Key norisoprenoid compounds in wines from early-harvested grapes in view of climate change

Aroma evaluation of wines from early-harvested grapes in view of climate change

Andriani Asproudi [a], Alessandra Ferrandino[b], Federica Bonello[a], Enrico Vaudano[a], Matteo Pollon[b], Maurizio Petrozziello[*a]

[a]Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria (Italy) – Centro di ricerca Viticoltura ed Enologia - CREA – VE, via P. Micca 35, Asti, Italy.

[b]Dipartimento di Scienze Agrarie, Forestali e Alimentari – Università di Torino, Largo Braccini 2, 10095 Grugliasco (TO), Italy

[*]Corresponding Author

Email: maurizio.petrozziello@crea.gov.it

Phone:+39 0141433811

Abstract

In view of climate change, the scheduling of an early harvest may be an agronomic option to limit wine alcohol provided that, a satisfactory content of secondary metabolites is ensured in grapes. In order to better understand the link between grape ripening, seasonal trend and wine aroma, the aromatic expression of Barbera and Pinot Noir wines produced with early-harvested grapes was assessed. Major attention was focused on norisoprenoids during both alcoholic fermentation and after three months of storage. At the end of fermentation, the highest β-damascenone content was detected in wines obtained from less ripe grapes, then its content increased significantly after 3 months of storage. Inversely, the levels of β-ionone decreased significantly during the same period. The reduction of wine alcohol assessed by harvesting earlier especially for Barbera, was associated to optimal aromatic levels as well as to good technological parameters.

Keywords

β-damascenone; β-ionone; Pinot noir; Barbera; carotenoids; early-harvest; wine, climate change

1. Introduction

Global warming and related climate change, mainly linked to anthropogenic factors, represent one of the most important world issue. The consequences of these changes involve agriculture and have considerable consequences both from a social and economic point of view (Barros, V. R., Field, C. B., Dokke, D. J., Mastrandrea, M. D., Mach, K. J., Bilir, T. E. et al., 2014). Viticulture is one of the agricultural sector more susceptible to these changes mainly due to its strict interaction with environment, soil, human choices addressed to drive viticultural techniques, and tradition. If global change could exert its influence on, for instance, cultivar distribution, the well-known and well-established combination variety-environment could fail and many other aspects, such as cultivar distribution, phenological phases, vine productivity, vine pathologies could be influenced (Palliotti et al 2014; Sacchelli, Fabbrizzi, & Menghini, 2016).

Numerous studies have pointed out that during the last decades there has been an advance of the phenological phases (Webb et al., 2012; Webb, Whetton, & Barlow, 2007), in particular flowering and veraison, compared to what was considered "normal" for the vine and for a specific area (van Leeuwen & Darriet, 2016). The increase in average temperatures of summer months as major consequence of climate change, as well as the different distribution of rainfall during the ripening phase, led both to a higher concentration of sugar and to a general change of the acidic profile of grapes, due, in particular, to the reduction of malic acid concentration. Microbiologically, the must pH increase can facilitate the development of bacterial contamination in wine, whereas the high sugar content may induce stuck fermentations or high production of unwanted by-products such as acetic acid and glycerol (De Orduna, 2010).

Furthermore, in several viticulture areas, ripening occurs during the hottest part of the season, when both color and aroma profile can be adversely affected (Mori et al., 2007, Asproudi et. al., 2016). At high temperatures, vine metabolism is inhibited, leading to a lower accumulation of polyphenols and a lack of synchrony among the timing of sugar/acid balance and polyphenolic optimum, especially in Mediterranean conditions (Mori et al 2007; Tomasi, Jones, Giust, Lovat, & Gaiotti, 2011). Moreover, the improvement of vineyard management

(clonal choice, rootstocks, agronomic practices) together with the viticulturists' and political choices, oriented to reduce yield per vine and to increase quality (sugars and polyphenols), combined with the series of anomalous and warm summers, further contributed to increase the sugar content in grapes.

The excessive alcohol content of wines, resulting from exceptionally sugary grapes, has become an unwelcome feature for the consumers. Nowadays the consumer orientation is directed to drinks with a moderate level of alcohol, as a result both of health concerns and of significant changes in people preferences, mainly addressed to more fresh and fruity wines (Caballero & Segura, 2017). From the sensorial point of view, the high alcohol wine content has numerous organoleptic consequences such as the decrease in freshness and a change in the perception of the aromatic bouquet. In fact, ethanol may enhance the perception of sweetness and bitterness while reducing that of acid, saltiness and sourness. Moreover, ethanol influences headspace partitioning of volatiles (Robinson et al., 2009) decreasing volatility of the aromatic compounds (Le Berre, Atanasova, Langlois, Etiévant, & Thomas-Danguin, 2007). Thus, climate-change related variations of grape ripeness can cause modification in the aromatic perception of wines, directly, with the formation of compounds characterized by overripe fruit notes, the reduction of vegetal, fresh and flowery notes (Pons et al., 2017) or indirectly, through the sensitive modification of their aromatic profile, due to the increase in alcohol content.

Nowadays, one of the major challenges in oenology and viticulture is how to mitigate and respond to the effects of climate change (Mozell & Thach, 2014), in order to preserve the specific and distinctive olfactory and gustatory notes that link wines to their territory of origin. In this regard, the early harvest of grapes to limit wine alcohol level may be an alternative to the use of subtractive cellar technologies, often invasive and which may cause compositional alterations, penalizing the aromatic quality of the product. Previous studies have doubted that, from the aromatic point of view, wines produced with early-harvested grapes could be endowed with a high acidity and an excessive content of C6 compounds (six carbon atom aldehydes and alcohols, known as leaf alcohols), to which vegetal notes are attributed (Longo et al., 2017). Nevertheless, the high sugar content of the grapes recorded during the last vintages has made early-harvest of renewed interest, especially for the warmer areas, provided that early-harvested grapes show an optimal balance between the different qualitative components of the berry, especially volatile and polyphenol concentrations and profiles.

To the authors' knowledge, little investigation on key aroma compounds such as norisoprenoids and their content in wines produced from sequential grape harvests has been reported and only some research linked technological and aromatic maturity of the grapes to the aroma of finished wines. Norisoprenoids derive from carotenoid degradation through both non-specific and enzymatic mechanisms, involving Carotenoid Cleavage Dioxygenases (CCDs) whose expression is strictly correlated to climatic and agronomic parameters (Chen et al., 2017). The most interesting norisoprenoids from the aromatic point of view are megastigmane, notably β-ionone with typical violet notes and β-damascenone, characterized by notes of quince and flowers (Mendes-Pinto, 2009). Especially β-damascenone is a strong flavor found in many foods and beverages (Pineau, Barbe, Van Leeuwen, & Dubourdieu, 2007). It has a complex aroma, reminiscent of honey, tropical fruit, quince, apple that is differently expressed depending on matrix and concentration. Some researchers suggested that β-damascenone also has an indirect impact on wine aroma by enhancing fruity notes of ethyl esters (Escudero, Campo, Fariña, Cacho, & Ferreira, 2007). Clarifying the relationship between aromatic precursors in grapes (carotenoids) and norisoprenoids in wines, could be relevant to understand the phenomena that can influence wine quality as the complexity of the transformations that control these phenomena has provided no clear answers, yet. Authors' previous research showed that in wines obtained from grapes with high total acidity, the concentration of β-damascenone was higher than that of wines produced with grapes harvested when fully ripe (Petrozziello M., 2012). More information is needed to define the optimum harvest time coupling together strategies able to enhance the wine aroma and meeting contemporarily the demand for wines with both reduced alcohol content and balanced organoleptic properties.

