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Abstract

The changes in the volatile composition of Moscato bianco grapes were evaluated during ripening. Grape berries were sampled during five weeks (16-20 ºBrix) and sorted for each date in ten density classes (1.05-1.12 g/cm^3). The highest total concentration of free terpenes was found at 19.3 ºBrix, however total concentration of the bound fraction increased significantly throughout ripening. Response surface methodology was used to assess the simultaneous effect of sampling time and berry density on the volatile composition, which was satisfactorily fitted to regression models for some key terpene compounds. Total free and bound terpenes were more affected by grape density than by sampling date. The same behavior was observed for free and bound linalool and bound nerol, whereas the stronger effect of sampling date was exhibited for bound *t*-rose oxide, *c*-rose oxide and geraniol. The results showed that the sampling strategy impacted strongly on the aroma quality of berries.

Keywords: terpenes, ripening, berry density, wine grapes, response surface methodology.

1. Introduction

Grape-originated aroma makes an important contribution to the varietal feature of the final wine flavor. Understanding the changes in secondary metabolism during berry ripening may provide predictive information about the link between grape and wine aroma. Aroma compounds in grape berries are present as free and glycosidically-bound forms. These odorless sugar conjugates can undergo acid or enzyme hydrolysis during the wine making and aging process, releasing free volatiles and potentially enhancing the aroma of the wine (Günata, Bayonove, Baumes, & Cordonnier, 1986). Other precursors such as fatty acids, amino acids and carotenoids can go through even more complicated biosynthesis processes to form aroma compounds during grape ripening (González-Barreiro, Rial-Otero, Cancho-Grande, & Simal-Gándara, 2015).

Terpenes are responsible for the characteristic varietal aroma of Muscat and other aromatic white cultivars (Pisarnitskii, 2001; Zalacain, Marín, Alonso, & Salinas, 2007). Several researchers have investigated the changes in the concentration of terpene compounds during grape ripening (Coombe & Iland, 2004; Kalua & Boss, 2010; Vilanova, Genisheva, Bescansa, Masa, & Oliveira, 2012). Free and glycosylated terpenes are accumulated in grapes from veraison. Normally, the increase of terpene concentration is observed from the first stages of grape ripening to maturity or over-ripeness. Some authors have reported a continuous accumulation of terpenes even after maturity (Schwab & Wüst, 2015), while others observed that terpene concentration starts to decrease before the maximum sugar concentration is reached in grapes (Lasanta, Caro, Gómez, & Pérez, 2014).

During grape ripening, it is well known the internal variability among clusters within the same vine and among berries within the same cluster for different physical and chemical parameters (Letaief, Rolle, & Gerbi, 2008; Tarter & Keuter, 2005, 2008). In

particular, some authors have observed variability for the aromatic composition of skin and flesh between shoulder and tip berries of the clusters (Noguerol-Pato, González-Barreiro, Cancho-Grande, Santiago, Martínez, & Simal-Gándara, 2012). This variation can involve differences not only in the concentration of sugars but also in the aromatic composition between varietal wines made from berries differently positioned in the cluster. Therefore, any procedure for classifying the grape berries would be advantageous, such as density sorting. On the other hand, the multifunctional ability of terpene synthases to form multiple products, and their spatial and temporal regulation during development and in response to biotic and abiotic factors, contribute to the time-variable formation of a diverse group of terpene metabolites (Tholl, 2006). In grapes, there is an interaction between the environment and gene expression of enzymes involved in aroma biosynthesis, which is genotype dependent and may lead to a differential accumulation of free and glycosylated terpenes (Wen, Zhong, Gao, Lan, Duan, & Pan, 2015).

The exploitation of the possible variations in the aromatic composition of grape berries would allow to maximize their aroma potential and/or to produce different wine types with particular aroma characteristics using density sorting equipments (Rolle, Torchio, Giacosa, Río Segade, Cagnasso, & Gerbi, 2012). Therefore, the main aim of this study was to better understand the evolution of both free and glycosylated terpenes during berry ripening, and to assess the effect of grape density and harvest date using response surface methodology (RSM). The study was carried out on the Moscato bianco aromatic white cultivar, which is used to produce the renowned Asti DOCG sweet sparkling wine.

2. Materials and methods

2.1. Grape samples

Grape samples of the Moscato bianco cultivar (*Vitis vinifera* L.) were collected from a vineyard located in the Piedmont wine region (Asti province, North-West Italy, 44°43' N, 8°10' E) during five consecutive weeks in 2014, from August 20th to September 11th. For each sampling date, about 20 kg of grape berries were randomly collected from 500 vines by picking the berries one by one and/or in small bunches (three or four berries) from different parts of each cluster. A set of 1000 berries was randomly selected and used for subsequent analysis in order to study the evolution of the chemical and volatile composition in the vineyard with the harvest date.

All the other single berries with attached short pedicels were densimetrically sorted by flotation in saline solutions of ten different concentrations (from 80 to 170 g/L sodium chloride, corresponding to densities comprised between 1.05 and 1.12 g/cm^3) (Kontoudakis, Esteruelas, Fort, Canals, De Freitas, & Zamora, 2011; Rolle et al., 2012). Afterwards, all berry groups were weighed for obtaining the distribution percentages of berries in density classes. The berries belonging to all the represented classes were then washed with water. The berry groups corresponding to different density classes and sampling dates were treated separately for all subsequent analysis in order to assess the variability in the chemical and volatile composition in the vineyard during ripening.

Two subsamples of 100 berries were used for the determination of the technological ripeness parameters. Another two subsamples of 200 berries were used for the determination of free and glycosylated volatile compounds.

2.2. Technological ripeness parameters

For each subsample, the grape juice was obtained by manual crushing and

centrifugation. Total soluble solids concentration (ºBrix, as SSC) was measured using an Atago 0-32 ºBrix temperature compensating refractometer (Atago Corporation, Tokyo, Japan), pH was determined by potentiometry using an InoLab 730 pH meter (WTW, Weilheim, DE), and titratable acidity (g/L tartaric acid, as TA) was estimated using the International Organization of Vine and Wine method (OIV, 2008). Organic acids (citric acid, tartaric acid and malic acid) (g/L) were determined using a HPLC system equipped with a diode array detector (DAD) set to 210 nm (Giordano, Zecca, Belviso, Reinotti, Gerbi, & Rolle, 2013).

