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ORIGINAL RESEARCH ARTICLE

Influence of harvest date on multi-targeted metabolomic profile and sensory attributes of Ribolla Gialla base and sparkling wines

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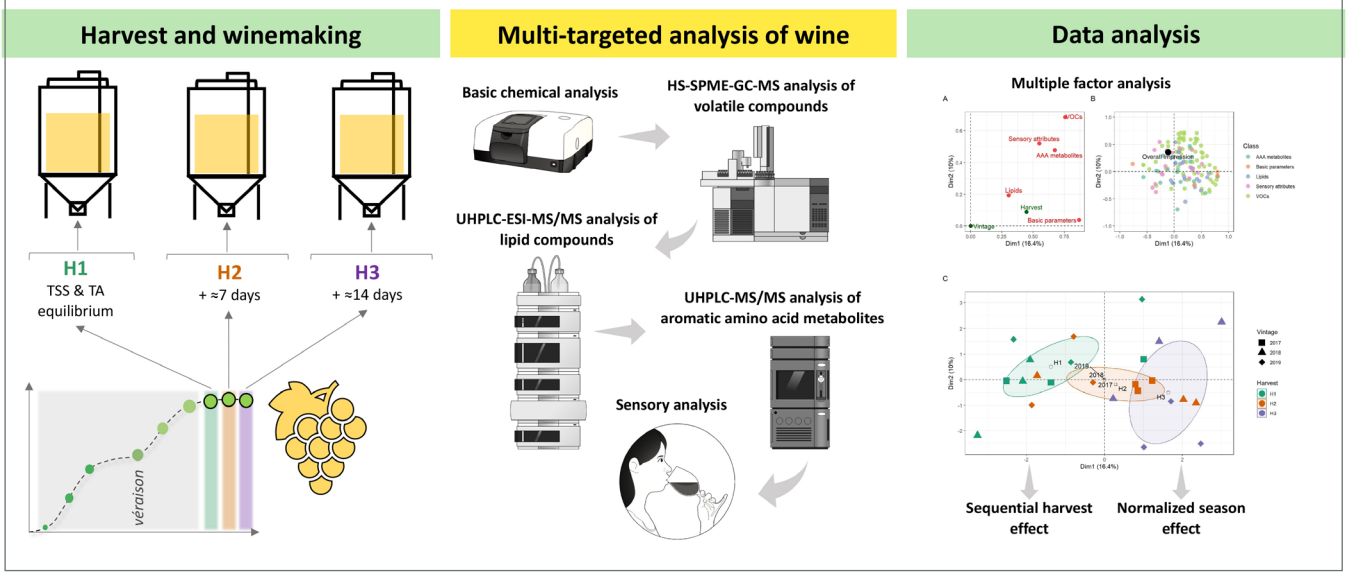
ABSTRACT

In addition to a balanced production level, the timing of grape harvest is a crucial factor to be considered for the winemaking process of sparkling wines. A sufficient accumulation of sugars and an optimal level of acidity in grapes throughout ripening is necessary not only to achieve the desired alcohol levels in the wine but also to guarantee the biosynthesis of the aromatic precursors. To target optimal grape ripeness and maximise the positive sensory attributes of the wine produced, the work presented herein deals with a study on whether an extended harvest date might have a greater positive organoleptic impact and lead to an increase in important odour–impact compounds. In the resulting Ribolla Gialla monovarietal sparkling wines, a one-week delay after reaching technological maturity of the grapes expressed an improvement in the aromatic profile in the obtained samples by altering the profile and abundance of grape-derived compounds and fermentative esters. This was consequently reflected in the sensory evaluation, as the wines achieved higher scores for 'floral', 'citrus fruit', and 'yeast' sensory descriptors when the grapes were harvested a week after the minimum compromise between total acidity concentration and total soluble solids. Moreover, an extension of the harvest date is not necessarily correlated with the formation of untypical ageing off-flavour substances that could be detrimental to the quality of sparkling wines. Conversely, the lipid content proved to be more dependent on the climatic factors of the individual vintage compared to subsequent harvest time. By merging a multi-targeted approach of exploring wine metabolites and sensory characteristics, it is thus possible to predict an optimal harvest date for obtaining high-quality Ribolla Gialla sparkling wines.

KEYWORDS: Sparkling wine, harvest date, grape maturity, volatile organic compounds, lipids, aromatic amino acid metabolites, sensory analysis

GRAPHICAL ABSTRACT



INTRODUCTION

A combination of intrinsic (i.e., variety) and extrinsic factors (e.g., climate, soil conditions, and cultural practices) represent one of the main predispositions that determine the sensory properties of wine (Lasanta *et al.*, 2014). Moreover, alongside a balanced production level, the timing of grape harvest plays an important role within the set of viticultural determinants (Šuklje *et al.*, 2019). Monitoring the grape ripening is thus extremely important for winegrowers since grapes undergo a number of physical and chemical changes from *véraison* onwards (Coelho *et al.*, 2007). To determine the optimal date of harvest, classical parameters based on the assay of colour, grape sugar content, titratable acidity, and pH are frequently used. Nonetheless, special attention is required when dealing with fruit destined for sparkling wine production, as the grapes are generally harvested with a lower grape ripeness than fruit for still wines, with relatively low pH, higher titratable acidity, and lower soluble sugars (Jones *et al.*, 2014; Martínez-Lapuente *et al.*, 2016). In addition to basic chemical analyses that are used to determine fruit maturity, more specific profiling of other chemical classes can be applied, with a view to better discriminating the varietal grape characteristics and enhancing the product quality. Therefore, the analysis of phenolics (Cadot *et al.*, 2012; Gil *et al.*, 2012; Pérez-Magariño and González-San José, 2006), carotenoids (Crupi *et al.*, 2010; Yuan and Qian, 2016), and volatile compounds (Bowen *et al.*, 2016; Fang and Qian, 2006; Kalua and Boss, 2010), has been previously used to underline the influence of different harvest dates.

Due to the occurrence of multiple biochemical processes at different maturing stages, many key grape-derived compounds do not necessarily track with sugar accumulation, thus, affecting the sensory profile of wines (Bindon *et al.*, 2013; Jackson and Lombard, 1993). It has been previously shown that delayed harvest decreased the concentration of C_6 alcohols and their derivatives, thereby reducing the perception of the sensory attribute of 'herbaceous' or 'green'; the descriptors that also characterise methoxypyrazines, nitrogen-containing heterocyclic compounds (Escudero *et al.*, 2007). Therefore, the concentration ratio between the varietal volatiles and C_6 compounds could serve as a criterion to define the harvesting moment, as proposed previously (Salinas *et al.*, 2004). Moreover, grape crushing can potentially generate additional C_6 derivatives from fatty acid precursors via the lipoxygenase pathway, which can be furthermore converted to acetate esters (e.g., hexyl acetate) during fermentation (Bindon *et al.*, 2013). This may, in turn, alter the aromatic profile of wines and characterise them with more 'fruity' descriptors (Dennis *et al.*, 2012), even though yeast-derived esters remain the greatest contributors to the wine 'fruitiness', which may be amplified by the presence of norisoprenoids or dimethyl sulfide in low concentrations (Escudero *et al.*, 2007). One study carried out on sparkling wines from the Ribolla Gialla variety showed that β-damascenone and fermentative esters were the main chemical compounds contributing to the aroma characteristics of this variety (Voce *et al.*, 2019).

Environmental factors and climate have a significant impact not only on the aromatic potential of grapes and, subsequently, wine but also on the lipid composition of these matrices. Linolenic (C18:3) and linoleic acid (C18:2) are the major components of total lipids in grape berries, and, therefore, it is important how their content changes during the vegetative growth of grapes (Pérez-Navarro *et al.*, 2019). These two poly-unsaturated fatty acids (PUFAs) are also susceptible to oxidation in the presence of lipoxygenase, which can be converted into low-molecular-weight compounds known as oxylipins, responsible for certain wine organoleptic defects (Pilati *et al.*, 2014; Zamora *et al.*, 1985). However, it is not completely clear how the composition of fatty acids changes during the vegetative cycle of grapes. Some authors claimed that the alternations of total lipid content occur from the *véraison* to the end

of the ripening (Barron *et al*., 1989; Bombai *et al*., 2017), while others reported consistent accumulation in saturated fatty acids and linoleic acid proportions with ripening (Millán *et al*., 1992).

Finally, tryptophan (TRP) and its metabolites are potential precursors of 2-aminoacetophenone (2-AAP), an aroma compound responsible for the untypical ageing off-flavour (Hoenicke *et al*., 2001). Its presence in the wine is considered detrimental, as it characterises the wines with 'naphthalene', 'floor-polish', 'washing-soap' or 'acacia-blossom' odour-like taints (Hoenicke *et al*., 2002). Ruiz-Rodríguez *et al*. (2017) compared the levels of TRP during grape ripening, and they showed that the TRP levels decreased in the reference sample during ripening, while an additional date for the post-harvest study showed that grapes on vines produced 12 % higher levels of TRP. However, according to Hoenicke *et al*. (2001), the amounts of bound indole 3-acetic acid (IAA) and free and bound TRP in grapes increased significantly at the stage of maturity, which confirms that the nitrogen or amino acid contents of the grape musts and wines rise with the ripeness of the harvested grapes. It could, therefore, be expected that wines from a later harvest stage will be more prone to develop the untypical ageing off-flavour (UTA); on the other hand, Hoenicke *et al*. (2002) claimed that UTA appearance is not directly correlated to the amount of IAA present in the must or wine, as it is more likely to be related to a nitrogen deficiency of the harvested grapes. Considering sparkling wines, it has been shown that they can contain a much lower amount of TRP than the other types of wines, which could be the consequence of secondary fermentation or earlier harvest time (Arapitsas *et al*., 2018).