To this purpose, the effect of different grape ripening levels on the aroma of wines made under the same fermentation conditions was investigated, using two non-floral varieties, the international Pinot noir, and Barbera an important Italian variety grown in the same vineyard in Piedmont.

Pinot noir is a non-floral international grape variety widely planted around the world mostly in cool climate areas. Berries generally accumulate low amounts of phenolic compounds, including anthocyanins whose profile is characterized by the total absence of acylated-derivatives. When young, wines made from Pinot Noir tend to have red-fruit aromas, such as cherries, raspberries and strawberries and overall Pinot noir wine

characteristics significantly vary with grape maturity (Fang & Qian, 2005, 2006; Miranda-Lopez, Libbey, Watson, & Mc Daniel, 1992). Barbera is an Italian cultivar producing berries with high titratable acidity that, in the past, made its cultivation a valued planting in warm climate regions where acidification was usually needed. Traditionally, some viticulturists used to delay harvest, if the seasonal climatic conditions were favorable, to increase sugar levels to balance Barbera wine acidity, despite a natural predisposition to a high sugar accumulation. From the aromatic point of view previous studies pointed out that Barbera grapes were characterized by important amounts of volatiles, including terpenes and β-ionone (Carlomagno et al., 2012).

In this work main technological parameters of Pinot noir and Barbera grapes harvested at three different ripening stages (-15d, -7d and 0d, indicating the days before full-ripeness) were assessed, together with important key aromas of must and wines. Attention was focused on β-damascenone, β-ionone and α-ionone which were quantified by stable isotope dilution assay (SIDA) and HS-SPME-GCMS quantification, whereas the most important free fermentative aromatic compounds were extracted and quantified respectively by Solid phase Extraction Gas chromatography coupled with mass spectrometry (SPE/GC-MS).

2. **Materials and methods**

2.1 Vineyard site

Grape samples of cv Pinot noir and Barbera were collected at the DISAFA (*Università degli Studi di Torino*) experimental vineyard located in Grugliasco (45°03'N, 7°35'E; in Piedmont, Italy), in 2015. Vine density was 4400 vines/ha (0.90 m x 2.50 m), vines were vertical shoot positioned (VSP) and trained to the Guyot pruning system. The vineyard is located at 293 m above s.l.in a plain area and vines were planted in 2008; Pinot noir plants were grafted onto 1103P while Barbera plants onto SO4. The vineyard was organized into randomized blocks of 10 plants each. Three blocks for each variety were used as biological replicates (namely: A, B and C). Starting from bud-burst, the main phenological phases of the plant were observed (flowering, veraison and ripening). The first sampling of Pinot Noir was carried out at veraison (50 % of colored berries) and grapes were then sampled again on the 13th, 19th and 25th of August 2015. Barbera was firstly sampled at veraison and then harvested sequentially on the 25th, 31st of August and on the 7th September 2015.

2.2 Grape sampling

Approximately 30 clusters for each biological replicate (A, B and C). were harvested manually at each sampling date (veraison, and 3 ripening levels). For each replicate, 500 berries were sampled for the analysis of the main chemico-physical parameters namely, berry weight, pH, titratable acidity (TA), total soluble solids (TSS). Remaining berries were opportunely prepared to obtain grape extracts for polyphenol, anthocyanin and total flavonoid measurements; two further replicates of 50 g of grapes were stored in the dark at -80°C for carotenoid compound assessment.

2.3 Microvinifications

Vinification trials at laboratory scale were carried out in triplicate for each maturation point for a total of 9 fermentations per variety. Grapes (about 2 Kg per replica) were manually destemmed, crushed and placed into three liters Erlenmeyer flask. Inoculum (5 x 106 cells g^{-1}) was done using *Saccharomyces cerevisiae* yeast strain ISE 167 belonging to CREA-VE culture collection after a preventive growth in YPG (Yeast Peptone Glucose) medium. Fermentations were performed at 25 °C, and two punching per day were carried out to simulate a standard red vinification. Fermentations were followed by daily monitoring of the flasks weight loss, indirectly calculating the consumed sugar. Sampling was carried out at crushing (day 0), 50% of fermented sugars (approximately day 3 for all trials) and at the end of fermentation (day 8). pH, AT, TSS and polyphenolic index measurements were assessed at crushing, at half time and at the end of the fermentation. Final alcohol content was determined for each wine. The measurement of TSS, total acidity, pH, of grape musts as well as the analysis of reducing sugars at the end of alcoholic fermentation, density, total dry extract and ethanol in wines were carried out according to official EC methods (Commission Regulation No. 2676/90 determining Community methods for the analysis of wines, 1990) .The evolution of norisoprenoid compounds was thoroughly investigated during fermentation, namely the determination of α-ionone β-ionone and β-damascenone has been carried out at crushing, at mid-fermentation, at the end of alcoholic fermentation (FFA) and finally after 3 months of wine storage in cellar at 4°C. All analysis were carried out twice.

2.4 Meteorological assessments

The vineyard had meterological station equipped with a thermohygrometer and a rain gauge, managed by the Agrometerological Serivice of the Piedmont Region. Part of the data were found on-line from the database of the Department of Physics of the University of Turin-DF station (45° 03′ N , 7°40 E, 254 above s.l, Turin).

2.5 Grape, must and wine determinations

2.5.1 Extraction and determination of polyphenols in grapes, musts and wines

Extraction of the polyphenolic fraction from the grapes was performed according to Di Stefano (Di Stefano & Cravero, 1991). Briefly, 20 frozen berries were peeled, and the skins were placed in 50 mL of tartaric buffer at pH 3.20 (5 g of tartaric acid, 22 mL of 1N NaOH, 120 mL of ethanol and 2 g of sodium metabisulphite brought up to 1L with distilled water). After 4 hours, the skins were homogenized and collected in a centrifuge tube. After centrifugation (4000 rpm for 15 min), the supernatant was collected in a 100 mL flask. The pellet was added of few mL of buffer and centrifuged for a second time, the supernatant collected in the same flask. Then the volume was adjusted up to 100 mL using the tartaric buffer; samples were stored in -20°C until analysis were carried out.

