxycinnamic (HCAs) acids were measured by
371 HPLC-DAD and, among HBAs (gallic, vanillic and syringic acids), only grapes
372 belonging to the GH group showed the presence of gallic acid, whose concentration
373 was under the limit of quantification (LOQ: 2.66 ppm; data not shown). None of the
374 other acids considered was detected in any of the grape extracts analysed. In
375 agreement with other authors (Artés-Hernandez et al. 2007; Boselli et al. 2010), the
376 most abundant HCAs in all grape samples are the hydroxycinnamic acid derivatives
377 caffeoyl tartaric acid (caftaric acid) and *p*-coumaroyl tartaric acid (coutaric acid). The
378 amount of these compounds decreased significantly after postharvest treatments (Fig.
379 1a). The highest decrease, compared to the harvest grapes, was recorded for GNO
380 samples (caftaric acid: -38.8%; coutaric acid: -26.1%), followed by GC ones (caftaric
381 acid: -25.1%; coutaric acid: -19.7%). As it has been suggested earlier, this result could
382 be due to a decreased synthesis of phenolic acids also. Besides, ozone in air caused
383 only a slight, but significant ($p < 0.008$), decrease in HCA derivate content, more
384 pronounced for coutaric acid, compared to that at harvest (caftaric acid: -5.9%;
385 coutaric acid: -10.4%). Our findings agree with those of other authors (Artés-
386 Hernandez et al. 2007). Among the other HCAs analysed (chlorogenic,
387 dihydrocaffeic, caffeic, ferulic, 4-coumaric, 2-coumaric and 3-coumaric acids), GH
388 samples showed very low levels of caffeic and p-coumaric acids, whose

389 concentrations, however, were under their limit of quantification (LOQ: 1.11 and 1.34
390 ppm, respectively; data not shown), whereas chlorogenic, ferulic, 3-coumaric and 2-
391 coumaric were not detected. These results are confirmed by the findings of Boselli et
392 al. (2010), who did not detect caffeic acid in free run juice of Grechetto grapes. Cold
393 storage and ozone in air were the only treatments that increased, to the same extent,
394 the level of dihydrocaffeic acid (DHCA) in the grapes compared to that found at
395 harvest (+26.7%, $p < 0.008$; Fig. 2). By contrast, GNO samples showed a content of
396 DHCA halved compared to GH ones (0.17 and 0.29 mg/g DM, respectively; $p <$
397 0.008). Interestingly, DHCA has been reported to be a more efficient radical
398 scavenger than its parent caffeic acid (Nenadis et al. 2003). Artés-Hernandez et al.
399 (2007) found a significant decrease in hyroxycinnamic acid derivatives exposed to an
400 ozone concentration higher than 100 ppb, while Torres et al. (2011) found that
401 ozonisation of apple juice at higher ozone concentration dramatically reduced (up to
402 99.8% in some cases) the concentrations of chlorogenic, caffeic and cinnamic acids.
403 HCAs possess the lowest redox potential among other phenolic compounds present in
404 the grapes and are subjected to both enzymatic and non-enzymatic oxidation
405 (Fernández-Zurbano et al. 1998; Robards et al. 1999; Artés-Hernandez et al. 2007)
406 and this can explain the reduction in HCA content and, as a consequence, the
407 observed reduction of TPC, of grapes exposed to highly oxidative postharvest
408 treatments. One of the main consequences of these phenomena is the browning of the
409 samples (Robards et al. 1999). In the present study, the degree of browning (%BR) of
410 grapes was highly correlated to the amounts of HCA derivates ($r_s = -0.82$ and -0.92
411 for caftaric and coutaric acid, respectively; $p < 0.001$). Besides, a high negative
412 correlation ($r_s = -0.82$ and -0.80 for $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, respectively), significant at
413 0.01 level, was found between DHCA and EC_{50} values, confirming the findings of

414 Nenadis et al. (2003) on the radical-scavenging properties of this compound, while no
415 correlation was found between AC and the other HCAs analysed.

