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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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Flavour analysis of grape is a key step in quality evaluation. The Stir Bar Sorptive Extraction technique (SBSE, ‘Twister’®) was used to assess varietal and pre-fermentative volatile accumulation in ‘Nebbiolo’ berries, from véraison to harvest. Grapes were collected in three vineyards, representing different ‘crus’ in the cultivation areas of Barolo, Barbaresco and Roero (North-western Italy). Volatile constituents of grapes were identified and quantified by GC-MS.

We demonstrate the influence exerted by the growing location on volatile concentration and profile, as well as on the timing of volatile accumulation. The accumulation of certain classes of compounds, considered favourable for defining berry quality, followed common patterns, and was negatively correlated to that of compounds with herbaceous and grassy notes, such as the C6 compounds.

PCA analysis shows that the concentrations of varietal and pre-fermentative volatiles were more effective in separating growing areas than dates of harvest. Grapes from the Barbaresco area, showing higher values of the concentration ratio between favourable and unfavourable compounds throughout ripening, could be statistically separated from grapes from the other areas.

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

1. Introduction

Hundreds of volatiles have been identified in *Vitis vinifera* berries. Compounds with a C6-moiety are products of the lipoxygenase pathway and are the major volatile constituents of varietal and pre-fermentative aroma in many varieties (Gomez, Martinez & Laencina, 1995; Kalua & Boss, 2010; Yang et al., 2009). They have grassy, herbaceous odours, generally considered unpleasant in wines if they are present in high concentrations (Baumes, Cordonnier, Nitz & Drawert, 1986). Several other aliphatic and aromatic alcohols and aldehydes, norisoprenoids and terpenes have been identified in grapes (Sefton, Francis & Williams, 1993) . Some aliphatic aldehydes such as octanal, decanal and (*Z*)-2-heptenal have citrus-like odour whereas furfural and benzaldehyde are responsible for almond aroma. Apocarotenoid volatiles (β-ionone and β-damascenone) are among the most important contributors to fruity and floral notes in many fruits, including grapes, together with furanones, like 4-hydroxy-2,5-dimethyl-3(2H)-furanone and lactones (Klee, 2010).

As grapes ripen, a number of changes occur, including sugar and flavonoid accumulation, modifications in the content of organic acids, and changes in the concentration of volatile substances. The modifications of the berry volatile composition are still little known in ‘neutral’ varieties, which represent most of winemaking grapes, as investigations have generally been focused on ’aromatic’ varieties, very rich in both free and in glycoconjugated volatiles, terpenes in particular. Neutral grapes, however, possess a number of free and glycosylated volatile organic compounds, generally at lower concentrations respect to the aromatic ones. When grapes are thawed and grinded a number of volatiles develop: these molecules were named ‘varietal and pre-fermentative related volatile compounds’ (Coehlo, Rocha, Delgadillo & Coimbra, 2006).

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

Volatile analysis is a key step in grape quality evaluation. Volatiles have traditionally been detected by GC/MS on grape extracts, which requires a prior time- and solvent-consuming preparation. Many methods, using different solvents and extractants have been proposed up to now (Sefton, Francis & Williams, 1993; Cabrita, Costa Freitas, Laureano & Di Stefano, 2006). In 1999 Baltussen, Sandra, David & Cramers proposed a novel extraction technique for aqueous samples based on the use of a magnetic stir bar coated with polydimethylxylosane (PDMS). This technique, based on the concept of 'sorption' is known with the name of Stir Bar Sorptive Extraction (SBSE) and is commercially available under the brand name 'Twister'®. Sorption offers a number of advantages above traditional adsorption such as: 1) it is gentler as analytes are not retained on an active surface, and degradation of unstable compounds is reduced or absent; 2) desorption can be performed at lower temperatures, minimizing the losses of thermolabile compounds; 3) the retaining capacity of PDMS is not influenced by the sample matrix, in particular by the presence of different amounts of water (the main constituent of grape homogenates) or of other analytes, since each solute has his own partitioning equilibrium into PDMS; 4) degradation fragments originating from the PDMS stir bar coating give mass-spectra that can be very easily recognized and discarded (Baltussen, Sandra, David & Cramers, 1999). This technique has been successfully used in the detection of volatiles in many matrices but at the moment few applications have dealt with grapes (Luan, Mosandl, Munch & Wust, 2005; Pedroza, Zalacain, Lara, Salinas, 2010). Salinas, Zalacain, Pardo & Alonso (2004) were the first to propose the use of this technique for detecting volatiles in grape berries.