2.5.2 Total polyphenols index (TPI)

Total polyphenol content was determined using the Folin-Ciocalteau reagent (Di Stefano, Cravero, & Gentilini, 1989). Briefly, must or grape extract obtained as described above, were previously acidified with H_2SO_4 and passed through a 500 mg C18 cartridge to retain the compounds of interest that were successively eluted with 3 mL of methanol in a 20 mL flask. As to wines, due to the lower SO_2 content respect to berry extracts, no cartridge passage was required. Total polyphenols were determined by measuring the absorbance of the extract at 700 nm and expressed as mg equivalent of (+)-cathechin per kg of berries as to grapes and per L of wine.

2.5.3 Total antocyanin and flavonoid indexes (TAI and TFI)

Determination of flavonoids and anthocyanins was carried out spectrophotometrically as described by Di Stefano and coworkers (Di Stefano et al., 1989). The grape extracts or musts were filtered onto 0.45 μm polypropylene membrane, then opportunely diluted with “hydrochloric ethanol” a mixture of ethanol/H_2O/HCl 37% (70: 30: 1). Subsequently, the sample absorbance spectrum was acquired from 230 to 700 nm, using a 10

mm path step cuvette. Total flavonoid index was determined through graphical correction applied to UV peak with a maximum of 280 nm and expressed as mg equivalent of (+)-cathechin per kg of berries. Total anthocyanin index was determined by measuring the absorbance of the extract at 540 nm and expressed as mg equivalent of malvidin-3-O-glucoside chloride per kg of berries.

2.5.4 Extraction and determination of grape carotenoids

Carotenoid extraction procedure was adapted from Crupi and coworkers (Crupi, Milella, & Antonacci, 2010). Approximately 50 g of berries, without seeds, added of BHA (Butylated hydroxyanisole) were homogenized for 2 min in the presence of magnesium carbonate basic. The homogenate was spiked with 200 μL of 180 mg L^{-1} of β-apo-8-carotenal (Fluka, Porto, Portugal, ref. 10810) as internal standard and diluted with 40 mL of ultrapure (UP) water obtained from a MilliQ purification system (Millipore Bedford, MA, USA). A liquid-liquid extraction was carried out with ether/hexane (1:1, v/v), repeated three times for 30 min each. The resulting upper layer was separated each time, thus the final combined extract was concentrated to dryness at 20° C (Laborota 4001, Heidolph instruments) and resuspended in 1 mL of acetone/hexane (1:1, v/v) for HPLC determination. Each sample was injected in duplicate. Sample handling, homogenization and extraction were carried out on ice under dim yellow light to minimize light-induced isomerization and oxidation of carotenoids.

An Agilent Model 1200 quaternary solvent system, equipped with a quaternary pump solvent delivery and an UV-visible photodiode array detector was used (Agilent Technologies, Santa Clara, CA, US). The absorption spectra were recorded at 447 nm and the sample injection volume was 20μL. The column was an YMC30, 250 x 4,6 mm, with a pre-column YMC pack C30 (3 x 20mm, 5 μm). Mobile phase was performed with three different solvents as described by Crupi and coworkers (Crupi et. al 2010). The flow was set at 0.35 mL min^{-1}. The analytical gradient started with 40%A, 60%B, and 0%C and then linear gradients as follows: to 20% A, 80% B, 0%C in 5 min; to 4%A, 81%B, 15%C in 10 min; to 45%A, 11%B, 85%C in 60 min. Acquisition time was 70 min and equilibration time was 10 min.

The most relevant carotenoids were identified by comparison of spectra with those of commercially available standards, violaxanthin, lutein epoxide, neoxanthin from CaroteNature (Lupsingen, *Switzerland*) and β-

carotene and lutein from Extrasynthèses (Lyon, Genay, France), matching also different information such as position of absorption maxima (λ_{max}) and the degree of vibration fine structure (% III/II) (Crupi et al., 2010). Quantification of individual compounds was done by calibration curves using the respective standards. The results were expressed as mg per kilogram of grape berries.

2.5.5 Determination of β-damascenone, α-ionone and β-ionone

The chemical standards for this analysis were obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA) at the maximum purity grade available, except β-damascenone, which was generously supplied by Firmenich (Genève, Switzerland). β-damascenone β-ionone and α-ionone were quantified in musts and wines using a sTable isotope dilution assay (SIDA)_HS-SPME/GC-MS method as described by Petrozziello and co-workers (Petrozziello, Borsa, Guaita, Gerbi, & Bosso, 2012). Briefly, a SPME fibre (CAR/PDMS/DVB da 30/50 µm, Supelco, Bellefonte, PA, USA). was conditioned daily before use for 30' at 270 °C. For each analysis, 10 mL of sample (must or wine) was placed into a 20 mL vial, added of 3 g of ammonium sulfate. Four µL of internal standard containing [$^2H_4$]-β-damascenone (final concentration: 2.36 µg L^{-1}), [$^2H_3$]-β-ionone (final concentration: 11.8 µg L^{-1}) and [$^2H_3$]-α-ionone (final concentration: 24.3 µg L^{-1}). The vial was capped with a crimp seal with a PTFE/silicone septum and the sample was left to equilibrate in agitation for at least 15 min at 40 °C before the analysis. The extraction time was 1h at 40°C and then the compounds were thermo-desorbed from the fiber for 3 min into the GC injector held at 250 °C. The analyses were performed in splitless mode, and the purge valve was opened after 3 min. Finally, to eliminate the carryover phenomena, the fiber was cleaned at 250 °C in the needle heater device for 10 min after each analysis and for an additional 3 min time before the following injection. All the operations were automated by a multipurpose sampler MPS 2XL (Gerstel Applications, Brielle, The Netherlands).

GC-MS analyses were performed with a 6980 Agilent gas chromatograph interfaced to a mass selective detector 5973N (Agilent Technologies, Palo Alto, CA,USA). A HP-Innowax column, polyethylene glycol, 30 m x 0.25 mm x 0.25 µm (J&W Scientific, Folsom, CA, USA) was used. Helium was the carrier gas and the column flow was maintained at 1.2 mL min^{-1}. Transfer line was set at 230 °C. The oven temperature was held

at 45 °C for 2 min, raised to 80°C at a rate of 30 °C min $^{-1}$, then raised from 80 to 230 °C at a rate of 5 °C min $^{-1}$ and, finally was held at 230 °C for 17 min. The ionization voltage was at 70 eV, the quadrupole was set at 230 °C and the source at 250 °C. Mass spectra were acquired in Selective Ion Monitoring (SIM) mode using a dwell time of 100 µs. Identification of these megastigmane compounds was performed by comparing recorded mass spectra and retention time with those of authentic standards. β-damascenone standard was kindly offered by Firmenich, (Swizerland). Quantifying ions were 190 and 194 m/z for β-damascenone and [$^{2}H_4$]-β-damascenone respectively; 136 and 139 m/z for α-ionone and [$^{2}H_3$]-α-ionone, respectively and 177 and 180 m/z for β-ionone and [$^{2}H_3$]-β-ionone, respectively, using a calibration curve for each compound.

