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

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Abstract

The evolution of pre-fermentative volatiles and of the global aroma potential in three Italian neutral varieties ('Nebbiolo', 'Barbera' and 'Dolcetto') was assessed from véraison to harvest by SBSE-GC/MS. C6 and C9 compounds, benzene derivatives, bound monoterpenes and sesquiterpenes showed differences among varieties in quantity and profiles during berry ripening. Quantitatively, the most of total monoterpenes, C-13 norisoprenoids and sesquiterpenes were detected after acid hydrolysis. Among pre-fermentative norisoprenoids, exclusively β-ionone was detected with different kinetics among varieties. Monoterpene accumulation started around véraison with the exception of (E)-geranylacetone, whose content was already high at véraison. (E)-geranylacetone, deriving from the degradation of carotenoids, could become a target molecule to study indirectly the accumulation of carotenoids.

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

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

Introduction

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

Two other major classes of grape berry volatiles include terpenoids and C-13 norisoprenoids, whose flavor characterizes fresh berries, musts and wines of many genotypes. They are present in berries as free or glycosylated forms: the former can be released from the latter following the action of grape and yeast enzymes, or by acid-catalyzed reactions in the wine. To analyze grape volatile precursors there are two main strategies: enzymatic hydrolysis and acid hydrolysis. The efficacy of these methods is related to the chemical family of compounds. The main criticism to acid hydrolysis, raised in the past, is that it can induce rearrangements of the chemical structures of some aglycones, such as cyclation in monoterpenes. However Loscos *et al*. (2009)[10] found that several monoterpenes, such as linalool, α-terpineol, geraniol, nerol and β-citronellol formed during acid hydrolysis were closely correlated with analogues formed during alcoholic fermentation. Moreover, acid hydrolysis was found efficient to study norisoprenoids [11] and the levels of hydrolytically liberated β-damascenone in grapes could closely predict the levels of free β-damascenone in the corresponding wines after one year of ageing [12]. Volatiles released after acid hydrolysis represent the grape global aroma potential and were effectively used in the characterization of neutral grapes [13]. Deglycosylation allowed the identification of some important C13-norisoprenoids, such as vitispirane, β-damascenone [14], Riesling acetale and TDN [15]. Both aglycones in the free form and acid hydrolysis-derived norisoprenoids have been used to characterize grapevine varieties [11, 13].

A crucial point of volatile determination in grape berries is the extraction method used as different extraction techniques can minimize or maximize the extraction of peculiar classes of volatiles [16]. A semi-rapid technique, based on the use of stir bars packed with polydimethylsiloxane (PDMS-SBSE) has been employed to assess pre-fermentative varietal volatiles [2, 17, 18] and global aroma potential [13] in *Vitis vinifera* grapes. The effectiveness of the SBSE technique use in different matrix, including grape and must, has recently been reviewed [19].

Nebbiolo, Dolcetto, and Barbera are the most cultivated red grape varieties in Piedmont (North-Western Italy). Nebbiolo is the basis of high quality wines defined by the growing area: 'Barolo' DOCG (Denomination of Controlled and Guaranteed Origin), 'Barbaresco' DOCG, 'Nebbiolo d'Alba' DOC (Denomination of Controlled Origin) and 'Roero' DOCG. Dolcetto is a red early-ripening cultivar of Piedmont, giving rise to several VQPRD wines: 'Dogliani' and 'Diano d'Alba' DOCG, 'Dolcetto d'Alba' DOC, all arising from the Langhe district. Barbera is one of the most important red-grape variety grown in Italy; in Piedmont Barbera is the base cultivar for the production of some appreciated red wines, such as 'Barbera d'Alba' DOC, 'Barbera del Monferrato' and 'Barbera d'Asti' DOCG.. Despite their economical importance, at present there is little information about the profile and evolution of volatiles in grapes from these varieties, even though knowing the volatile concentration and potential at different stages of ripening could help to optimize the date of harvest [2, 20],in match with other maturity indices (*i.e.* sugar/acidity ratio, phenolic maturity).

The aim of this study was to characterize the concentration of pre-fermentative and acid-released volatiles of 'Nebbiolo', 'Dolcetto' and 'Barbera' by SBSE-GC/MS. To this aim we collected grapes from commercial vineyards from véraison to harvest; each variety was studied in its typical cultivation site, corresponding to a specific DOC or DOCG wine. Our results describe the accumulation kinetics of volatiles in the three genotypes, and offer new insights for the study of key steps of volatile biosynthesis in grapes. Moreover, we propose some molecules as chemical markers of each variety and we point out possible differences among genotypes.

Materials and Methods

Vineyard description and sampling.