The concentrations of volatiles change during grape ripening and information on these changes, and the factors which induce them, is of high technological importance as it allows harvesting grapes with specific aroma profiles. Little has been published up to now about the evolution of volatiles during neutral grape berry ripening even though several reports deal with the detection of volatiles at harvest. Studies dealing with the detection of grape volatiles during ripening are relatively recent and mostly based on adsorption (Kalua & Boss, 2009; Coehlo, Rocha, Barros, Delgadillo & Coimbra, 2007) or sorption methods (Salinas, Zalacain, Pardo & Alonso, 2004). Another well-known, but little detailed, factor affecting aromatic composition of neutral grapes (and possibly of the derived wines) is the so-called 'terroir' where the vines are grown, each terroir giving rise to specific aromatic notes for a given vine genotype. However, very limited information is available about the influence exerted by the growing location on the volatile composition of neutral grapes (Bureau, Razungles & Baumes 2000; Ji & Dami, 2008).

In the present study we report the results of varietal and pre-fermentative volatile assessment from véraison to harvest in berries of the neutral cultivar Nebbiolo, which is widely grown in North-western Italy for the production of premium wines. Volatiles were detected using the SBSE technique on grapes collected in three vineyards, each located in one of three terroirs of Piedmont (Barbaresco, Barolo and Roero). Our aim was to identify individual molecules or classes of molecules able to distinguish growing areas. Moreover, we assessed the timing of accumulation of some class of volatiles in Nebbiolo grapes during ripening. Our results allow i) to gain information regarding the aroma potential of Nebbiolo when harvested at different developmental stages; ii) to assess the influences exerted by environmental conditions on the timing of flavour accumulation in grapes and iii) to contribute to knowledge about the key steps of flavour biosynthesis in grape berries.

2. Materials and Methods

2.1 Samples

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

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

2.2 SBSE methodology

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

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

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

0.10 min, ramp rate of 120°C/min to 280°C, and 280°C for 1.00 min. The desorbed analytes were cryo-focused at 0 °C (maintained by the use of liquid CO_2) in a PTV injector (CIS, Gerstel, Germany) for the total desorption time, then ramped at 12°C/s until 300°C and held at that temperature for 6.0 min. The analytes were separated on a DB-WAX column (J&W 122-7032; 30 m * 0.25 micron * 0.25 mm ID), using He as gas-carrier at a flow rate of 1 ml/min. The GC/MS was an Agilent Technologies, GC 7890A, MS 5975C; the MS ionization energy was set at 70 eV and masses were acquired from 19 to 400 *m/z* in full scan acquisition mode. The oven GC initial temperature was set at 40 °C for 10 minutes, then at 180 °C with a ramp rate of 2.5 °C/min. Temperature increased to 200°C at 1°C/min and was maintained for 10 minutes. The transfer line temperature was 280 °C. After each desorption the magnetic stir bars were cleaned by immersion in methanol for 24 hours (stirring during the first hour).

2.2.1 Qualitative analysis

Volatile compounds were identified comparing mass spectra with the data system library (NIST-05a), by comparison with spectra found in literature (NIST Chemistry WebBook, webbook.nist.gov/chemistry/), and/or according with the volatile constituents found in previous studies on Nebbiolo grapes and isolated by means of liquid extraction (Di Stefano, Bottero, Pigella, Borsa, Bezzo & Corino, 1998). In the case of comparison with the data system library, positive characterization was accepted when a compound was identified with a probability higher than 85% in all replicates. 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.