2.3. Extraction of free volatile compounds

For each subsample, the berries were treated following the procedure described by Rolle, Torchio, Giacosa and Río Segade (2015). After crushing the berries with a laboratory blender (Waring Laboratory, Torrington, USA) under a nitrogen atmosphere for 1 min, a 5 mL-aliquot of the supernatant obtained by centrifugation (7000 x g, 15 min, 4 ºC) was diluted with an equal volume of deionized water (Purelab Classic system, Elga Labwater, Marlow, United Kingdom), adjusted at pH 5 and transferred to a 20 mL glass headspace sampling vial containing 2 g of sodium chloride. 1-Heptanol (Sigma-Aldrich, Milan, Italy) was added as internal standard (200 μL of 1.55 mg/L solution in 10 % v/v ethanol).

2.4. Extraction of glycosidically-bound volatile compounds

Glycosylated volatile compounds were extracted according to the method proposed by Wang, Kang, Xu and Li (2011) slightly modified. Briefly, a 10 mL-aliquot of the supernatant previously obtained by centrifugation was loaded onto a 1 g Sep-Pak C18 reversed-phase solid phase extraction cartridge (Waters Corporation, Milford, MA,

USA). The cartridge was then rinsed with 30 mL of deionized water. The free fraction was eluted with 10 mL of dichloromethane (Sigma-Aldrich) and discarded. After washing the cartridge with 10 mL of deionized water, the glycosylated fraction was eluted with 10 mL of methanol (Sigma-Aldrich). In all cases, the flow rate was approximately 2 mL/min. The methanolic extract was evaporated to dryness using a vacuum rotavapor (Buchi R-210, Switzerland) at 35 °C. The dried extract obtained was dissolved in 5 mL of 0.2 M citrate-phosphate buffer (pH 5). The enzymatic hydrolysis was carried out using 50 mg of an AR-2000 commercial preparation with glycosidase side activities (DSM Oenology, The Netherlands) with incubation at 40 °C for 24 h. Afterwards, the extract was diluted with an equal volume of deionized water (5 mL), and transferred to a 20 mL glass headspace sampling vial containing 2 g of sodium chloride and 200 µL of 1.55 mg/L 1-heptanol solution in 10% v/v ethanol (internal standard).

2.5. HS-SPME and GC-MS conditions

The vials were sealed using silicone septa from Supelco (Bellefonte, PA, USA) with 18 mm diameter screw caps, and were then shaken to dissolve sodium chloride. A 50/30 µm DVB/CAR/PDMS fibre from Supelco (Sánchez-Palomo, Díaz-Maroto, & Pérez-Coello, 2005) was exposed to the headspace of the capped vial for 20 min at 40 ºC. SPME injections were performed in the splitless mode at 250 ºC for 5 min for the thermal desorption of analytes from the fibre.

The GC–MS system and chromatographic conditions were previously reported by Sánchez-Palomo et al. (2005) with some modifications (Rolle et al., 2015). The GC-MS system consisted of an Agilent 7890C gas chromatograph (Little Falls, DE, USA) coupled to an Agilent 5975 mass selective detector. A DB-WAXETR capillary column

(30 m x 0.25 mm, 0.25 µm, J&W Scientific Inc., Folsom, CA, USA) was used, starting at the temperature of 40 ºC for 5 min, increasing at a rate of 2 ºC/min to 200 ºC for 10 min and 5 ºC/min to 220 ºC, and holding at 220 ºC for 5 min before returning to the initial temperature. Helium was used as carrier gas at a flow-rate of 1 mL/min. The injection port temperature was 250 ºC, the ion source temperature was 150 ºC, and the interface temperature was 280 ºC. For the detection, electron impact mass spectrometry was used in total ion current (TIC) mode with an ionization energy of 70 eV. The mass acquisition range was *m/z* 35–350.

Semiquantitative determinations (µg/L) were performed by measuring the relative peak area of each identified compound, according to pure standards and/or the NIST database (http://webbook.nist.gov/chemistry/), in relation to that of the internal standard. The chemical standards used were rose oxide (mixture of isomers), linalool oxide (mixture of isomers), linalool, α-terpineol, citronellol, nerol, geraniol, geranic acid, benzyl alcohol and 2-phenylethanol, which were purchased from Sigma-Aldrich. The identification of hotrienol and *cis*- and *trans*-isomers of rose oxide, furan linalool oxide and pyran linalool oxide was based on the NIST library. Each replicate was analyzed in duplicate.

2.6. Experimental design and statistical analysis

The independent variables used were harvest date (X_1) and berry density (X_2). The mean values for each parameter determined at each harvest date-berry density combination were fitted using regression analysis to the following second-order polynomial model:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2$$

where Y is the response variable, X_1 and X_2 correspond to the independent variables, b_0

is the value estimated in the initial conditions (first harvest date and lowest berry density), b_1 and b_2 represent the principal effects associated with each variable, b_{11} and b_{22} are the squared effects, and b_{12} is the interaction effect. The regression models were obtained using the Statistica software package version 7.0 (Statsoft Inc., Tulsa, OK, USA), and the corresponding surface plots were represented using RSM. A statistical analysis was performed to evaluate the goodness of the fit.

The Tukey-b test at $p < 0.05$ was performed to establish significant compositional differences by one-way analysis of variance (ANOVA) using the SPSS Statistics software package version 19.0 (IBM Corporation, Armonk, NY, USA).

3. Results and discussion

3.1. Evolution of technological ripeness parameters in the vineyard with the harvest date

Table 1 shows the technological ripeness parameters of Moscato bianco grapes at five harvest dates (I-V). A significant effect of ripening state was found for all parameters studied with the exception of citric acid. At harvest dates I, II and IV, the berries showed a significantly lower sugar accumulation (SSC), corresponding the highest richness in sugars to the last harvest date. Moscato bianco berries with SSC values higher than 19 ºBrix are considered ripe. After a hot and dry August, the sample IV was collected few days after the first heavy rains, which could have affected the usual accumulation of sugars in the berries. As the harvest date was delayed, the values of pH increased whereas those of titratable acidity decreased due to the progressive and significant decrease in the concentrations of malic and tartaric acids. These results agreed with those found in other studies previously published. The variations in the technological ripeness parameters decreased at the last stages of grape ripening, being

more accused for the acidity than for the sugar accumulation (Lasanta et al., 2014). The decrease in the value of titratable acidity with the harvest date was significant even in grapes having the same concentration of reducing sugars (Rolle et al., 2011). Nevertheless, this behavior can be variety dependent (Esteruelas et al., 2015).

