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**Varietal and pre-fermentative volatiles during ripening of *Vitis vinifera* cv Nebbiolo berries from three growing areas**

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# UNIVERSITÀ DEGLI STUDI DI TORINO

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19 **VARIETAL AND PRE-FERMENTATIVE VOLATILES DURING RIPENING OF VITIS**  
20 **VINIFERA CV NEBBIOLO BERRIES FROM THREE GROWING AREAS.**

21

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27

28 Flavour analysis of grape is a key step in quality evaluation. The Stir Bar Sorptive Extraction

29 technique (SBSE, ‘Twister’®) was used to assess varietal and pre-fermentative volatile

30 accumulation in ‘Nebbiolo’ berries, from véraison to harvest. Grapes were collected in three

31 vineyards, representing different ‘crus’ in the cultivation areas of Barolo, Barbaresco and Roero

32 (North-western Italy). Volatile constituents of grapes were identified and quantified by GC-MS.

33 We demonstrate the influence exerted by the growing location on volatile concentration and

34 profile, as well as on the timing of volatile accumulation. The accumulation of certain classes of

35 compounds, considered favourable for defining berry quality, followed common patterns, and

36 was negatively correlated to that of compounds with herbaceous and grassy notes, such as the C6

37 compounds.

38 PCA analysis shows that the concentrations of varietal and pre-fermentative volatiles were more

39 effective in separating growing areas than dates of harvest. Grapes from the Barbaresco area,

40 showing higher values of the concentration ratio between favourable and unfavourable

41 compounds throughout ripening, could be statistically separated from grapes from the other areas.

42

43 Keywords: SBSE/GC-MS, flavours, grapes, principal component analysis.

44

## 45 **1. Introduction**

46 Hundreds of volatiles have been identified in *Vitis vinifera* berries. Compounds with a C6-moiety  
47 are products of the lipoxygenase pathway and are the major volatile constituents of varietal and  
48 pre-fermentative aroma in many varieties (Gomez, Martinez & Laencina, 1995; Kalua & Boss,  
49 2010; Yang et al., 2009). They have grassy, herbaceous odours, generally considered unpleasant  
50 in wines if they are present in high concentrations (Baumes, Cordonnier, Nitz & Drawert, 1986).  
51 Several other aliphatic and aromatic alcohols and aldehydes, norisoprenoids and terpenes have  
52 been identified in grapes (Sefton, Francis & Williams, 1993) . Some aliphatic aldehydes such as  
53 octanal, decanal and (*Z*)-2-heptenal have citrus-like odour whereas furfural and benzaldehyde are  
54 responsible for almond aroma. Apocarotenoid volatiles ( $\beta$ -ionone and  $\beta$ -damascenone) are among  
55 the most important contributors to fruity and floral notes in many fruits, including grapes,  
56 together with furanones, like 4-hydroxy-2,5-dimethyl-3(2H)-furanone and lactones (Klee, 2010).  
57 As grapes ripen, a number of changes occur, including sugar and flavonoid accumulation,  
58 modifications in the content of organic acids, and changes in the concentration of volatile  
59 substances. The modifications of the berry volatile composition are still little known in ‘neutral’  
60 varieties, which represent most of winemaking grapes, as investigations have generally been  
61 focused on ‘aromatic’ varieties, very rich in both free and in glycoconjugated volatiles, terpenes  
62 in particular. Neutral grapes, however, possess a number of free and glycosylated volatile organic  
63 compounds, generally at lower concentrations respect to the aromatic ones. When grapes are  
64 thawed and grinded a number of volatiles develop: these molecules were named ‘varietal and  
65 pre-fermentative related volatile compounds’ (Coehlo, Rocha, Delgadillo & Coimbra, 2006).

66 They have been associated to C6 alcohols and aldehydes (Gomez, Martinez & Laencina, 1995),  
67 benzene derivatives, esters, and non-glycosylated monoterpenes and sesquiterpenes (Kalua &  
68 Boss, 2010); these compounds are present to such low concentrations that, when instrumental  
69 sensitiveness is low, they are seldom detected. This is probably a major reason of the scarcity of  
70 published data on the volatile composition of neutral grapes (Salinas, Zalacain, Pardo & Alonso,  
71 2004).

72 Volatile analysis is a key step in grape quality evaluation. Volatiles have traditionally been  
73 detected by GC/MS on grape extracts, which requires a prior time- and solvent-consuming  
74 preparation. Many methods, using different solvents and extractants have been proposed up to  
75 now (Sefton, Francis & Williams, 1993; Cabrita, Costa Freitas, Laureano & Di Stefano, 2006). In  
76 1999 Baltussen, Sandra, David & Cramers proposed a novel extraction technique for aqueous  
77 samples based on the use of a magnetic stir bar coated with polydimethylxylosane (PDMS). This  
78 technique, based on the concept of 'sorption' is known with the name of Stir Bar Sorptive  
79 Extraction (SBSE) and is commercially available under the brand name 'Twister'®. Sorption  
80 offers a number of advantages above traditional adsorption such as: 1) it is gentler as analytes are  
81 not retained on an active surface, and degradation of unstable compounds is reduced or absent; 2)  
82 desorption can be performed at lower temperatures, minimizing the losses of thermolabile  
83 compounds; 3) the retaining capacity of PDMS is not influenced by the sample matrix, in  
84 particular by the presence of different amounts of water (the main constituent of grape  
85 homogenates) or of other analytes, since each solute has his own partitioning equilibrium into  
86 PDMS; 4) degradation fragments originating from the PDMS stir bar coating give mass-spectra  
87 that can be very easily recognized and discarded (Baltussen, Sandra, David & Cramers, 1999).  
88 This technique has been successfully used in the detection of volatiles in many matrices but at the

89 moment few applications have dealt with grapes (Luan, Mosandl, Munch & Wust, 2005;  
90 Pedroza, Zalacain, Lara, Salinas, 2010). Salinas, Zalacain, Pardo & Alonso (2004) were the first  
91 to propose the use of this technique for detecting volatiles in grape berries.

92 The concentrations of volatiles change during grape ripening and information on these changes,  
93 and the factors which induce them, is of high technological importance as it allows harvesting  
94 grapes with specific aroma profiles. Little has been published up to now about the evolution of  
95 volatiles during neutral grape berry ripening even though several reports deal with the detection  
96 of volatiles at harvest. Studies dealing with the detection of grape volatiles during ripening are  
97 relatively recent and mostly based on adsorption (Kalua & Boss, 2009; Coehlo, Rocha, Barros,  
98 Delgadillo & Coimbra, 2007) or sorption methods (Salinas, Zalacain, Pardo & Alonso, 2004).

99 Another well-known, but little detailed, factor affecting aromatic composition of neutral grapes  
100 (and possibly of the derived wines) is the so-called 'terroir' where the vines are grown, each  
101 terroir giving rise to specific aromatic notes for a given vine genotype. However, very limited  
102 information is available about the influence exerted by the growing location on the volatile  
103 composition of neutral grapes (Bureau, Razungles & Baumes 2000; Ji & Dami, 2008).

104 In the present study we report the results of varietal and pre-fermentative volatile assessment  
105 from véraison to harvest in berries of the neutral cultivar Nebbiolo, which is widely grown in  
106 North-western Italy for the production of premium wines. Volatiles were detected using the  
107 SBSE technique on grapes collected in three vineyards, each located in one of three terroirs of  
108 Piedmont (Barbaresco, Barolo and Roero). Our aim was to identify individual molecules or  
109 classes of molecules able to distinguish growing areas. Moreover, we assessed the timing of  
110 accumulation of some class of volatiles in Nebbiolo grapes during ripening. Our results allow i)  
111 to gain information regarding the aroma potential of Nebbiolo when harvested at different

112 developmental stages; ii) to assess the influences exerted by environmental conditions on the  
113 timing of flavour accumulation in grapes and iii) to contribute to knowledge about the key steps  
114 of flavour biosynthesis in grape berries.

115

## 116 **2. Materials and Methods**

### 117 *2.1 Samples*

118 Nebbiolo grapes from three cultivation areas of Piedmont in North-West Italy (Barolo,  
119 Barbaresco and Roero) were collected from early véraison to harvest in 2010. The 20/25 year old  
120 vines were trained to the vertical trellis system and Guyot pruned. Canopies were routinely and  
121 similarly managed during spring and summer accordingly to the standard cultural practices of the  
122 cultivation area. In addition, crop load was controlled and standardized with cluster removal in  
123 the pre-véraison period. Grapes were collected fortnightly from three homogenous replicate  
124 groups of 20 adjacent vines in each vineyard. Berries were detached from the rachis in small  
125 groups of 3 to 5 respectively from the upper, middle and bottom part of the cluster, to avoid  
126 possible effects of scalar maturity inside the cluster. 250-300 berries were collected from each  
127 replicate group of vines and from both sides of the row to overcome possible effects of light  
128 exposure and temperature on secondary metabolite accumulation. Berries were stored in portable  
129 refrigerators at 5°C and transported to the lab within 4 hrs. In the lab, berries were separated from  
130 the rachis with small scissors and a subgroup of 200 berries per replicate was weighed and stored  
131 at -80 °C until volatile analysis. Must was obtained by crushing the remaining berries and total  
132 soluble solids (TSS) were determined with a digital refractometer (ATAGO, PR-32).