2.2.2 Semi-quantitative analysis of volatiles

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

2.3 Statistical analysis

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

3. Results

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

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

3.1 Varietal and pre-fermentative volatile profiles

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

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

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

3.2.1 Total aldehydes and alcohols

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

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

3.2.2 Esters

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

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

3.2.3 Terpenes and sesquiterpenes

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

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

3.2.4 Norisoprenoids

The importance of norisoprenoids in grape aroma is well known; they have been intensively studied as glycosides in many varieties but they have also been reported as components of varietal and pre-fermentative flavours (Salinas, Zalacain, Pardo & Alonso, 2004; Coehlo, Rocha, Barros, Delgadillo & Coimbra, 2007; Kalua & Boss, 2010). Salinas and co-workers (2004) detected vomifoliol, 3-oxo-α-ionol and β-ionone whereas Coehlo and co-workers (2007) detected β-damascenone and β-ionone and Kalua & Boss (2010) exclusively β-ionone at precise moments of ripening. In Nebbiolo grapes, except for trace amounts of TDN found in grapes from Roero at 24 and 38 dpv (Tab. 4), we exclusively detected β-ionone (Table 2, 3 and 4), similarly to Pedroza, Zalacain, Lara and Salinas (2010) and Kalua & Boss (2010) did. β-ionone biosynthesis started before véraison and increased throughout ripening, peaking at 10 to 20 dpv depending on the cultivation site. From 20 dpv onwards, norisoprenoid concentration decreased in all vineyards. The incidence of norisoprenoids over total varietal and pre-fermentative volatile amount was very low, ranging from 0.014 to 2.6 %.

3.2.5 Benzene derivatives and lactones

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

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

3.2.6 Total varietal volatiles and favourable/unfavourable volatile compound ratios

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

3.2.7 PCA analysis

To clarify possible relations among the different classes of compounds and identify general parameters able to distinguish growing locations, we performed a principal component analysis (PCA) on nine variable normalized data (Table 5); with the first three principal components (Prin) the model proposed justified 89 % of the total variance (Table 5). According to the eigenvalues, 5 variables were associated to Prin 1, namely the percentages of norisoprenoids, volatile total concentrations, the percentages of benzene derivatives, total aldehydes and sesquiterpenes. One variable (the percentage of total terpenes) was associated to the second Prin; alcohols were positively associated to the third Prin, although with an eigenvalue slightly lower than 1 (0.97; Tab. 5).

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

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

4. Discussion

The flavour differences among ‘Barbaresco’, ‘Barolo’ and ‘Roero’ DOCG wines are well-known to wine consumers and they have also been characterized through GC/MS analysis and multivariate statistical tools (Marengo, Aceto & Maurino, 2001). At the last sampling date (that was coincident with harvest for viticulturists) significant differences among the three vineyards were found not only for esters but also for aliphatic alcohols, terpenes, benzene derivatives and total volatile concentration (Table 6). Grapes from the Barbaresco area were distinguishable from those from Barolo and Roero and very often they showed similarity with those from the Barolo area. At the wine level Marengo, Aceto & Maurino (2001) pointed out that wines that underwent a more prolonged ageing (Barbaresco and Barolo) were associated with each other whereas they were quite distant from wines, such as those from Roero, that underwent a reduced ageing period; differences were tied to a series of compounds but the most implicated compounds were esters. In this study we show that Barbaresco and Barolo grapes accumulated more esters than Roero grapes, thus the differences previously detected at the wine level could have a previous origin in grapes rather than be exclusively due to the numerous fermentative reactions that induce ester formation during winemaking and ageing.

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

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

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

The use of crushed grapes together with prolonged time of SBSE/grape homogenate contact have probably promoted the formation of C6 compounds as it was the case in other studies using SBSE or SPME techniques and implying grinding. C6 compounds accounted for 14 to 71 % of total varietal volatiles with increasing values from véraison to harvest in Barolo and Barbaresco grapes; in Roero grapes the incidence of C6 compounds over total was already high (49 %) at the first sampling and it increased to 69 % at harvest. At harvest, independently from the cultivation area, C6 compounds represented the major class of grape volatiles, consistent with previous findings (Kalua & Boss, 2009; Kalua & Boss, 2010; Yang et al., 2009). Temporal analysis of the ratio between favourable and unfavourable (C6 aldehydes and alcohols) compounds shows that in grapes from Barbaresco and Barolo the optimum values were reached at 42 and 44 dpv, respectively, thus about 15 days before the date of effective harvest. Oliveira, Faria, Sá, Barros & Araújo (2006) discovered the effectiveness of C6 compounds, particularly of the ratio (E)-3-hexenol/(Z)-3-hexenol in discriminating wine origin in monovarietal wines. In Nebbiolo we found, besides hexanol, (Z)-3-hexenol and (E)-2-hexen-1-ol, and we were thus able to calculate their ratio, which at harvest was 0.6 for Roero, 1.2 for Barolo and 1.3 for Barbaresco grapes. The classificatory capacity of this ratio is thus evident and once more an association between Barbaresco and Barolo grapes on one side and Roero grapes on the other, emerges. Nevertheless further studies will confirm the capability of ratios such as this to discriminate growing locations. Calculation of this or other ratios could represent an important start point for finding in the wide range of varietal and pre-fermentative volatiles some target compounds that could be considered as a possible ‘fingerprints’, not only of the growing locations but also of the variety. Further studies are ongoing in our lab on other varieties and over more years.

