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1 **Screening and evolution of volatile compounds during ripening of ‘Nebbiolo’,**
2 **‘Dolcetto’ and ‘Barbera’ (*Vitis vinifera* L.) neutral grapes by SBSE-GC/MS.**

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16 **Abstract**

17 The evolution of pre-fermentative volatiles and of the global aroma potential in three Italian neutral
18 varieties (‘Nebbiolo’, ‘Barbera’ and ‘Dolcetto’) was assessed from véraison to harvest by SBSE-GC/MS.

19 C6 and C9 compounds, benzene derivatives, bound monoterpenes and sesquiterpenes showed differences
20 among varieties in quantity and profiles during berry ripening. Quantitatively, the most of total
21 monoterpenes, C-13 norisoprenoids and sesquiterpenes were detected after acid hydrolysis. Among pre-
22 fermentative norisoprenoids, exclusively β -ionone was detected with different kinetics among varieties.
23 Monoterpene accumulation started around véraison with the exception of (E)-geranylacetone, whose
24 content was already high at véraison. (E)-geranylacetone, deriving from the degradation of carotenoids,
25 could become a target molecule to study indirectly the accumulation of carotenoids.

26 Data allowed to measure the global aroma potential and the pre-fermentative volatiles of grapes: result
27 interpretation suggested a number of implications on biosynthetic processes that have been addressed.

28

29 Keywords: pre-fermentative volatiles; global aroma potential; C6 compounds; monoterpenes;
30 sesquiterpenes; norisoprenoids.

31

32 **Introduction**

33

34 Volatiles of grape berries include molecules from different chemical classes that are essential for wine
35 quality and typicality; many of these compounds are final or intermediate compounds of different
36 metabolite pathways and play important ecological roles in plants. These compounds are present mainly
37 in grape skin [1] and their concentration depends on many factors such as grape variety, vine physiology,
38 soil management and growing area. Some grape genotypes show relatively high flavor (in particular
39 monoterpene) concentration in the berry skins (“aromatic varieties”, *e.g.* Muscat), whereas others have a
40 lower, albeit perceptible, content (“neutral varieties”). Many investigations have dealt with monoterpene
41 profile in muscat-flavored varieties since longtime, whereas studies on volatiles of neutral varieties are
42 more recent [2,3]. Most grape volatiles are ascribed to the chemical classes of benzenoids ([with an
43 important ecological role in plant interactions \[4\]](#)), aliphatic aldehydes and alcohols, and lipid derivatives.
44 Aldehyde and alcohol lipid derivatives (C6 and C9 compounds) are produced in plants by hydroperoxide
45 lyase in response to wounding and play an important role in plant defense strategies [5]. They are
46 produced at the crushing of berries and represent the majority of varietal pre-fermentative (*i.e.* determined
47 in berry tissues before alcoholic fermentation) grape volatiles [3,6,7]. Oliveira and co-workers (2006) [8]
48 have attributed to C6 aldehydes and alcohols important roles in wine classification, indicating the ratio
49 between (*E*)-3-hexenol and (*Z*)-3-hexenol as a useful tool to distinguish monovarietal wines. Recently, the
50 expression of two hydroperoxide lyases (*VvHPL1* and *VvHPL2*), has been characterized in Cabernet
51 Sauvignon berries and was shown to peak at veraison [9].

52 Two other major classes of grape berry volatiles include terpenoids and C-13 norisoprenoids, whose
53 flavor characterizes fresh berries, musts and wines of many genotypes. They are present in berries as free
54 or glycosylated forms: the former can be released from the latter following the action of grape and yeast
55 enzymes, or by acid-catalyzed reactions in the wine. To analyze grape volatile precursors there are two
56 main strategies: enzymatic hydrolysis and acid hydrolysis. The efficacy of these methods is related to the
57 chemical family of compounds. The main criticism to acid hydrolysis, raised in the past, is that it can
58 induce rearrangements of the chemical structures of some aglycones, such as cyclation in monoterpenes.
59 However Loscos *et al.* (2009)[10] found that several monoterpenes, such as linalool, α -terpineol, geraniol,
60 nerol and β -citronellol formed during acid hydrolysis were closely correlated with analogues formed