To target optimal grape ripeness and maximise positive attributes of the wine produced, the present study aimed to explore whether an extended harvest date can lead to an increased amount of important odour-impact compounds, thus potentially impacting sensory/organoleptic perception. For this purpose, the production of sparkling wine from the locally important variety Ribolla Gialla was selected to identify the alternations of multi-targeted metabolomic profile throughout a maximum of three different harvest times over the course of three consecutive years.

MATERIALS AND METHODS

1. Chemicals and reagents

All chemicals used were of analytical grade. For the analysis of free volatile compounds, sodium chloride (NaCl) and 2-octanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). C_9–C_{30} n-alkane solution in n-hexane for linear retention index (LRI) determination was purchased from Supelco (Bellefonte, PA, USA). For the determination of lipids, methanol (CH_3OH) and acetonitrile (ACN) were LC–MS-graded and were purchased from Sigma-Aldrich (St. Louis, MO, USA), together with 2-propanol (IPA), chloroform ($CHCl_3$), formic acid (HCOOH), ammonium formate (NH_4COOH) and 3,5-di-tert-4-butylhydroxytoluene (BHT). Internal standards (i.e., cholesterol-d7 and stearic acid-d3) were obtained from CDN Isotopes (Quebec, QC, Canada). For the analysis of aromatic amino acid metabolites, the HPLC-grade CH_3OH and 3-nitro-*L*-tyrosine were provided from Sigma-Aldrich (St. Louis, MO, USA).

2. Vineyard site

The harvest timing experiment was carried out in the Friuli–Venezia Giulia region (Italy) across three consecutive vintages (2017–2019). The commercial vineyard of *Vitis vinifera* L. cv. Ribolla Gialla used for this trial was located in Corno di Rosazzo, situated in the Friuli Colli Orientali and Ramandolo district (N 46.005306, E 13.441833) at 94 m above sea level, on silt–clay–loam soil with no coarseness and on a moderate slope hill. No possibility of irrigation was present in that vineyard. The spacing of Ribolla Gialla vines was 2.7 m × 1.1 m, which resulted in 3367 plant/ha of planting density. The clone used for this trial was the VCR 100, grafted onto the Kober 5BB rootstock (Vivai Cooperativi, Rauscedo, PN, Italy). All vines presented a single-arched Guyot training system, and the vineyard rows were East-West oriented.

3. Weather data

Weather data were retrieved from the ARPA–OSMER online database (*ARPA FVG–OSMER, http://www.meteo.fvg.it/*) and the measurements were recorded by the ARPA-OSMER weather stations of Cividale del Friuli. Daily temperatures and rainfall from the beginning of April through the end of October (i.e., 214 days) across all three vegetative seasons were sourced from the database.

4. Sequential grape harvest and sparkling wine production

Grapes intended for wine production and subsequent chemical analysis were harvested manually from a vineyard in up to three different stages of ripeness. According to the results of the fruit maturity monitoring to determine the normal maturity of Ribolla Gialla, the first harvest (H1) was set when a minimum compromise was reached between the accumulation of total soluble solids (TSS) and the level of titratable acidity (TA) in the grapes. The second (H2) and the third harvest (H3) were positioned about one and two weeks later, respectively, after H1, based on the meteorological conditions of each growing season. H3 was carried out only in 2018 and 2019, since in 2017, the excessive level of rainfall prevented the harvest of healthy grapes. Details of the grape juice composition at harvest are presented in the Supplementary material (Supplementary Table 1). Inside the vineyard, three biological replicates were selected, and at each harvest time, approximately 30 kg of grapes were picked and immediately transported to the experimental winery of the University of Udine, where the visual inspection of grapes was performed. Once the negligible percent of rotten berries were removed, the rest of the grapes were pressed at 2-bar pressure using the A20 pneumatic press provided by Grifo Macchine Enologiche (Piadena, CR, Italy).

More detailed information on the vinification process of sparkling wines has been previously published elsewhere (Škrab *et al*., 2021). Briefly, 15–18 L of grape juice, derived from pressing, was recovered and placed in glass carboys, where the base wine production was initiated by inoculation with *Saccharomyces bayanus* commercial yeast strain Mycoferm IT07 (400 mg/L), purchased from Ever (Pramaggiore, VE, Italy). The vinification temperature was set at 20 °C until the end-point of the alcoholic fermentation. After the tartaric stabilisation (about two weeks at 4 °C), the base wines were elaborated following the Martinotti–Charmat method in the 7-L stainless-steel autoclaves, maintained at 18 °C. The pressure was measured continuously by a manometer positioned on the outlet valve of the autoclaves and monitored daily, and when the pressure reached a value close to 4.5 bars, the residual sugars were analysed. Fermentation was completed after approximately 40 days, followed by cold stabilisation and isobaric bottling. The sparkling wines were, thus, stored at a constant temperature (4 °C) and were degassed for 2 min in the ultrasonic bath sonicator prior to the chemical analysis, except in the case of basic chemistry where wine samples were filtered by vacuum filtration on Whatman 1 filter paper (Sigma-Aldrich, St. Lois, MO, USA) to eliminate carbon dioxide.

5. Basic chemical analysis

A WineScan FT-120 Fourier transform infrared (FTIR) spectroscopy instrumentation (FOSS, Hillerød, Denmark) was used for the determination of alcoholic strength (% v/v), reducing sugars (g/L), and titratable acidity (expressed as g/L of tartaric acid) for wine samples. Additionally, a manual refractometer (ATC-1, Atago, Tokyo, Japan) was utilised to measure TSS (°Brix) in grape juice. Two technical replicas were measured for analysis purposes, and the mean value was considered for the data exploration.

6. HS-SPME-GC-MS analysis of volatile compounds

The wine volatiles were determined as described previously by Carlin *et al*. (2016) and Škrab *et al*. (2021), using 1 mL of CO_2-free wine, spiked with 50 µL of alcoholic solution of 2-octanol at 2.13 mg/L as internal standard, united with 1 mL of Milli-Q graded H_2O and 1.5 g of NaCl in the 20-mL glass headspace vial, and securely closed with an air-tight magnetic screw cap, equipped with PTFE/silicone septa. Five minutes of sample equilibration at 35 °C was then followed by 30 min HS-SPME extraction of volatiles in a PAL combi-xt agitator (CTC, Zwingen, Switzerland), using 2 cm long 50/30 divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) StableFlex SPME fibre (Supelco, Bellefonte, PA). After the splitless desorption of extracted compounds in the gas chromatograph (GC) inlet, maintained at 250 °C for 3 min, the separation of volatiles occurred in the Thermo Trace Ultra GC, equipped with a VF-Wax column (30 m × 0.25 mm × 0.25 µm, Agilent J&WScientific Inc., Folsom, CA, USA). Oven temperature programme conditions were as follows: initial temperature of 40 °C for 2 min, programmed at 6 °C/min up to 250 °C, where it remained for 5 min. Helium was used as a carrier gas at a constant flow rate of 1.2 mL/min. The detection was carried out on a Thermo Quantum XLS mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), operated in positive electron ionisation (EI) mode at 70 eV, and all spectra were acquired in full scan mode with a mass range of *m*/*z* 30–350. The ion source temperature was set at 230 °C. The processing of acquired chromatograms was performed using the manufacturer's ThermoXcalibur software 1.1.1.03 (Thermo Fisher Scientific, Waltham, MA, USA). Analysed volatile compounds were confirmed by comparison of mass spectra and their retention times to those available in the literature and NIST database (www.webbook.nist.gov/chemistry) and, where possible, by injection of pure analytical standards and by calculating the linear temperature-programmed retention indices for a series of n-alkanes. Finally, a semi-quantitation analysis of obtained data was carried out, and the results were expressed as µg equivalents of the internal standard per L of wine.