2.5.6 Extraction and determination of free volatiles in wines:

150 mL of wine, added of 150 µL of internal standard (1-heptanol, 73.43 mg L^{-1}), were passed through a 5 g C18-RP cartridge (Biotage AB, Uppsala, Sweden), previously activated with 20 mL of methanol and equilibrated with 50 mL of UP water. After washing the cartridge with 50 mL of water, free varietal compounds and fermentative compounds were recovered with 30 mL of dichloromethane. Glycoside compounds were recovered with 25 mL of methanol (Sigma Aldrich Co., St. Louis, MO, USA). Dicloromethane was dried using anhydrous Na_2SO_4 and evaporated to about 200µL under a gentle stream of nitrogen; an aliquot of 1 µL was injected into the GC-MS.

The hydrolysis of glycosides by exogenous enzyme was carried out accordingly to Cabrita and collaborators (Cabrita, Costa Freitas, Laureano, Borsa, & Di Stefano, 2007). Briefly, the methanolic phase was evaporated to dryness under vacuum and the residue dissolved in 5 mL of citrate-phosphate buffer (pH 5.0, 51.5 % v/v of 0.2 M sodium phosphate and 48.5 % v/v of 0.1 M citric acid). 100 mg of polyvinylpolypyrrolidone (PVPP) was added and then the enzymatic hydrolysis was carried out with 0.2 mL of Pectinol (Genencor, Palo Alto , CA, USA) with glycosidase-side activities at 40 °C for 24 h. After hydrolysis, 0.1 mL of 1-octanol as internal standard was added and the hydrolyzed extract was passed through a 1 g C18-RP cartridge (Biotage AB, Uppsala, Sweden) to isolate the aglycons. The free-released compounds were eluted with 12 mL of dichloromethane. The organic layer was dried using anhydrous Na_2SO_4, and reduced to a small volume (about

500 µL) under a gentle stream of nitrogen at room temperature. The analysis of the aglycons was carried out by GC-MS. Two replicates of all samples were analyzed.

All compounds were analyzed by GC-MS using an Agilent 7890A GC, equipped with an Agilent 5975C Mass Selective triple Axis Detector. The samples (1µL) were manually injected at 250 °C, in splitless mode. The column was a Zebron ZB-WAX column (30 m, 0.25 mm i.d., 0.25 µm film thickness; Phenomenex, Torrance, Calif., U.S.A.). The oven temperature was set at 45 °C for 2 min, then raised to 60 °C at a rate of 30 °C min^{-1}, from 60 to 230 °C at a rate of 2 °C min^{-1}, and held at 230 °C for 20 min. The carrier gas was helium with a constant flow of 1 mL min^{-1}. The transfer line was set at 230 °C. The ionization voltage was 70 eV, the quadrupole was set at 230 °C and the source at 250 °C. The acquisition of mass spectra for the analysis of compounds was carried out in total ion current mode (TIC) and a 29-300 m/z range was recorded. Identification of volatile compounds was performed by comparing recorded mass spectra with those of the WILEY275 database and retention index with those of authentic standards, if available, or by comparison with the gas chromatographic retention index LRI (Bianchi, Careri, Mangia, & Musci, 2007) and with the mass spectrometric data reported in literature. The semi-quantitative analysis was carried out by comparing the areas of individual chromatographic peaks with that of the internal standard.

2.6 Statistical analysis

Data from chemico-physical analyses were statistically elaborated using the software SPSS Windows version 15.0 (SPSS Inc., Chicago, IL, USA), and XLstat (XLSTAT 2017: Data Analysis and Statistical Solution for Microsoft Excel. Addinsoft, Paris, France, 2017). Both for Barbera and Pinot noir, the evolution study of C13-norisoprenoids during the grape maturation, the fermentation process and the interaction between these two factors were treated with a linear mixed effect regression model (lme) performed with R 3.4.3 (R Foundation for Statistical Computing, Vienna, Austria). Linear mixed effects model was choice in order to manage the random factors of the analytical design and the fermentation repeated measures. Each of the three vineyard

rows (A, B, C) and the three fermentation replications were included in the model as random factors. In case of lme ANOVA (p-value < 0.05) was calculated and significant differences among means were analyzed with least mean square with Bonferroni's correction.

3. Results and Discussion

3.1 Climatic trend 2015 vintage

Main meteorological conditions of 2015 are shown in Table 1. A mild winter characterized the season; this led, early in the season, to the sum of temperatures necessary to the vine to bud-burst and to a general advance of the phenological phases that was maintained throughout the entire season. The month of June was particularly rainy either as frequency of rainy days and as mm of rainfalls. Because of these peculiar climatic conditions (mild winter and water availability in June), veraison was much anticipated and it happened on July the 23rd and the 27th in Pinot noir and Barbera, respectively. Grape technological ripening was set to about 21 °Brix for Pinot noir and to about 24 °Brix for Barbera, on the basis of average ripening level used for the two varieties in the cultivation area (Piedmont, North-West Italy). As a consequence, the sampling carried on the 13th of August corresponded to 15 days before full ripening for Pinot noir (early-ripening grapevine cultivar), whereas for Barbera the sampling carried on the 25th of August corresponded to 15 days before full ripening. Samplings performed on the 19th and 31st of August, represented harvests at seven days (-7d) before full ripeness, respectively for Pinot noir and Barbera grapes.

Table 1

3.2 Chemical-physical characteristics of grapes

A relevant increase of berry weight for both varieties was noticed from veraison until the first sampling of the ripening period. Berry weight of Pinot Noir grapes increased constantly until the last sampling date (August, the 25th), *vice versa* Barbera berry weights increased earlier and since the 25th of August (15 days prior to full ripeness) they did not vary anymore. At full ripeness, as expected, Pinot noir grapes were about 60% lighter berries than Barbera ones. (Table 2).

Pinot noir grape TSS did not vary from the first to the second sampling and it increased slightly at full ripeness. Pinot noir TA decreased since the second sampling and it was almost half respect to that measured in Barbera berries. Berry pH was consequently higher in Pinot noir respect to Barbera but no major differences were detected among harvest dates, regardless the cultivar. The constant and linear increase of TSS during the last stage of ripening in Barbera allowed to obtain grapes that differed of about 2 degrees °Brix at each sampling date (-15 d, -7 d and full ripeness). TA values above 13.1 g L^{-1} were reached in the first sampling (-15 d) and slowly decreased afterwards, maintaining, however, high levels until full ripeness (Table 2).

Table 2

3.3 Trend of the polyphenolic component during the last stages of ripening

Table 2 also reports the changes in the main parameters related to the polyphenolic composition of Pinot and Barbera grapes during maturation. The accumulation of polyphenols in the berries followed a different trend in the two varieties: in Pinot noir grapes a progressive decrease in the total polyphenol index and an increase in the colored fraction was observed from veraison to the first sampling (-15 d) with a substantial constant trend of the total flavonoid index. As to all measured indices, no relevant differences were noticed during the last stages of ripening (-15d, -7d, and full ripeness) in Pinot noir grapes. In Barbera grapes there was a progressive increase of total polyphenols and anthocyanins and the total anthocyanin index (TAI) reached satisfactory values already 7 days before the theoretical and scheduled harvest (full ripeness). On average, the total polyphenol index of Barbera grapes was lower than that of Pinot for the first two samplings.