61 during alcoholic fermentation. Moreover, acid hydrolysis was found efficient to study norisoprenoids [11]
62 and the levels of hydrolytically liberated β -damascenone in grapes could closely predict the levels of free
63 β -damascenone in the corresponding wines after one year of ageing [12]. Volatiles released after acid
64 hydrolysis represent the grape global aroma potential and were effectively used in the characterization of
65 neutral grapes [13]. Deglycosylation allowed the identification of some important C13-norisoprenoids,
66 such as vitispirane, β -damascenone [14], Riesling acetale and TDN [15]. Both aglycones in the free form
67 and acid hydrolysis-derived norisoprenoids have been used to characterize grapevine varieties [11, 13].
68 A crucial point of volatile determination in grape berries is the extraction method used as different
69 extraction techniques can minimize or maximize the extraction of peculiar classes of volatiles [16]. A
70 semi-rapid technique, based on the use of stir bars packed with polydimethylsiloxane (PDMS-SBSE) has
71 been employed to assess pre-fermentative varietal volatiles [2, 17, 18] and global aroma potential [13] in
72 *Vitis vinifera* grapes. The effectiveness of the SBSE technique use in different matrix, including grape and
73 must, has recently been reviewed [19].
74 Nebbiolo, Dolcetto, and Barbera are the most cultivated red grape varieties in Piedmont (North-Western
75 Italy). Nebbiolo is the basis of high quality wines defined by the growing area: ‘Barolo’ DOCG
76 (Denomination of Controlled and Guaranteed Origin), ‘Barbaresco’ DOCG, ‘Nebbiolo d’Alba’ DOC
77 (Denomination of Controlled Origin) and ‘Roero’ DOCG. Dolcetto is a red early-ripening cultivar of
78 Piedmont, giving rise to several VQPRD wines: ‘Dogliani’ and ‘Diano d’Alba’ DOCG, ‘Dolcetto d’Alba’
79 DOC, all arising from the Langhe district. Barbera is one of the most important red-grape variety grown
80 in Italy; in Piedmont Barbera is the base cultivar for the production of some appreciated red wines, such
81 as ‘Barbera d’Alba’ DOC, ‘Barbera del Monferrato’ and ‘Barbera d’Asti’ DOCG.. Despite their
82 economical importance, at present there is little information about the profile and evolution of volatiles in
83 grapes from these varieties, even though knowing the volatile concentration and potential at different
84 stages of ripening could help to optimize the date of harvest [2, 20], in match with other maturity indices
85 (*i.e.* sugar/acidity ratio, phenolic maturity).
86 The aim of this study was to characterize the concentration of pre-fermentative and acid-released volatiles
87 of ‘Nebbiolo’, ‘Dolcetto’ and ‘Barbera’ by SBSE-GC/MS. To this aim we collected grapes from
88 commercial vineyards from véraison to harvest; each variety was studied in its typical cultivation site,
89 corresponding to a specific DOC or DOCG wine. Our results describe the accumulation kinetics of
90 volatiles in the three genotypes, and offer new insights for the study of key steps of volatile biosynthesis

91 in grapes. Moreover, we propose some molecules as chemical markers of each variety and we point out
92 possible differences among genotypes.

93

94 **Materials and Methods**

95 *Vineyard description and sampling.*

96

97 The study was carried out in 2010 in three vineyards, one of ‘Nebbiolo’, one of ‘Dolcetto’ and one of
98 ‘Barbera’; each vineyard was located within one of the Denomination of Origin areas of the variety,
99 respectively in the sites of Barbaresco-Montestefano for ‘Nebbiolo’ (Barbaresco DOCG, Ca’ Neuva
100 Winery), Treiso for ‘Dolcetto’ (Dolcetto d’Alba DOC, Pellissero Luigi winery) and Monforte d’Alba for
101 ‘Barbera’ (Barbera d’Alba DOC, Podere Ruggeri Corsini winery).

102 ‘Nebbiolo’ vines were grafted onto ‘Kober 5 BB’, planted at a spacing of 2.40 by 0.90 m; the vineyard
103 was South-exposed with East-West row orientation. ‘Dolcetto’ vines were grafted onto ‘420 A’, planted
104 at 2.50 × 0.90 m; the vineyard was West-exposed with North-South row orientation. ‘Barbera’ (clone
105 CVT 83) vines were grafted onto ‘420 A’; vines were planted with a spacing of 2.50 × 0.70 m with
106 NNW-SSE row orientation and East exposure. The vines of the three vineyards were vertically shoot
107 positioned (VSP) trained and pruned according to the Guyot system. In 2010 climatic conditions were
108 similar in Barolo and Barbaresco whereas in Treiso temperatures were cooler, resulting in a lower GGD
109 over the vegetative period (March-October, 1645 GDD), and the weather was rainier (about 100 mm of
110 rain more than in Barolo and Barbaresco).

111 For each vineyard, three field replicates of 20-25 contiguous vines in a row were established; 250-300
112 berries were collected from each field replicate from both sides of the canopy, to avoid the influence of
113 different exposure to solar radiation on volatile accumulation [26]. Berries were detached from the rachis
114 in small groups of 3 to 5 each from the upper, the middle and the bottom part of each cluster (about 60
115 clusters sampled per each field replicate). Berries were stored in portable refrigerators and transported to
116 the laboratory; berries were severed from the rachis and a subgroup of 200 berries was weighed and
117 stored at – 20°C until volatile analysis. The remaining berries were crushed and the must soluble solids
118 were measured with a digital refractometer (ATAGO, PR-32).

119

120 *Determination of volatile compounds by stir bar sorptive extraction gas*
121 *chromatography-mass spectrometry (SBSE-GC/MS).*

122

123 For the analysis of pre-fermentative volatiles, frozen berries were crushed for 2 min in a common robot
124 for domestic use without breaking seeds. 10 g of homogenized grapes were diluted to 100 mL with
125 distilled water and a solution of 2-heptanol ($\geq 97\%$, Sigma-Adrich, St. Louis, MO) was added as internal
126 standard for semi-quantification. After 30 min of extraction, 20 mL of the aqueous grape extract was
127 transferred into a screw-cap vial and stirred with a PDMS-coated stir bar (0.5 film thickness, 10 mm
128 length, Twister®, Gerstel, Mulheim and der Ruhr, Germany) for 6 hours at room temperature (20°C)
129 [2,18]. The stir bar was then removed from the sample, rinsed with distilled water, dried with soft paper,
130 and transferred into a thermal desorption unit for GC/MS analysis. Attention was paid to the time spent
131 for each sample preparation to avoid that samples were subjected to different periods of de-freezing and
132 extraction.

133 To measure the global aroma potential of grapes, we measured the concentration of volatiles released by
134 acid hydrolysis as reported in Pedroza *et al.* 2010 [13]. To this aim, we added to 20 mL of the aqueous
135 grape extract a citric acid solution 2 M to reach pH 2.5. For quantitative purposes, 2-heptanol was used as
136 internal standard. The acidified suspension was stirred at 600 rpm with Twister® for 2 hours at 70°C in a
137 water bath [13]. At the end of the extraction, the stir bar was removed from the sample, rinsed with
138 distilled water, dried with soft paper and transferred into a thermal desorption unit for GC/MS analysis.