7. UHPLC-ESI-MS/MS analysis of lipid compounds

The method for the analysis of hydrophobic/amphiphilic small molecules present in the wine samples was adopted from Della Corte *et al*. (2015) and adapted accordingly, as reported by Škrab *et al*. (2021). Briefly, a two-step extraction of 0.5 mL of degassed wine was performed using CH_3OH (1.5 mL), $CHCI_3$ (3 mL) containing BHT (500 mg/L) as a preventative agent for free radical-mediated oxidation, and water of high purity (1.25 mL). Stearic acid-d3 was used as an internal standard (10 µL at 100 µg/L). Once collected, the lipid-rich layer was dried under the stream of N_2 and finally reconstituted in 300 µL of ACN/IPA/H_2O (65:30:5 *v*/*v*/*v*) with pre-added cholesterol-d7 (1 µg mL). Dissolved extracts were finally filtered through 0.22 µm PTFE filters into a 250 µL glass vial insert with polymer feet. Subsequently, 5 µL of each sample were injected into Dionex 3000 liquid chromatograph (LC, Thermo Fisher Scientific, Waltham, MA, USA), equipped with reversed phase C18 Ascentis Express column (2.7 µm, 150 × 2.1 mm, Sigma-Aldrich, Milan, Italy), set to 55 °C. Further chromatographic separation was carried out as described by Della Corte *et al*. (2015). The LC system was coupled to an API 5500 QqQ mass spectrometer (MS) provided by Sciex (Concord, Vaughan, ON, Canada). Detailed instrumental parameters used in the analytical method were retrieved from Della Corte *et al*. (2015). Analyst software (Applera Corporation, Norwalk, CT, USA) and MultiQuant 2.1 (Sciex, Concord, Vaughan, ON, Canada) were used for data acquisition and data processing, respectively. Quantification was carried out by constructing calibration curves for each analyte, and the results were expressed as mg/L after normalisation by reference feature (i.e., internal standard, stearic acid-d3) as a general-purpose adjustment for systematic differences among samples, as reported previously (Ruocco *et al*., 2017).

8. UHPLC-MS/MS analysis of aromatic amino acid metabolites

De-gassed samples, intended for the analysis of aromatic amino acid (AAA) metabolites, were filtered at 0.22 µm by a Millex-GV filter unit (Merc, Darmstadt, Germany) directly into 2 mL HPLC certified amber vials and spiked with 20 µL of the 3-nitrotyrosine as the internal standard (10 mg in 10 mL of CH_3OH). Samples were thus loaded onto the auto-sampler plate and kept at 6 °C until injection. Once injected (10 µL), the separation of the metabolites was achieved on a Waters Acquity HSS T3 column 1.8 µm particle size, 150 mm × 2.1 mm (Milford, MA, USA), conditioned at 40 °C. The column was installed on a Waters Acquity UPLC system (Milford, MA, USA). H_2O and ACN contained 0.1 % of HCOOH and represented mobile phases A and B, respectively. More detailed information on the gradient profile is presented elsewhere (Arapitsas *et al*., 2018). A triple quadrupole mass spectrometer equipped with an electrospray (ESI) source (Waters Xevo TQMS, Milford, MA, USA) was used to detect AAA metabolites. The ion spray voltage was set at 3500 V for positive mode and −2700 V for negative mode. The source temperature was set at 150 °C. Instrument control and data acquisition were performed using Waters MassLynx 4.1 software, whereas data was processed on Waters TargetLynx, supplied by the manufacturer. For each identified compound, a calibration curve was constructed following the protocol of Arapitsas *et al*. (2018), and the results were expressed as mg/L. To control the instrumental variability, quality control (QC) samples were prepared as a pooled mix of all studied samples (each 100 µL) and were injected every ten sample injections. Relative Standard Deviations (RSD) were calculated for each compound of the QC samples. The compounds with RSD values above 20 % were removed from the dataset (Dudzik *et al*., 2018).

9. Sensory analysis

Sensory evaluation of wines produced was undertaken each tested year separately, approximately nine months after bottling. The wines were evaluated by a median panel of ten subjects, considering the three-year period. The panellists were recruited according to their motivation and availability among staff and students from the University of Udine, as well as local oenologists and producers, presumably well acquainted with the studied cultivar and its products. The University of Udine did not require any ethical statement for this sensory evaluation, and no biological samples or clinical data were collected from panellists. However, the personal data and affirmative consent of the panellists have been processed in accordance with the university's privacy policy (https://www.uniud.it/en/uniud-international/privacy-policy). The products tested were safe for consumption. Each panel of judges carried out the sensory analysis of the Ribolla Gialla sparkling wines in different tasting sessions. In the first session, thirty-three randomly selected testing commercial wines were evaluated; the panel was asked to identify the most perceivable attributes referred to as odour, taste, and aftertaste for the development of the scorecard. The final list consisted of eighteen attributes: eleven referred to aroma ('floral', 'dry vegetable', 'citrus fruit', 'yeast', 'green apple', 'tropical', 'oxidation notes', 'liquorice', 'herbaceous vegetable', 'dried fruit'), seven were mouthfeel attributes ('acidity', 'astringency', 'bitterness', 'flavour', 'body', 'the finesse of foam', 'flint') and 'overall impression' that referred to global perception. Thereafter, the identified attributes were used in the following sessions. Furthermore, before each session, a wine sample was used to standardise the evaluation of the panel, and panellists were asked to quantify the level of each attribute in an open session to fine-tune the evaluation. After this initial training, which was repeated at each sensory session, the wines were evaluated in triplicate and presented according to a completely randomised block design. Finally, the samples were judged for selected descriptors on a 10 cm scale anchored with "low" and "high" intensity.

10. Data processing and statistical analysis

Before analysing the data obtained by spectrometric methods, the missing values were imputed with a random value between zero and LOQ, where the calibration curves were constructed using a custom R Statistics software (R Foundation for Statistical Computing, Vienna, Austria) script. For the GC-MS semi-quantitative data, missing values were replaced by a random value between zero and half of the corresponding minimum value for each metabolite. For data obtained via LC-MS and GC-MS analysis, the imputation was performed only if there were less than 10 % of missing values for the corresponding variables. In the case of a higher percentage of missing values, the variables were excluded from further statistical processing. Chemical data was then subjected to Student's *t*-test for comparison of only two harvest times in the 2017 season, while a one-way Analysis of Variance (ANOVA) was carried out to compare the means of three different harvest times. Significance level according to statistical tests was indicated by '***' if $p < 0.001$, '**' if $p < 0.01$, and '*' if $p < 0.05$. If $p > 0.05$, the differences were considered not significant and 'NS' was used. The multiple testing issue was addressed by adjusting the false positive rate by adjusting the raw *p* values. For this, the Benjamini and Hochberg method was used. Following ANOVA or Student's *t*-test, significant differences among the means of studied variables were separated using the Student–Newman–Keuls method as a post-hoc test. Assumptions for homogeneity of variances and normality were tested using Levene's test and the Shapiro–Wilk test, respectively. The Principal Component Analysis (PCA) was used for exploratory analysis of standardised and normalised datasets (variable standard deviation equal to one). This was performed using a 'FactoMineR' package (Husson *et al*., 2020), and the results were extracted and visualised using 'factoextra' (Kassambara and Mundt, 2020) and 'ggplot2' packages (Wickham *et al*., 2020). All three R packages were also used for Multiple factor analysis (MFA) that was applied as a data fusion method for multi-targeted datasets of VOC composition, lipids, and aromatic amino acid metabolites and plotted as a single projection over the multivariate partial axes. To eliminate the effects on variance resulting from three harvests, each dataset was normalised