133 Meteorological data were obtained from three automatic weather stations, each located within a 5  
134 km range from one of the vineyards.

135

136 *2.2 SBSE methodology*

137 Sample preparation for the detection of volatiles involved grinding and homogenization of  
138 grapes. A common robot for domestic use was employed to crush berries without breaking seeds;  
139 from each sample of 200 homogenized berries, 10 g were diluted to 100 ml with distilled water  
140 and 30  $\mu\text{L}$  of 2-heptanol, 104.38  $\text{mg L}^{-1}$ , were added as internal standard for semi-quantification.  
141 Samples were left at room temperature for 30 minutes, and during this time lapse they were  
142 manually shaken three times; 20 ml of the aqueous grape extract were transferred into a cap-  
143 screw vial and stirred (700 rpm) with a PDMS stir bar (0.5 film thickness, 10 mm length,  
144 Twister®, Gerstel, Mulheim and der Ruhr, Germany) for 6 hours at room temperature (Salinas,  
145 Zalacain, Pardo & Alonso, 2004).

146 All grape samples were analyzed within 6.5 hours from grinding and thawing to avoid possible  
147 artefacts that may have arisen from reactions due to the acid conditions of the juice, to  
148 endogenous enzymes and to other biochemical events, such as fermentation (Kalua and Boss,  
149 2008; Salinas, Zalacain, Pardo & Alonso, 2004). However, as crushing grapes induces formation  
150 of some compounds that probably are not constitutive of grapes, the detected compounds were  
151 named ‘varietal and pre-fermentation related volatile compounds’ similarly to what was  
152 previously proposed by Coehlo and co-workers (2007), thus indicating both compounds  
153 constitutive of grapes and compounds which arise in the grape homogenate prior to fermentation.

154 After the stirring step, the bar was picked up from the aqueous grape homogenate, rinsed with  
155 distilled water, dried with paper, transferred into a glass thermal desorption tube and introduced  
156 into the thermal-desorption unit (TDU, Gerstel, Mulheim and der Ruhe, Germany) in the splitless  
157 mode. Thermal desorption was carried out with the following temperature program: 30 °C for

158 0.10 min, ramp rate of 120°C/min to 280°C, and 280°C for 1.00 min. The desorbed analytes were  
159 cryo-focused at 0 °C (maintained by the use of liquid CO<sub>2</sub>) in a PTV injector (CIS, Gerstel,  
160 Germany) for the total desorption time, then ramped at 12°C/s until 300°C and held at that  
161 temperature for 6.0 min. The analytes were separated on a DB-WAX column (J&W 122-7032; 30  
162 m \* 0.25 micron \* 0.25 mm ID), using He as gas-carrier at a flow rate of 1 ml/min. The GC/MS  
163 was an Agilent Technologies, GC 7890A, MS 5975C; the MS ionization energy was set at 70 eV  
164 and masses were acquired from 19 to 400 *m/z* in full scan acquisition mode. The oven GC initial  
165 temperature was set at 40 °C for 10 minutes, then at 180 °C with a ramp rate of 2.5 °C/min.  
166 Temperature increased to 200°C at 1°C/min and was maintained for 10 minutes. The transfer line  
167 temperature was 280 °C. After each desorption the magnetic stir bars were cleaned by immersion  
168 in methanol for 24 hours (stirring during the first hour).

169

### 170 *2.2.1 Qualitative analysis*

171 Volatile compounds were identified comparing mass spectra with the data system library (NIST-  
172 05a), by comparison with spectra found in literature (NIST Chemistry WebBook,  
173 [webbook.nist.gov/chemistry/](http://webbook.nist.gov/chemistry/)), and/or according with the volatile constituents found in previous  
174 studies on Nebbiolo grapes and isolated by means of liquid extraction (Di Stefano, Bottero,  
175 Pigella, Borsa, Bezzo & Corino, 1998). In the case of comparison with the data system library,  
176 positive characterization was accepted when a compound was identified with a probability higher  
177 than 85% in all replicates. Furthermore, for qualitative identification purposes, Kovats indices of  
178 identified compounds were calculated using an alkane standard mixture C10-C40 (Sigma-  
179 Aldrich, St. Louis, MO) as reference for retention times.

180

181 *2.2.2 Semi-quantitative analysis of volatiles*

182 Volatile compounds were quantified only when they were present in at least two replicates out of  
183 the three of each sample. Concentrations of each identified compound were calculated by  
184 comparing each compound peak area response to that of the internal standard; data were  
185 expressed as µg equivalents of 2-heptanol per kg of fresh berries. The ratio between favourable  
186 and unfavourable compounds was calculated by dividing the sum of the concentrations of  
187 volatiles which in the literature are considered favourable to the human senses and that of C6  
188 compounds, to whom unpleasant herbaceous notes are attributed. Favourable compounds  
189 included aliphatic aldehydes and alcohols other than C6, aromatic aldehydes, esters, terpenes,  
190 sesquiterpenes, benzene derivatives, lactones and norisoprenoids.

191

192 *2.3 Statistical analysis*

193 A separate extraction and analysis was done from each replicate. For each treatment, the data of  
194 the three replicates for each class of detected compounds were averaged and the standard error  
195 was calculated. Data collected at comparable phenological stages underwent an analysis of  
196 variance (Anova) to find out significant differences among locations (Duncan test for  $P \leq 0.05$   
197 and  $P \leq 0.01$ ). A principal component analysis (PCA) was performed on normalized data; all  
198 statistics were carried out with SAS 8.2 for Windows, (SAS Institute, Cary, USA).

199

200 **3. Results**

201 Some important meteorological differences were detected among the three growing locations.  
202 The vegetative season (April-October) in the Barbaresco area was characterized by a higher  
203 cumulated global solar radiation (4200 MJ/m<sup>2</sup> against 3382 and 3492 MJ/m<sup>2</sup> in Barolo and

204 Barbaresco, respectively). The vegetative period in the Roero area was cooler and received more  
205 rainfall than in the other two areas (1695 growing degree days base 10 °C, versus 1864 in  
206 Barbaresco and 1939 in Barolo; 626 mm rainfall, versus 561 in Barbaresco and 528 in Barolo).

207

### 208 3.1 Varietal and pre-fermentative volatile profiles

209 The varietal and pre-fermentative volatiles of Nebbiolo identified were 43, and could be grouped  
210 in 9 chemical classes: aliphatic aldehydes (8 compounds), aromatic aldehydes (2 compounds),  
211 aliphatic alcohols (7 compounds), monoterpenes (6 compounds), benzene derivatives (7  
212 compounds), esters (7 compounds), lactones (2 compounds), sesquiterpenes (2 compounds) and  
213 norisoprenoids (2 compounds; Table 1). C6 compounds (hexanal, (*E*)-2-hexenal, 1-hexanol, (*Z*)-  
214 3-hexenol and (*E*)-2-hexenol) were identified within aliphatic aldehydes and alcohols. The  
215 volatiles of Nebbiolo grapes were mostly common to the three growing areas, except a few  
216 compounds that were typical of the Roero vineyard, namely (*E, E*)-2,4-heptadienal, (*E*)-2-octen-  
217 1-ol, the norisoprenoid TDN and  $\gamma$ -butyrolactone (Tables 2, 3 and 4). Alpha-terpineol was  
218 exclusively found at 19 days post véraison (dpv) in grapes from Barbaresco (Table 2).

219 The majority of varietal and pre-fermentative volatiles found in Nebbiolo grapes have been  
220 previously identified in berries from other *Vitis* varieties except penthyl-hexanoate, butyl-  
221 hexadecanoate, two sesquiterpenes (longicyclene and junipene), and a furanone-type compound  
222 (Table 1). The two sesquiterpenes and the furanon-like compound were tentatively identified as  
223 such on the basis of NIST 05a; for all of them the probability of matching identification was  
224 around 99 %.