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

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

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

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

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

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

Volatile compounds sorbed on the Twister® were desorbed in a thermal desorption unit (TDU, Gerstel, Mulheim and der Ruhr, Germany) in the splitless mode. The temperature program for thermal desorption was the following: 30°C for 6 seconds, then ramping at 120°C/min to 280°C, than 280°C for 1 min. The desorbed analytes were cryo-focused at 0°C using liquid CO_2, in a programmed temperature vaporization (PTV) injector (CIS 4, Gerstel, Germany); the cryo-focalized analytes were transferred to the GC column 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 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 performed using a 7890A gas chromatograph interfaced with 5975 C mass spectrometer (Agilent Technologies). The oven GC initial temperature was set at 40°C for 10 min, rose to 180°C at a rate of 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 transfer line temperature was 280°C. After each desorption the magnetic stir bars were cleaned by immersion in acetonitrile for 24 hours (stirring during the first hour).

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

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

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

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

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

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

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

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

Statistical analysis.

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

Results

Total pre-fermentative and acid hydrolysis-released volatiles.

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

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

Pre-fermentative C6 compounds.

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

Other pre-fermentative(non C6) aliphatic aldehydes.

At 50 dpv N grapes displayed the highest aldehyde concentration and, in general, showed a constant accumulation during ripening with a subsequent reduction in correspondence of harvest, whereas in D grape aldehyde concentration was more or less constant(Fig. 1 c). In B grapes a rapid decrease of aldehyde concentration was detected immediately after véraison followed by a peak of maximum concentration around 30 dpv (Fig. 1 c).

Pre-fermentative alcohols.

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

Pre-fermentative benzenoids .

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

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

Pre-fermentative and acid hydrolysis-released monoterpenes.

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

Pre-fermentative and acid hydrolysis-released norisoprenoids.

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

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

Pre-fermentative and acid hydrolysis-released sesquiterpenes.

At harvest total pre-fermentative sesquiterpene concentration (Tab. 1) was higher in B grapes respect to D which, conversely showed the highest concentration of acid hydrolysis-released Sesquiterpenes (Tab. 2): 417.5 µg/Kg against 21.6 µg/Kg for N and 23.9 µg/Kg for B.

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

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

Discussion

In this work we identified and quantified some volatile precursors after acid hydrolysis, namely monoterpenes, norisoprenoids and sesquiterpenes whereas aldehydes and alcohols, including C6 and C9 derivatives and benzene derivatives, were found exclusively without acid hydrolysis so they were classified as pre-fermentative volatiles. As studies focused on sesquiterpene accumulation in *Vitis vinifera* are a few and quite recent [21] at present there are no information about the efficacy of acid hydrolysis to assess them. In berries sesquiterpenes were measured both from the headspace [21] and after homogenization (in strawberries) [22]. Our data indicate the existence of sesquiterpenes in low amounts as pre-fermentative volatiles whereas they were present in higher concentration after acid hydrolysis, probably indicating they mainly exist as glycosides.