139 Volatile compounds sorbed on the Twister® were desorbed in a thermal desorption unit (TDU, Gerstel,
140 Mulheim and der Ruhr, Germany) in the splitless mode. The temperature program for thermal desorption
141 was the following: 30°C for 6 seconds, then ramping at 120°C/min to 280°C, than 280°C for 1 min. The
142 desorbed analytes were cryo-focused at 0°C using liquid CO₂, in a programmed temperature vaporization
143 (PTV) injector (CIS 4, Gerstel, Germany); the cryo-focalized analytes were transferred to the GC column
144 by ramping at 12°C/s until 300°C (held for 6.00 min). Helium was used as the carrier gas, at a flow rate of
145 1 mL/min, in a DB-WAX J&W 122-7032 (30 m × 0,25 µm × 0,25 mm ID) column. GC-MS analysis was
146 performed using a 7890A gas chromatograph interfaced with 5975 C mass spectrometer (Agilent
147 Technologies). The oven GC initial temperature was set at 40°C for 10 min, rose to 180°C at a rate of
148 2.5°C/min, then to 200°C at a rate of 1°C/min, and was finally maintained at 200°C for 10 min. The

149 transfer line temperature was 280°C. After each desorption the magnetic stir bars were cleaned by
150 immersion in acetonitrile for 24 hours (stirring during the first hour).

151 The identification of compounds was performed using NIST and Wiley libraries spectra (NIST-05a;
152 Wiley7). Furthermore, for qualitative identification purposes, Kovats indices of identified compounds
153 were calculated using an alkane standard mixture C10–C40 (Sigma–Aldrich, St. Louis, MO) as reference
154 for retention times. Volatile compounds were quantified only when they were present in at least two
155 replicates out of the three for each sample. The results were expressed as microgram equivalents of
156 internal standard per Kg of fresh berry weight.

157 When a compound was detected both as pre-fermentative volatile and as global aroma potential its
158 concentration as acid-released form was calculated by subtracting its free-form concentration from that
159 detected after acid hydrolysis [as suggested by Pedroza *et al.* \(2010\) \[13\]](#).

160 On the basis of their mass-spectrum profile and with the aid of Nist and Wiley libraries we attempted to
161 identify these sesquiterpenes:

162 Sesquiterpene 1: 43.97 min.; mass spectrum: 119 105 133 41 93 91 107 55 204 121; MW 204; C₁₅H₂₄;
163 α -longipinene;

164 Sesquiterpene 2: 44.04 min.; mass spectrum: 41 161 91 93 105 107 204 79 69 133; MW 204; C₁₅H₂₄;
165 (+)-aromadendrene;

166 Sesquiterpene 3: 50.48 min.; mass spectrum: 157 147 142 173 91 55 77 69 115 200 ; MW 200; C₁₅H₂₀;
167 not identified;

168 Sesquiterpene 4: 60.40 min.; mass spectrum: 161 189 204 41 105 91 119 133 27 55; MW 204; C₁₅H₂₄;
169 cadinene;

170 Sesquiterpene 5: 61.83 min.; mass spectrum: 183 198 168 184 153 165 152 167 169 141; MW 198;
171 C₁₅H₁₈; cadalene.

172

173 *Statistical analysis.*

174 One separate extraction and analysis was performed for each [field](#) replicate. The data of each replicate
175 were averaged and standard errors of averages were calculated. [Results are shown as the mean of the](#)
176 [three field replicates. On data reported in tables 1 and 2, we performed an analysis of variance \(SPSS](#)
177 [Statistics 22.0, IBM ®\) using Tukey-b as a post-hoc setting \$\alpha = 0.05\$ to assess significance.](#)

178

179 **Results**

180 *Total pre-fermentative and acid hydrolysis-released volatiles.*

181

182 From véraison to harvest, the pre-fermentative total volatile compounds of Nebbiolo (N) constantly
183 increased (Fig. 1a), whereas in Dolcetto (D) grapes total pre-fermentative volatiles increased until 30 dpv
184 with a successive decrease until harvest (Fig. 1 a). Barbera (B) grapes displayed a plateau phase between
185 30 and 50 dpv (Fig. 1 a).

186 The accumulation trend of acid hydrolysis-released products showed a peak at 10 dpv in N, followed by a
187 decreasing trend until 30 dpv and by a successive increase until harvest (Fig. 2 a). D showed a linear
188 accumulation trend from 30 dpv onwards, whereas no major differences were detected in B during the
189 examined period. However, at harvest (about 50 dpv) no significant differences were detected among
190 varieties (Fig. 2 a).

191

192 *Pre-fermentative C6 compounds.*

193

194 C6 compounds were detected throughout the berry ripening (Fig. 2 a); C6 compound concentration
195 increased in the three varieties over the studied period and at harvest D showed the lowest concentration
196 in comparison with N and B. The accumulation of hexanal increased from véraison to harvest in N and B
197 (Fig. 3 a). N and D did not accumulate (Z)-3-hexenal in contrast to B, where it appeared 30 days after
198 veraison (Fig. 3 c). Furthermore, in N, (Z)-3-hexen-1-ol was detected, whereas it was not found in B and
199 D (Fig. 3 e). N and B showed a higher concentration of (E)-2-hexenal than D around 30 and 50 dpv,
200 respectively (Fig. 3 b). Hexyl-acetate was exclusively accumulated in B grapes (Fig. 3 h).