Fig. S1 shows the distribution as weight percentage of the berries in different density classes at the five dates studied during ripening for the Moscato bianco cultivar. For all harvest dates, high heterogeneity can be observed in the berry density according to a Gaussian bell-shaped distribution. The most representative density classes were 1.075 and 1.081 g/cm^3 for the sampling dates I (56.3 % of the berries belonging to these two density classes), II (53.1 %), III (41.4 %) and IV (42.4 %) and to 1.081 and 1.088 g/cm^3 for the sampling date V (51.9 %). In-field grape variability was lower at the first sampling date, whereas the berries were more heterogeneously distributed in density classes at ripening stage III. As ripening advanced, the percentage of denser berries was higher (Kontoudakis et al., 2011; Rolle et al., 2011).

Different studies have found great variability in berry density along the vineyard even at the same sampling date during the ripening process (Kontoudakis et al., 2011; Río Segade et al., 2013; Rolle et al., 2011, 2012, 2015). Variability among berries demonstrates that the ripening rate of a grape crop can be strongly influenced by the degree of asynchrony in the development rate of the individual berries in the crop. This high variability among berries in their development can be a serious negative factor for grape and wine quality (Coombe & Iland, 2004) or can be exploited to produce wines of different characteristics (Rolle et al., 2012).

3.2. Evolution of terpene composition in the vineyard with the harvest date

The changes in total concentration of free and glycosylated terpenes during grape

ripening are shown in Fig. 1. Total free terpenes had the significantly higher concentration at ripening stage III (19.3 ºBrix), decreasing until the last ripening stage V (20.2 ºBrix). However, the total concentration of bound terpenes increased significantly during ripening, showing the maximum value at stage V (20.2 ºBrix).

Terpenes are responsible for the characteristic varietal aroma of a number of winegrapes, especially white cultivars belonging to the Muscat family (Pisarnitskii, 2001; Zalacain et al., 2007). Several studies have shown no relationship between sugar levels and accumulation of grape berry flavorants. Thus, the concentration of sugars cannot be the best ripeness index, particularly regarding aroma potential (Combe & Iland, 2004; Wilson, Strauss, & Williams, 1984). Some authors suggested that the synthesis of aroma compounds occurs independently of the sugar accumulation by processes within the berry itself at the last stages of ripening (Coombe & Iland, 2004). In grapes with a significant contribution of terpenoids to overall aroma, the concentration of the free fraction is correlated with the organoleptic assessment of the resulting wine, but rarely with juice SSC, TA or pH (Reynolds & Wardle, 1997). Studies performed by Vilanova et al. (2012) on white cultivars from Galicia (Spain) have found significant differences in free and bound monoterpenes during ripening, and these differences were dependent of the cultivar. The evolution of volatiles during grape ripening was not related to the changes in the sugar concentration, showing that the technological and aromatic maturities did not occur at the same time in these cultivars (Vilanova et al., 2012).

Usually the bound fraction of terpenes is higher than the free fraction, which does not contribute directly to the aroma but can be an additional reservoir of flavor because further volatiles are released by enzymatic or acid hydrolysis during vinification or wine storage (Cabrita, Costa, Freitas, Laureano, & Di Stefano, 2006; Günata, Bayonove,

Tapiero, & Cordonnier, 1990; Mateo & Jimenez, 2000; Sefton, Francis, & Williams, 1994; Torchio et al., 2012). In our study, the bound fraction of terpenes was 2.5, 3.3, 3.2, 4.1 and 4.7 times more abundant than the free fraction at harvest dates I, II, III, IV and V, respectively. The differences in the terpene concentration between the bound and free fractions increased along ripening, achieving the maximum value in the last ripening stage. Similar results were found for Muscat Hamburg grapes during ripening (Fenoll, Manso, Hellín, Ruiz, & Flores, 2009). Other work showed that at harvest the highest concentration of the three major monoterpenes (linalool, geraniol and nerol) in Muscat of Alexandria grapes occurred as glycosides, and the fluctuation during grape development seemed to correspond to changes in temperature. However the response of the free monotepenes was less to temperature changes than that of the bound forms (Park, Morrison, Adams, & Noble, 1991).

3.2.1. Free volatile compounds

Table 2 shows the evolution of the individual free terpenes for the Moscato bianco cultivar at the five grape ripening stages. A total of 12 free terpene compounds were identified and quantified. Free terpenes showed significant differences among harvest dates with the exception of *c*-furan linalool oxide, hotrienol and citronellol+pyran linalool oxide. All significant free terpenes reached their maximum concentration at ripening stage III with the exception of geraniol, with maximum concentration at ripening stage IV.

Geranic acid, linalool, nerol, geraniol and citronellol were the most abundant free terpenes in the Muscat of Sorso-Sennori cultivar, synonym for Moscato bianco from Sardinia (del Caro, Fanara, Genovese, Moio, Piga, & Piombino, 2012), while linalool and geraniol also were in Moscato bianco grapes grown in a mountain area (Giordano et

al., 2013). In our study, linalool, geraniol, nerol and *t*-rose oxide were the predominant free terpenes in Moscato bianco grapes. Linalool was the major terpene compound in the free fraction, showing significantly higher concentrations at harvest date III (19.3 ºBrix) when accounting for 74.20 % of total concentration of free terpenes. Similar results were found in the Weisser Riesling cultivar, where the highest concentration of linalool was observed at 18.7 ºBrix (Marais & Wyk, 1986). In contrast, free linalool concentration in the Blanco lexítimo white aromatic cultivar from Galicia (Spain) showed a significant increase during ripening, from 5.58 % of total free tepenes at 20.0 ºBrix to 32.91 % at 22.2 ºBrix (Vilanova et al., 2012). Following to linalool, geraniol accounted for 11.13 % and 17.35 % of total free terpenes in harvest dates III and IV, respectively, when the highest accumulation occurred. These results agreed with those previously reported (Marais & Wyk, 1986). The concentrations of nerol and *t*-rose oxide remained practically stable during the harvest dates II, III and IV with significantly higher values compared to dates I and V. It is important to take into account that headspace technique avoids the possible formation, degradation and interconversion of some volatile free monoterpenols such as geraniol, nerol, linalool and α-terpineol during their determination (Sejer Pedersen, Capone, Skouroumounis, Pollnitz, & Sefton, 2003). Regarding rose oxide, it has been proposed as a useful indicator for the identification of Muscat cultivars because its presence and Muscat flavor are highly correlated (Ruiz-García, Hellín, Flores, & Fenoll, 2014).

Other free volatile compounds studied, such as benzyl alcohol and 2-phenylethanol, were present at low concentrations, which were not significantly different among ripening stages. This agreed with the evolution found for Muscat Hamburg grapes during ripening (from 100 to 150 g/L sugars) (Fenoll et al., 2009).