416 Three main flavonols, named hyperoside (quercetin-3-O-galactoside), rutin
417 (quercetin-3-O-rutinoside) and kaempferol-3-O-glucoside were found in all grape
418 extracts analysed (Fig. 1b). Total flavonol content increased significantly ($p < 0.008$),
419 in an almost linear manner, from GH (1.79 mg/g DM) to GNO samples (2.13 mg/g
420 DM) (data not shown). These results are quite different from those reported by Artés-
421 Hernandez et al. (2003), who found that ozone and cold storage kept the total flavonol
422 content of grapes at harvest. However, in this case, the authors analysed table grapes
423 with a much lower sugar content (15.7° Brix), submitted to a different ozone
424 treatments from that reported herein. Among different analysed flavonols, at harvest,
425 grapes showed the highest content of hyperoside (0.86 mg/g DM), followed by rutin
426 (0.52 mg/g DM) and kaempferol-3-O-glucoside (0.41 mg/g DM). Grapes exposed to
427 ozone-enriched atmosphere (both in air and nitrogen) accumulated hyperoside to a
428 greater extent compared to the GH (+25.3% and +22.3% for GO and GNO,
429 respectively; $p < 0.008$) and GC samples (+5.78% and +3.4% for GO and GNO,
430 respectively; $p < 0.008$). Quettier-Deleu et al. (2000) found that hyperoside is more
431 effective than rutin against hydrogen peroxide and superoxide anion. Moreover,
432 several authors (Piao et al. 2008; Xing et al. 2011) demonstrated that hyperoside
433 enhanced the cellular antioxidant defence system (i.e. induction of catalase and
434 glutathione peroxidase activities) and significantly inhibits overproduction of
435 intracellular ROS.

436 Our results also pointed out a significant effect of ozone in nitrogen treatment
437 on the kaempferol-3-O-glucoside content of grapes compared to the other postharvest
438 treatments (+2.1% and +4.1% compared to GC and GO samples, respectively; p <

439 0.008) and to the grapes at harvest (+16.3%, $p < 0.008$). To the best of our
440 knowledge, there is no literature information about the effect of ozone on kaempferol
441 content of grapes. However, Foy et al. (1995) pointed out that in soybean, ozone
442 tolerance is associated with the presence of kaempferol glycosides. As regards the
443 levels of rutin, all postharvest treatments had a positive effect on its accumulation,
444 which was more pronounced in the case of cold storage in air (GC; +15.8% compared
445 to GH, $p < 0.008$) and similar, but significantly different, to that obtained in the case
446 of ozone in nitrogen exposure (GNO; +13.7% compared to GH, $p < 0.008$).

447 Interestingly, only a weak correlation (r_s = -0.61 and -0.63 for $DPPH^{\bullet}$ and
448 $ABTS^{\bullet+}$, respectively, significant at 0.05 level) was found between rutin and EC_{50}
449 values, while no correlation was found between AC and the other flavonols analysed,
450 supporting the findings of other authors that these compounds contrast the oxidative
451 stress not by directly scavenging the ROS but rather stimulating the endogenous
452 defence mechanism of the cells.

453 Both cold storage and ozone in air treatments significantly increased catechin
454 content compared to that in the grapes at harvest (+54.6% and +35.7% for GC and
455 GO, respectively; $p < 0.008$) (Fig. 1c). Our results agreed with those of Artés-
456 Hernandez et al. (2007), who found that O_3 increased the total flavan-3-ol content,
457 quantified by HPLC-DAD, after cold storage. No significant changes in catechin
458 content occurred in GNO samples compared to GH ones (0.83 and 0.91 mg/g DM,
459 respectively).

460 Both O_3 treatments had a negative effect on epicatechin levels, which resulted
461 lower than those of GH samples (-13.0% and -15.9% for GO and GNO, respectively;
462 $p < 0.008$), but not statistically different between each other. Non-parametric
463 correlation analysis pointed out very poor correlations between FLC and both

464 catechin (r_s = 0.34) and epicatechin (r_s = 0.43), probably due to the several critical
465 issues of the colorimetric assay used (Sun et al. 1998). Flavan-3-ols are known as the
466 best free radical scavengers present in grapes and wines. In the present study, we
467 found a very good correlation between EC_{50} values and catechin (r_s = -0.84 and -0.90
468 for $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, respectively, significant at 0.01 level), while epicatechin
469 correlated to a lesser extent with AC (r_s = -0.64 and 0.68 for $DPPH^{\bullet}$ and $ABTS^{\bullet+}$,
470 respectively, significant at 0.05 level).
471
472 Differentiation of post harvest treatments by principal component analysis
473
474 In the current study, PCA was used to visualize the differentiation among post
475 harvest treatments and to highlight those metabolites that could be employed as
476 biomarkers of oxidative stress.
477 PCA was conducted on selected variables, chosen on the basis of the analysis of
478 the correlation and anti-image correlation matrices obtained from the raw dataset
479 (Field 2009), with orthogonal rotation (Varimax model). The Kaiser-Meyer-Olkin
480 measure verified the sampling adequacy for the analysis (KMO = 0.76; Field, 2009).
481 Bartlett's test of sphericity ($p < 0.001$) indicated that correlations between considered
482 items were sufficiently large for PCA. On the basis of eigenvalues > 1 (Kaiser's
483 criterion) and of the scree plot, three principal components (PCs) accounting for
484 95.2% of the total variance were considered significant. Component loadings after
485 rotation were reported in Table 3 showing the major variables differentiating the
486 grape samples submitted to the different postharvest treatments. Each number in the
487 table represents the partial correlation between the variable and the rotated factor. As
488 we can see, HCA derivates and flavonols loaded highly on factor 1, explaining most