201

202 *Other pre-fermentative(non C6) aliphatic aldehydes.*

203

204 At 50 dpv N grapes displayed the highest aldehyde concentration and, in general, showed a constant
205 accumulation during ripening with a subsequent reduction in correspondence of harvest, whereas in D
206 grape aldehyde concentration was more or less constant(Fig. 1 c). In B grapes a rapid decrease of

207 aldehyde concentration was detected immediately after véraison followed by a peak of maximum
208 concentration around 30 dpv (Fig. 1 c).

209

210 *Pre-fermentative alcohols.*

211

212 D showed a more complex qualitative profile than N and B, accumulating 2-methyl-4-octanol and
213 dodecanol, during ripening (Tab. 1; Tab. 4 in supplementary data). D showed the highest alcohol
214 concentration during all stages of ripening, whereas N and B showed comparable concentration over
215 ripening (Fig. 1 d).

216

217 *Pre-fermentative benzenoids .*

218

219 These compounds showed the tendency to decrease (in N and D) or to remain stable (B) during ripening
220 (Fig. 1 e). Qualitative differences were detected among varieties, as shown in table 1 and tables 3, 4 and 5
221 (supplementary data).

222 After hot acid hydrolysis, zingerone (Tab. 6 in supplementary data), a methoxyphenol compound
223 involved in wine aroma definition, was detected exclusively in N grapes at 47 dpv.

224

225 *Pre-fermentative and acid hydrolysis-released monoterpenes.*

226

227 Total pre-fermentative monoterpenes showed different concentrations and accumulation trends in the
228 three examined varieties (Fig. 1 f). Qualitative differences were detected among varieties (Tab. 1 and
229 supplementary Tables 3, 4 and 5). In N grapes the total concentration of acid hydrolysis-released
230 monoterpenes was already high 10 dpv; then, the lowest concentrations were concomitant with the 2nd
231 and the 3rd sampling dates, followed by a successive increase of concentration until harvest (Fig. 2 b) . B
232 and D showed similar accumulation trends and concentrations of acid hydrolysis-released monoterpenes,
233 however their concentration was much more lower than that detected in N grapes in the first stage of
234 ripening (Fig. 2 b). At harvest the concentrations of monoterpene precursors, released after acid
235 hydrolysis was much higher than that of pre-fermentative forms in all three examined varieties (Tab. 2).

236

237 *Pre-fermentative and acid hydrolysis-released norisoprenoids.*

238

239 β -ionone was the only pre-fermentative detected norisoprenoid. N grapes showed a decrease of β -ionone
240 concentration since 10 dpv to harvest (Fig. 1 g). D and B showed a lower concentration respect to N at 12
241 dpv and in pre-véraison (-5 dpv), respectively (Fig. 1 g). However, D showed a decreasing trend whereas
242 B displayed an increase from 23 to 32 dpv and a successive decrease until harvest (Fig. 1 g).

243 The three varieties did not show any difference in terms of quality profile of bound norisoprenoids, except
244 for α -ionene which was exclusively detected in B at 23 dpv (Tab. 8 in supplementary data).

245

246 *Pre-fermentative and acid hydrolysis-released sesquiterpenes.*

247

248 At harvest total pre-fermentative sesquiterpene concentration (Tab. 1) was higher in B grapes respect to D
249 which, conversely showed the highest concentration of acid hydrolysis-released Sesquiterpenes (Tab. 2):
250 417.5 $\mu\text{g/Kg}$ against 21.6 $\mu\text{g/Kg}$ for N and 23.9 $\mu\text{g/Kg}$ for B.

251 In this study we did not observe the presence of pre-fermentative sesquiterpenes in N grapes, whereas D
252 accumulated sesquiterpene 3 and B sesquiterpene 2 (Tab. 1; Tab. 3 and 4 in supplementary data).
253 Conversely, B exclusively accumulated sesquiterpene 2 since 23 dpv until harvest, with a constant
254 accumulation trend over the studied period (Tab. 1; Tab 5 in supplementary data).

255 Sesquiterpenes released after acid hydrolysis in N and B showed a constant plateau phase from véraison
256 to harvest whereas D displayed an important increase (Fig. 2 d). The profile of bound sesquiterpenes was
257 different among the studied varieties, as shown in table 2 and tables 6, 7 and 8 in supplementary data.

258

259 **Discussion**

260

261 In this work we identified and quantified some volatile precursors after acid hydrolysis, namely
262 monoterpenes, norisoprenoids and sesquiterpenes whereas aldehydes and alcohols, including C6 and C9
263 derivatives and benzene derivatives, were found exclusively without acid hydrolysis so they were
264 classified as pre-fermentative volatiles. As studies focused on sesquiterpene accumulation in *Vitis vinifera*
265 are a few and quite recent [21] at present there are no information about the efficacy of acid hydrolysis to
266 assess them. In berries sesquiterpenes were measured both from the headspace [21] and after

267 homogenization (in strawberries) [22]. Our data indicate the existence of sesquiterpenes in low amounts
268 as pre-fermentative volatiles whereas they were present in higher concentration after acid hydrolysis,
269 probably indicating they mainly exist as glycosides.

270 During ripening, in Nebbiolo and in Barbera a significant positive correlation between sugar and total
271 pre-fermentative volatile accumulation ($R^2 = 0.62$ for Nebbiolo; $R^2 = 0.92$ for Barbera) was detected, in
272 agreement with a previous study [2] on the colored varieties Monastrell. On the other hand, in Dolcetto we
273 could not detect any correlation between sugars and total pre-fermentative volatiles ($R^2 = 0.05$) as
274 maximum pre-fermentative volatile accumulation was reached before maximum sugar content. This
275 pattern was also previously observed. Versini *et al.* (1981) [23] indicated that the maximum 'aroma' can
276 be attained before sugars have been accumulated. Vilanova *et al.* (2012) [7] reported that flavor maturity
277 and technological maturity are not simultaneous, because they did not find any correlation between
278 volatile evolution and total soluble solid accumulation in cv. Agudelo, Blanco lexitimo, Godello and
279 Serradelo. In the white varieties Airen, Macabeo and Chardonnay, a non-uniform evolution of volatiles
280 during ripening was described [24], highlighting the difficulty to establish grape maturity on the basis of
281 volatile accumulation.