225

### 226 3.2 Accumulation trends of varietal and pre-fermentative volatiles during ripening

### 227 3.2.1 Total aldehydes and alcohols

228 Total aldehydes regularly accumulated throughout ripening in grapes from Barolo whereas in  
229 grapes from Barbaresco and Roero they reached a peak of maximum accumulation followed by a  
230 decrease (Fig. 1). Total aldehydes were the most abundant compounds from 20days post véraison  
231 (dpv), similarly to what was previously detected in neutral Monastrell grapes (Salinas, Zalacain &  
232 Pardo, 2004). Their concentration ranged from 30% before véraison to about 70% of total  
233 volatiles at the last sampling date. Among the detected aldehydes, C6 aldehydes (hexanal and  
234 (E)-2-hexenal) were the most abundant; the other aliphatic aldehydes were generally absent at the  
235 first sampling at véraison, but they started to accumulate in the period between 6 and 18 dpv  
236 (Tables 2, 3 and 4).

237 Aliphatic alcohols constantly accumulated in grapes from Barbaresco whereas they reached a  
238 peak of maximum concentration in grapes from Roero (at 25 dpv) and from Barolo (at 45 dpv,  
239 Fig. 1), followed by a decrease. At the first time point, independently from the growing area,  
240 aliphatic alcohols (except octanol and (E)-2-octen-1-ol, whose accumulation began later) were  
241 already present (Tables 2, 3 and 4). Among aliphatic alcohols, 1-hexanol, (Z)-3-hexenol and (E)-  
242 2-hexen-1-ol belong to the sub-group of C6 compounds whose concentration decreased during  
243 ripening in grapes from Roero (Table 4), whereas it increased in grapes from Barbaresco and  
244 Barolo (Tables 2 and 3). C6 aldehydes and alcohols are known to provide the green, grassy notes  
245 of many fruits (Klee, 2010). They are formed subsequently to the crushing of berries thanks to  
246 the berry constitutive lipoxygenase activity (Gunata, Bayonove, Baumes & Cordonnier, 1985).

247

### 248 3.2.2 Esters

249 The total ester content increased during ripening; ester biosynthesis started 25 dpv in grapes from  
250 Barbaresco and Barolo, earlier in grapes from Roero (Fig. 1). No ethyl esters were detected  
251 whereas methyl- and pentyl- esters of butanoic, nonanoic, decanoic and hexadecanoic acids  
252 were identified (Table 1). Among ethyl-esters we exclusively found ethyl-hexadecanoate in trace  
253 amounts and exclusively in grapes from the Roero area (Table 4). The fact that no ethyl-esters  
254 were detected in homogenates of Nebbiolo grapes could indicate that no fermentation of the juice  
255 started during grinding and extraction with the SBSE.

256 Some important differences were found in the type of accumulated esters: grapes from the Barolo  
257 area were characterized by the presence of ethyl-hexanoate, methyl-3-OH-butanoate and methyl-  
258 nonanoate (Table 3); conversely, grapes from Barbaresco accumulated methyl-decanoate and  
259 butyl-hexadecanoate (Table 2). At harvest, ester total concentrations were similar in grapes from  
260 Barbaresco and Barolo whereas quantities detected in Roero grapes were significantly lower (Fig.  
261 1; Table 4).

262

### 263 *3.2.3 Terpenes and sesquiterpenes*

264 The trend of total terpene accumulation in Nebbiolo showed important differences among  
265 vineyards. The biosynthesis of terpenes started before véraison in all vineyards but peaks of  
266 maximum accumulation were reached in different moments depending on the growing area (Fig.  
267 1). The importance and the sensorial impact of terpenes in floral grapes is well known (Gunata,  
268 Bayonove, Baumes & Cordonnier, 1985). Terpenes possess very pleasant ‘sweet’ and ‘floral’  
269 aromas and a very low olfactory threshold that allows them to be easily recognizable even at very  
270 low concentration. In non-floral cv Monastrell, Salinas and co-workers (2004) reported the  
271 existence of a series of non-glycosilated terpenes; in Nebbiolo grapes we identified D-limonene,

272  $\alpha$ -terpineol (a distinctive marker found exclusively in grapes from Barbaresco at 19 dpv, Table  
273 2), (*Z*)-citral,  $\beta$ -citronellool and (*E*)-geranylacetone (Tables 2, 3 and 4); in agreement with Salinas  
274 and co-workers (2004) no linalool was detected, showing that the existence of trace amounts of  
275 this terpene as varietal volatile should be more properly attributed to artefact formation after the  
276 activation of constitutive grape glycosidases, which were not activated with the method we used.  
277 Sesquiterpenes possess important biological roles as attractant of insects for pollination or as  
278 defence molecules against fungi. The most known sesquiterpene molecule investigated up to now  
279 in viticulture/enology is rotundone, associated to the pepper aroma of Shiraz wines (Wood, et al.,  
280 2008); although little is known about the implications on odour and taste induced by other  
281 sesquiterpene molecules in grapes and wines, some sesquiterpenes have recently been described  
282 in cv Baga (Rocha, Delgadillo & Coimbra, 2006), and in Cabernet Sauvignon and Riesling  
283 (Kalua & Boss, 2010). Sesquiterpene accumulation trend was very characteristic as no  
284 sesquiterpenes were detected before véraison; accumulation started since 20 dpv in fruit from  
285 Barolo and Barbaresco, and earlier, around véraison, in grapes from Roero (Fig. 1). These  
286 observations are in line with those previously reported by Coelho and co-workers (2006) in cv  
287 Baga where sesquiterpenes were detected from 14 dpv onwards and with the fact that *Vitis*  
288 *vinifera* sesquiterpene synthase transcript were exclusively detected during the last phases of  
289 berry ripening (Lücker, Bowen & Bohlmann, 2004).

290

#### 291 3.2.4 Norisoprenoids

292 The importance of norisoprenoids in grape aroma is well known; they have been intensively  
293 studied as glycosides in many varieties but they have also been reported as components of  
294 varietal and pre-fermentative flavours (Salinas, Zalacain, Pardo & Alonso, 2004; Coelho, Rocha,

295 Barros, Delgadillo & Coimbra, 2007; Kalua & Boss, 2010). Salinas and co-workers (2004)  
296 detected vomifoliol, 3-oxo- $\alpha$ -ionol and  $\beta$ -ionone whereas Coehlo and co-workers (2007) detected  
297  $\beta$ -damascenone and  $\beta$ -ionone and Kalua & Boss (2010) exclusively  $\beta$ -ionone at precise moments  
298 of ripening. In Nebbiolo grapes, except for trace amounts of TDN found in grapes from Roero at  
299 24 and 38 dpv (Tab. 4), we exclusively detected  $\beta$ -ionone (Table 2, 3 and 4), similarly to  
300 Pedroza, Zalacain, Lara and Salinas (2010) and Kalua & Boss (2010) did.  $\beta$ -ionone biosynthesis  
301 started before véraison and increased throughout ripening, peaking at 10 to 20 dpv depending on  
302 the cultivation site. From 20 dpv onwards, norisoprenoid concentration decreased in all  
303 vineyards. The incidence of norisoprenoids over total varietal and pre-fermentative volatile  
304 amount was very low, ranging from 0.014 to 2.6 %.

305

### 306 *3.2.5 Benzene derivatives and lactones*

307 Benzene derivatives were synthesized already before véraison; their accumulation trend was  
308 greatly influenced by the growing area (Fig. 1). At harvest, benzene derivatives accounted for 9.6  
309 % in grapes from Barbaresco, for 5.3 % in grapes from Barolo and for 7.3 % in grapes from  
310 Roero. Benzyl alcohol was exclusively detected in grapes from Barolo at harvest (Table 3). The  
311 accumulation of benzene derivatives peaked 20 dpv in grapes from Barbaresco and Roero,  
312 whereas a completely different behaviour characterized grapes from Barolo (Fig. 1).

313 A furanone-type compound was found in grapes from all three vineyards; Roero grapes were also  
314 characterized by the accumulation of  $\gamma$ -butyrolactone (Table 2, 3 and 4). The biosynthesis of  
315 lactones started before véraison; similar trends were detected in grapes from Barolo and Roero (a  
316 progressive slow increase of concentrations) whereas a peak of maximum accumulation was  
317 reached 45 dpv in grapes from Barbaresco (Fig. 1).

318

319 *3.2.6 Total varietal volatiles and favourable/unfavourable volatile compound ratios*

320 Total varietal volatiles showed an accumulation trend during ripening in grapes from Barolo and  
321 Barbaresco whereas a phase of plateau since 15 dpv characterised berries from the Roero area  
322 that globally accumulated lower quantities of varietal and pre-fermentative volatiles (Fig. 1).  
323 Total volatile maximum concentration was reached at about 45 dpv in grapes from Barbaresco  
324 and Barolo, earlier in grapes from Roero (Fig. 1). However, at harvest total amounts of volatiles  
325 in Roero grapes were similar to those of berries from the other two areas (Table 4). In grapes  
326 from Barolo a final concentration reduction was detected before harvest and this was due to the  
327 concomitant reduction of aliphatic alcohol, ester and terpene concentrations (Fig. 1). In Barolo  
328 and Barbaresco grapes, the maximum volatile varietal content occurred in a very short period,  
329 about 45 dpv, coincident with the highest values of the favourable/unfavourable compound ratio  
330 (Fig. 1). The ratio between favourable and unfavourable varietal and pre-fermentative volatiles,  
331 calculated since 20 dpv, when phenological stages were comparable among the studied  
332 vineyards, showed values ranging from 0.2 to 4.2. It was constantly over 1 in grapes from  
333 Barbaresco due to the lower amounts of C6 compounds and to the progressive accumulation of  
334 favourable compounds.