4. Conclusions

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

Barbaresco, Barolo and Roero wines. The influence exerted by the growing location on volatile concentration and profile was analysed, as well as on the timing of volatile biosynthesis.

One of the main aims of this study was to verify the existence of differences in the volatile profile of the same genotype, grown in different areas or terroirs. The results show that, even in a relatively homogenous geographic area like southern Piedmont, the growing locations induce differences in the concentration and accumulation trend of several classes of compounds, in particular esters. On the contrary, certain classes of compounds such as sesquiterpenes and norisoprenoids showed no differences in the pattern of accumulation in the different areas. Varietal and pre-fermentative volatiles seemed to be more effective in separating growing areas than dates of sampling: as a matter of fact grapes from the Barbaresco area, showing higher values of the favourable/unfavourable compound ratio throughout ripening, were separated from grapes from the other areas.

The concentrations of certain classes of compounds such as terpenes, norisoprenoids and benzene derivatives were generally correlated among them so that this opens the possibility to follow the 'aromatic ripening' of grapes by following the evolution of a unique class of compounds.

Further studies, over more years and also on global aroma potential, i.e. including glyco-conjugate volatile detection, will be necessary to better identify Nebbiolo varietal volatiles, to deepen knowledge about volatile concentrations and profiles and to investigate their implications on wine quality as influenced by the growing area.

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CAPTIONS

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

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

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

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

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

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

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

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

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

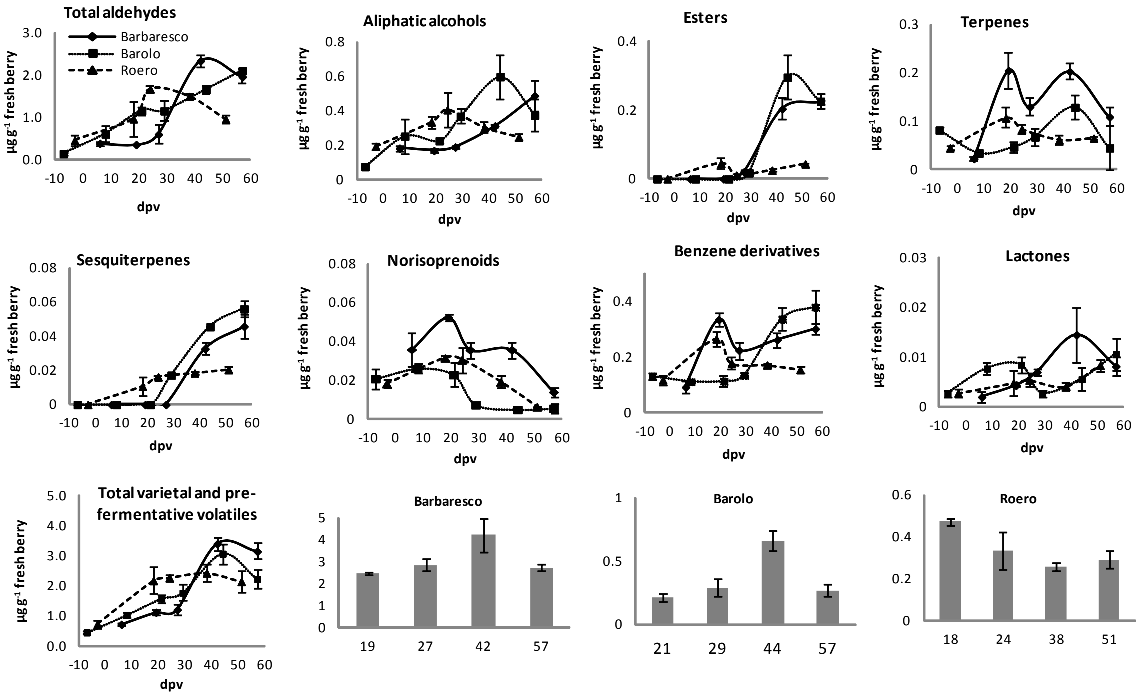
Total aldehydes
Barbaresco
Barolo
Roero
Aliphatic alcohols
Esters
Terpenes
Sesquiterpenes
Norisoprenoids
Benzene derivatives
Lactones
Total varietal and pre-fermentative volatiles
Barbaresco
Barolo
Roero
μg g^{-1} fresh berry
dpv
-10
0
10
20
30
40
50
60
19
27
42
57
21
29
44
57
18
24
38
51