282 Volatiles derived from oxydation of lipids were detected in all stages of ripening: it is known that
283 lipoxygenation of fatty acids is a plant response to biotic and abiotic stress and leads to the formation of
284 the so-called 'oxylipins' that include the phytohormone jasmonic acid, hydroxy-, oxo- or keto-fatty acids
285 and volatile aldehydes [25]. The three varieties examined in this study showed diversity in the profile and
286 evolution of these compounds, underlying the existence of lipoxygenases with different activity,
287 activation timing and, probably, acting on different substrates. Hexanal and E-2-hexenal, the most
288 important product of lipoxygenation, were much more concentrated in Nebbiolo and Barbera than in
289 Dolcetto; on the contrary hexanal increased during ripening in all genotypes, in agreement with Kalua and
290 Boss (2010) [3]. In Cabernet Sauvignon berries, the expressions of VvHPL1 acting on 13-hydroperoxides
291 and forming C6 compounds and of VvHPL2 acting on both 13- and 9-hydroxyperoxides and forming C6
292 and C9 compounds were detected about 2 weeks after flowering and peaks of activity were at 12 and 14
293 weeks after flowering, respectively; C6 compounds were accumulated in correspondence until 10 weeks
294 after flowering and thereafter a reduction, probably due to the transformation of aldehydes into the
295 correspondent alcohols, was detected [9]. In the varieties we studied, the accumulation trend during
296 ripening was in line with the timing of enzyme expression in Cabernet Sauvignon, but the final reduction

297 of C6 compound concentration was not detected; this could be ascribed to differences in alcohol
298 dehydrogenase activity due to the genotype or to the cultivation environment. In Nebbiolo, in particular,
299 the absence of (Z)-3-hexenal (Fig. 3c) but the presence of (Z)-3-hexen-1-ol(Fig. 3e) suggests the specific
300 activity of an alcohol dehydrogenase, whereas this enzyme may absent or not expressed in Barbera
301 (where (Z)-3-hexen-1-ol was absent). In a previous work on Nebbiolo grapes from three different
302 growing locations (Z)-3-hexenal was never detected [18], suggesting that the absence of the aldehyde is
303 more a genetic mark than an environmental effect. In effect, (Z)-3-hexen-1-ol concentrations in berries
304 have been previously reported to be cultivar-dependent [3, 6, 26]. The high concentration of (E)-2-
305 hexenal in Nebbiolo and Barbera throughout ripening (Fig. 3b), suggests an important role of enal
306 isomerases in these two varieties, as suggested by Kalua and Boss(2010) [3] in Riesling and Cabernet
307 Sauvignon. Besides, the lipoxygenase activity on linolenic acid (C18:3) is evidenced by the accumulation
308 of (Z)-3-hexenal (only in B), E-2-hexenal, (Z)-3-hexen-1-ol (only in N) which, on the contrary, could not
309 be active in D where (Z)-3-hexenal and (Z)-3-hexen-1-ol were not accumulated. The high concentration of
310 (E,Z)-2,6-nonadienal (Fig. 3 g), a product of linolenic acid peroxidation via the formation of 9-
311 hydroperoxides, could suggest a high expression of *VvHPL2* in Nebbiolo. The contents of (E)-2-
312 nonenal and (E,Z)-2,6-nonadienal (Tab. 3 , 4, 5 in supplementary data) were rather low respect to C6
313 volatiles, in line with data reported for Cabernet Sauvignon and they were almost absent in B, confirming
314 what was described by Zhu *et al.* (2012) [9] and suggested by Kalua and Boss (2010) [3] that the
315 degradation of fatty acids is mainly due to 13-LOXs and to 13-HPLs (which lead to the biosynthesis of
316 C6) rather than to 9-LOXs and 9-HPLs. Interestingly, we noticed that Barbera berries did not accumulate
317 C9 (except nonadienal at harvest; [Tab.1](#)), suggesting a very strong varietal influence on this metabolism.
318 The presence of hexyl acetate (a C6-moiety ester) (Fig. 3h) limited to Barbera grapes suggests the activity
319 of an alcohol acetyl transferase (AAT) on hexan-1-ol in this genotype. Moreover, this compound showed
320 a decrease during ripening, implying that AAT activity decreased after véraison. To the best of our
321 knowledge, nothing is known in *Vitis* on the specificity of alcohol acyltransferases; in *Malus domestica*
322 the existence of a varietal effect on this enzyme was suggested as different enzyme haplotypes were
323 detected in different varieties able to attain high or low ester concentrations [27]. Besides, an effect of
324 MdAAT2 on the response to biotic and abiotic stress was detected in transformed tobacco leaves [28].
325 Differences among varieties were found in concentration and profile of benzene derivatives.
326 Benzaldehyde was detected in all varieties, but the derived benzylalcohol was present only in Nebbiolo

327 and Barbera grapes, consistently with a cultivar specificity observed in previous studies [3,24,29].This
328 finding suggests a varietal influence on the dehydrogenation pathway from benzaldehyde to the
329 corresponding alcohol. In terms of quality of derived wines, these concentration aspects are important
330 because sensory attributes of benzene derivatives depend on their concentration and on their reciprocal
331 ratio [30]. Other benzenoid compounds may help to discriminate neutral grapevine varieties, though the
332 biosynthetic origin of many of them is not known. For instance, Nebbiolo (Tab 1 and Tab. 3 in
333 supplementary data) did not accumulate cinnamaldehyde, Dolcetto (Tab.1 and Tab. 4 in supplementary
334 data) and Barbera (Tab. 1 and Tab. 5 in supplementary data) did not accumulate 2-phenoxy-ethanol (rose
335 ether); methyl vanillate was present only in Dolcetto grapes (Tab. 1). Eugenol was detected exclusively at
336 harvest in Barbera berries (Tab. 1); correspondingly, in a previous study on Nebbiolo grapes from
337 different growing locations, no eugenol was detected [18].