The same spectrophotometric indices, but referred to the single berry (data not reported), showed for Barbera, a peak of accumulation coinciding with the second sampling followed by a plateau phase thereafter. For Pinot noir, when expressing data on a per berry basis, the highest values of total anthocyanin and flavonoid indices were observed at the third sampling date while the total polyphenol index remained almost constant all along the considered harvesting period.

3.4 Trend of carotenoids during the last stages of ripening

Pinot noir. According to literature, β-carotene and lutein contents in Pinot noir grapes tended to decrease markedly within veraison, whereas violaxanthin showed a short period of accumulation before veraison Lutein, β-carotene, and neoxanthin continued to decrease during berry development until harvest. Small differences as regards neoxanthin in the last day of ripening were highlighted (Yuan and Qian, 2016). Our results showed an important degradation of lutein from veraison to the first harvest (-15d) without any differences afterwards. Similar levels of β−carotene were observed during ripening and a slight decrease at the last sampling. As regards violaxanthin and neoxanthin a decrease from veraison until the first sampling time was noticed, but no notable differences were detected, afterwards. The lutein/ß−carotene ratio in Pinot noir grapes was found to be higher than one (Table 2).

Barbera. A previous research, concerning the content in carotenoid compounds of Barbera during berry development (Giovanelli and Brenna 2007), showed that lutein concentration followed a discontinuous and fluctuating trend, whereas the content in β-carotene tended to decrease gradually. The data reported here (Table 2) showed for Barbera grapes a lutein concentration decrease from veraison until the first sampling (-15 d) and a slight reduction afterwards; no variations over the considered period were noticed for β-carotene. The ratio lutein/β-carotene in Barbera grapes was lower than 1 at full ripeness similarly to Giovannelli and Brenna (2007). However, this ratio was found to be dependent on the variety: in fact, it was found to be higher than 1 in Pinot Noir, whereas in other varieties these two carotenoids showed similar concentrations or, vice versa, β-carotene concentration was higher than that of lutein, resulting in ratio lower than 1 (Bunea et al., 2012).

The concentration of minor xanthophylls, determined for the first time in Barbera grapes, had heterogeneous behaviors depending on the compound. Violaxanthin and lutein-5,6-epoxide followed a similar trend, that agreed with what reported in literature for other varieties (Razungles, Babic, Sapis, & Bayonove, 1996; Winterhalter & Ebeler, 2013) and that highlighted a content increase from veraison until the first sampling (-15 d) and almost constant concentrations afterwards. On the contrary, neoxanthin concentrations decreased constantly from the first (-15 d) to the last sampling date (full ripeness).

3.5 Chemical-physical characteristics of musts

In Pinot noir musts, no major differences were detected as to TSS, pH and TA during the three-consecutive samplings (Table 2). Pinot noir grapes reached a ripening degree correspondent to a satisfactory technological harvest, already on August the 13th, fifteen days before the scheduled harvest and the last days of ripening did not contribute to modify significantly the main technological parameters. Moreover, this ripening trend was also favored by the lowering of the temperatures and the rainy conditions that characterized that period. As to Barbera musts, the differences between the three different harvest dates were relevant; TSS increased and TA decreased from the first (-15 d) to the third grape sampling date (full ripeness).

3.6 Norisoprenoids in musts and wines during and at the end of fermentation

Pinot Noir wines. The linear mixed effect model applied to the entire Pinot dataset showed significant changes in norisoprenoid concentrations in wines obtained from different harvests. The comparison among modelized means of the three norisoprenoids highlighted that the concentrations of β-damascenone and β-ionone decreased on average in dependence of the ripening level of grapes while, α-ionone was less influenced by the grape ripening level (Table 3).

Table 3

Fig. 1 shows the trends of free norisoprenoids during fermentation in Pinot noir. β-ionone average content of 90 ng/L was measured at crushing (day 1) then its concentration increased linearly throughout fermentation reaching final average values of more than 1.417 μg L^{-1} corresponding to what reported in literature (Oliveira et al., 2006; Yuan & Qian, 2016). It is worth mentioning that this concentration of β-ionone is well above its perception threshold in aqueous medium (Tempere et al., 2011). Comparing β-ionone content in wines at the end of fermentation (Fig. 2), it emerged that those obtained with more mature grapes reached slightly lower concentrations of β-ionone (-15 d = 1.521a μgL^{-1}; -7 days = 1.467a μgL^{-1}; full ripeness = 1.263b μgL^{-1}). These results show a trend in contrast to previous researches (Fang & Qian, 2006), likely linked to the fact that, in the present study, the ripening of Pinot Noir grapes was already accomplished at the first sampling (-15 d), due to the net anticipation of the phenological phases detected in the studied season.

Fig.1

373 α-ionone tended to accumulate during fermentation rather quickly, following a trend similar to β-ionone (Fig.
374 1). At mid-fermentation, the average concentrations recorded for this compound were similar to those
375 measured at the end of fermentation. The ripening level did not display any significant impact on α-ionone
376 concentration at the end of alcoholic fermentation (Fig. 2).

377 β-damascenone increased differently depending on time of harvest during fermentation; in the case of musts
378 from less mature grapes (-15 days) there was a rapid increase, whereas in musts from more mature grapes the
379 trend was more linear and less rapid during fermentation (Fig. 1). The average values measured at the end of
380 fermentation were very similar between treatments reflecting the small ripening differences already
381 highlighted in the grapes of origin. (Fig. 2).

382 *Barbera wines.* Considering all the data collected for Barbera, the linear mixed effect model showed statistical
383 significance for each factor considered. Also, the interaction between time of harvest and fermentation step
384 was significant. As regards "time of harvest" levels (-15 days, -7 days and full ripeness or 0 days), a statistically
385 significant decreasing trend for β-damascenone in dependence of time of harvest was found in finished wines
386 (Table 3). The same trend was visible comparing exclusively the average values at the end of fermentation,
387 namely the last two sampling dates (Fig. 2). β-damascenone content resulted 15% higher in the wines at end
388 of fermentation from early harvested grapes (-7 days) than in the wine from fully ripe grapes. Also for α-ionone
389 it was possible to observe a decrease in concentration from -7 to zero point, finally resulting in a significantly
390 lower concentration in wines from fully-ripe berries (Fig. 1). No differences at end of fermentation were
391 observed for β-ionone from -7d and 0d (Table 3, Fig. 2).