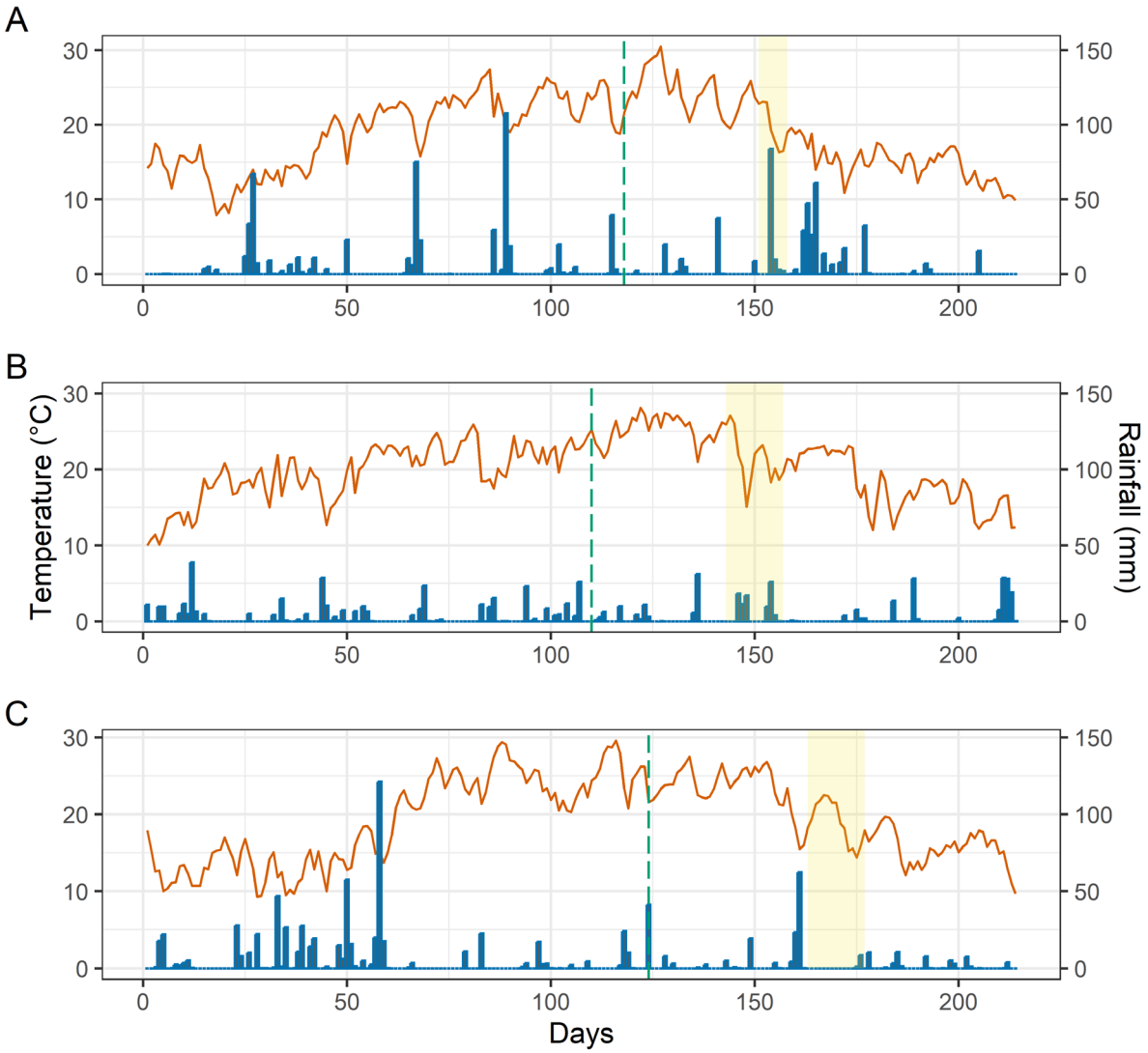


FIGURE 1. Meteorological data from 01 April to 30 September in Cividale del Friuli during 2017 (A), 2018 (B) and 2019 (C). The blue bars indicate the rainfall quantity, measured in mm, while the red lines on the plot annotate average temperature (°C). The yellow area shows the harvest window, determined for each harvest separately, while the green dotted line marks the determined onset of grapevine berry ripening.

by season (z-transformation) and recalculated back using the average and the standard deviation of the three-season dataset. The MFA considers the contribution of all groups of variables to define the distance between harvest times. This, of course, requires a balanced approach since influences may vary from one dataset to another. The MFA first computes a PCA of each data table and normalises them by dividing them by the first singular value from PCA. These normalised data tables are subsequently merged into a grand table that is analysed via a (non-normalised) PCA to obtain factor scores and loadings for observations and variables, respectively (Abdi *et al.*, 2013). For the purposes of analysis, the merged dataset was divided into five different subsets: the variables from basic quality parameters, multi-targeted metabolomic compounds (i.e., VOC, AAA metabolites, and lipids), and sensory attributes. The packages 'corrr' (Kuhn *et al.*, 2022) and 'ggraph' (Pedersen, 2022) were used to calculate Pearson coefficients and plot the arc diagram, respectively. Finally, data obtained after sensory analysis were normalised by the panellists, subjected to the statistical comparison of the means as per chemical data, and the results were plotted as lollipop plots.

RESULTS AND DISCUSSION

1. Weather conditions

The three seasons considered in the present investigation reported several differences in terms of temperatures and precipitation, although they could be considered representative of the recent meteorological trend in the region of Friuli Venezia Giulia, Italy (Figure 1).

Out of the three seasons, 2017 was characterised by the highest rainfall, mainly concentrated in June (275 mm) and September (345 mm) (Figure 1A). Because of the

concentration of rain during grape maturation, the occurrence of bunch rots made the third harvest impossible in this season. As regards the temperature trends, the lowest values were registered in April and, mainly, in September, a phenomenon related to the number of rainy days during the month. The average monthly temperature and the number of days above 30 °C were comparatively lower than in the other seasons. To date, over 37 days, reported temperatures exceeded 30 °C, mainly in July and August.

Moving onto the following season, 2018 (Figure 1B), one could easily argue that the distribution of rainfalls was homogeneous across months, except for September (45 mm), which proved to be the driest month. By comparing the three years, the sum of accumulated rainfall in 2018 was the lowest (665 mm), which resulted in a slightly higher number of rain-free days than the average if the three years are evaluated together. As regards the mean temperatures, April (15.8 °C), May (19.2 °C), and September (20.0 °C) were the hottest months of the three-year period of observation, and the number of days exceeding 30 °C added up to 50 days, mostly concentrated in August, making the 2018 vintage the second hottest season in the last 20 years.

In the last season, 2019, the distribution of rainfalls appeared to be unbalanced between spring and summer (Figure 1C), with 409 mm in May, followed by a scarcity of rains in June (38 mm), July (69 mm), and August (81 mm). At the beginning of September, an intense storm cumulated 90 mm of rain, but again, after these two days, the rain was scattered and allowed the harvests to be without problems of rot. As regards temperatures, April and May were characterised by low values of this parameter due to the high number of rainy days that occurred. Subsequently, in June (24.4 °C), July (24.4 °C), and August (24.5 °C), the average temperatures reported the highest values compared to the other two vintages. It is important to mention that during June and July, three heat waves were responsible for a transient significant increase in temperature. The number of days with maximum temperatures shooting past the 30 °C threshold added up to 65, almost equally divided between June (19 days), July (23 days), and August (20 days).

Considering together all the meteorological data described above, the 2017 and 2019 seasons were similar in terms of temperatures, whereas as regards rainfalls, the summer part of the vegetative cycles revealed similarities between the years 2018 and 2019. The particular meteorological behaviour of the three seasons was responsible for some of the differences in the compositional parameters that are described in the following paragraphs.

2. Basic chemical parameters of grapes and sparkling wines

The maturity parameters, such as TSS, TA, and pH value, were analysed in grape juice to compare the differences between two or three harvest timings (Supplementary Table 1), as these parameters are among the most important parameters in determining the harvest date for sparkling wine production (Jones *et al*., 2014). Moreover, the acid versus sugar balance is fundamental to constructing a grape's distinctive flavour and ultimately contributes to the wine quality (Costa *et al*., 2020).

Considering the only two harvest timings (H1 versus H2) in 2017, the results did not reveal any significant differences for TSS, TA, and pH. However, the trend indicated that H2 was characterised by a slightly higher °Brix and pH, and a consequently lower TA value. When comparing the effects of three harvest times in 2018 and 2019, later dates resulted in a non-significant increase in TSS, especially when comparing H2 and H3 in the 2018 season. Conversely, the trends of titratable acidity were consistent in both 2018 and 2019, with values that slightly decreased from H1 to H3. In the case of pH, while in the first season, 2018, a trend towards an increase alongside maturation was discernible, in the following season, this parameter remained quite stable with a slight reduction in H2 and H3. Bowen and Reynolds (2015) reported that during grape maturation, there is a reduction of TA and a simultaneous increase of TSS concentration, in agreement with the results of our study. As far as the 2019 growing season is concerned, the TSS fluctuation from H1 through H2 to H3 was particularly insignificant, although there was a noticeable trend of increasing acidity and decreasing pH. This trend can be easily explained since, during maturation, the berry weight showed a reduction due to shrinking, responsible for the increased °Brix, but also for a concentration of acidity that could be the reason for the lower values of pH in H2 and H3.

The parameters analysed in sparkling wines mostly correlated with the grape analysis mentioned just above. When comparing H1 and H2 of the basic sparkling wine composition from 2017, the results of extended harvest showed a slight increase of alcohol in H2-produced sparkling wines. Conversely, lower TA from H2 led to a higher pH value. The effect of an additional third harvest was analysed in the two following seasons. The TA content proved to be the only parameter where the differences between H1, H2, and H3 were statistically different in both seasons. By contrast, although there were slight increases in alcohol content in sequential harvests, only in the 2019 season were significantly higher values ascertained with respect to H3. On average, the 2019 season differed from the previous vintage in higher wine TA content (7.62 g/L versus 6.57 g/L) and ultimately low pH (3.09 versus 3.27). This result can be related to the delayed harvest that was performed at lower temperatures, whereupon the respiration of the malic acid was less pronounced (Bindon *et al*., 2013).

3. Chemical characterisation of base wines and sparkling wines by multi-targeted metabolic profile

In the following paragraphs, the multi-targeted approach (i.e., volatile and lipid composition, as well as the metabolites obtained from aromatic amino acids) is discussed for Ribolla Gialla base and sparkling wines obtained from each harvest date as well as for each respective vintage.

3.1. VOC profiling of base and sparkling wines

The results of the semi-quantitative characterisation of volatile organic compounds (VOCs) found in the base and sparkling wines are summarised in Supplementary Table 2 and Supplementary Table 3, respectively. HS-SPME-GC-MS analysis revealed a total of 62 compounds that characterised the aroma profile of Ribolla Gialla base wines, while the number of compounds detected in sparkling wines was 66. The compounds were subsequently separated according to their affiliation with different chemical classes (monoterpenes, C_{13}-norisoprenoids, aldehydes, alcohols, esters, acids, and ketones). Student's *t*-test and one-way ANOVA were then applied to investigate whether the means from each harvest time were significantly different within each harvest season separately.