489 of its variance (42.0%; rotated solution), while the variables loaded highly on factor 2,
490 which accounted for 37.7% (rotated solution) of the total variance, seemed all related
491 to different aspect of antiradical capacity of berries. Interestingly, hyperoside
492 accounted for 66.8% of the total variance in factor 3 (15.5% of the total variance in
493 the rotated solution). Fig. 2 and Fig. 3 (a and b) depict the scores for samples analysed
494 in a two-dimensional plot. The studied samples were clustered into four different
495 groups, which corresponded to the different postharvest treatments applied (Fig. 2).
496 Furthermore, these plots reveal the influence of individual secondary metabolites in
497 differentiating between treatments when the entire dataset is considered. In fact, GC
498 and GON samples were mainly separated each other on PC2 on account of their
499 overall antiradical properties, and from GH and GO samples, on PC1, for the lower
500 content of HCA derivates (Fig. 3a). Finally, GO was differentiated from GH along
501 PC3 for the higher content of hyperoside (Fig. 3b).
502 According to PCA analysis, hyperoside appears to be an useful indicator of the
503 ozone effect on the grape physiology, while TPC become more pronounced when
504 grapes are cold stored.
505
506 **Conclusions**
507 Ozone is characterized by a high oxidative potential, which could cause an
508 oxidative burst in treated plant tissue, and consequently compromising the nutritional
509 value and the technological attitude of white wine grapes. The present findings
510 indicate that the use of ozone in air atmosphere during cold storage preserves fruit
511 quality as well as the overall nutraceutical properties of treated grapes compared to
512 that of grapes at harvest. However, ozone proves to have a great impact on secondary
513 metabolites, especially when used in an inert atmosphere. In fact, the use of ozone in

nitrogen atmosphere resulted in high reduction of HCAs, while increasing the overall content of flavonols. These results suggest that abiotic stressors (alone or in combination between them) can be used to selectively target the increase of specific healthy compounds. However, in order to clarify the ozone oxidant effect, further studies would be needed employing different ozone concentrations and times of treatment.

Moreover, PCA allowed to differentiate samples on the basis of the postharvest treatments applied and to find out the variables that contribute most to this differentiation. From the reported results, secondary metabolites (i.e. flavonols and hydroxycinnamic acid derivates), related to the oxidative stress response, appear as good candidates to be further considered as biomarkers of the physiological status of the grapes.

Finally, the research findings indicate that the vinification process may benefit from a postharvest ozone treatment because, besides its very well known sanitizing effect, ozone also has the potential to increase the content of compounds that are important for wine production and quality, such as hyperoside and flavanols.

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689
690 **Captions of figures**

691 **Fig. 1** Effect of different postharvest treatments on the polyphenolic fingerprinting of
692 Grechetto grapes. **a**: hydroxycinnamic acids: DHCA: dihydrocaffeic acid; CAFT:
693 caftaric acid; COUT: coutaric acid; **b**: flavonols: HYP: hyperoside, KAEMP:
694 kaempferol-3-O-glucoside, RUT: rutin; **c**: flavan-3-ols: CAT: catechin, EPICAT:
695 epicatechin. Data are expressed in mg/kg (ppm) dry weight. Each value is the mean of
696 three replicates ± SD.
697

698 **Fig. 2** Score plot of the principal components PC2 and PC3. GH: grapes at harvest;
699 GC: grapes in air atmosphere; GO: grapes fumigated with ozone in air atmosphere;
700 GNO: grapes fumigated with ozone in nitrogen atmosphere.
701

702 **Fig. 3** Score plot of the principal components PC1 and PC2 (**a**), and PC1 and PC3 (**b**).
703 GH: grapes at harvest; GC: grapes in air atmosphere; GO: grapes fumigated with
704 ozone in air atmosphere; GNO: grapes fumigated with ozone in nitrogen atmosphere.
705

706