392 Unlike what observed in Pinot noir, β-ionone did not increase during Barbera fermentation. After the mid of
393 process, the concentrations of this compound remained constant on values between 1.915 μg L^{-1} for the wine
394 obtained from -15 d-grapes and 1.629 μgL^{-1} for wine from fully-ripe grapes. Similarly to Pinot noir, β-
395 damascenone increased rapidly in the first stage of fermentation in musts obtained from less mature grapes (-
396 15 d), whereas a slow increase was observed in those from fully ripe grapes (Fig. 1).

397 **Fig.2**

3.7 Aroma profile of wines after three month of storage

After three months of storage both free and glycoside compounds were quantified (supplementary material Tables 1S-4S). As to Barbera free volatiles, ethyl ester concentration increased with the berry ripening level and C6 alcohol concentration decreased correspondently. This trend could be correlated with the level of nitrogen readily assimilable of the musts, equal to 176 mg L^{-1} for -15 d point sampling, 213 mg L^{-1} for -7 d point sampling and 211 mg L^{-1} for 0 d point sampling. As to Pinot noir, we observed a reduction of medium chain fatty acid ethylic esters, and a more marked reduction of C6 alcohols, that was probably correlated to berry over-ripening and to a slight decrease in assimilable nitrogen from the first to the last sampling (data not reported). Overall Barbera wines glycosylated compounds, tended to decrease while increasing the grape maturity grade, this fact was particularly evident for terpene and norisoprenoid forms (supplementary material Tables 1S-4S).

3.7.1 Norisoprenoids in wines after three months of storage

A clear difference in free norisoprenoid profile between Barbera and Pinot noir.wines was detected (Fig. 2). Pinot noir wines were characterized by a higher concentration in β-damascenone, consistently with the highest average neoxanthin concentration at veraison (Table 1). β-ionone and α-ionone measured in all wines were much lower than those reported at the end of alcoholic fermentation (Fig. 2). According to literature, β-ionone tends to degrade in the presence of oxygen (Silva Ferreira & Guedes de Pinho, 2004), whereas sulfur dioxide could protect this compound from phenomena of oxidative degradation. Conceivably, the significant decrease observed in our case for both α- ionone and β- ionone may be due to the conservation conditions that did not provide a strict oxidation protection (Fig. 2). Differently, β-damascenone content, after 3 months, resulted higher than that recorded at the end of alcoholic fermentation. The presence of some glycosilate precursors, extracted during the alcoholic fermentation and that can undergo acid catalyzed degradation during wine conservation could have been the responsible of β-damascenone concentration increase. Actually, previous

studies have shown that wine acidity, more than other variables, plays a fundamental role in determining the increase of β-damascenone concentration during wine storage from its glycosylate precursors (Silva Ferreira & Guedes de Pinho, 2004).

4. Conclusions

This research work focused on the investigation of key aroma compounds such as megastigmane C13 norisoprenoids (α-ionone, β-ionone and β-damascenone) and their resulting content in wines produced from the vinification of sequential grape harvests. Meteorologically, 2015 was characterized by a very mild winter and a large amount of rain in June, which led to a significant advance of the vine phenological phases. Under these climatic conditions, a rapid accumulation of sugars was highlighted during ripening for Barbera whereas in Pinot noir no differences were observed during ripening. We observed that carotenoid degradation in grapes was not linked to an increase of key-norisoprenoids in respective wines, but these compounds decreased in wines concordantly with grape ripeness; namely β-damascenone and β-ionone decreased in dependence of grape maturity while, α-ionone was less influenced by the grape ripening level. This was particularly evident in wines from Barbera grapes. Actually, Barbera grapes collected 7 days earlier respect to full ripeness allowed to obtain wines with a lower alcohol of 2% v/v and a higher content of β-damascenone of about 15%. The reduction in alcohol content, obtained by harvesting grapes earlier was associated, especially in Barbera, to an optimal composition in terms of acidity and of polyphenolic content.

After 3 months of storage β-damascenone content, resulted higher than that measured at the end of fermentation, probably due to the presence of some glycosylate precursors extracted during vinification. Generally, Pinot noir wines were characterized by a higher concentration in β-damascenone than Barbera wines, consistently with the higher levels of neoxanthin at veraison.

β-damascenone, besides being *per-se*, an important wine-flavour, is also an aromatic enhancer of fruity notes thus, especially for Barbera, early-harvesting can indirectly impact on wine quality having a positive sensorial impact on the final wine aroma.