338 Concerning monoterpenes, Nebbiolo showed a lower concentration respect to Barbera and Dolcetto; these
339 latter two exhibited a more complex profile characterized by a number of specific molecules (isomenthol
340 in Barbera and β -myrcene in Dolcetto). Monoterpene accumulation started around véraison with the
341 exception of (E)-geranylacetone, whose content was already high at véraison. This aspect might depend
342 on the different biosynthetic origin of this molecule respect to the other terpenes: indeed, (E)-
343 geranylacetone derives from phytoene by carotenoid cleavage dioxygenase 1 (CCD1) [30], so timing and
344 type of its biosynthesis could be rather different from those of other terpene compounds whose
345 biosynthesis was ascribed to monoterpene-synthases at flowering [31] and to other specific terpene-
346 synthases activated during ripening [32]. (E)-geranylacetone deriving from the degradation of carotenoids
347 (like abscissic acid, ABA) could become a target molecule to study indirectly the accumulation of
348 carotenoids, thus a possible indicator of the vine early response to abiotic conditions, light in particular,
349 being known that light has a direct influence on carotenoid accumulation [33, 34]. Currently, no
350 information is available on the sensorial role of (E)-geranylacetone in grapes and derived wines, and
351 about its fate during wine aging, even though a floral aroma descriptor was associated to its isomer (Z)-
352 geranylacetone [35].

353 Monoterpene glycosides reached higher concentration than pre-fermentative forms during all stages of
354 ripening, as noted in other grape genotypes [36, 37]. In a previous study, Di Stefano *et al.* (1998) [38]
355 showed that Barbera grapes at harvest had few monoterpenes in the bound form compared to Nebbiolo. In
356 this study similar concentrations of bound monoterpenes were detected at harvest among varieties, but

357 major differences were detected at early stages of berry ripening. The complexity of terpene profiles from
358 acid hydrolysis was much higher in Nebbiolo respect to the other genotypes, which probably justifies the
359 typical flavor fingerprint of Nebbiolo wines, also after long term storage. Grape juice heat treatment gives
360 rise to changes in the terpene composition: Williams *et al.*, (1980) [39] described reaction mechanisms for
361 the production of some monoterpenes from linalool as a precursor. Moreover, it was assessed that
362 temperature and acid hydrolysis can induce the rearrangement of bound monoterpenes into free
363 monoterpenes [39]. From data of the present study, however, as we treated grapes from the three varieties
364 in the same way we can conclude 1) that both pre-fermentative and acid hydrolysis monoterpenes are
365 cultivar related and 2) by exploiting the chemical transformation of terpenes following heat treatments at
366 low pH, we were able to detect a number of compounds (among which cyclic α -terpineol) whose
367 concentration depends on the concentration of other terpene molecules from which they derive due to
368 chemical cyclization.

369 The varietal volatile fingerprint of neutral grapes (and their corresponding monovarietal wines), also
370 depends on norisoprenoid concentrations. The only pre-fermentative form detected in the three varieties
371 was β -ionone. This molecule is important in vegetables due to its floral aroma [40] and it possesses a low
372 sensorial threshold of 0.09 $\mu\text{g/L}$ [26]. Nebbiolo and Dolcetto showed a decrease in free β -ionone
373 concentration during ripening whereas Babera displayed a later reduction, between 32 dpv and harvest.
374 Kalua and Boss (2010) [3] reported the presence of norisoprenoids in grape prior to véraison. In tomato
375 Goff and Klee (2006) [41] imputed the role of these apocarotenoids in signaling ripeness and attracting
376 seed-dispersing organism, including humans, because of their absence from vegetative tissues: this was
377 confirmed in our lab in leaves of *Vitis vinifera* where we did not find norisoprenoids whereas we found
378 them in tendrils, that are homologue organs to flowers (data not shown). The accumulation trend of
379 norisoprenoids also depends on environmental condition [42] and on plant water status [43, 44]; in our
380 case, we cannot exclude that the different kinetics detected were influenced not only by the different
381 genotypes, but also by the different growing areas (*i.e.* water availability).