3.2.2. Glycosylated volatile compounds

The changes in the individual bound terpenes for the Moscato bianco cultivar at the five grape ripening stages are shown in Table 2. Only 5 from 12 bound terpenes identified and quantified have shown significant differences among harvest dates (linalool, hotrienol, citronellol+pyran linalool oxide, nerol and geraniol). Nerol, geraniol and linalool were the most abundant bound terpenes, particularly nerol. These compounds were also found at high concentrations in Moscato bianco grapes grown in a mountain area (Giordano et al., 2013). Their concentrations increased progressively and significantly during the ripening period monitored. In fact, the maximum concentration of nerol, geraniol and linalool was found at the last ripening stage, accounting for 42.44 %, 29.60 % and 22.27 % of total concentration of bound terpenes, respectively. In ripe Muscat Hamburg grapes, nerol and geraniol were also the predominant bound terpenes (Fenoll et al., 2009). Other studies reported the increase of bound nerol, geraniol and linalool concentration of white cultivars from Galicia (Spain) during ripening (Vilanova et al., 2012). Bound ho-trienol showed the highest concentration at the first ripening stage I and then decreased slightly, whereas the richness in bound citronellol+pyran linalool oxide was higher at the last stages of ripening (harvest dates III, IV and V).

Del Caro et al. (2012) observed that bound nerol and geraniol were present at concentrations greater than twice the concentration of the free fraction in the Muscat cultivar from Sardinia. The same results were found in Muscat of Alexandria (Günata et al., 1986; Selli, Cabaroglu, Canbas, Erten, & Nurgel, 2003). In our study, this difference was higher at ripening stage V, bound nerol and geraniol concentrations being 121 and 15.5 times free nerol and geraniol ones, respectively, at the last ripening date.

In the same way that in the free fraction, other volatile compounds such as glycosylated benzyl alcohol and 2-phenylethanol did not show significant differences in their

concentrations among ripening stages. The concentrations of these glycosylated volatile alcohols were higher than those of free forms at all ripening stages in agreement with other studies carried out on Muscat Hamburg grapes (Fenoll et al., 2009).

3.3. Assessment of the combined effect of grape density and harvest date on chemical and volatile composition using response surface methodology

To better investigate the simultaneous effect of sampling date and grape density on the chemical and volatile composition of Moscato bianco grapes during ripening, the 3D plot of the response surface was obtained (Fig. 2 and 3). Berry density was the main factor affecting technological ripeness parameters (SSC, pH, titratable acidity and malic acid), the density effect being particularly stronger than the date effect for SSC. Other studies confirmed that the pulp composition directly influences the berry density (Rolle et al., 2012). In fact, the highest values of SSC corresponded to the densest berries at any sampling date (Fig. 2a). pH increased with increasing the two independent variables, reaching the highest values in the densest berries harvested at the latest sampling dates (Fig. 2b). Titratable acidity and malic acid showed similar trends (Fig. 2c and 2d). The highest values of these two parameters corresponded to the first sampling date and the lowest berry density, whereas those lowest were associated to berries with intermediate values of density that were sampled at the end of the ripening process. For the parameters related with acidity (pH, titratable acidity and malic acid concentration), the density effect was stronger at the beginning of the ripening process, and the date effect was more evident at lower berry density. The equations obtained are shown in Table 3 for those parameters whose experimental data were adequately fitted into second-order polynomial models ($R^2 > 0.60$). High determination coefficients ($R^2 > 0.90$) suggest goodness-of-fit for the models proposed to assess the changes in the

chemical parameters SSC, pH, titratable acidity and malic acid. These results agreed with those previously reported for the evaluation of the separate effect of the two independent variables (Rolle et al., 2011).

The relationship between the variability within the bunches and berries position in the rachis was reported by several authors (Noguerol-Pato et al., 2012; Pisciotta, di Lorenzo, Barbagallo, & Hunter, 2013; Tarter & Keuter, 2005). Nevertheless, the heterogeneity in the ripeness level among berries may depend upon the variety.

To our knowledge, this is the first time that the simultaneous effect of sampling date and berry density was studied for volatile compounds. According to Fig. 3, the accumulation of all free terpenes was more affected by the berry density than by the sampling date. The total concentration of free terpenes increased with increasing the berry density or delaying the sampling date up to reach intermediate values of the two independent variables (around 1.09 g/cm^3 and from the 5th day after the first sampling), and then only decreased with the density (Fig. 3a). Free linalool, one of the most representative volatile compounds of Muscat cultivars, showed a similar trend but, from the 15th day, its concentration also decreased slightly with the sampling date (Fig. 3b). The decrease observed in linalool concentration at higher values of berry density or longer ripening times could be due to free and bound forms of terpenols reach the maximum concentrations just prior to hexose sugar peak levels being attained (Noguerol-Pato et al., 2012). High determination coefficients suggest goodness-of-fit for the models proposed to evaluate the changes in the concentration of total free terpenes and free linalool (R^2 = 0.85 and 0.77, respectively).

The behavior of free nerol and geraniol was quite similar to linalool (Figures not shown), although the highest concentrations were favored by the increase of density until 1.09-1.10 g/cm^3, particularly from day 17 after the first sampling date. Free *c*-rose

oxide and *t*-rose oxide were much more influenced by the berry density than by the sampling date, the highest concentrations corresponding to the densest berries harvested at the latest date (Figures not shown). The effect of density on the concentrations of free nerol, *c*-rose oxide and *t*-rose oxide was higher when the sampling date was delayed, and the date effect was more accused at higher berry densities. Although these three free terpenes and geraniol are largely present in Moscato bianco grapes, there is a lack-of-fit for the equations obtained by regression analysis ($R^2 < 0.70$). The other free volatile compounds were found in concentrations below odor threshold values, as also happened for nerol (Fenoll et al., 2009). Figures were not shown for these compounds because of lack-of-fit and/or low sensory contribution to the aroma.

Rolle et al. (2015) observed similar results in Muscat Hamburg table grapes, where free nerol, *c*-rose oxide and *t*-rose oxide were more abundant in the berries with the higher density (1.09 g/cm^3). Fenoll et al. (2009) found a significant increase in the content of free linalool with increasing berry density from 100 to 150 g/L also for Muscat Hamburg.