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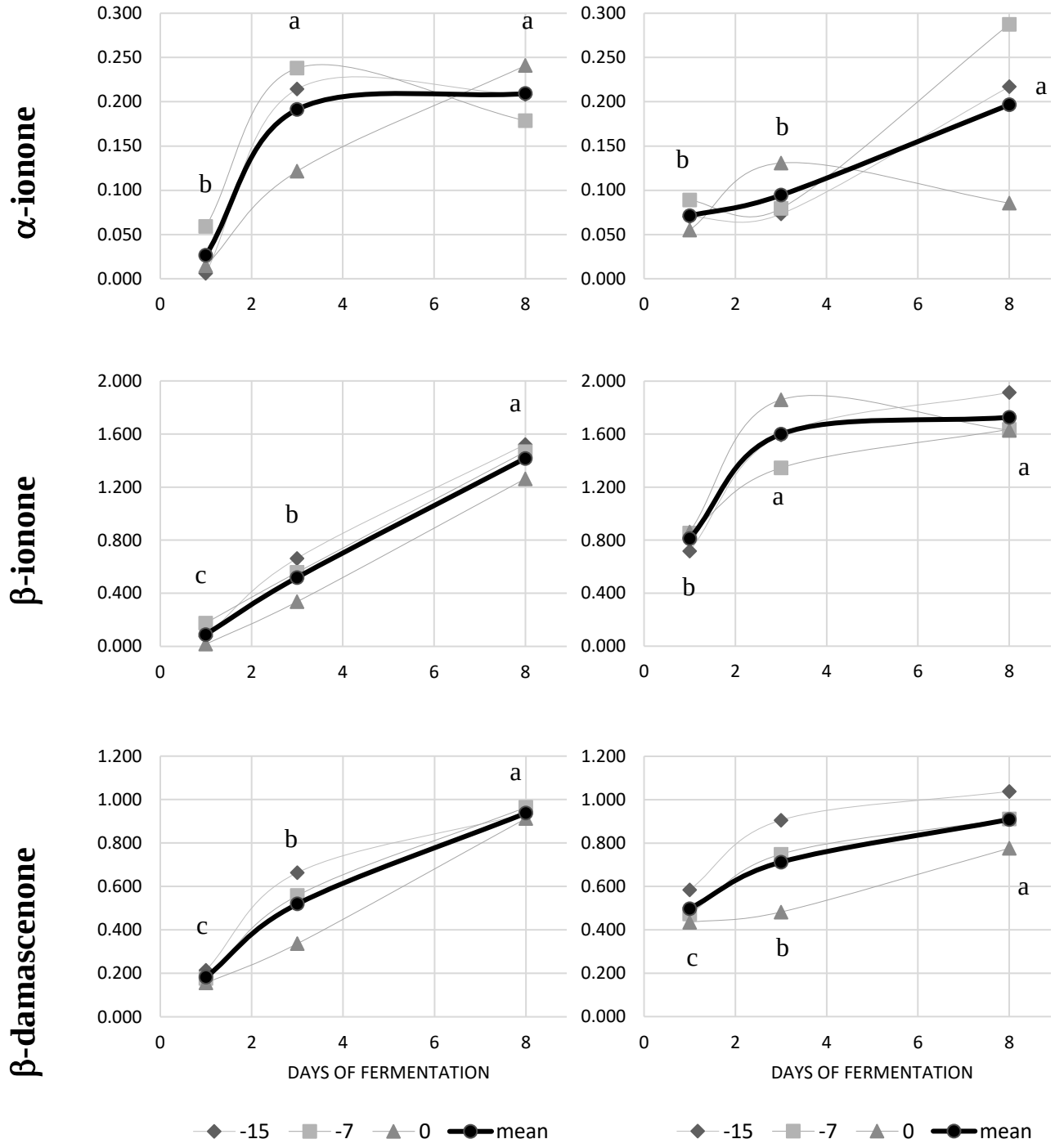
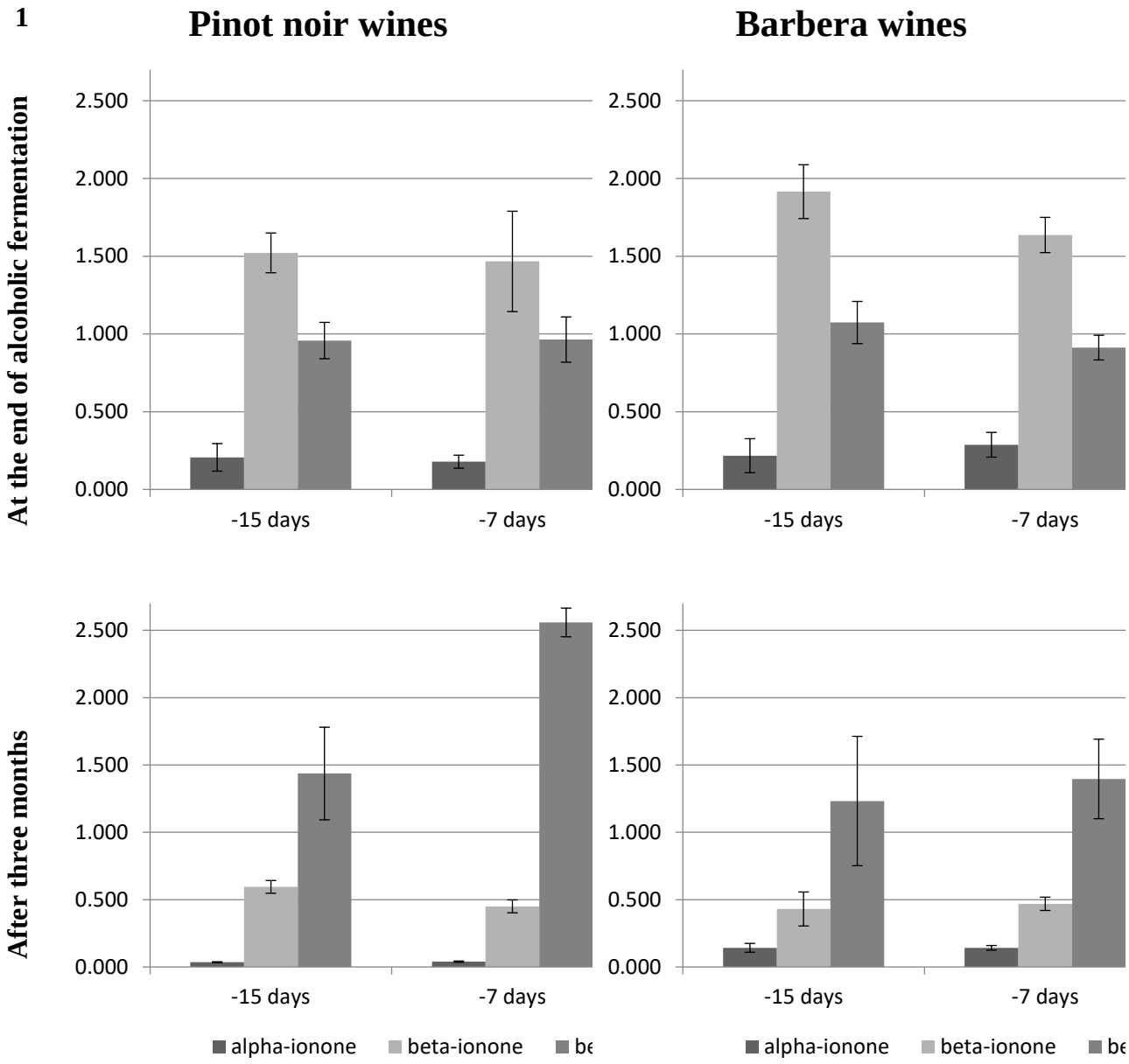
Pinot noir **Barbera**



568 Figure 1

569

1



570 Figure 2

571

572 **Figure 1** – Concentration of megastigmane norisoprenoids during fermentation of Pinot noir and Barbera
573 wines obtained from grapes and collected at different ripening levels (-15 and -7 days before full-ripeness,
574 0). All concentrations are expressed in μg L^{-1}. Bold line represents the average values; light colored lines are
575 referred to different harvest time. Different means are indicate with different letters, Sign.= ANOVA on
576 linear mixed effects model significativity; *= p-value < 0.05, **= p-value < 0,01, *** = p-value < 0.001.

577 **Figure 2** - Average concentrations (μg L-1) of α-ionone, β-ionone and β-damascenone in wines, obtained
578 from grapes at different ripening levels, at the end of alcoholic fermentation and after a three-month
579 period storage. Averages ± standard deviation (n=3).