From the results of base wines (Supplementary Table 2), it can be observed that the concentration of volatile compounds in general increased in wines obtained, postponing the harvest. Since Ribolla Gialla is considered to be neutral in terms of aromatic potential, it is therefore of utmost importance to note that prolonged maturation of grapes can eventually lead to the increased content of primary volatile compounds, as they are generally associated with 'floral', 'sweet fruit', and 'citrus' aromas (Zhao *et al*., 2019). These sensory descriptors are particularly valid for eight monoterpenes that were found in this study. The postponement of harvest for one week from H1 to H2 led to a significant increase in some of the monoterpenes (e.g., citronellol and nerol in the 2017 season), with the exception of the 2019 season, where the total amount of these compounds decreased. From that viewpoint, the results of the present study agree with the findings reported by some other authors, whereby the increased content of monoterpenes is correlated with the sugar accumulation in grapes (Bowen and Reynolds, 2015). However, one of the most distinctive contributors to the floral aroma of wines is linalool, and it is well known that its concentration increases until optimal grape maturity is achieved, followed by an immediate drop in concentration (Marais and van Wyk, 2017); this could be the reason why the amount of linalool in 2017 and 2018 decreased in H2 and H3. In addition to linalool, geraniol appeared to be equally important in base wines, but its concentration similarly began to decline in H3 wines. This probably occurred due to the increased activity of geraniol reductase towards the end of the ripening, which can produce high levels of citronellol (Luan *et al*., 2005). In addition, previous studies showed that the high geraniol disappearance during early fermentation is mainly linked to isomerisation by acid catalysis, reduction to citronellol by Old Yellow Enzyme 2 (Oye2) (Slaghenaufi *et al*., 2020; Steyer *et al*., 2013). In fact, H3 wines were characterised by a higher content of citronellol than the previous two harvest dates. In connection with the seasonal effect on the monoterpene content, it turned out that monoterpenes reached significantly higher values in the 2018 season, which was considered the warmest across the three-year average. A similar relationship was previously described by Pons *et al*. (2017), where higher temperatures appeared to be beneficial for the aroma and their precursor of 'fruity' and 'floral' nuances that are characteristic of terpenes. However, this was not the case for citronellol, whose average level was the highest in the 2019 season. Since this vintage resulted in high levels of sterols and unsaturated fatty acids (UFA), this could well lead to higher Oye2 activity, considering that these lipid compounds are essential for yeast adaptation to fermentation stressors, such as high sugar levels and ethanol toxicity, to avoid sluggish and stuck fermentations.

Overall, the concentration of monoterpenes proved higher in sparkling wines than in base wines (Figure 2); such a trend was clearly expressed only in 2019 due to the later harvest date and, consequently, lower average temperatures. Although the hydrolysis of glycosidic linkages and, thus, the release of free volatile compounds has not been the aim of this study, it is known that this process is facilitated during wine ageing. However, the extended harvest date led to an increased concentration of citronellol and a simultaneous decrease in the limonene content, as can be seen in Supplementary Table 3. Additionally, a higher concentration of primary aromas could be observed in the 2019 season for sparkling wines, which suggests that environmental factors also played a major role in the synthesis of VOCs. Considering that H3 was carried out in September 2019, the higher concentration of monoterpenes is in line with previous findings since there was a noticeable accumulation of these compounds throughout ripening (Yue *et al*., 2020). Besides terpenes, C_{13}-norisoprenoids also constitute primary aroma compounds that have a great impact on wine, are closely related to the quality of white wines, and may be used to differentiate monovarietal wines. The concentration of C_{13}-norisoprenoids tends to accumulate during ripening and starts to degrade once grapes reach full maturity (Waterhouse *et al*., 2016). This can be directly applied to Ribolla Gialla base and sparkling wines produced in 2018 and 2019, where the amount of norisoprenoids increased until H2. By adding the third harvest timing, the amount of all norisoprenoids decreased in H3 base wines and sparkling wines, except for vitispirane.

A similar observation was confirmed in other studies, especially in the case of β-damascenone and 1,1,6-trimethyl-1,2-dihydronapthalene (TDN) (Šuklje *et al*., 2019; Versini *et al*., 2002). β-damascenone was recognised as the most abundant C_{13}-norisoprenoid in sparkling wines, and its concentration nearly doubled the concentration detected in the commercial monovarietal sparkling wines from Ribolla Gialla, which is promising for the development of 'fruity' and 'honey' scents (Voce *et al*., 2019). Since norisoprenoids are inactive at the beginning of the winemaking process, their release from glycosides could enhance the differences between separate harvest stages. The evidence for such chemical rearrangement can be evident in the increased amount of C_{13}-norisoprenoids in sparkling wines compared to base wines (Figure 2). Similar to monoterpenes, increased sunlight exposure seems to encourage the development of carotenoids and consequently increase the levels of norisoprenoids in the finished wine. Additionally, higher temperatures promote

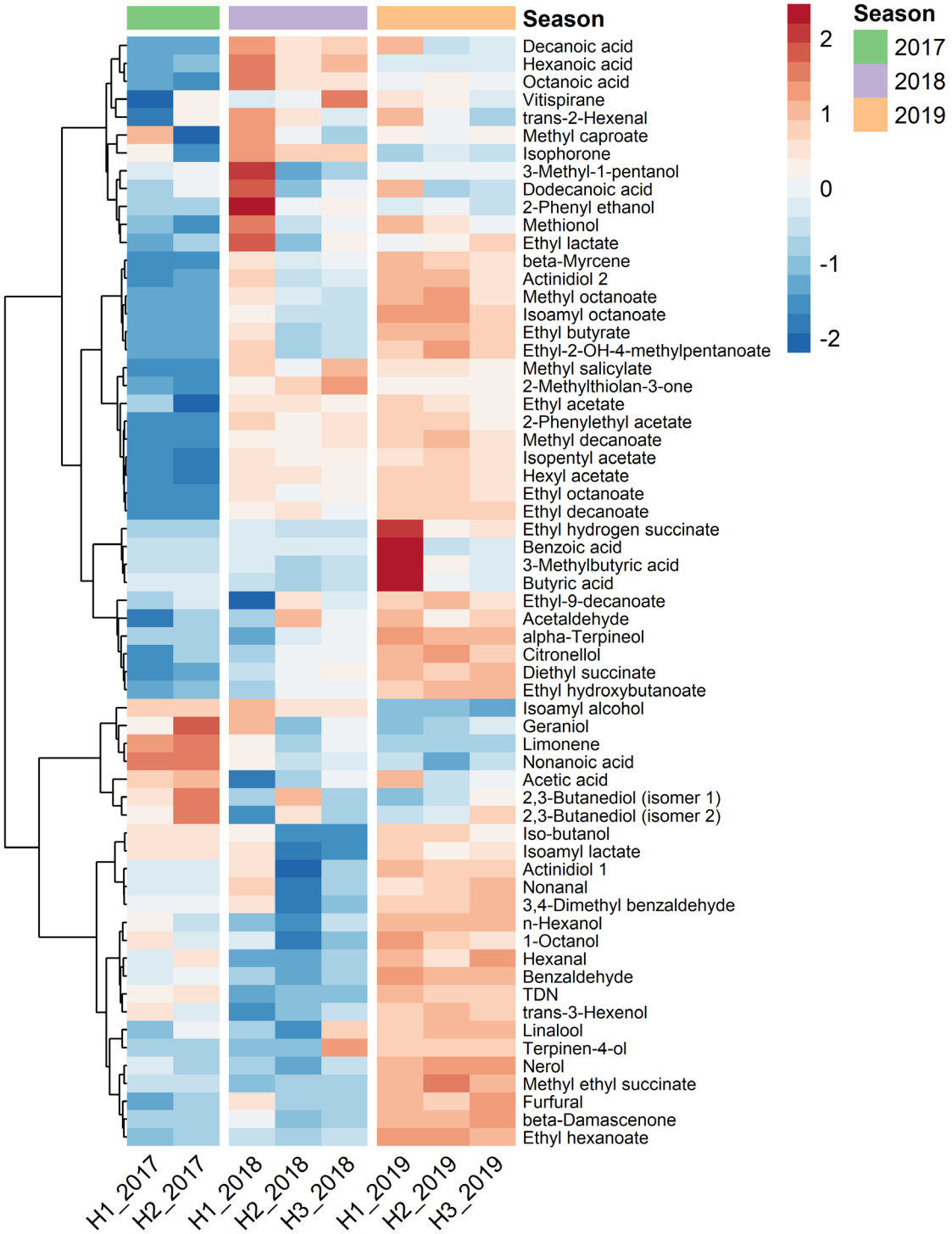


FIGURE 2. Heatmap presentation of the log2-fold change (sparkling wines/base wines) of the volatile compounds for each harvest time and throughout all three studied seasons. An average of three replicates for each harvest time was used for the analysis. Blue and red boxes indicate lower and higher concentrations in sparkling wines, compared to base wines, respectively. The results obtained after the log2-fold change calculations, were additionally scaled to unit variance to facilitate the presentation. Ward's method was applied for hierarchical cluster analysis of the single volatiles. *cis*-3-Hexenol, *trans*-2-hexenol, ethyl dodecanoate, and isobutyl acetate were left out from the analysis since they were identified only in sparkling wines.