Fig. 3 shows also the combined effect of sampling date and berry density on bound volatile compound concentration in Moscato bianco grapes. Bound total terpenes (Fig. 3c) and bound linalool (Fig. 3d) showed a similar behavior to that of the free fraction, for which density exhibited a higher effect than the sampling date. The highest concentrations were found at high densities (about 1.10 g/cm^3) and intermediate sampling dates (about day 15 after the first sampling). The bound nerol concentration also increased with increasing both berry density and sampling date, although the effect was much more evident for density (Fig. 3e). The maximum concentrations of nerol were reached in berries having a density of 1.10 g/cm^3 from day 10 after the first sampling, and they then decreased with the density. Nevertheless, the influence of

sampling date was important for the concentration of bound geraniol, having a positive effect at low berry densities and negative at high densities (Fig. 3f). Furthermore, the increasing effect of density on the concentration of bound geraniol was smaller when the sampling date was delayed. In fact, the highest concentration of this compound was obtained in the densest berries harvested at the first sampling date. Regarding bound *c*-rose oxide, the effect of the two independent variables was similar, reaching the highest concentration at intermediate values of grape density and sampling date (1.09 g/cm^3 and day 15 after the first sampling) (Fig. 3g). Finally, bound *t*-rose oxide was more influenced by the sampling date than the berry density. In this case, the berry density had a little positive effect whereas the sampling date had a strong negative effect on the concentration of bound *t*-rose oxide (Fig. 3h). Therefore, the berries harvested at the first sampling date were the richest in bound *t*-rose oxide, particularly for values of density about 1.09 g/cm^3. The other bound volatile compounds detected were present in concentrations below odor threshold values, and therefore the corresponding Figures were not shown.

The models were satisfactory (goodness-of-fit) for bound total terpenes, linalool, nerol, geraniol, *c*-rose oxide and *t*-rose oxide, according to determination coefficients ($R^2 >$ 0.70), particularly for bound total terpenes, linalool, nerol, and *t*-rose oxide with determination coefficients higher than 0.80.

The variations observed in the concentrations of glycosylated linalool, nerol, geraniol and isomers of rose oxide with the berry density agreed with those previously reported for Muscat Hamburg table grapes (Fenoll et al., 2009).

Variation within bunches obviously affects the sampling strategy (Barbagallo, Guidoni, & Hunter, 2011; Di Lorenzo, Barbera, Costanza, Pisciotta, Santangelo, & Barbagallo, 2007; Tarter & Keuter, 2008), grape composition, and wine style (Sousa de Melo,

2011). It is important to recognize the high variability in the fruit composition that exists within a vineyard. More information about berry development and its impact on grape and wine quality is necessary.

4. Conclusions

In-field variation among berries may suppose quality differences between varietal wines produced from grapes harvested at the same date. The present work permitted to assess the integrated effect of sampling date and berry density on the chemical and volatile composition of Moscato bianco berries during ripening. Significant differences in free and bound terpenes were observed among berries with different densities and/or sampled at various dates. Grape density was the main factor affecting the total concentration of free and bound terpenes, and the highest concentrations were reached at around 1.10 g/cm^3 and the 15th day after the first sampling. Individual volatile compounds were differently influenced by these two variables. Important terpenes in the Moscato bianco cultivar, such as the free and glycosylated forms of linalool and nerol and the free form of geraniol, *c*-rose oxide and *t*-rose oxide, showed changes in their concentrations with sampling date and berry density, although they were much more influenced by the berry density than by the sampling date. The glycosylated form of geraniol and *c*-rose oxide showed an evident combined effect of the two variables. Higher effect of sampling date was only observed on bound *t*-rose oxide. According to these results, in-field variation of the berries may be used to exploit the maximum varietal aroma potential in the vineyard by selecting the best harvest date and berry density. The berries classification based on the density could minimize the negative effects of the variability between and within the clusters. Furthermore, wines with different aromatic composition may be produced potentiating the presence of the target

compounds in the grapes using an adequate sampling strategy. Further studies could be done to know if the behavior of Moscato bianco during ripening in different vintages is similar.

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Table 1. Technological ripeness parameters for Moscato bianco grapes harvested at different dates.

Chemical parameters	I (20/08)	II (24/08)	III (29/08)	IV (05/09)	V (11/09)	Sign.
SSC (°Brix)	16.6±0.1a	17.4±0.1a	19.3±0.2b	17.2±0.8a	20.2±0.2b	**
pH	3.10±0.01a	3.14±0.01a	3.30±0.01b	3.31±0.02b	3.33±0.02 b	***
Titratable acidity (g/L tartaric acid)	8.3±0.1c	7.6±0.1bc	7.3±1.1bc	5.9±0.1ab	5.4±0.1a	*
Citric acid (g/L)	0.2±0.1	0.2±0.1	0.3±0.1	0.2±0.1	0.2±0.1	ns
Tartaric acid (g/L)	7.1±0.0c	7.0±0.1c	6.6±0.2b	6.1±0.2a	6.2±0.1a	**
Malic acid (g/L)	3.6±0.0d	2.9±0.0c	2.5±0.1b	2.4±0.1b	1.9±0.1a	***

Within each row different letters indicate significant differences among harvest dates according to the Tukey test at $p < 0.05$.
Sign: *, **, *** and ns indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ and not significant, respectively.
SSC, total soluble solids concentration.

Table 2. Free and glycosylated volatile composition of Moscato bianco grapes harvested at different dates.