Figure 1

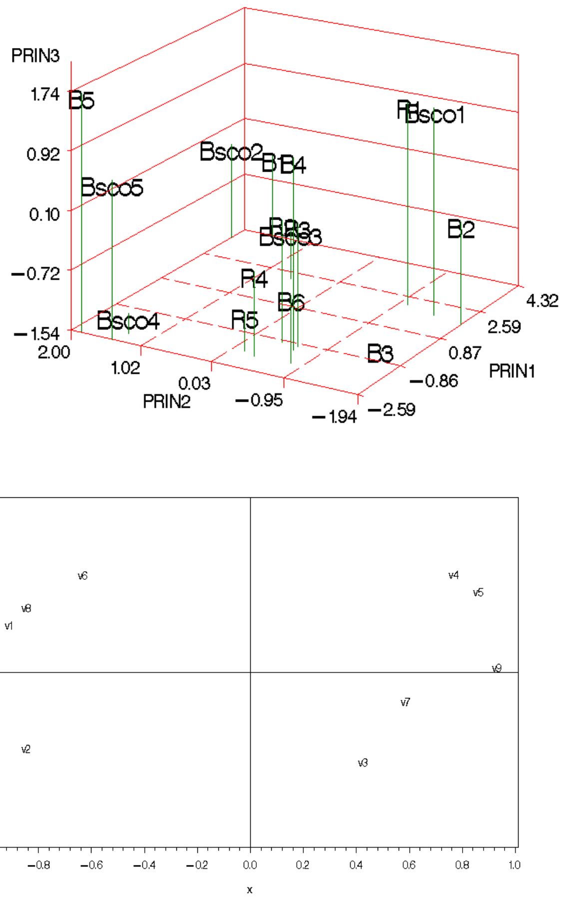
PRIN3
1.74
0.92
0.10
−0.72
−1.54
B5
Bsco5
Bsco2
B1
B4
R1
Bsco1
B2
R2
R3
Bsco3
R4
B6
R5
Bsco4
B3
2.00
1.02
0.03
−0.95
−1.94
PRIN2
4.32
2.59
0.87
−0.86
−2.59
PRIN1
y
1.0
0.8
0.6
0.4
0.2
0.0
−0.2
−0.4
−0.6
−0.8
−1.0
v6
v8
v1
v2
v4
v5
v9
v7
v3
x