335

336 *3.2.7 PCA analysis*

337 To clarify possible relations among the different classes of compounds and identify general  
338 parameters able to distinguish growing locations, we performed a principal component analysis  
339 (PCA) on nine variable normalized data (Table 5); with the first three principal components  
340 (Prin) the model proposed justified 89 % of the total variance (Table 5). According to the

341 eigenvalues, 5 variables were associated to Prin 1, namely the percentages of norisoprenoids,  
342 volatile total concentrations, the percentages of benzene derivatives, total aldehydes and  
343 sesquiterpenes. One variable (the percentage of total terpenes) was associated to the second Prin;  
344 alcohols were positively associated to the third Prin, although with an eigenvalue slightly lower  
345 than 1 (0.97; Tab. 5).

346 By observing the distribution of individuals in the x-y-z graph (Fig. 2) we noticed that  
347 exclusively the first date of sampling was discriminated on Prin 1; the PCA model proposed was  
348 effective in the discrimination of samples from the Barbaresco area (except the first sampling), all  
349 individuals being well associated to the positive values of Prin 2 and thus characterized by higher  
350 terpene content incidence over total concentrations respect to grapes from the other locations.  
351 Moreover, samples from the Roero area were well grouped around nil values of Prin 2 (except the  
352 first sampling); the third principal component, that alone justified the 10 % of total variance, was  
353 not so effective in individual discrimination even though it partially discriminated some  
354 Barbaresco and Barolo cases associated to its positive values (Fig. 2).

355 As to variable correlation (Fig. 2) we found that the percentage of total aldehydes was negatively  
356 correlated with that of benzene derivatives ( $R = -0.96$ ), norisoprenoids ( $R = -0.80$ ) and terpenes  
357 ( $R = -0.86$ ); these last two in particular are classes of compounds that largely contribute to  
358 positive notes of berry aroma. Moreover, benzene derivatives, terpenes and norisoprenoids were  
359 positively correlated with each other, with correlation coefficients ( $R$ ) always higher than 0.7.

360

#### 361 **4. Discussion**

362 The flavour differences among 'Barbaresco', 'Barolo' and 'Roero' DOCG wines are well-known  
363 to wine consumers and they have also been characterized through GC/MS analysis and

364 multivariate statistical tools (Marengo, Aceto & Maurino, 2001). At the last sampling date (that  
365 was coincident with harvest for viticulturists) significant differences among the three vineyards  
366 were found not only for esters but also for aliphatic alcohols, terpenes, benzene derivatives and  
367 total volatile concentration (Table 6). Grapes from the Barbaresco area were distinguishable from  
368 those from Barolo and Roero and very often they showed similarity with those from the Barolo  
369 area. At the wine level Marengo, Aceto & Maurino (2001) pointed out that wines that underwent  
370 a more prolonged ageing (Barbaresco and Barolo) were associated with each other whereas they  
371 were quite distant from wines, such as those from Roero, that underwent a reduced ageing period;  
372 differences were tied to a series of compounds but the most implicated compounds were esters. In  
373 this study we show that Barbaresco and Barolo grapes accumulated more esters than Roero  
374 grapes, thus the differences previously detected at the wine level could have a previous origin in  
375 grapes rather than be exclusively due to the numerous fermentative reactions that induce ester  
376 formation during winemaking and ageing.

377 As shown from the interpretation of the PCA model, grapes from the Barbaresco area, except  
378 those of the first sampling date, were well separated on the second principal component axis,  
379 representing the incidence of terpenes over total volatiles; previous studies (Bureau, Razungles &  
380 Baumes 2000) reported that artificial bunch shading, by reducing illumination of clusters,  
381 decreased the concentration of free terpenols and norisoprenoids respect to sun-exposed berries.  
382 The Barbaresco growing area was characterized by a higher cumulated global radiation, and by  
383 the accumulation of higher quantities of varietal and pre-fermentative terpenes and  
384 norisoprenoids, confirming the fundamental role of light on of the biosynthesis of these  
385 secondary metabolites.

386 Elaboration of the PCA model proposed suggests that the first sampling date was distinguished  
387 from the others, and associated to the positive values of the first principal component axis (Fig.  
388 2), whereas the successive sampling dates were not discernable anymore, suggesting that at  
389 véraison the biosynthesis of varietal volatiles was not influenced by the growing area, but  
390 differences among areas occurred later. Thus the steps of flavour biosynthesis more influenced by  
391 climatic conditions seem to be successive to véraison, even though nothing can be said about the  
392 steps before véraison as we started sampling at véraison; the only study dealing with the  
393 accumulation of varietal volatiles before véraison concluded that a crucial step of volatile  
394 biosynthesis occurs around 4 weeks after flowering in Cabernet Sauvignon and Riesling (Kalua &  
395 Boss, 2010). Our data suggest that at véraison there are no crucial steps in Nebbiolo grape flavour  
396 biosynthesis but they take place later and the growing area largely influences them.

397 The evolution of volatile classes during ripening suggests some further general patterns of  
398 biosynthesis. The negative correlation between aldehydes (on average 91 % represented by C6  
399 compounds) on one side and terpenes, norisoprenoids and benzene derivatives on the other, could  
400 be an analytical evidence of the fact that the accumulation of 'negative' or 'positive' varietal  
401 volatile is antithetical. The positive correlation terpenes/norisoprenoids ( $R = 0.70$ ) could be  
402 explained as an analytical evidence of the known common steps of monoterpenes and carotenoids  
403 (the precursor of norisoprenoids) biosynthetic pathways (Mathieu, Wirth, Sauvage, Lepoutre,  
404 Baumes & Gunata, 2009).

405 The use of crushed grapes together with prolonged time of SBSE/grape homogenate contact have  
406 probably promoted the formation of C6 compounds as it was the case in other studies using  
407 SBSE or SPME techniques and implying grinding. C6 compounds accounted for 14 to 71 % of  
408 total varietal volatiles with increasing values from véraison to harvest in Barolo and Barbaresco

409 grapes; in Roero grapes the incidence of C6 compounds over total was already high (49 %) at the  
410 first sampling and it increased to 69 % at harvest. At harvest, independently from the cultivation  
411 area, C6 compounds represented the major class of grape volatiles, consistent with previous  
412 findings (Kalua & Boss, 2009; Kalua & Boss, 2010; Yang et al., 2009). Temporal analysis of the  
413 ratio between favourable and unfavourable (C6 aldehydes and alcohols) compounds shows that in  
414 grapes from Barbaresco and Barolo the optimum values were reached at 42 and 44 dpv,  
415 respectively, thus about 15 days before the date of effective harvest. Oliveira, Faria, Sá, Barros &  
416 Araújo (2006) discovered the effectiveness of C6 compounds, particularly of the ratio (E)-3-  
417 hexenol/(Z)-3-hexenol in discriminating wine origin in monovarietal wines. In Nebbiolo we  
418 found, besides hexanol, (Z)-3-hexenol and (E)-2-hexen-1-ol, and we were thus able to calculate  
419 their ratio, which at harvest was 0.6 for Roero, 1.2 for Barolo and 1.3 for Barbaresco grapes. The  
420 classificatory capacity of this ratio is thus evident and once more an association between  
421 Barbaresco and Barolo grapes on one side and Roero grapes on the other, emerges. Nevertheless  
422 further studies will confirm the capability of ratios such as this to discriminate growing locations.  
423 Calculation of this or other ratios could represent an important start point for finding in the wide  
424 range of varietal and pre-fermentative volatiles some target compounds that could be considered  
425 as a possible ‘fingerprints’, not only of the growing locations but also of the variety. Further  
426 studies are ongoing in our lab on other varieties and over more years.