Free terpenes (µg/L)	I	II	III	IV	V	Sign.
t-Rose oxide	13.5 ± 0.3a	18.4 ± 0.4b	22.0 ± 1.5b	20.0 ± 1.7b	14.4 ± 0.5a	**
c-Rose oxide	4.0 ± 0.1a	5.1 ± 0.7ab	6.4 ± 0.3b	5.8 ± 0.3b	4.2 ± 0.2a	**
t-Furan linalool oxide	3.5 ± 0.1b	3.3 ± 0.1b	4.0 ± 0.1c	2.8 ± 0.1a	3.7 ± 0.1bc	***
c-Furan linalool oxide	3.1 ± 0.8	2.8 ± 0.1	2.9 ± 0.3	3.0 ± 0.2	3.3 ± 0.8	ns
Linalool	478.1 ± 38.2b	389.0 ± 5.1a	555.1 ± 8.8c	379.8 ± 14.5a	457.8 ± 15.7b	**
Hotrienol	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	ns
α-Terpineol	0.07 ± 0.00bc	0.05 ± 0.00a	0.08 ± 0.00c	0.07 ± 0.01bc	0.08 ± 0.01c	*
Pyran linalool oxide	0.05 ± 0.01b	0.04 ± 0.01a	0.06 ± 0.01c	0.04 ± 0.01a	0.05 ± 0.01b	***
Citronellol+Pyran linalool oxide	0.10 ± 0.04	0.13 ± 0.04	0.13 ± 0.05	0.12 ± 0.06	0.10± 0.01	ns
Nerol	48.2 ± 3.8b	69.1 ± 4.1c	73.9 ± 1.8c	73.5 ± 5.3c	9.7 ± 2.7a	***
Geraniol	45.2 ± 6.7a	80.0 ± 5.3b	83.3 ± 3.7bc	101.9 ± 7.6c	52.7 ± 5.7a	**
Geranic acid	0.06 ± 0.01ab	0.06 ± 0.01ab	0.08 ± 0.01b	0.07 ± 0.01ab	0.04 ± 0.01a	*
Other free compounds (µg/L)						
Benzyl alcohol	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	ns
2-Phenylethanol	0.09 ± 0.03	0.10 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	ns
Glycosylated terpenes (µg/L)						
t-Rose oxide	44.8 ± 16.9	54.2 ± 16.0	81.8 ± 21.6	78.7 ± 22.4	70.8 ± 14.1	ns
c-Rose oxide	11.2 ± 1.7	19.2 ± 4.9	29.0 ± 6.5	28.1 ± 6.6	25.3 ± 4.5	ns
t-Furan linalool oxide	22.6 ± 3.2	24.0 ± 2.2	32.9 ± 5.9	32.9 ± 5.2	38.6 ± 6.1	ns
c-Furan linalool oxide	4.7 ± 0.9	5.1 ± 0.4	6.1 ± 1.1	6.1 ± 1.2	5.6 ± 1.0	ns
Linalool	220.2 ± 25.5a	263.7 ± 26.2a	530.2 ± 10.4b	484.0 ± 23.2b	615.9 ± 15.3c	***
Hotrienol	2.5 ± 0.2b	1.5 ± 0.1ab	1.4 ± 0.2ab	0.9 ± 0.6a	1.5 ± 0.3ab	*
α-Terpineol	2.0 ± 0.3	2.3 ± 0.3	3.0 ± 0.5	3.2 ± 0.6	3.6 ± 0.6	ns
Pyran linalool oxide	0.8 ± 0.2	0.9 ± 0.1	1.3 ± 0.3	1.2 ± 0.3	1.2 ± 0.2	ns
Citronellol+Pyran linalool oxide	5.1 ± 0.4a	6.0 ± 0.3b	6.6 ± 0.1bc	7.1 ± 0.1c	6.6 ± 0.1bc	**
Nerol	655.2 ± 18.0a	842.0 ± 9.3b	969.7 ± 48.0 c	1028.2 ± 47.5c	1173.8 ± 21.2d	***
Geraniol	534.9 ± 40.1a	644.2 ± 25.7ab	728.4 ± 40.7bc	751.0 ± 46.7bc	818.9 ± 27.4c	**
Geranic acid	3.0 ± 0.1	3.7 ± 1.6	3.3 ± 0.1	3.3 ± 0.6	3.4 ± 0.6	ns
Other glycosylated terpenes (µg/L)						
Benzyl alcohol	1.8 ± 0.3	1.6 ± 0.6	2.4 ± 0.3	2.5 ± 0.4	2.2 ± 0.1	ns
2-Phenylethanol	2.9 ± 0.5	3.1 ± 0.3	4.1 ± 0.5	4.0 ± 0.7	3.7 ± 0.2	ns

Within each row different letters indicate significant differences among harvest dates according to the Tukey test at $p < 0.05$.
Sign: *, **, *** and ns indicate significance at $p < 0.05$, $p < 0.01$, $p < 0.001$ and not significant, respectively.

Table 3. Second-order polynomial models by Response Surface Methodology.

Parameter (Y)	R^2	Variable coefficient					
			X_1	X_2	X_1^2	X_1X_2	X_2^2
Technological ripeness parameters							
SSC (°Brix)	0.990	-201.28	0.99	141.62	6.51E-04	-0.91	57.14
pH	0.963	-132.45	0.14	241.18	-1.86E-04	-0.12	-107.06
Titratable acidity (g/L)	0.903	2259.02	-0.91	-4080.87	-1.39E-04	0.77	1848.19
Malic acid (g/L)	0.964	-734.76	-1.30	-1300.13	-2.50E-04	1.16	576.53
Free terpenes (µg/L)							
c-Rose oxide	0.688	-1.28E+03	-2.88	2.07E+03	-3.77E-03	2.85	-815.63
t-Rose oxide	0.649	-67.13	-0.49	32.13	-2.32E-05	0.47	30.69
t-Furan linalool oxide	0.868	-460.99	1.35	732.57	-1.38E-03	-1.24	-279.78
c-Furan linalool oxide	0.758	3.39E+03	2.57	-6.38E+03	-2.45E-03	-2.41	3.00E+03
Linalool	0.775	-4.88E+05	19.98	8.92E+05	-0.35	-12.29	-4.08E+05
Hotrienol	0.637	-152.05	1.90	194.89	-1.48E-03	-1.76	-47.48
α-Terpineol	0.686	-556.25	1.80	918.43	-9.13E-04	-1.64	-369.08
Nerol	0.679	-9.80E+04	-61.44	1.80E+05	-0.10	60.83	-8.26E+04
Geraniol	0.653	-9.22E+04	-7.62	1.70E+05	-0.09	12.22	-7.81E+04
Total free terpenes	0.850	-6.89E+05	-48.07	1.26E+06	-0.60	62.28	-5.76E+05
Glycosylated terpenes (µg/L)							
c-Rose oxide	0.791	-7.82E+04	-41.45	1.44E+05	-0.36	48.13	-6.66E+04
t-Rose oxide	0.814	-2.49E+04	-9.09	4.59E+04	0.02	6.02	-2.11E+04
t-Furan linalool oxide	0.689	785.31	-1.29	-1.84E+03	-0.04	2.19	1.05E+03
c-Furan linalool oxide	0.655	-6.13E+03	26.61	1.08E+04	0.04	-26.61	-4.70E+03
Linalool	0.819	-2.92E+05	77.27	5.32E+05	-0.28	-60.39	-2.41E+05
α-Terpineol	0.769	-3.71E+03	21.02	6.05E+03	-0.04	-18.78	-2.40E+03
Pyran linalool oxide	0.701	1.96E+03	6.19	-3.80E+03	-0.01	-5.52	1.84E+03
Citronellol	0.673	-2.13E+04	-17.66	3.88E+04	-0.04	17.37	-1.76E+04
Nerol	0.815	-6.16E+05	59.06	1.13E+06	-0.14	-47.66	-5.13E+05
Geraniol	0.703	-8.85E+04	289.32	1.56E+05	-5.80E-04	-263.88	-6.75E+04
Total glycosylated terpenes	0.813	-1.29E+06	537.69	2.34E+06	-1.31	-459.43	-1.06E+06

X_1, sampling days; X_2, berry density.
SSC, total soluble solids concentration.