Figure 2

	Compound	KI	Reference	
Aliphatic aldehydes	Hexanal^1	1064	Kalua & Boss, 2010	
	(*E*)-2-hexenal^1	1213	Kalua & Boss, 2010	
	octanal	1291	Kalua & Boss, 2010	
	(*Z*)-2-heptenal	1319	Caven-Quantrill & Buglass, 2007	
	(*E,E*)-2,4-heptadienal	1485	Yang et al., 2011	*
	decanal	1497	Salinas et al., 2004	
	(*E*)-2-nonenal	1530	Ruberto et al., 2008	
	(*E,Z*)-2,6-nonadienal	1580	Sotiroudis et al., 2009 in cocumber	
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^1	1363	Kalua & Boss, 2010	
	(Z)-3-hexenol^1	1387	Sefton et al., 1993	
	(*E*)-2-hexen-1-ol^1	1410	Coehlo et al., 2007	
	2-ethyl-hexanol	1501	Caven-Quantrill & Buglass, 2007	
	1-octanol	1570	Yang et al., 2009	
	(*E*)-2-octen-1-ol	1628	La Guerche et al., 2006 from *Botrytis cinerea* infected grapes	*
Terpenes	D-limonene	1085	Coehlo et al., 2007	
	B-cyclocitral	1608	Coehlo et al., 2006	*
	isomenthol	1648	Caven-Quantrill & Buglass, 2007	
	(*E*)-geranylacetone	1662	Salinas et al., 2004	
	α-terpineol	1707	Sefton et al., 1993	*
	β-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		*
	penthyl-hexanoate	1515		
	methyl-decanoate	2035		*
	methyl-hexadecanoate	2061	Caven-Quantrill & Buglass, 2007	
	butyl-hexadecanoate	2253		*
Lactones	γ-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^+ hydrolysis	*
	β-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.

		6 dpv	19 dpv	27 dpv	42 dpv	57 dpv
	SSC (°Brix)	11.3	18.4	19.3	24.1	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
Aromatic aldehydes	furfural	0.0660 ± 0.0153	0.1737 ± 0.0386	0.1295 ± 0.0033	0.3737 ± 0.2831	0.0974 ± 0.0278
	benzaldehyde	0.0039 ± 0.0008	0.0054 ± 0.0000	0.0033 ± 0.0011	0.0098 ± 0.0017	0.0230 ± 0.0142
Aliphatic alcohols	1-butanol	0.0159 ± 0.0016	0.0047 ± 0.0020	0.0043 ± 0.0008	0.0197 ± 0.0161	0.0447 ± 0.0127
	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-olo	ND	ND	ND	ND	ND
Terpenes	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
	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
Benzene derivatives	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
	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
Esters	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
	penthyl-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
Lactones	γ-butyrolactone	ND	ND	ND	ND	ND
	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	ND
	β-ionone	0.0359 ± 0.0088	0.0524 ± 0.0015	0.0357 ± 0.0040	0.0358 ± 0.0034	0.0141 ± 0.0025

Table 2

		-7 dpv	8 dpv	21 dpv	29 dpv	44 dpv	57 dpv
	SSC (°Brix)	6.6	14.8	21.4	22.6	24.5	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
Aromatic aldehydes	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
	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-olo	ND	ND	ND	ND	ND	ND
Terpenes	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
Benzene derivatives	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
	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-buthyl-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
Esters	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
	methyl-hexadecanoate	ND	ND	ND	ND	0.0285 ± 0.0096	0.0085 ± 0.0041
	butyl hexadecanoate	ND	ND	ND	ND	ND	ND
Lactones	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
Norisoprenoids	TDN	ND	ND	ND	ND	ND	ND
	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

Table 3

		-3 dpv	18 dpv	24 dpv	38 dpv	51 dpv
	SSC (°Brix)	12.6	19.9	20.6	23.6	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
Aromatic aldehydes	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-olo	ND	0.009 ± 0.005	0.011 ± 0.001	0.006 ± 0.001	0.007 ± 0.000
Terpenes	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
	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
Benzene derivatives	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-buthyl-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
	penthyl-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

Table 4

	Prin 1	Prin 2	Prin 3
V1	0.28	**0.48**	0.09
V2	0.18	**0.60**	0.12
V3	**-0.39**	0.15	0.09
V4	0.30	0.41	-0.25
V5	**-0.39**	0.08	-0.17
V6	**0.39**	-0.29	-0.08
V7	-0.23	0.11	-0.35
V8	**-0.40**	0.04	0.06
V9	0.02	-0.05	**0.85**
V10	**0.34**	-0.35	-0.13
Eingenvalues	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
1	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
2	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
3	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
4	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
1	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
2	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
3	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
4	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
1	1.13	1.58	2.18
2	1.21 bB	1.77 abAB	2.28 aA
3	3.41	3.08	2.43
4	3.15 a	2.24 b	2.15 b

Table 6