427

#### 428 **4. Conclusions**

429 The SBSE was effectively used for the first time to detect varietal and pre-fermentative volatiles  
430 in grapes of cv Nebbiolo, an important neutral Italian variety, base cultivar for the production of

431 Barbaresco, Barolo and Roero wines. The influence exerted by the growing location on volatile  
432 concentration and profile was analysed, as well as on the timing of volatile biosynthesis.  
433 One of the main aims of this study was to verify the existence of differences in the volatile profile  
434 of the same genotype, grown in different areas or terroirs. The results show that, even in a  
435 relatively homogenous geographic area like southern Piedmont, the growing locations induce  
436 differences in the concentration and accumulation trend of several classes of compounds, in  
437 particular esters. On the contrary, certain classes of compounds such as sesquiterpenes and  
438 norisoprenoids showed no differences in the pattern of accumulation in the different areas.  
439 Varietal and pre-fermentative volatiles seemed to be more effective in separating growing areas  
440 than dates of sampling: as a matter of fact grapes from the Barbaresco area, showing higher  
441 values of the favourable/unfavourable compound ratio throughout ripening, were separated from  
442 grapes from the other areas.  
443 The concentrations of certain classes of compounds such as terpenes, norisoprenoids and benzene  
444 derivatives were generally correlated among them so that this opens the possibility to follow the  
445 ‘aromatic ripening’ of grapes by following the evolution of a unique class of compounds.  
446 Further studies, over more years and also on global aroma potential, i.e. including glyco-  
447 conjugate volatile detection, will be necessary to better identify Nebbiolo varietal volatiles, to  
448 deepen knowledge about volatile concentrations and profiles and to investigate their implications  
449 on wine quality as influenced by the growing area.

450  
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458

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543

544 CAPTIONS

545 Table 1 - Varietal and pre-fermentative volatile compounds found in *Vitis vinifera* cv Nebbiolo  
546 grapes by SBSE/GC-MS.

547  
548 Table 2 – Evolution of varietal and pre-fermentative volatiles in *Vitis vinifera* cv Nebbiolo grapes  
549 (as  $\mu\text{g g}^{-1}$  fresh berries) from the Barbaresco area, during ripening. Mean values of three  
550 replicates  $\pm$  standard errors. Dpv = days post-véraison; SSC = soluble solid content; ND = not  
551 detected.

552  
553 Table 3 – Evolution of varietal and pre-fermentative volatiles in *Vitis vinifera* cv Nebbiolo grapes  
554 (as  $\mu\text{g g}^{-1}$  fresh berries) from the Barolo area, during ripening. Mean values of three replicates  $\pm$   
555 standard errors. Dpv = days post-véraison; SSC = soluble solid content; ND = not detected.

556  
557 Table 4 – Evolution of varietal and pre-fermentative volatiles in *Vitis vinifera* cv Nebbiolo grapes  
558 (as  $\mu\text{g g}^{-1}$  fresh berries) from the Roero area, during ripening. Mean values of three replicates  $\pm$   
559 standard errors. Dpv = days post-véraison; SSC = soluble solid content; ND = not detected.

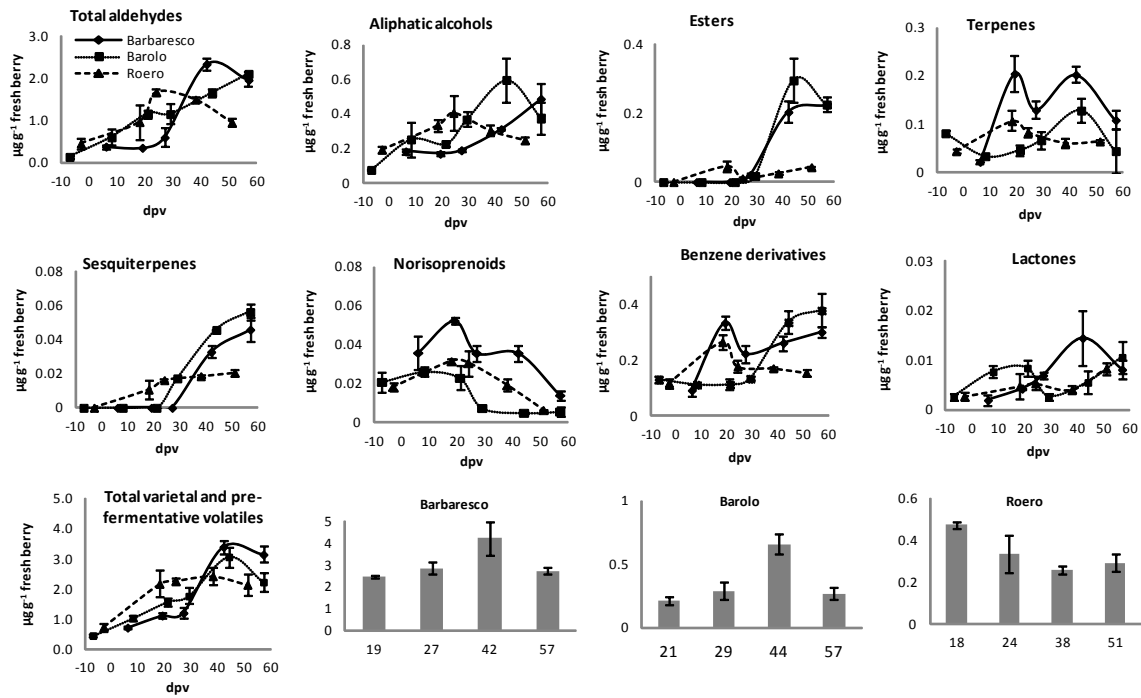
560  
561 Table 5 - Eigenvectors of the examined variables on the three principal components (Prin 1, Prin  
562 2 and Prin 3). Eigenvalues of the three Prins and their contribution to total variance. In bold  
563 letters the variables associated to the appropriate Prin.

564  
565 Table 6 – Analysis of variance of averages of the main classes of varietal and pre-fermentative  
566 volatiles of *Vitis vinifera* cv Nebbiolo grapes from three different growing locations at

567 comparable phenological stages (1 = 19 days post véraison, dpv; 2 = 27 dpv; 3 = 41 dpv; 4 = 55  
568 dpv). Within the same phenological stage, averages were subjected to the analysis of variance  
569 and means were separated by the Duncan's test. Means followed by different letters are  
570 significantly different for  $P \leq 0.05$  (lowercase letters) and  $P \leq 0.01$  (uppercase letters).

571  
572 Figure 1 – Accumulation trend of the main classes of varietal and pre-fermentative volatiles in  
573 *Vitis vinifera* cv Nebbiolo grapes from three different growing areas in Piedmont (North-West  
574 Italy). Average values of three replicates  $\pm$  standard errors. Histograms of the  
575 favourable/unfavourable compound ratio at similar phenological stages.

576  
577 Figure 2 – Distribution of individuals on the x-y-z axis. Bsc0 = Barbaresco; B = Barolo; R =  
578 Roero. The numbers after the acronym indicating the growing area stand for the different picking  
579 times. x-y plot of the nine variables used to run the principal component analysis. See footnote of  
580 Table 5 for variable identification.

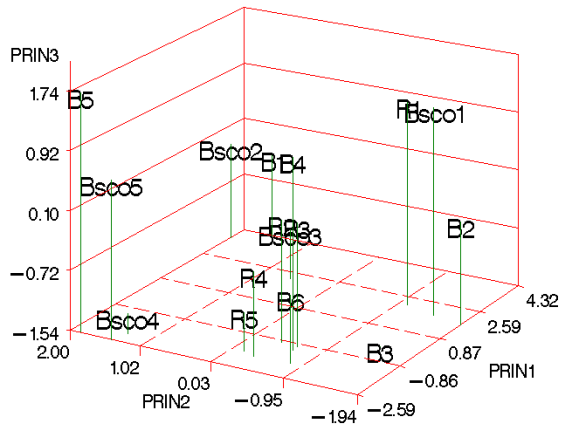


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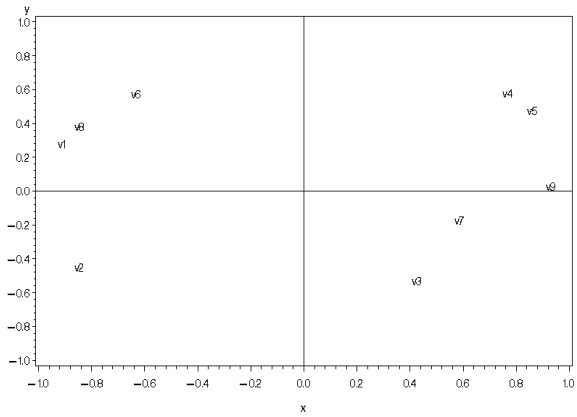
582 Figure 1