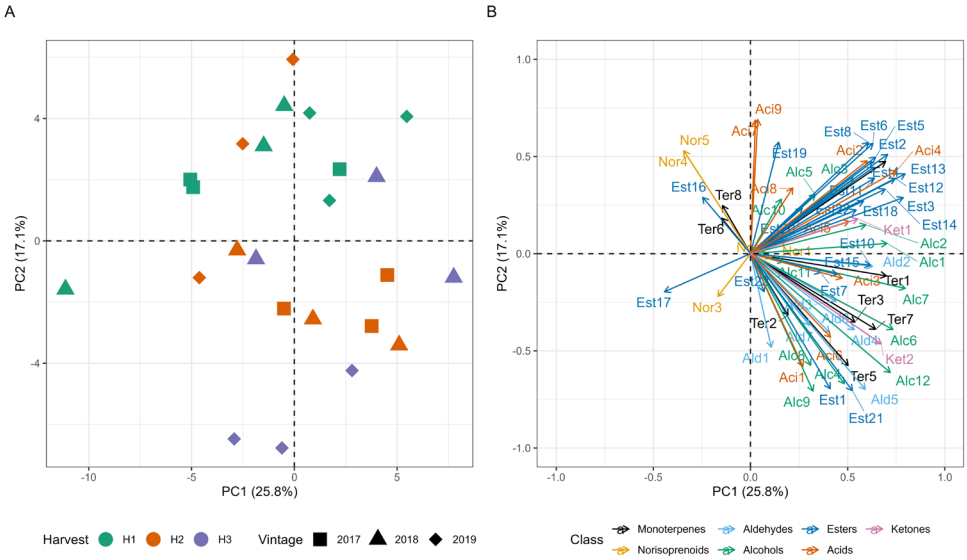


FIGURE 3. Principal Component Analysis (PCA) score plot (A) and loading plot (B) of the VOC profiles of three different harvest times (H1, H2, and H3) from three harvesting seasons (2017, 2018, and 2019), obtained after normalizing the season. Each point in the score plot represents a single replicate, while the label of each compound in the loading plot corresponds to a denomination listed in Supplementary Table 3.

the synthesis of norisoprenoids, as was observed in the case of Glera grapes (Alessandrini *et al*., 2017). This is also the reason why the total amount of norisoprenoids was higher in the warm 2019 season, especially in sparkling wine.

The presence of *Saccharomyces* species at the onset of alcoholic fermentation has great potential to contribute to the liberation of some aglycons from the flavourless precursor glycoside during fermentation. Nevertheless, it is well known that yeasts are also producers of VOCs; in wine, the main groups of compounds that form the fermentation bouquet are acids, alcohols, and esters, while aldehydes and ketones contribute to aroma development to a lesser extent (Swiegers *et al*., 2005). The last two classes were among the least represented also in the case of Ribolla Gialla wines, although *trans*-2-hexanal and isophorone were the most abundant, respectively. Generally, postponing the harvest led to an increase in the concentration of aldehydes, especially in the case of base wines from H1 to H2, with a substantial drop in concentration through the introduction of a third harvest in 2018 and 2019. The decreased amount of *trans*-2-hexenal is ultimately desirable since it can contribute to the herbaceous note in the wine aroma profile, something that can turn out to be detrimental and undesirable to the consumer if it occurs in high concentrations (Herraiz *et al*., 1990). Among fermentative compounds, twelve higher alcohols were detected. This did not place them among the most numerous, although the expressed concentration proved to be a factor to be considered. In certain studies, where authors compared the effect of sequential harvest timings, higher alcohols accounted for 86 % of the total volatile composition (Zhao *et al*., 2019). Environmental stresses, in particular water deficiency, can activate the alcohol dehydrogenase (ADH) activity that is responsible for catalysing the reduction of aliphatic aldehydes to alcohols (Moreno Luna *et al*., 2018). This proved to be in conflict with our observations since the concentration of n-hexanol was higher in 2017, where June was characterised by an abundant amount of precipitation (275 mm) compared to the other two seasons (74 mm versus 38 mm for 2018 and 2019, respectively). In the case of sparkling wines, the differences appeared to be more limited when comparing the three sequential harvest dates. However, by taking into consideration only two harvests spanning the 2017–2018 seasons, it turned out that the concentration of C_6 alcohols derived from the lipoxygenase (LOX) pathway (e.g., hexanol and *trans*-3-hexenol) increased in the later stage of ripening. Conflicting results have been previously published arguing that the concentration of *trans*-3-hexenol and its configurational isomer have decreased in wines produced from the later harvests (Antalick *et al*., 2015; Fang and Qian, 2012; Šuklje *et al*., 2019).

Second only to higher alcohols, esters also directly influence the aromatic profiles and sensory perception of wines. An increased °Brix level in grapes from later harvest dates leads to the enhanced production of ethanol as well as higher alcohols, therefore increasing the content of acetate esters (Moreno Luna *et al*., 2018). The delayed harvest showed an increased value for a large number of esters in base wines, where ethyl acetate, isopentyl acetate, and hexyl acetate were displaying an increasing trend towards H2. Moving on to sparkling wines, the later harvests have shown, in many cases, a decreasing trend in the concentration of esters. Nevertheless, it is worth mentioning that major ethyl esters of fatty acids and higher alcohol acetates are strictly fermentative compounds produced by wine microorganisms (Pons *et al*., 2017). Therefore, the results show large differences in ester content between base and sparkling wines (Figure 3). The loss of esters during the second fermentation and subsequent ageing period is a consequence of chemical

hydrolysis and thermodynamic instability. Despite this, the results of sparkling wines showed that the concentration of diethyl succinate increased in 2019 compared to base wines, a finding in agreement with the results reported by Ubeda *et al*. (2019). Moreover, researchers have found that in dry, hot seasons, aggravated sunshine and daytime temperature on berry clusters could be the main causative factor for a reduction in the levels of C_6-derived esters (He *et al*., 2020). This is also true for the present study since the 2018 and 2019 seasons were characterised by high average temperatures due to the large number of days with temperatures above 30 °C. For instance, the amount of ethyl hexanoate in 2017 was 1007 µg/L, while 761 µg/L and 433 µg/L were measured in 2018 and 2019, respectively.

Considering the concentration of volatile acids in our study, harvest time had a different effect on the composition of base wines and sparkling wines. In the case of base wines, the harvest date was extended for approximately one week, increasing the total concentration of all acids. As far as sparkling wines are concerned, the later harvests did not significantly modify the concentration of medium carbon chain acids (i.e., hexanoic, octanoic, and decanoic acid), thus potentially preserving the wine aroma of 'cheese-like' negative notes (Ferreira *et al*., 2000). Additionally, when H3 results were studied, an increased amount of acetic acid was produced in the extended harvest date, especially in 2019.

To additionally explore the dataset, PCA was used to extract and characterise the most influential factors that affected the sparkling wine volatilome. The results are shown as a score plot in Supplementary Figure 1, where the projection of the first principal component (PC1) versus the second principal component (PC2) accounts for 43.7 % of the total variance. Moreover, the plot shows a clear separation between the samples harvested in each respective season. This was somewhat expected, given that abiotic factors greatly influence the biogenesis of secondary metabolites in plants (Gao *et al*., 2019; Pavarini *et al*., 2012). For this reason, all metabolites were mean-centred by year to remove the seasonal effect prior to reinvestigation of the data matrix by performing the PCA. The results are summarised in Figure 3, where the separation between the two or three harvest times is clear in the PC1 versus PC2 plane, accounting for 42.9 % of the overall variance. PC1 proved to be particularly important in separating the two harvest times in 2017, while PC2 clearly separated the H1 from H2 in the final vintage of the trial. The length and direction of the variables in the loading plot (Figure 3B) show that H1 from 2019 was consistently richer in almost all analysed esters detected, which corresponds to one-way ANOVA results.

3.2. Lipid composition

In wines, the lipid profile is crucial for the synthesis of some volatile compounds that are derived from them. As regards lipid composition in Ribolla Gialla base wines and sparkling wines, it has been observed that the delayed harvest date contributed to a very reduced number of statistically significant compounds (Supplementary Table 4 and Supplementary Table 5). Instead, the results showed that the seasonal factor affected most of the nineteen lipids analysed, arranged in several chemical classes (glycerolipids, sterols, unsaturated and saturated fatty acids, fatty esters, and prenols). Among those, the changes in fatty acid composition were the most evident. It turned out that the concentration of saturated fatty acids (SFA) outstripped the concentration of unsaturated fatty acids (UFA) in all three years of the experiment. The sum of average concentrations of SFA was at its highest in the 2019 winegrowing season, and a similar observation was also shown for polyunsaturated fatty acids (PUFAs), linolenic acid (C18:3), and linoleic acid (C18:2), which are considered the major components of total lipids in grapes (Pérez-Navarro *et al*., 2019). Other authors have shown that the content of SFA and UFA is highly affected by the ripening period of grapes as well as by climate factors. Tociu *et al*. (2017) argued that the harvesting time is an important factor in the ripening of grapes, and, thus, the amount of Mamaia grape seed oil increased with the delayed harvest. Moreover, the authors reported increasing values of mono unsaturated fatty acids (MUFA) in the years with high precipitation during the ripening period. Conversely, the concentration of polyunsaturated fatty acids (PUFA) content was higher, and the SFA content appeared to be lower in the dry years. In our case, the summer temperatures of 2019 increased above average values compared to the other two vintages; however, the ratio between SFA and UFA remained unchanged in favour of the higher SFA concentration (Supplementary Figure 2). The ratio between SFA and UFA composition is important in wines, as both have an impact on the aroma characteristics of wine. Liu *et al*. (2019) reported that low concentrations of linoleic acid enhanced the production of certain free fatty acids (e.g., octanoic and decanoic acid), while oleic acid promoted the isoamyl acetate biosynthesis. Interestingly, a low amount of linolenic acid had no effect on acetate ester production, while higher supplementation enhanced the production of C_6 alcohols (1-hexanol) and higher alcohols (isobutyl alcohol and 2,3-butanediol). Our results proved to be in accordance with this case since the 2018 season was marked by the lowest amount of linolenic acid, which consequently led to lower LOX activity and lower C_6 alcohol production. The SFA/UFA ratio can, moreover, have a major influence on wine foaming in sparkling wines (Gallart *et al*., 2002; Pueyo *et al*., 1995). Unsaturated linoleic acid can exhibit a positive correlation with foam stability, while saturated palmitic acid can show a strong relation to foam height. Although palmitic acid was proven to be the most abundant among SFA, this was not the case for linolenic acid concentration compared to other UFA substances in our study. Nevertheless, both compounds were higher in the 2019 season, and it can therefore be expected that wines from that particular vintage will be characterised by positive descriptors related to foam.