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

Volatiles derived from oxydation of lipids were detected in all stages of ripening: it is known that lipoxygenation of fatty acids is a plant response to biotic and abiotic stress and leads to the formation of the so-called 'oxylipins' that include the phytohormone jasmonic acid, hydroxy-, oxo- or keto-fatty acids and volatile aldehydes [25]. The three varieties examined in this study showed diversity in the profile and evolution of these compounds, underlying the existence of lipoxygenases with different activity, activation timing and, probably, acting on different substrates. Hexanal and E-2-hexenal, the most important product of lipoxygenation, were much more concentrated in Nebbiolo and Barbera than in Dolcetto; on the contrary hexanal increased during ripening in all genotypes, in agreement with Kalua and Boss (2010) [3]. In Cabernet Sauvignon berries, the expressions of VvHPL1 acting on 13-hydroperoxides and forming C6 compounds and of VvHPL2 acting on both 13- and 9-hydroxyperoxides and forming C6 and C9 compounds were detected about 2 weeks after flowering and peaks of activity were at 12 and 14 weeks after flowering, respectively; C6 compounds were accumulated in correspondence until 10 weeks after flowering and thereafter a reduction, probably due to the transformation of aldehydes into the correspondent alcohols, was detected [9]. In the varieties we studied, the accumulation trend during ripening was in line with the timing of enzyme expression in Cabernet Sauvignon, but the final reduction of C6 compound concentration was not detected; this could be ascribed to differences in alcohol dehydrogenase activity due to the genotype or to the cultivation environment. In Nebbiolo, in particular, the absence of (Z)-3-hexenal (Fig. 3c) but the presence of (Z)-3-hexen-1-ol(Fig. 3e) suggests the specific activity of an alcohol dehydrogenase, whereas this enzyme may absent or not expressed in Barbera (where (Z)-3-hexen-1-ol was absent). In a previous work on Nebbiolo grapes from three different growing locations (Z)-3-hexenal was never detected [18], suggesting that the absence of the aldehyde is more a genetic mark than an environmental effect. In effect, (Z)-3-hexen-1-ol concentrations in berries have been previously reported to be cultivar-dependent [3, 6, 26]. The high concentration of (E)-2-hexenal in Nebbiolo and Barbera throughout ripening (Fig. 3b), suggests an important role of enal isomerases in these two varieties, as suggested by Kalua and Boss(2010) [3] in Riesling and Cabernet Sauvignon. Besides, the lipoxygenase activity on linolenic acid (C18:3) is evidenced by the accumulation of (Z)-3-hexenal (only in B), E-2-hexenal, (Z)-3-hexen-1-ol (only in N) which, on the contrary, could not be active in D where (Z)-3-hexenaland (Z)-3-hexen-1-ol were not accumulated. The high concentration of (E,Z)-2,6-nonadienal (Fig. 3 g), a product of linolenic acid peroxidation via the formation of 9-hydroxyperoxides, could suggest a high expression of *VvHPL2* in Nebbiolo. The contents of (E)-2-nonenal and (E,Z)-2,6-nonadienal (Tab. 3 , 4, 5 in supplementary data) were rather low respect to C6 volatiles, in line with data reported for Cabernet Sauvignon and they were almost absent in B, confirming what was described by Zhu *et al*. (2012) [9] and suggested by Kalua and Boss (2010) [3] that the degradation of fatty acids is mainly due to 13-LOXs and to 13-HPLs (which lead to the biosynthesis of C6) rather than to 9-LOXs and 9-HPLs. Interestingly, we noticed that Barbera berries did not accumulate C9 (except nonadienal at harvest; Tab.1), suggesting a very strong varietal influence on this metabolism.

The presence of hexyl acetate (a C6-moiety ester) (Fig. 3h) limited to Barbera grapes suggests the activity of an alcohol acetyl transferase (AAT) on hexan-1-ol in this genotype. Moreover, this compound showed a decrease during ripening, implying that AAT activity decreased after véraison. To the best of our knowledge, nothing is known in *Vitis* on the specificity of alcohol acyltransferases; in *Malus domestica* the existence of a varietal effect on this enzyme was suggested as different enzyme haplotypes were detected in different varieties able to attain high or low ester concentrations [27]. Besides, an effect of MdAAT2 on the response to biotic and abiotic stress was detected in transformed tobacco leaves [28].

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

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

Monoterpene glycosides reached higher concentration than pre-fermentative forms during all stages of ripening, as noted in other grape genotypes [36, 37]. In a previous study, Di Stefano *et al*. (1998) [38] showed that Barbera grapes at harvest had few monoterpenes in the bound form compared to Nebbiolo. In this study similar concentrations of bound monoterpenes were detected at harvest among varieties, but major differences were detected at early stages of berry ripening. The complexity of terpene profiles from acid hydrolysis was much higher in Nebbiolo respect to the other genotypes, which probably justifies the typical flavor fingerprint of Nebbiolo wines, also after long term storage. Grape juice heat treatment gives rise to changes in the terpene composition: Williams *et al*., (1980) [39] described reaction mechanisms for the production of some monoterpenes from linalool as a precursor. Moreover, it was assessed that temperature and acid hydrolysis can induce the rearrangement of bound monoterpenes into free monoterpenes [39]. From data of the present study, however, as we treated grapes from the three varieties in the same way we can conclude 1) that both pre-fermentative and acid hydrolysis monoterpenes are cultivar related and 2) by exploiting the chemical transformation of terpenes following heat treatments at low pH, we were able to detect a number of compounds (among which cyclic α-terpineol) whose concentration depends on the concentration of other terpene molecules from which they derive due to chemical cyclization.