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584



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Figure 2

588

	Compound	KI	Reference	
Aliphatic aldehydes	Hexanal <sup>1</sup>	1064	Kalua & Boss, 2010	
	( <i>E</i> )-2-hexenal <sup>1</sup>	1213	Kalua & Boss, 2010	
	octanal	1291	Kalua & Boss, 2010	
	( <i>Z</i> )-2-heptenal	1319	Caven-Quantrill & Buglass, 2007	
	( <i>E,E</i> )-2,4-heptadienal	1485	Yang et al., 2011	*
	decanal	1497	Salinas et al., 2004	
	( <i>E</i> )-2-nonenal	1530	Ruberto et al., 2008	
	( <i>E,Z</i> )-2,6-nonadienal	1580	Sotiroudis et al., 2009 in cucumber	
Aromatic aldehydes	furfural	1457	Caven-Quantrill & Buglass, 2007	
	benzaldehyde	1510	Sefton et al., 1993	
Aliphatic alcohols	1-butanol	1159	Caven-Quantrill & Buglass, 2007	
	1-hexanol <sup>1</sup>	1363	Kalua & Boss, 2010	
	( <i>Z</i> )-3-hexenol <sup>1</sup>	1387	Sefton et al., 1993	
	( <i>E</i> )-2-hexen-1-ol <sup>1</sup>	1410	Coehlo et al., 2007	
	2-ethyl-hexanol	1501	Caven-Quantrill & Buglass, 2007	
	1-octanol	1570	Yang et al., 2009	
	( <i>E</i> )-2-octen-1-ol	1628	La Guerche et al., 2006 from <i>Botrytis cinerea</i> infected grapes	*
Terpenes	D-limonene	1085	Coehlo et al., 2007	
	B-cyclocitral	1608	Coehlo et al., 2006	*
	isomenthol	1648	Caven-Quantrill & Buglass, 2007	
	( <i>E</i> )-geranylacetone	1662	Salinas et al., 2004	
	$\alpha$ -terpineol	1707	Sefton et al., 1993	*
	$\beta$ -citronellol	1781	Coehlo et al., 2007	
	Benzene derivatives	acetophenone	1640	Caven-Quantrill & Buglass, 2007
benzyl alcohol		1686	Sefton et al., 1993	*
benzothiazole		1898	Sefton et al., 1993	
phenol		1831	Sefton et al., 1993	
2-phenoxy ethanol (rose ether)		2105	Caven-Quantrill & Buglass, 2007	
para-buthyl-cresol		2053	Sefton et al., 1993	
trimethyl-tetrahydro-benzofuranone		2124	Caven-Quantrill & Buglass, 2007	
Esters		ethyl-hexanoate	1240	Yang et al., 2009; Ruberto et al., 2008
	methyl-3-OH-butanoate	1483	Yang et al.[6]	*
	methyl-nonanoate	1495		*
	pentyl-hexanoate	1515		
	methyl-decanoate	2035		*
	methyl-hexadecanoate	2061	Caven-Quantrill & Buglass, 2007	
	butyl-hexadecanoate	2253		*
	Lactones	$\gamma$ -butyrolactone	1609	Sefton et al., 1993
furanone type compound		1710		
Sesquiterpenes	sesquiterpene 1	1480		
	sesquiterpene 2	1551		
Norisoprenoids	TDN	1735	Sefton et al., 1993 after H <sup>+</sup> hydrolysis	*
	$\beta$ -ionone	1740	Salinas et al., 2004	

Table 1 KI = Kovats index. (1) Compounds attributable to the sub-group of C6 compounds. Compound identification was performed by comparison with mass spectra given by NIST05a library or with available spectra in literature. Compounds followed by the asterisk were detected exclusively in grapes from one vineyard. Sesquiterpenes 1 and 2 were tentatively identified as longicyclene and junipene, respectively.

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SSC (°Brix)		6 dpv 11.3	19 dpv 18.4	27 dpv 19.3	42 dpv 24.1	57 dpv 22.0
Aliphatic aldehydes	hexanal	0.0895 ± 0.0104	0.0038 ± 0.0008	0.0089 ± 0.0040	0.6815 ± 0.1051	0.6677 ± 0.0403
	(E)-2-hexenal	0.1595 ± 0.0328	0.1136 ± 0.0130	0.3979 ± 0.2153	1.1076 ± 0.2763	0.9895 ± 0.0547
	octanal	0.0029 ± 0.0016	ND	0.0074 ± 0.0032	0.0064 ± 0.0004	0.0317 ± 0.0112
	(Z)-2-heptenal	0.0655 ± 0.0225	0.0626 ± 0.0205	0.0528 ± 0.0066	0.0167 ± 0.0050	ND
	(E,E)-2,4-heptadienal	ND	ND	ND	ND	ND
	decanal	ND	ND	ND	0.0111 ± 0.0001	0.0273 ± 0.0086
	(E)-2-nonenal	ND	ND	ND	0.0838 ± 0.0102	0.0832 ± 0.0140
	(E,Z)-2,6-nonadienal	ND	ND	0.0043 ± 0.0024	0.0504 ± 0.0132	0.0440 ± 0.0093
	furfural	0.0660 ± 0.0153	0.1737 ± 0.0386	0.1295 ± 0.0033	0.3737 ± 0.2831	0.0974 ± 0.0278
Aromatic aldehydes	benzaldehyde	0.0039 ± 0.0008	0.0054 ± 0.0000	0.0033 ± 0.0011	0.0098 ± 0.0017	0.0230 ± 0.0142
	1-butanol	0.0159 ± 0.0016	0.0047 ± 0.0020	0.0043 ± 0.0008	0.0197 ± 0.0161	0.0447 ± 0.0127
Aliphatic alcohols	1-hexanol	0.0580 ± 0.0174	0.0881 ± 0.0109	0.0634 ± 0.0035	0.1318 ± 0.0182	0.1213 ± 0.0097
	(Z)-3-hexenol	0.0607 ± 0.0071	0.0299 ± 0.0023	0.0208 ± 0.0021	0.0607 ± 0.0069	0.1342 ± 0.0434
	(E)-2-hexen-1-ol	0.0423 ± 0.0041	0.0382 ± 0.0066	0.0753 ± 0.0136	0.0700 ± 0.0014	0.1090 ± 0.0231
	2-ethyl-hexanol	0.0068 ± 0.0010	0.0092 ± 0.0002	0.0103 ± 0.0021	0.0195 ± 0.0014	0.0570 ± 0.0104
	1-octanol	ND	ND	0.0164 ± 0.0091	0.0108 ± 0.0008	0.0218 ± 0.0075
	(E)-2-octen-1-ol	ND	ND	ND	ND	ND
	isomenthol	ND	ND	ND	0.0071 ± 0.0007	0.0070 ± 0.0015
	D-limonene	0.0085 ± 0.0016	0.0235 ± 0.0071	0.0162 ± 0.0043	0.0126 ± 0.0030	0.0233 ± 0.0060
Terpenes	β-cyclocitral	ND	0.0550 ± 0.0138	0.0168 ± 0.0139	ND	ND
	α-terpineol	ND	ND	0.0234 ± 0.0017	ND	ND
	β-citronellol	ND	0.0181 ± 0.0074	0.0162 ± 0.0050	ND	ND
	(E)-geranylacetone	0.0147 ± 0.0018	0.1087 ± 0.0175	0.0575 ± 0.0133	0.1836 ± 0.1250	0.0792 ± 0.0186
	acetophenone	0.0588 ± 0.0141	0.1245 ± 0.0066	0.0629 ± 0.0077	0.1279 ± 0.0145	0.1610 ± 0.0104
	benzyl alcohol	ND	ND	ND	ND	ND
	benzothiazole	ND	0.0153 ± 0.0028	0.0356 ± 0.0248	0.0145 ± 0.0005	0.0116 ± 0.0027
	phenol	0.0119 ± 0.0017	0.0238 ± 0.0037	0.0135 ± 0.0011	ND	ND
	2-phenoxy ethanol (rose ether)	0.0163 ± 0.0081	0.1272 ± 0.0026	0.0823 ± 0.0101	0.0789 ± 0.0059	0.0865 ± 0.0122
Benzene derivatives	para-buthyl-cresol	ND	0.0159 ± 0.0007	0.0104 ± 0.0026	0.0413 ± 0.0004	0.0424 ± 0.0071
	trimetil-tetrahydro-benzofuranone	0.0061 ± 0.0011	0.0276 ± 0.0035	0.0198 ± 0.0009	ND	ND
	ethyl-hexanoate	ND	ND	0.0058 ± 0.0008	ND	ND
	methyl-3-OH-butanoate	ND	ND	ND	ND	ND
	methyl-nonanoate	ND	ND	ND	ND	ND
	pentyl-hexanoate	ND	ND	ND	0.0143 ± 0.0024	0.0119 ± 0.0061
	methyl-hexadecanoate	ND	ND	ND	0.0390 ± 0.0022	0.0331 ± 0.0150
	butyl-hexadecanoate	ND	ND	ND	0.1024 ± 0.0023	0.0927 ± 0.0163
	γ-butyrolactone	ND	ND	ND	ND	ND
Lactones	furanone type compound	0.0020 ± 0.0120	0.0043 ± 0.0001	0.0700 ± 0.0006	0.0354 ± 0.0216	0.0082 ± 0.0018
Sesquiterpenes	sesquiterpene 1	ND	ND	ND	0.0082 ± 0.0004	0.0073 ± 0.0011
	sesquiterpene 2	ND	ND	ND	0.0245 ± 0.0026	0.0385 ± 0.0068
	Norisoprenoids	TDN	ND	ND	ND	ND
β-ionone		0.0359 ± 0.0088	0.0524 ± 0.0015	0.0357 ± 0.0040	0.0358 ± 0.0034	0.0141 ± 0.0025