382 It has been proposed [42] that glycosylation, which occur between véraison and maturity, is responsible
383 for the decrease of the concentration of free norisoprenoids. This hypothesis could help to explain the
384 reduction of β -ionone in Dolcetto during ripening, because it showed a correspondent accumulation in the
385 bound form after véraison, but not in Nebbiolo that showed a decrease after véraison. Among acid
386 hydrolysis-released norisoprenoids, we found *trans* β -damascenone, which contributes to the floral and

387 fruity notes of wine and has a very low sensorial threshold in model solutions (45 ng/L) [41]. The higher
388 concentration of vitispirane and 1,1,6-trimethyl-1,2-dihydronaftalene (TDN), known to give camphor and
389 kerosene notes in wines [45], in Dolcetto grapes could explain the tendency of Dolcetto wines to present
390 these notes. Sefton *et al.* (1989) [46], reported the acid-catalized mechanism formation of these molecules
391 from megastigmane precursors and Winterhalter *et al.* (1991) [15] suggested that the potential levels of
392 TDN upon aging may be predicted by analysis of the corresponding aglycone released at acid pH.
393 Together with the genotype, factors such as cluster exposure to sunlight could have influenced the
394 accumulation of TDN and vitispirane in Dolcetto [47]; as a matter of fact in Dolcetto, the North-South
395 row orientation in a vineyard with West exposure, together with an early leaf removal were probably able
396 to favour TDN and vitispirane accumulation in berries. We found differences in the qualitative profile and
397 in the accumulation kinetics of sesquiterpenes. In literature, data about accumulation of these compounds
398 are not always in agreement; Coelho *et al.* (2006) [48] reported that sesquiterpene accumulation in cv.
399 Baga, from véraison to post-ripening, showed its maximum expression at maturity and then remained
400 constant until post-ripening, whereas in cv. Riesling and Cabernet Sauvignon it was reported that
401 sesquiterpenes significantly decreased towards harvest [3]. Our data show that the kinetics of these
402 compounds depend on the terroir (genotype \times environment interaction); the same molecule, namely
403 sesquiterpene 5, displayed different kinetics in the three varieties: its concentration was constant during
404 ripening in Nebbiolo and Barbera, whereas it increased in Dolcetto. Lückner *et al.* (2004) [31] identified
405 two sesquiterpene synthases in grapevine flowers and berries; these Authors reported that sesquiterpene
406 synthase and monoterpene synthase transcripts were not detected in the mesocarp and exocarp during
407 early stages of fruit development, because they are expressed only during late ripening. May *et al.* (2013)
408 [49] demonstrated that sesquiterpene biosynthesis and accumulation in grape berries is restricted to the
409 exocarp, particularly to wax layers. As we homogenized the entire berry we cannot indicate where
410 sesquiterpenes were accumulated; however, finding no or trace amounts of sesquiterpenes as free pre-
411 fermentative volatiles we can conclude that in grape berries the most of sesquiterpenes exist as
412 glycosides.

413 The present study allowed to point out that C6 and C9 compounds, benzene derivatives, bound
414 monoterpenes and sesquiterpenes showed differences in quantity and profiles during berry ripening (from
415 véraison to harvest) among varieties. The fate of specific molecules such as (E)-geranylacetone, could be
416 indicative of stress conditions, being known that this molecule, easily detectable by SBSE-GC/MS,

417 derives from carotenoid degradation. Quantitatively, the most of total monoterpenes, C-13 norisoprenoids
418 and sesquiterpenes were detected after acid hydrolysis, showing that in neutral grapes they mostly exist as
419 glycosides. This aspect is well known for monoterpenes and C-13 norisoprenoids but it has not been
420 largely investigated as to sesquiterpenes.

421 Pre-fermentative norisoprenoids did not differ among varieties as exclusively β -ionone was accumulated
422 (table 1), but differences were detected as to kinetics (figure 1). Further research should be devoted to
423 investigate the possible role of β -ionone as a target molecule for signaling ripeness in *Vitis vinifera*
424 reproductive tissues, similarly to other plant species.

425

426 **Conclusions**

427 Data allowed to study the kinetic of pre-fermentative volatiles and of global aroma potential in the berries
428 of three economical important grape varieties: result interpretation suggested a number of implications on
429 biosynthetic processes that have been addressed. For instance (E)-geranylacetone, deriving from the
430 degradation of carotenoids, could become a target molecule to study indirectly the accumulation of
431 carotenoids.

432 Data showed a high complexity of volatile compounds in all three cultivars, despite being neutral flavor
433 varieties. Moreover, this study revealed differences in the accumulation kinetics of single molecules and
434 differences in terms of qualitative profile. This aspect is very important for the technological choices and
435 for typical varietal productive performance, but also to discriminate monovarietal wines with chemical
436 markers. The results showed a considerable contribute of volatile in the free-form to define the typical
437 aromatic composition; the free-forms are characterized especially by lipid derivatives, quantitatively very
438 important as pre-fermentative compounds in the fresh must. Moreover, this study revealed the importance
439 of sesquiterpenes, in free and bound forms, to discriminate non aromatic varieties; still, the sensorial role
440 of these molecules in berry tasting and the influence of biotic and abiotic factors on their accumulation
441 remain to be clarified.

442

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448 delle componenti aromatiche’.

449

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Table 1

Pre-fermentative volatile concentration (mean of three field replicates \pm standard errors) at harvest time of 'Nebbiolo', 'Dolcetto' and 'Barbera' grape berry. Data obtained by SBSE-GC/MS and expressed as $\mu\text{g Kg}^{-1}$ of 2-heptanol equivalents; dpv = days post veraison; TSS = total soluble solids; bw = berry weight; KI = Kovats Index; nd = not detected. The data marked by different letters are significantly different according to the test Tukey-b ($\alpha = 0.05$); ns = no significant differences.