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

It has been proposed [42] that glycosylation, which occur between véraison and maturity, is responsible for the decrease of the concentration of free norisoprenoids. This hypothesis could help to explain the reduction of β-ionone in Dolcetto during ripening, because it showed a correspondent accumulation in the bound form after véraison, but not in Nebbiolo that showed a decrease after véraison. Among acid hydrolysis-released norisoprenoids, we found *trans* β-damascenone, which contributes to the floral and fruity notes of wine and has a very low sensorial threshold in model solutions (45 ng/L) [41]. The higher concentration of vitispirane and 1,1,6-trimethyl-1,2-dihydronaftalene (TDN), known to give camphor and kerosene notes in wines [45], in Dolcetto grapes could explain the tendency of Dolcetto wines to present these notes. Sefton *et al*. (1989) [46], reported the acid-catalized mechanism formation of these molecules from megastigmane precursors and Winterhalter *et al*. (1991) [15] suggested that the potential levels of TDN upon aging may be predicted by analysis of the corresponding aglycone released at acid pH. Together with the genotype, factors such as cluster exposure to sunlight could have influenced the accumulation of TDN and vitispirane in Dolcetto [47]; as a matter of fact in Dolcetto, the North-South row orientation in a vineyard with West exposure, together with an early leaf removal were probably able to favour TDN and vitispirane accumulation in berries. We found differences in the qualitative profile and in the accumulation kinetics of sesquiterpenes. In literature, data about accumulation of these compounds are not always in agreement; Coelho *et al*. (2006) [48] reported that sesquiterpene accumulation in cv. Baga, from véraison to post-ripening, showed its maximum expression at maturity and then remained constant until post-ripening, whereas in cv. Riesling and Cabernet Sauvignon it was reported that sesquiterpenes significantly decreased towards harvest [3]. Our data show that the kinetics of these compounds depend on the terroir (genotype × environment interaction); the same molecule, namely sesquiterpene 5, displayed different kinetics in the three varieties: its concentration was constant during ripening in Nebbiolo and Barbera, whereas it increased in Dolcetto. Lücker *et al*. (2004) [31] identified two sesquiterpene synthases in grapevine flowers and berries; these Authors reported that sesquiterpene synthase and monoterpene synthase transcripts were not detected in the mesocarp and exocarp during early stages of fruit development, because they are expressed only during late ripening. May *et al*. (2013) [49] demonstrated that sesquiterpene biosynthesis and accumulation in grape berries is restricted to the exocarp, particularly to wax layers. As we homogenized the entire berry we cannot indicate where sesquiterpenes were accumulated; however, finding no or trace amounts of sesquiterpenes as free pre-fermentative volatiles we can conclude that in grape berries the most of sesquiterpenes exist as glycosides.

The present study allowed to point out that C6 and C9 compounds, benzene derivatives, bound monoterpenes and sesquiterpenes showed differences in quantity and profiles during berry ripening (from véraison to harvest) among varieties. The fate of specific molecules such as (E)-geranylacetone, could be indicative of stress conditions, being known that this molecule, easily detectable by SBSE-GC/MS, derives from carotenoid degradation. Quantitatively, the most of total monoterpenes, C-13 norisoprenoids and sesquiterpenes were detected after acid hydrolysis, showing that in neutral grapes they mostly exist as glycosides. This aspect is well known for monoterpenes and C-13 norisoprenoids but it has not been largely investigated as to sesquiterpenes.

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

Conclusions

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

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

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Table 1

Pre-fermentative volatile concentration (mean of three field replicates ±standard errors) at harvest time of 'Nebbiolo', 'Dolcetto' and 'Barbera' grape berry. Data obtained by SBSE-GC/MS and expressed as µg $Kg^{-1}$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 (α= 0.05); ns = no significant differences.

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

Table 2

Bound volatile concentration (mean of three field replicates ±standard errors) at harvest time of 'Nebbiolo', 'Dolcetto' and 'Barbera' grape berry. Data obtained by SBSE-GC/MS and expressed as µg $Kg^{-1}$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 (α= 0.05); ns = no significant differences.

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	KI			
		Nebbiolo	**Dolcetto**	**Barbera**
Monoterpenes				
γ-terpinene	1218	19.3±3.2	nd	nd
p-cymene	1270	39.6±14.2 ns	57.3±24.5 ns	nd
dehydro-p-cymene	1422	31.4±3.5 ns	88.9±36.8 ns	nd
ho-trienol	1615	38.7±3.0	nd	nd
α-terpineol	1703	nd	74.3±2.0	nd
Z-geranylacetone	1831	nd	16.3±8.1	nd
E-geranylacetone	1859	442.6±23.9 b	322.8±51.8 b	697.3±42.8 a
C13-Norisoprenoids				
vitispirane	1515	136.1±18.0 ns	694.3±260.9 ns	306.8±42.1 ns
TDN	1731	49.5±9.2 ns	327.6±141.6 ns	194.7±85.1 ns
trans-β-damascenone	1817	265.2±83.0 a	62.8±23.0 b	48.2±26.1 b
β-ionone	1936	56.6±14.7 b	23.0±2.1 c	101.3±0.9 a
Sesquiterpenes				
sesquiterpene 1	1790	nd	334.6±117.6	nd
sesquiterpene 4	2346	nd	44.2±18.5	nd
sesquiterpene 5	2226	21.6±4.6 ns	38.7±17.0 ns	23.9±4.5 ns