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Table 2

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SSC (*Brix)		-7 dpv 6.6	8 dpv 14.8	21 dpv 21.4	29 dpv 22.6	44 dpv 24.5	57 dpv 24.5	
Aliphatic aldehydes	hexanal	0.0231 ± 0.0049	0.1167 ± 0.0425	0.4282 ± 0.0470	0.3551 ± 0.1121	0.5293 ± 0.0201	0.8161 ± 0.1412	
	(E)-2-hexenal	ND	0.4245 ± 0.1363	0.6484 ± 0.0360	0.7062 ± 0.1248	0.8282 ± 0.0144	0.8863 ± 0.1343	
	octanal	0.0035 ± 0.0004	0.0265 ± 0.0131	0.0231 ± 0.0024	0.0056 ± 0.0000	0.0546 ± 0.0136	0.0816 ± 0.0639	
	(Z)-2-heptenal	0.1122 ± 0.0346	0.0381 ± 0.0058	0.0355 ± 0.0098	0.0159 ± 0.0008	0.0000 ± 0.0000	0.0000 ± 0.0000	
	(E,E)-2,4-heptadienal	ND	ND	ND	ND	ND	ND	
	decanal	ND	ND	ND	0.0055 ± 0.0001	0.0749 ± 0.0423	0.1361 ± 0.1149	
	(E)-2-nonenal	ND	ND	ND	0.0465 ± 0.0003	0.0535 ± 0.0113	0.0484 ± 0.0267	
	(E,Z)-2,6-nonadienal	ND	ND	0.0230 ± 0.0126	0.0078 ± 0.0018	0.0271 ± 0.0137	0.0133 ± 0.0008	
	furfural	ND	ND	ND	0.0057 ± 0.0010	0.0552 ± 0.0091	0.0834 ± 0.0158	
	benzaldehyde	0.0049 ± 0.0025	0.0020 ± 0.0003	0.0062 ± 0.0009	0.0052 ± 0.0008	0.0253 ± 0.0089	0.0210 ± 0.0114	
Aliphatic alcohols	1-butanol	0.0100 ± 0.0057	ND	ND	0.0156 ± 0.0008	0.0405 ± 0.0036	0.0430 ± 0.0297	
	1-hexanol	0.0123 ± 0.0007	0.0936 ± 0.0491	0.1136 ± 0.0064	0.1288 ± 0.0194	0.1561 ± 0.0366	0.1668 ± 0.0240	
	(Z)-3-hexenol	0.0536 ± 0.0068	0.0548 ± 0.0161	0.0390 ± 0.0132	0.0469 ± 0.0009	0.1628 ± 0.0485	0.2631 ± 0.1761	
	(E)-2-hexen-1-ol	ND	0.0970 ± 0.0419	0.0697 ± 0.0025	0.1537 ± 0.0214	0.1671 ± 0.0217	0.1725 ± 0.0722	
Terpenes	2-ethyl-hexanol	0.0009 ± 0.0009	0.0068 ± 0.0005	0.0018 ± 0.0001	0.0138 ± 0.0001	0.0462 ± 0.0151	0.0850 ± 0.0664	
	1-octanol	ND	0.0011 ± 0.0011	0.0025 ± 0.0025	0.0096 ± 0.0003	0.0245 ± 0.0065	0.0496 ± 0.0443	
	(E)-2-octen-1-ol	ND	ND	ND	ND	ND	ND	
	isomenthol	ND	ND	ND	0.0034 ± 0.0023	0.0088 ± 0.0001	0.0100 ± 0.0072	
	D-limonene	0.0087 ± 0.0038	0.0073 ± 0.0030	0.0079 ± 0.0026	0.0291 ± 0.0066	0.0151 ± 0.0016	0.0637 ± 0.0433	
	β-cyclocitral	ND	ND	ND	ND	ND	ND	
	α-terpineol	ND	ND	ND	ND	ND	ND	
	β-citronellol	ND	0.0038 ± 0.0022	0.0134 ± 0.0029	0.0025 ± 0.0004	0.0000 ± 0.0000	0.2493 ± 0.2460	
	(E)-geranylacetone	0.0735 ± 0.0076	0.0238 ± 0.0037	0.0262 ± 0.0055	0.0329 ± 0.0085	0.1053 ± 0.0210	0.0867 ± 0.0700	
	acetophenone	0.0566 ± 0.0058	0.0434 ± 0.0010	0.0573 ± 0.0099	0.0592 ± 0.0022	0.1775 ± 0.0190	0.1826 ± 0.1302	
Benzene derivatives	benzyl alcohol	ND	ND	ND	ND	ND	0.0236 ± 0.0149	
	benzothiazole	0.0007 ± 6.89E-05	0.0016 ± 0.00064	0.0020 ± 0.00088	0.0065 ± 0.0005	0.0125 ± 0.0018	0.0169 ± 0.0094	
	phenol	0.0104 ± 0.0019	0.0095 ± 0.0008	0.0093 ± 0.0019	0.0141 ± 0.0036	0.0127 ± 0.0127	0.0424 ± 0.0275	
	2-phenoxy ethanol (rose ether)	0.0564 ± 0.0098	0.0480 ± 0.0018	0.0298 ± 0.0050	0.0467 ± 0.0015	0.0946 ± 0.0128	0.0876 ± 0.0581	
	para-butyl-cresol	ND	0.0034 ± 0.0005	0.0067 ± 0.0016	0.0080 ± 0.0003	0.0392 ± 0.0063	0.0256 ± 0.0153	
	trimetil-tetrahydro-benzofuranon	0.0077 ± 0.0038	0.0071 ± 0.0014	0.0089 ± 0.0007	ND	ND	ND	
	ethyl-hexanoate	ND	ND	ND	ND	ND	ND	
	methyl-3-OH-butanoate	ND	ND	ND	ND	0.0791 ± 0.0385	0.0207 ± 0.0113	
	methyl-nonanoate	ND	ND	ND	ND	0.0843 ± 0.0201	0.0550 ± 0.0432	
	penthyl-hexanoate	ND	ND	ND	0.0176 ± 0.0028	0.104 ± 0.0096	0.11444 ± 0.1107	
Esters	methyl-hexadecanoate	ND	ND	ND	ND	0.0285 ± 0.0096	0.0085 ± 0.0041	
	butyl hexadecanoate	ND	ND	ND	ND	ND	ND	
	γ-butyrolactone	ND	ND	ND	ND	ND	ND	
	furanone type compound	0.0026 ± 0.0008	0.0077 ± 0.0011	0.0085 ± 0.0015	0.0027 ± 0.0007	0.0056 ± 0.0024	0.0106 ± 0.0027	
	Sesquiterpenes	sesquiterpene 1	ND	ND	ND	0.0023 ± 0.0006	0.0019 ± 0.0007	0.0017 ± 0.0012
		sesquiterpene 2	ND	ND	ND	0.0149 ± 0.0000	0.0438 ± 0.0012	0.0543 ± 0.0436
		TDN	ND	ND	ND	ND	ND	ND
	Norisoprenoids	β-ionone	0.0209 ± 0.0053	0.0261 ± 0.0025	0.0230 ± 0.0064	0.0075 ± 0.0002	0.0051 ± 0.0005	0.0057 ± 0.0022