3.3. Aromatic amino acid metabolites in Ribolla Gialla wines

The results presented on TRP metabolites highlight a substantially positive role in the extended harvest date.

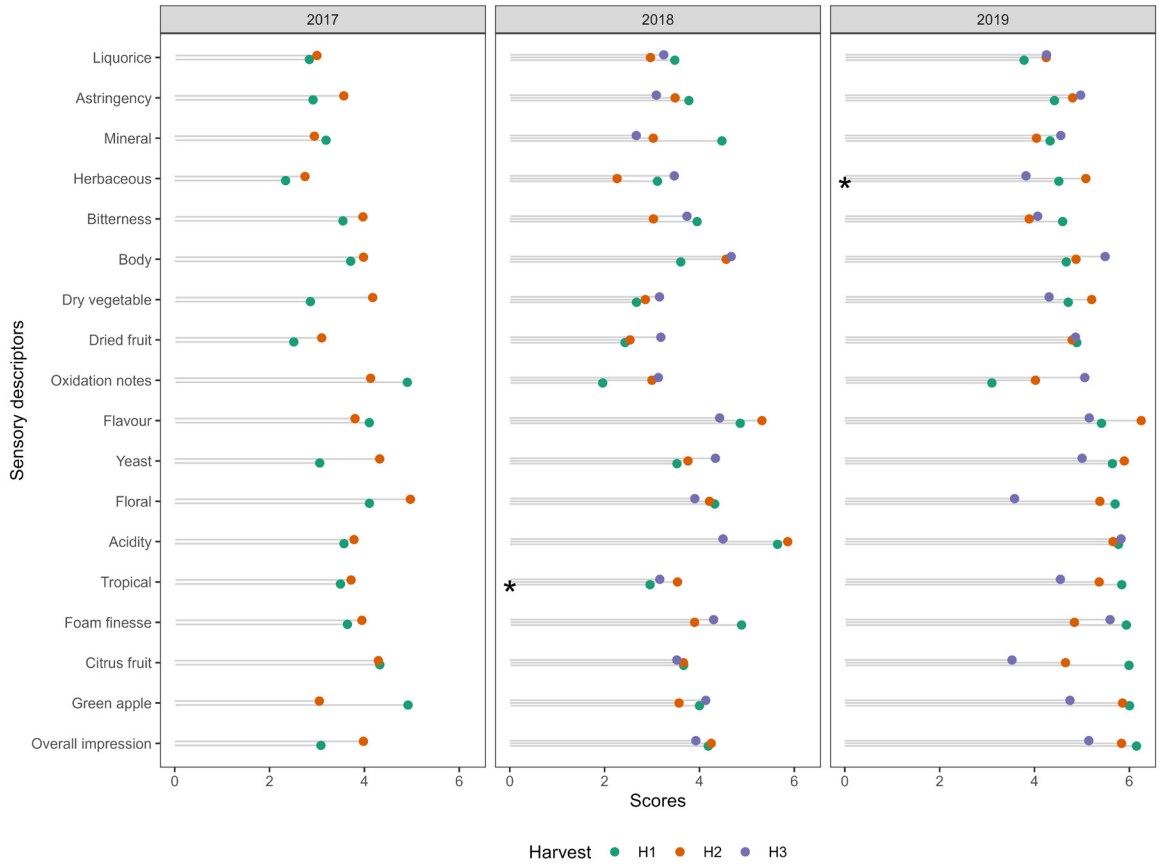


FIGURE 4. Lollipop plots represent the effect of two and three different harvest dates on the organoleptic characteristics of Ribolla Gialla sparkling wines obtained in the 2017–2019 harvest seasons. Each point represents an average of triplicate, while a final score was normalized by panellists. Significance of one-way ANOVA is indicated with asterisk '*' that corresponds to '$p < 0.05$'.

Firstly, the TRP did not show any significant differences when comparing H1 to H2 and finally to H3. Moreover, the trend in concentration has proven to be quite inconsistent. However, despite very few significant changes regarding the amount of TRP in different harvest dates (Supplementary Table 6 and Supplementary Table 7), our results for tyrosine (TYR) and phenylalanine (PHE) showed that their concentration increased in the samples of base wines with an extended harvest date (Supplementary Table 6). This observation was in line with the fact that the nitrogen or amino acid contents of the grapes, musts, and wines increase with the ripeness of the grapes (Hoenicke *et al.*, 2001). Concomitantly, with an increase of the amino acid TYR in the base wines, the concentration of its catabolites tyrosine ethyl ester (TYR-EE), N-acetyl-tyrosine ethyl ester (N-TYR-EE), and tyrosol also increased. Both ethyl esters are produced during alcoholic fermentation by *S. cerevisiae*, and they play an important role in the yeast mechanism and, thus, in the quality of fermented food products. However, very little is known about the formation of amino acid-derived esters during wine production; therefore, the Pearson correlation coefficient of analysed esters belonging to VOCs and AAA metabolites was calculated for sparkling wine samples (Supplementary Figure 3A) to hypothesise the possible generation of TYR esters. From the results obtained, it is possible to observe a significant correlation between TYR-EE and ethyl lactate, as well as isoamyl lactate (Supplementary Figure 3B). Generally, esters can be synthesised from the reaction of acid and alcohol by ester synthase catalysis, but in yeast cytoplasm, the catalytic reaction of alcohol acyltransferases is the preferred pathway for the synthesis of esters (Park *et al.*, 2009). Analogously, ethyl lactate can be synthesised from the reaction of lactoyl-CoA and ethanol catalysed by acyltransferases (Ren *et al.*, 2020). Further in-depth studies are required for a more precise explanation of the formation of TYR-originated esters. The importance of these compounds is significant for wine quality since it has been previously observed that N-TYR-EE actively participate in the inhibition of TRP synthesis and metabolism in the yeast, as well as being a mediator in the production of tryptophol (Antonia Álvarez-Fernández *et al.*, 2019). This may be crucial for the aromatic profile of wines since an off-flavour is known to be formed in white wines and is associated with the aroma compound 2-AAP, which formation is conditioned by the presence of precursors TRP and IAA. This off-flavour is often described by aromas such descriptors as 'acacia blossom', 'furniture polish', 'wet wool', 'mothball', and 'fusel alcohol', and usually leads to the