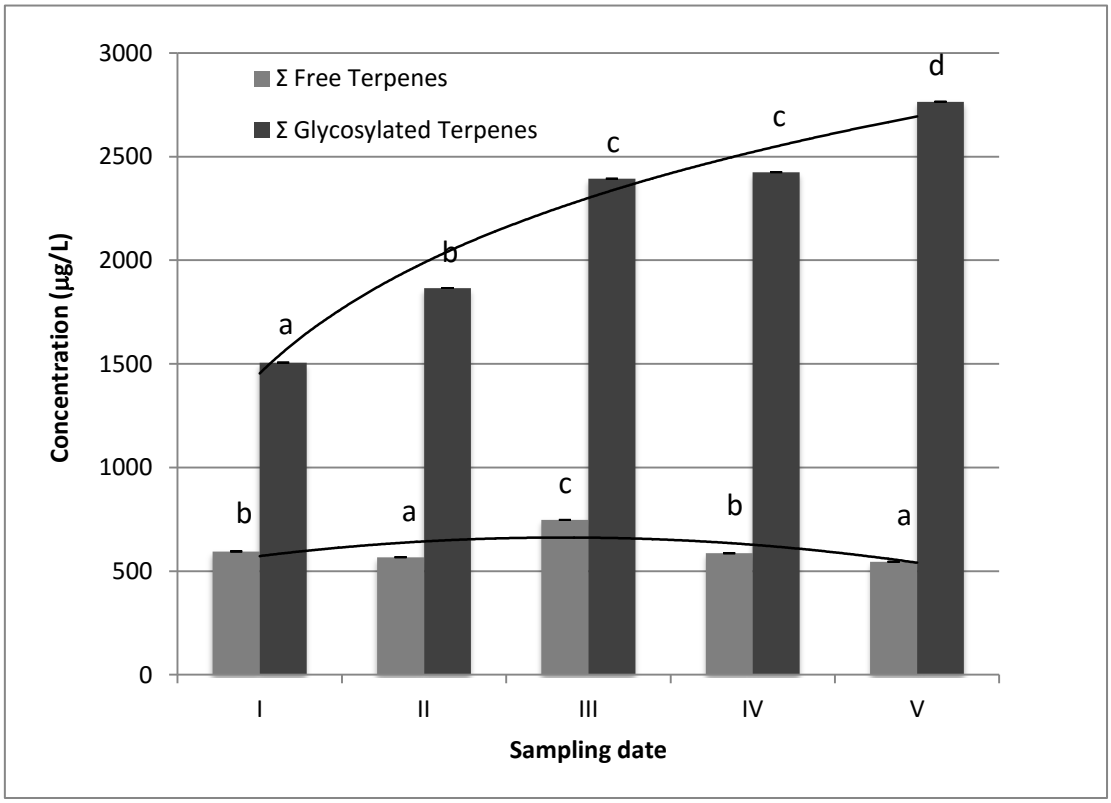

Figure 1. Total free and glycosylated terpene concentration of Moscato bianco grapes harvested at different dates.

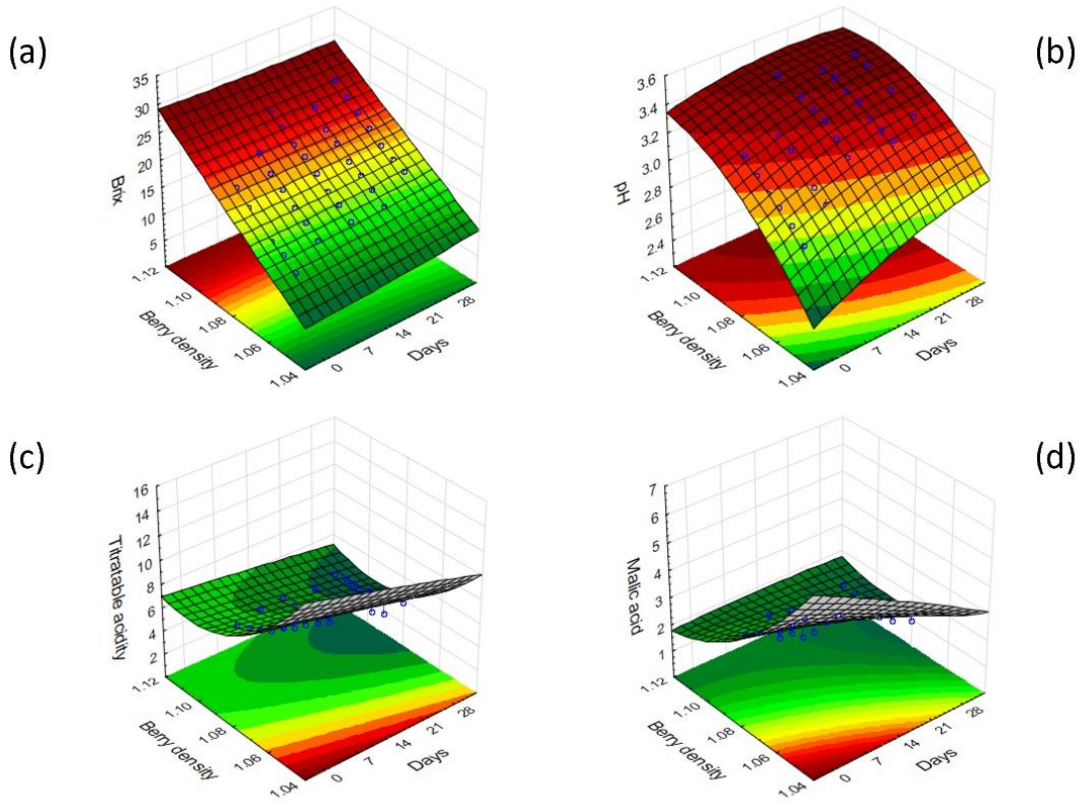

Figure 2. Response surface plot showing the effect of sampling date and berry density on technological ripeness parameters of Moscato bianco grapes: (a) SSC (ºBrix), (b) pH, (c) titratable acidity (g/L tartaric acid) and (d) malic acid concentration (g/L).