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596 Table 3

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SSC (*Brix)		-3 dpv 12.6	18 dpv 19.9	24 dpv 20.6	38 dpv 23.6	51 dpv 24.3
Aliphatic aldehydes	hexanal	0.097 ± 0.044	0.432 ± 0.064	0.422 ± 0.054	0.776 ± 0.134	0.566 ± 0.136
	(E)-2-hexenal	0.218 ± 0.075	0.772 ± 0.232	0.963 ± 0.141	0.899 ± 0.116	0.908 ± 0.174
	octanal	ND	0.018 ± 0.007	0.007 ± 0.001	0.005 ± 0.002	0.004 ± 0.000
	(Z)-2-heptenal	0.042 ± 0.002	0.062 ± 0.009	0.039 ± 0.006	0.025 ± 0.009	0.015 ± 0.005
	(E,E)-2,4-heptadienal	ND	0.006 ± 0.003	0.005 ± 0.001	0.004 ± 0.001	0.000 ± 0.000
	decanal	ND	0.033 ± 0.022	0.008 ± 0.001	0.005 ± 0.003	0.006 ± 0.000
	(E)-2-nonenal	ND	0.045 ± 0.023	0.035 ± 0.018	0.059 ± 0.017	0.039 ± 0.002
	(E,Z)-2,6-nonadienal	ND	0.034 ± 0.017	0.036 ± 0.011	0.026 ± 0.010	0.036 ± 0.001
	furfural	ND	0.038 ± 0.017	0.018 ± 0.014	0.016 ± 0.012	0.018 ± 0.011
	benzaldehyde	ND	0.026 ± 0.009	0.013 ± 0.002	0.014 ± 0.003	0.006 ± 0.000
Aliphatic alcohols	1-butanol	0.011 ± 0.003	0.008 ± 0.004	0.020 ± 0.003	0.007 ± 0.004	0.014 ± 0.002
	1-hexanol	0.073 ± 0.009	0.105 ± 0.015	0.191 ± 0.068	0.163 ± 0.020	0.108 ± 0.012
	(Z)-3-hexenol	0.066 ± 0.006	0.087 ± 0.016	0.048 ± 0.004	0.023 ± 0.007	0.031 ± 0.000
	(E)-2-hexen-1-ol	0.034 ± 0.006	0.089 ± 0.030	0.110 ± 0.035	0.075 ± 0.016	0.059 ± 0.007
	2-ethyl-hexanol	0.011 ± 0.001	0.024 ± 0.006	0.014 ± 0.001	0.015 ± 0.001	0.016 ± 0.000
	1-octanol	ND	0.013 ± 0.003	0.011 ± 0.002	0.013 ± 0.001	0.013 ± 0.003
	(E)-2-octen-1-ol	ND	0.009 ± 0.005	0.011 ± 0.001	0.006 ± 0.001	0.007 ± 0.000
	isomenthol	ND	0.003 ± 0.002	0.004 ± 0.002	0.009 ± 0.004	0.007 ± 0.003
	D-limonene	0.029 ± 0.003	0.023 ± 0.013	0.023 ± 0.004	0.019 ± 0.010	0.020 ± 0.003
	β-cyclocitral	ND	0.012 ± 0.002	0.008 ± 0.001	0.006 ± 0.001	0.001 ± 0.000
Terpenes	α-terpineol	ND	ND	ND	ND	ND
	β-citronellol	ND	0.005 ± 0.003	0.005 ± 0.000	0.005 ± 0.001	0.004 ± 0.001
	(E)-geranylacetone	0.016 ± 0.002	0.063 ± 0.003	0.044 ± 0.001	0.023 ± 0.005	0.033 ± 0.002
	acetophenone	0.054 ± 0.006	0.089 ± 0.013	0.067 ± 0.010	0.069 ± 0.008	0.067 ± 0.003
	benzyl alcohol	ND	ND	ND	ND	ND
	benzothiazole	ND	0.007 ± 0.001	0.007 ± 0.001	0.009 ± 0.000	0.003 ± 0.001
	phenol	0.010 ± 0.001	0.042 ± 0.024	0.013 ± 0.001	0.014 ± 0.001	0.027 ± 0.007
	2-phenoxy ethanol (rose ether)	0.043 ± 0.010	0.266 ± 0.152	0.075 ± 0.011	0.062 ± 0.006	0.042 ± 0.002
	para-butyl-cresol	ND	0.025 ± 0.015	0.008 ± 0.000	0.009 ± 0.001	0.010 ± 0.001
	trimetil-tetrahydro-benzofuranone	0.011 ± 0.001	0.040 ± 0.027	0.010 ± 0.001	0.008 ± 0.001	0.006 ± 0.000
Esters	ethyl-hexanoate	ND	ND	ND	ND	ND
	methyl-3-OH-butanoate	ND	ND	ND	ND	ND
	methyl-nonanoate	ND	ND	ND	ND	ND
	pentyl-hexanoate	0.000 ± 0.000	0.040 ± 0.020	0.011 ± 0.003	0.025 ± 0.004	0.028 ± 0.001
	methyl-hexadecanoate	ND	ND	ND	ND	0.002 ± 0.000
	butyl-hexadecanoate	ND	ND	ND	ND	0.013 ± 0.003
Lactones	γ-butyrolactone	ND	ND	0.001 ± 0.000	0.004 ± 0.002	ND
	furanone type compound	0.003 ± 0.001	0.005 ± 0.002	0.005 ± 0.001	0.004 ± 0.001	0.008 ± 0.001
Sesquiterpenes	sesquiterpene 1	ND	0.002 ± 0.001	0.003 ± 0.000	0.002 ± 0.001	0.004 ± 0.000
	sesquiterpene 2	ND	0.009 ± 0.005	0.014 ± 0.001	0.016 ± 0.001	0.016 ± 0.002
Norisoprenoids	TDN	ND	ND	0.007 ± 0.000	0.006 ± 0.001	ND
	β-ionone	0.018 ± 0.002	0.032 ± 0.002	0.023 ± 0.006	0.013 ± 0.003	0.006 ± 0.000

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Table 4

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	Prin 1	Prin 2	Prin 3
V1	0.28	<b>0.48</b>	0.09
V2	0.18	<b>0.60</b>	0.12
V3	<b>-0.39</b>	0.15	0.09
V4	0.30	0.41	-0.25
V5	<b>-0.39</b>	0.08	-0.17
V6	<b>0.39</b>	-0.29	-0.08
V7	-0.23	0.11	-0.35
V8	<b>-0.40</b>	0.04	0.06
V9	0.02	-0.05	<b>0.85</b>
V10	<b>0.34</b>	-0.35	-0.13
Eigenvalues	5.25	2.13	1.16
Total variance	0.52	0.21	0.11

V1 = total varietal and pre-fermentative volatile concentration of *Vitis vinifera* cv Nebbiolo; V2-V9 = percentages of esters, terpenes, sesquiterpenes, benzene derivatives, C6 compounds, other aliphatic aldehydes and alcohols, norisoprenoids, lactones, respectively; V10 = ratio between C6 and the sum of esters, terpenes, sesquiterpenes and norisoprenoids.

Table 5

Total aldehydes				Aliphatic alcohols			Esters			Terpenes		
	Barbaresco	Barolo	Roero	Barbaresco	Barolo	Roero	Barbaresco	Barolo	Roero	Barbaresco	Barolo	Roero
F <sub>1</sub>	0.36 b	1.16 a	1.47 a	0.17 bB	0.23 bAB	0.34 aA	0.00 b	0.00 b	0.04 a	0.21 aA	0.05 bB	0.11 bAB
F <sub>2</sub>	0.60 b	1.16 ab	1.55 a	0.19	0.37	0.41	0.02	0.02	0.01	0.13 a	0.07 b	0.08 ab
F <sub>3</sub>	2.34	1.66	1.83	0.31	0.60	0.30	0.20 aAB	0.30 aA	0.03 bB	0.20	0.13	0.06
F <sub>4</sub>	1.96	2.11	1.60	0.49 a	0.37 ab	0.25 b	0.22 aA	0.23 aA	0.04 bB	0.11 a	0.05 b	0.07 ab
Sesquiterpenes				Benzene derivatives			Norisoprenoids			Lactones		
	Barbaresco	Barolo	Roero	Barbaresco	Barolo	Roero	Barbaresco	Barolo	Roero	Barbaresco	Barolo	Roero
F <sub>1</sub>	0.00	0.00	0.01	0.33 aA	0.11 bB	0.27 aA	0.05 aA	0.02 bB	0.03 bAB	0.004	0.008	0.005
F <sub>2</sub>	0.00 b	0.02 aA	0.02 aA	0.22	0.13	0.18	0.04 a	0.01 b	0.03 a	0.007 a	0.003 b	0.005 ab
F <sub>3</sub>	0.03 bB	0.05 aA	0.02 cC	0.26 ab	0.34 a	0.17 b	0.04 aA	0.01 bB	0.02 cB	0.015 a	0.006 ab	0.004 b
F <sub>4</sub>	0.05 aA	0.06 aA	0.02 bB	0.30 a	0.38 a	0.16 b	0.01	0.01	0.01	0.008	0.011	0.008
Total varietal and pre-fermentative volatiles												
	Barbaresco	Barolo	Roero									
F <sub>1</sub>	1.13	1.58	2.18									
F <sub>2</sub>	1.21 bB	1.77 abAB	2.28 aA									
F <sub>3</sub>	3.41	3.08	2.43									
F <sub>4</sub>	3.15 a	2.24 b	2.15 b									

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Table 6