580

581

582 ***Table 1**: Main meteorological conditions of 2015 and of ten years before (2005-2014).*

583

	GDD 10 °C	HI	Rain	RD ≥ 1
January	4.5	20.8	21.0	4
February	0.0	4.9	103.6	10
March	43.8	106.4	126.6	7
April	138.8	231.0	81.0	6
May	274.2	367.7	46.8	5
June	386.7	489.5	141.6	10
July	542.3	654.8	27.4	4
August	425.9	529.6	132.2	10
September	264.4	353.3	66.4	5
October	108.1	183.5	197.2	13
November	47.7	108.9	2.6	0
December	0.0	3.6	2.8	0
Period April/September for GDD and HI Whole year for rain and RD ≥ 1	**2032.3**	**2625.9**	**949.2**	**74**
Averages of years 2005-2014 (period April – September for GDD and HI; whole year for rain and RD ≥ 1)	**1889.0**	**2436.7**	**1026.0**	**82**

584

585 *Monthly Growing Degree Days (GDD, base 10 °C), Huglin Index (HI), rain (mm) and number of rainy days with rainfall > 1 mm (RD≥1)*
586 *measured in Grugliasco in 2015. GDD and HI were calculated from the 1st of April to the 30th of September. Average values of the*
587 *period 2005-2014 measured in the same weather station (Grugliasco, node 144, Regione Piemonte). Meteorological data were kindly*
588 *provided by Dott. Spanna, Servizio Agrometereologico Regione Piemonte.*

589

590

591

	Pinot noir grapes				Barbera grapes			
Sampling date	**23-Jul**	**13-Aug**	**19-Aug**	**25-Aug**	**27-Jul**	**25-Aug**	**31-Aug**	**07-Sept**
	véraison	**-15d**	**-7d**	**Full ripeness**	**véraison**	**-15d**	**-7d**	**Full ripeness**
berry weight (g)	1.17 ± 0.06	1.54 ± 0.24	1.59 ± 0.23	1.80 ± 0.27	1.63 ± 0.15	2.66 ± 0.18	2.66 ± 0.34	2.66 ± 0.27
TSS (°Bx)	10.1 ± 0.6	19.5 ± 1.2	19.6 ± 0.8	20.4 ± 0.3	8.1 ± 2.8	19.8 ± 1.5	21.8 ± 1.5	23.8 ± 1.1
pH	2.91 ± 0.11	3.15 ± 0.16	3.50 ± 0.07	3.21 ± 0.02	2.58 ± 0.13	2.82 ± 0.11	3.01 ± 0.05	3.02 ± 0.03
TA (g L^{-1})	25.3 ± 3.8	8.8 ± 0.8	6.4 ± 0.2	6.9 ± 0.1	36.6 ± 0.7	13.1 ± 1.9	11.9 ± 1.4	11.2 ± 1.4
TPI (mg kg^{-1})	1895 ± 399	1451 ± 333	1147 ± 229	1145 ± 150	1073 ± 130	1196 ± 156	1341 ± 124	1349 ± 107
TAI (mg kg^{-1})	157 ± 94	510 ± 182	461 ± 137	616 ± 92	56 ± 37	908 ± 148	1132 ± 165	1163 ± 41
TFI (mg kg^{-1})	1647 ± 404	1576 ± 392	1323 ± 226	1446 ± 360	652 ± 23	1524 ± 243	1863 ± 250	1951 ± 68
Lutein (mg kg^{-1})	10.02 ±1.03	3.94 ± 0.28	3.64 ± 0.54	3.50 ± 0.48	4.17 ± 0.49	2.53 ± 0.10	2.55 ± 0.30	1.91 ± 0.28
β-carotene (mg kg^{-1})	4.13 ± 0.56	3.91 ± 0.39	3.63 ± 0.53	2.81 ± 0.68	2.56 ± 0.15	2.91 ± 0.26	3.27 ± 0.29	2.87 ± 0.32
Violaxanthin (mg kg^{-1})	1.67 ± 0.28	0.76 ± 0.05	0.83 ± 0.13	0.93 ± 0.13	0.60 ± 0.09	1.33 ± 0.12	1.06 ± 0.14	1.08 ± 0.15
Neoxanthin (mg kg^{-1})	1.33 ± 0.17	0.60 ± 0.07	0.54 ± 0.07	0.62 ± 0.09	0.74 ± 0.09	0.79 ± 0.11	0.50 ± 0.05	0.43 ± 0.07
Lutein epox. (mg kg^{-1})	0.71 ± 0.10	0.69 ± 0.09	0.71 ± 0.11	0.85 ± 0.08	0.04 ± 0.02	0.22 ± 0.02	0.24 ± 0.02	0.24 ± 0.04
Total carotenoids (mg kg^{-1})	17.84 ±2.14	9.89 ± 0.88	9.35 ± 1.38	8.70 ± 1.46	8.12 ± 0.84	7.79 ±0.61	7.64 ± 0.80	6.52 ± 0.86
		Pinot noir musts				**Barbera musts**		
		-15d	**-7d**	**Full ripeness**		**-15d**	**-7d**	**Full ripeness**
TSS (°Bx)	-	19.7 ± 0.3	20.8 ± 1.2	20.5 ± 0.5	-	19.8 ± 0.8	20.9 ± 2.1	24.0 ± 0.3
pH	-	3.41 ± 0.10	3.48 ± 0.09	3.39 ± 0.09	-	2.94 ± 0.08	3.02 ± 0.09	3.09 ± 0.06
TA (g L^{-1})	-	4.8 ± 0.4	5.5 ± 0.7	5.0 ± 0.3	-	11.5 ± 1.2	10.5 ± 1.8	9.1 ± 1.0
potential alcohol (% v/v)	-	10.8 ± 0.2	11.4 ± 0.6	11.3 ± 0.3	-	10.9 ± 0.4	11.50 ± 1.1	13.2 ± 0.2
		Pinot noir wines				**Barbera wines**		
		-15d	**-7d**	**Full ripeness**		**-15d**	**-7d**	**Full ripeness**
density	-	0.99593	0.99469	0.99459	-	0.99732	0.99526	0.99291
alcohol (% v/v)	-	10.9 ± 0.4	11.3 ± 0.4	11.4 ± 0.4	-	10.6 ± 0.8	12.1 ± 1.3	13.8 ± 0.6
extract (g L^{-1})	-	29.4 ± 1.4	27.6 ± 0.8	27.6 ± 1.1	-	32.1 ± 0.9	31.9 ± 0.1	31.4 ± 0.8
TPI (mg L^{-1})	-	1820 ± 367	1310 ± 390	1581 ± 168	-	1154 ± 206	1192 ± 210	1449 ± 219
TAI (mg L^{-1})	-	160 ± 31	170 ± 45	191 ± 59	-	331 ± 105	357 ± 130	461 ± 145
TFI (mg L^{-1})	-	1977 ± 481	1221 ± 422	1561 ± 239	-	1009 ± 218	1121 ± 281	1360 ± 327

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Table 3: Average concentration (µg L-1) and calculated on the whole database of ß-damascenone, α- and ß-ionone produced with grapes harvested at different ripening levels

		Time of harvest			
		-15 d	**-7 d**	**0 d**	**Sign.**
Pinot noir	β-damascenone	0.60 a	0.57 a	0.47 b	**
	α-ionone	0.14 a	0.16 a	0.13 a	ns
	β-ionone	0.75 a	0.73 a	0.54 b	**
Barbera	β-damascenone	0.84 a	0.71 b	0.56 c	***
	α-ionone	0.12 ab	0.15 a	0.09 b	**
	β-ionone	1.41 ab	1.28 b	1.45 a	*

*fully ripe = 0 d; seven and fifteen days before full ripening, -15 d and – 7 d, respectively. Values are. Sign.= ANOVA on linear mixed effects model significance; *= P value < 0.05, **= P value < 0.01, *** = P-value < 0.001.*