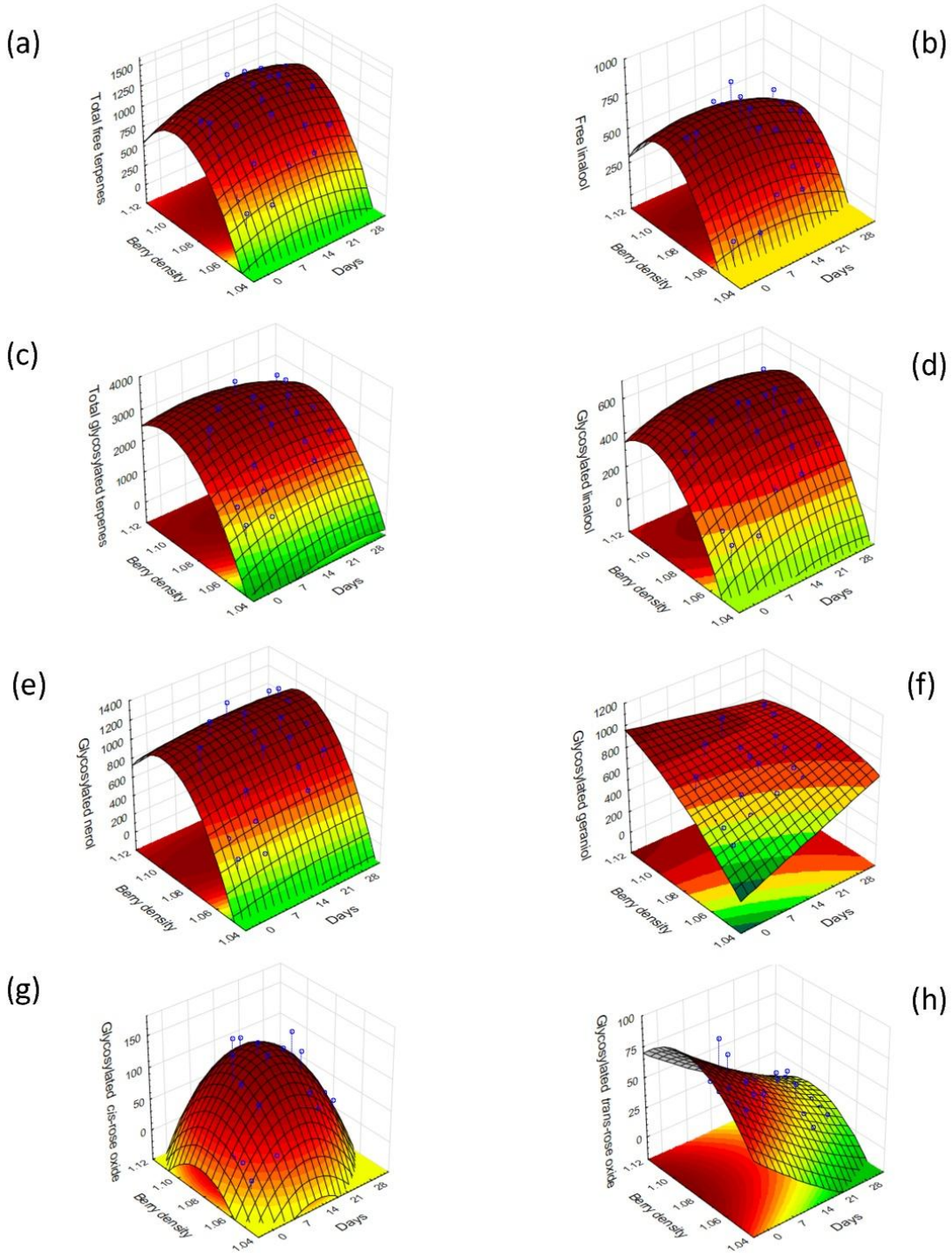

Figure 3. Response surface plot showing the effect of sampling date and berry density on the concentration of terpenes (µg/L) in Moscato bianco grapes: (a) total free terpenes and (b) free linalool, (c) total glycosylated terpenes, (d) glycosylated linalool, (e) glycosylated nerol, (f) glycosylated geraniol, (g) glycosylated *c*-rose oxide and (h) glycosylated *t*-rose oxide.

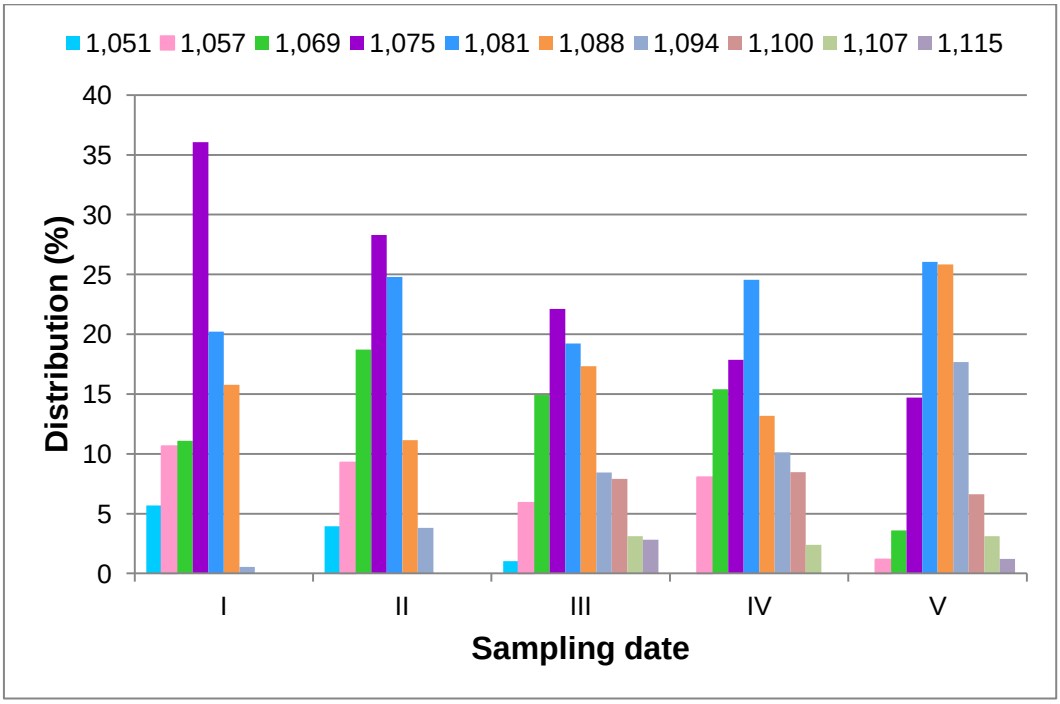

Figure S1. Weight percentage of Moscato bianco grape berries in density classes at different harvest dates.

Highlights

- Changes in the terpene composition of grape berries were evaluated during ripening
- The simultaneous effect of sampling time and berry density on terpenes was studied
- Response surface methodology was used to assess the combined effect time-density
- Grape density was the main factor affecting the total terpene concentration
- The sampling strategy may suppose different aroma quality of grape berries