	harvest time	1 st october 2010	17 th September 2010	23 rd September 2010
	dpv	55	43	46
	TSS (Brix)	24.2	18.0	25.5
	bw (g)	1.9	1.3	2.3
KI				
		Nebbiolo	Dolcetto	Barbera
Aldehydes				
octanal	1291	7.9 \pm 1.2 ab	5.1 \pm 0.2 b	11.0 \pm 1.9 a
Z-2-heptenal	1324	14.0 \pm 5.2 ns	37.2 \pm 5.7 ns	39.0 \pm 9.9 ns
nonanal	1386	nd	22.9 \pm 1.2 ns	27.2 \pm 7.9 ns
E-2-octenal	1412	nd	3.1 \pm 1.7 b	6.9 \pm 0.7 a
furfural	1457	113.9 \pm 9.2 ns	100.1 \pm 25.7 ns	112.5 \pm 3.4 ns
decanal	1498	3.9 \pm 1.2 ns	1.7 \pm 0.9 ns	12.2 \pm 4.1 ns
E-2-nonenal	1528	73.3 \pm 13.8	nd	nd
E,Z-2,6-nonadienal	1580	46.9 \pm 6.7 a	11.5 \pm 0.8 b	9.9 \pm 1.7 b
Alcohols				
2-ethyl-1-hexanol	1499	1.4 \pm 0.8 b	7.2 \pm 0.5 a	3.8 \pm 0.6 b
1-octanol	1568	nd	26.7 \pm 0.7	nd
E-2-octen-1-ol	1628	nd	23.6 \pm 3.1 ns	13.8 \pm 4.7 ns
furfurylic alcohol	1671	3.7 \pm 1.4 ns	6.9 \pm 1.3 ns	6.8 \pm 1.2 ns
2-methyl-4-octanol	1807-	nd	13.0 \pm 1.1	nd
Benzenoids				
benzaldehyde	1510	17.2 \pm 1.8 ns	9.5 \pm 0.9 ns	8.7 \pm 3.6 ns
cinnamaldehyde	1588	nd	5.4 \pm 0.1 a	3.2 \pm 0.5 b
acetophenone	1639	18.9 \pm 0.5 b	41.2 \pm 2.6 a	27.5 \pm 5.0 b
2-ethyl-benzaldehyde	1660	5.36 \pm 0.0 ns	nd	4.2 \pm 0.9 ns
benzyl alcohol	1887	20.7 \pm 3.1	nd	nd
phenol	2031	10.3 \pm 0.3 ns	12.0 \pm 0.4 ns	12.4 \pm 1.3 ns
eugenol	2172	nd	nd	4.2 \pm 2.1
2-phenoxy ethanol	2308	27.3 \pm 3.2	nd	nd
p-butyl-cresol	2258	6.1 \pm 1.2 ns	7.8 \pm 0.6 ns	9.7 \pm 0.6 ns
trimethyl-tetrahydro-benzofuranone	2324	5.7 \pm 1.1 ns	3.7 \pm 0.6 ns	4.3 \pm 0.5 ns
methyl vanillate	2390	nd	8.8 \pm 0.5	nd
Monoterpenes				
β -myrcene	1171	nd	13.0 \pm 0.9	nd
D-limonene	1206	3.8 \pm 1.9 b	13.7 \pm 1.1 a	9.6 \pm 1.9 ab
isomenthol	1648	nd	nd	2.3 \pm 1.7
geranial	1731	nd	8.2 \pm 0.7 a	4.8 \pm 0.9 b
β -citronellol	1783	10.2 \pm 2.1 b	41.3 \pm 3.2 a	12.3 \pm 2.0 b
nerol	1813	nd	26.5 \pm 2.0 a	7.6 \pm 1.1 b
E-geranyl acetone	1861	17.0 \pm 1.5 ns	13.2 \pm 2.6 ns	17.2 \pm 1.6 ns

geraniol	1864	nd	144.1±8.7 a	79.4±5.9 b
C13-Norisoprenoids				
β-ionone	1939	17.5±4.0 ns	25.1±1.0 ns	35.0±6.9 ns
Sesquiterpenes				
sesquiterpene 2	1706-	nd	nd	12.6±2.5
sesquiterpene 3	1906	nd	2.8±0.7	nd

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Table 2

Bound volatile concentration (mean of three field replicates \pm standard errors) at harvest time of 'Nebbiolo', 'Dolcetto' and 'Barbera' grape berry. Data obtained by SBSE-GC/MS and expressed as $\mu\text{g Kg}^{-1}$ of 2-heptanol equivalents; dpv = days post veraison; TSS = total soluble solids; bw = berry weight; KI = Kovats Index; nd = not detected. The data marked by different letters are significantly different according to the test Tukey-b ($\alpha = 0.05$); ns = no significant differences.

harvest time	1 st october 2010	17 th September 2010	23 rd September 2010
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TSS (Brix)	24.2	18	25.5
bw (g)	1.9	1.3	2.3
KI			
	Nebbiolo	Dolcetto	Barbera
Monoterpenes			
γ -terpinene	1218	19.3 \pm 3.2	nd
p-cymene	1270	39.6 \pm 14.2 ns	57.3 \pm 24.5 ns
dehydro-p-cymene	1422	31.4 \pm 3.5 ns	88.9 \pm 36.8 ns
ho-trienol	1615	38.7 \pm 3.0	nd
α -terpineol	1703	nd	74.3 \pm 2.0
Z-geranylacetone	1831	nd	16.3 \pm 8.1
E-geranylacetone	1859	442.6 \pm 23.9 b	322.8 \pm 51.8 b
C13-Norisoprenoids			
vitispirane	1515	136.1 \pm 18.0 ns	694.3 \pm 260.9 ns
TDN	1731	49.5 \pm 9.2 ns	327.6 \pm 141.6 ns
trans- β -damascenone	1817	265.2 \pm 83.0 a	62.8 \pm 23.0 b
β -ionone	1936	56.6 \pm 14.7 b	23.0 \pm 2.1 c
Sesquiterpenes			
sesquiterpene 1	1790	nd	334.6 \pm 117.6
sesquiterpene 4	2346	nd	44.2 \pm 18.5
sesquiterpene 5	2226	21.6 \pm 4.6 ns	38.7 \pm 17.0 ns

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