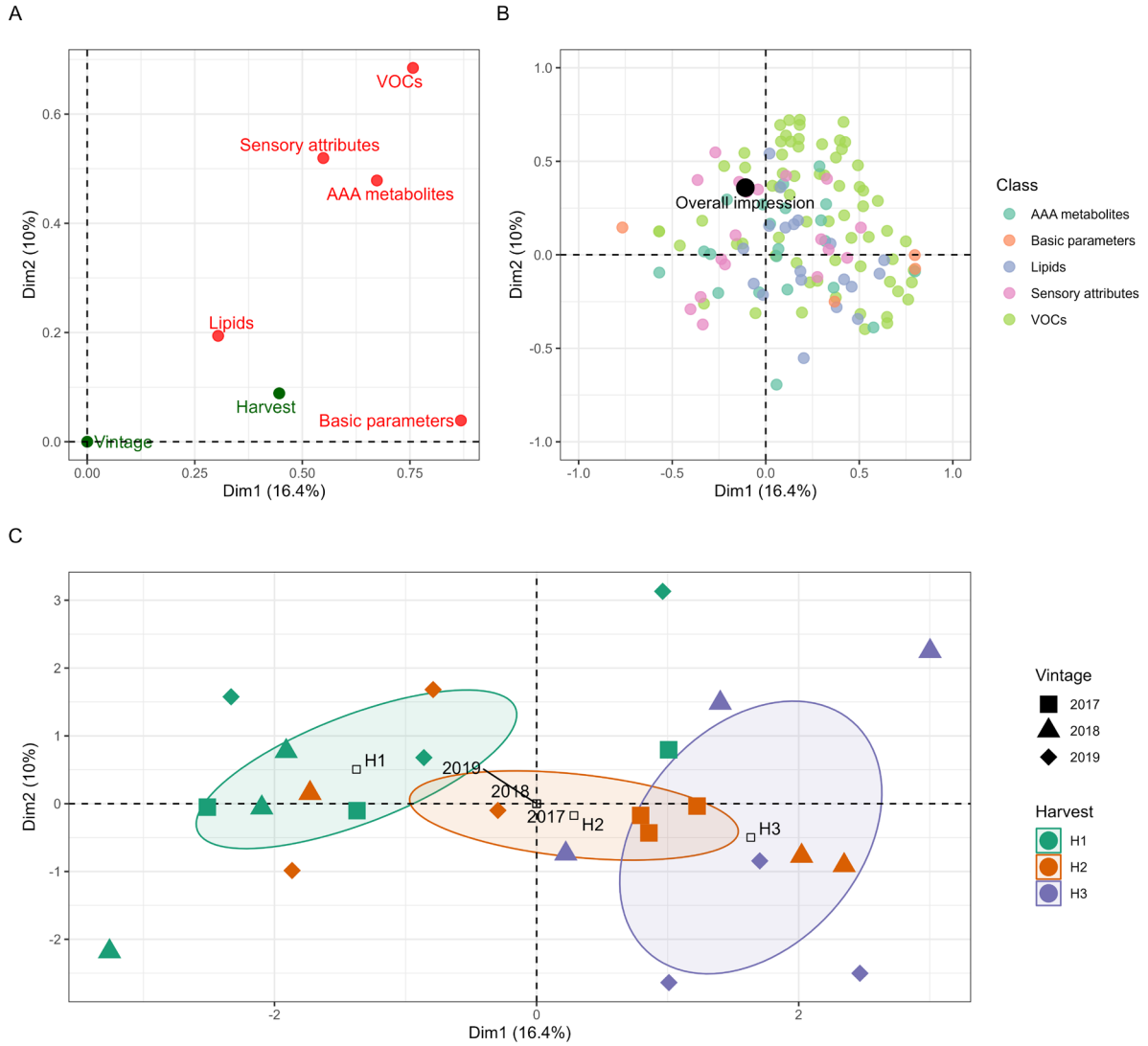


FIGURE 5. Plots of total variables (A), quantitative variables (B), and individuals (C) of Ribolla Gialla sparkling wines, obtained by Multiple factor analysis (MFA). The smaller dots on the plot of individuals represent single samples. The radius of ellipses drawn around centroid points of individuals for sequential harvests, demonstrate the confidence intervals.

loss of a typical bouquet of the grape variety (Arapitsas *et al*., 2018; Hoenicke *et al*., 2002). Since the 2-AAP has not been identified in our study, it was observed that IAA concentration predominated in sparkling wines during the 2019 harvest season, which had been characterised as one of the driest in the three-year average. The location of the vineyard where the experiment was carried out may have also contributed to the increased IAA concentration since there was less possibility of irrigation in the event of drought. Among the remaining precursors that can possibly lead to the formation of aromatic aminobenzene 2-AAP, there is also the glycosidic form of indole lactic acid (ILA-GLU), which accumulated in base wine and sparkling wine with an extended harvest date. Namely, when the glycosidic bond is hydrolysed in wine, the producing ILA can react with the present SO_2 to deliver the sulfonated form of ILA (ILA-SO_3H) since the sulfonation of indoles could be responsible for their degradation and the formation of aromatic aminobenzenes (e.g., 2-AAP), responsible for untypical ageing flavour emergence in wine. Similarly, the increase of tryptophol (TOL) concentration was observed in base wines, especially when comparing H1 wines with the H2 ones, thus increasing the risk of giving rise to products similar to 2-AAP (Arapitsas *et al*., 2018). Lastly, phenyl acetic acid (PhAA) also appeared to show a tendency to accumulate in the later stage of the harvest. The synthesis of PhAA is correlated with the occurrence of the grape sour, which often occurs towards the end of grape ripening when the grape skin becomes thinner and more susceptible to microorganism infections. A high quantity of precipitations and relatively high temperatures, typical for the 2018 harvest season, can, therefore, facilitate the development of grape sour, and this could contribute to the development of 'sweet-like' and 'honey' off-odour as a result

of high presence of PhAA (Campo *et al*., 2012; Pinar *et al*., 2017).

4. Sparkling wine sensory evaluation

The sensory attributes of obtained sparkling wines showed that the 'overall impression' was assigned to the wines from H2 when only two harvest times were compared in 2017 (Figure 4), characterised by 'floral' and 'yeast' sensory descriptors. Certain aldehydes can cause the phenomenon of oxidative aroma in wines, which was confirmed by this study, as the total concentration of aldehydes predominated in H1 wines (Mayr *et al*., 2015). In general, the addition of H3 did not greatly affect the results where subsequent harvesting was applied in 2019 since the wines were less preferable due to the increased assessment of the perception of oxidative aroma in wines, which could be related to rising content of nonanal, 3,4-dimethyl benzaldehyde, and furfural. This proved to be partially in line with the study of Zhao *et al*. (2019), where the total aldehyde and ketone contents in sequential harvest wines increased compared to control samples. Interestingly, the scores for 'citrus fruit' and 'floral' sensory descriptors decreased with each additional harvest. The disappearance of the total composition of monoterpenes and esters in H3 confirms this finding. An interesting result can be observed in 2018 since the wines from H2 were significantly characterised by a 'tropical' sensory descriptor. Traditionally, the compounds thought to cause tropical fruit aromas are volatile thiols; however, it has been found that wines that have low or no volatile thiol compounds can also have 'tropical' characteristics with fermentation esters (e.g., ethyl acetate in our study) being a possibility (Ferreira *et al*., 1995; Iobbi *et al*., 2023).

5. Multiple factor analysis (MFA) of measured multi-targeted datasets

To better understand how the combination of several multi-targeted datasets has an influence on the separation of different harvest times in Ribolla Gialla sparkling wines, a multivariate data analysis method was applied (Figure 5). Figure 5A illustrates the correlation between an active group of quantitative variables and categorical variables (i.e., harvest time and vintage). The coordinates of AAA metabolites and VOC composition on the first dimension (Dim1) are very similar, meaning that their contribution is comparable. Concerning the second dimension (Dim2), the basic chemical parameters have the lowest coordinate, indicating a small contribution to the separation of the Dim2. The graph of the variables, represented in Figure 5B, shows the relationship between all analysed variables, where it can be observed that the majority of VOC, as well as lipid compounds, are placed on the positive side of Dim1, suggesting a positive correlation. However, a close-up examination of the same plot displayed in Supplementary Figure 4 reveals that some important olfactory molecules (e.g., nerol and several ethyl esters) are positively correlated with 'floral' and 'citrus fruit' sensory descriptors, which probably contributes to the 'global impression' of analysed wines, placed in the same second quadrant. An interesting observation is the remarkably high contribution of hydroxytyrosol to the definition of the Dim2. It could be hypothesised that its antioxidant activity could preserve wine aroma during prolonged storage conditions, even though it does not have any particular direct influence on the volatile profile of wines (Raposo *et al*., 2016). Consequently, overall acceptance is positioned between H1 and H2, which is consistent with the findings from sensory analysis if the entire three-year period is taken into account (Figure 5C). As observed in the previous plot, Dim1 represents the vast majority of alcohols and aldehydes, which could imply poor acceptance of the wines among panellists. Nevertheless, the H3 wines, represented in Dim1, proved to be positively correlated with 'oxidation notes' despite positive contributions to the aroma profile from selected esters (e.g., fatty acid esters, 2-phenetheyl acetate, isobutyl acetate) and monoterpenes, such as β-myrcene, linalool, and terpinen-4-ol.

CONCLUSIONS

This work confirmed the importance of determining the harvest time of grapes intended for the production of quality sparkling wines. As far as the composition in volatile compounds is concerned, the results have shown a significant advantage in the transition from first (H1) to second (H2) harvest time, which resulted in enhanced production of esters, while no encouraging result emerged in the third (H3) harvest. In certain cases, the additional third harvest even meant a deterioration in the aromatic profile of the sparkling wines. In fact, the addition of an extra harvest date caused an increase in acetic acid, C8, and C9 volatile fatty acids.

The lipid composition of base wines and sparkling wines was not affected by the harvest time. However, the lipid content varied depending on seasonal factors. Thus, the hot season of 2019 was associated with higher content of SFA, in particular palmitic acid, which could positively affect the foam height of sparkling wines.

At the level of TRP metabolites, it has been clearly shown that the extension of the harvest date is not necessarily correlated with the formation of UTA substances that could compromise the quality of sparkling wines.

Lastly, the sensory evaluation of sparkling wines appeared to be in accordance with the chemical analysis since it revealed that the wines from the second harvest were rated with high 'overall impression' in 2017 and 2018; however, considering the results of the MFA analysis, the 'overall impression' is positioned in the same quadrant as H1 wines, considering all chemical analyses. On the contrary, the wines from the later harvest were evaluated as the least attractive from an olfactory, gustatory, and sensory point of view. In addition, the best harvest time for Ribolla Gialla destined for sparkling wine production should be calculated based on sugar loading, meaning that the grapes should be harvested between the phloem blockage and one week later since a postponement causes a decline in the aromatic potential.

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