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Effects of pre- and post-fermentative practices on oligomeric cyclic and non-cyclic condensed tannins in wine from Schiava grapes

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ABSTRACT

The effects of a) pre-fermentative freezing of the grapes (- 20 °C for two weeks); b) inoculation of the grape must with *Saccharomyces cerevisiae* yeast, or co-inoculation with *Saccharomyces cerevisiae* yeast and *Oenococcus oeni bacteria;* c) vinification with or without fermentative maceration, and d) cold stabilization with or without bentonite treatment, were studied on the profile of oligomeric condensed tannins (proanthocyanidins, PAC) with non-cyclic or macrocyclic structures in wines made from Schiava cv., a red grape variety. The samples were evaluated just before inoculation and at the bottling of the wine. Commercial Schiava wines from two different producers stored at six and eighteen months were also studied for the effect of artificially introduced dissolved oxygen, and half of these bottles were subjected to periodic mechanical stress for one year, to see the effects on the PAC profile. Freezing of the grapes increased the extraction of all non-cyclic PAC in the must, whereas tetrameric, pentameric, and hexameric cyclic procyanidins (m/z 1153, m/z 1441, m/z 1729, respectively) were not affected; only a tetrameric cyclic prodelphinidin (m/z 1169) showed a more similar trend to the non-cyclic PAC. In wines at bottling, cyclic procyanidins were higher in wines obtained by fermentative maceration (as well as most non-cyclic congeners); however, the significance of these differences depended on specific interactions between the factors. In contrast, no effect was found on the cyclic tetrameric prodelphinidin (m/z 1169). Bentonite treatment showed no significant effect on either oligomeric non-cyclic or cyclic PAC profiles. The addition of dissolved oxygen led to a significant decrease in non-cyclic trimeric and tetrameric PAC in the samples with respect to the control ones; however, the addition of dissolved oxygen did not influence the profile of the cyclic PAC. This study sheds new light on the substantial differences in the behaviour of the cyclic and non-cyclic oligomeric PAC in red wine in relation to the vinification process and in the bottle. Cyclic oligomeric PAC were more stable and less influenced by applied factors than linear PAC, again proving to be potential markers for the grape variety of wine.

1. Introduction

Condensed tannins (i.e., proanthocyanidins, herein abbreviated as PAC) are a class of phenolic compounds widespread in nature (Versari et al., 2013). Their wide natural distribution makes these compounds a major source of antioxidant phenolic compounds in food. PAC have been studied in relation to antioxidant, anti-inflammatory, cardiovascular, anti-diabetic, anti-infection, and anti-cancer properties (de la Iglesia et al., 2010). Because they are the product of oligo-/polymerizations of flavan-3-ols, they can include a wide variety of different substances; besides their molecular weight (i.e., the number of oligomerized monomeric units, i.e. degree of polymerization = DP), which for instance in wine can vary considerably from DP = 2 potentially up to DP > 50 (Merkytė et al., 2020b; Labarbe et al., 1999), their variability is related to differences in types of ring positions involved in the inter-flavanolic linkage (position and configuration – Fig. 1A), to

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different types of flavan-3-ol monomeric units (Fig. 1B) translating into different patterns of hydroxylation of the B-rings in the resulting PAC, to the presence of different linkage types (B-type linkage versus A-type linkage, Fig. 1C), or even to other modifications, such as 3O-esterifications (Fig. 1D), and to macrocyclic ring-closure (cyclic/crown PAC – Fig. 1E) (Zeng et al., 2019; Longo et al., 2018c). The macrocyclic (cyclic oligomeric or crown) PAC (herein c-PAC) and their resulting macromolecular structures greatly affect their abundance and evolution in grapes and wine. However, their physicochemical properties are still poorly understood (Longo et al., 2018c).

Analytically, investigating their profile requires the separation and determination of many different congeners, with very different concentration ranges (Merkytė et al., 2020a). On the other hand, their variability could be correlated to the grape variety or the application of certain winemaking conditions (Longo et al., 2018a; Longo et al., 2019; Arapitsas et al., 2020).

Recent studies have investigated the ability of c-PAC to be applied as varietal markers in authenticity studies (Longo et al., 2018a, 2019). These appealing results might have an impact on the development of anti-fraud models. Other classes of phenolics such as anthocyanins have been applied already for this goal (Merkytė et al., 2020b; Mattivi et al., 1990). However, their profile in wine is variable and can affect substantially the correlation between the profile and the grape variety. The anthocyanin profile of Schiava cv. grapes (germ. Vernatsch, the variety used in this study) is peculiar to this variety and is different from that of the wines made from it. Thus, a more stable set of chemical markers might be sought to complement authenticity models.

The class of c-PAC offer several potential advantages in this respect since they are more polar than most of the "conventional" non-macrocyclic B-type and A-type condensed tannins (Longo et al., 2018b, 2018c) with the same degrees of polymerization. Hence, they are even more quantitatively extracted from the grapes during the earlier vinification stages compared to linear condensed tannins in Pinot noir (Merkytė et al., 2020c), and in proportions that might have a great resemblance to the compositions in the grapes. Besides, it has been shown that c-PAC are more structurally resistant to acidic conditions (Zeng et al., 2019).

Further condensation reactions of (non-cyclic) PAC are common in wine over aging, leading to further oligomerizations, polymerizations, and eventually to precipitation. Conversely, preliminary studies have argued that the non-availability of a reactive C4 position in the condensed flavan-3-ol units of c-PAC is another major reason for their stability (Longo et al., 2018b, 2018c). In fact, being already occupied by an intra-flavanolic linkage, the C4 position is less likely to be involved in oligo-/polymerization processes that would lead to a chain extension. At the same time, their higher stability to acidic conditions could also slow down the rate of depolymerization. This increased stability has also the side effect to make all investigated c-PAC less easily fragmented in mass spectrometric MS/MS experiments (Longo et al.,2018b; Longo et al., 2018c); this feature was successfully exploited as another tool to differentiate these compounds from their non-cyclic congeners in MS/MS.

The applicability of c-PAC and of some related chemical indexes in classification models for grape varieties was proposed in previous works (Longo et al., 2018a, 2019). However, no attempts have been made so far to observe systematic winemaking factors that could affect the applicability of these models. Namely, different winemaking conditions, such as pre-fermentative treatments (Busse-Valverde et al., 2010), vinification methods (Smith et al., 2015), the use of additives or adjuvants, and wine stabilization treatments (Ghanem et al., 2017) are known to affect the phenolic profile and could influence the profile of c-PAC as well.

In this study, a controlled and systematic experimental plan has been applied to define the effects of specific winemaking practices on the PAC profile during the vinification of a monovarietal wine obtained from *Schiava grossa* grapes from the must to the bottled wine. The analysis of the must samples was done right before the inoculum of the yeast and also before any addition of pomace to the pressed musts (for red winemaking), in order to evaluate *i*) the effects of a pre-fermentative grape freezing treatment, and *ii*) the effect of an over-night clarification with pectolytic enzymes. Then, the produced wines were analysed at bottling, to study *i*) the effect of grape freezing, *ii*) the effect of a co-inoculum with *Oenococcus oeni*, and *iii*) the effect of fermentative maceration. Successively, all wines underwent a stabilization step at + 4 °C after addition of bentonite. Control samples, which did not undergo bentonite stabilization but only cold stabilization, were also analysed and compared.

The profile of PAC was also analysed in commercial Schiava wines from the same harvest from two different producers after six months and eighteen months of storage in bottle. It was also chosen to study the effect of applied mechanical stress on bottled wine against a control

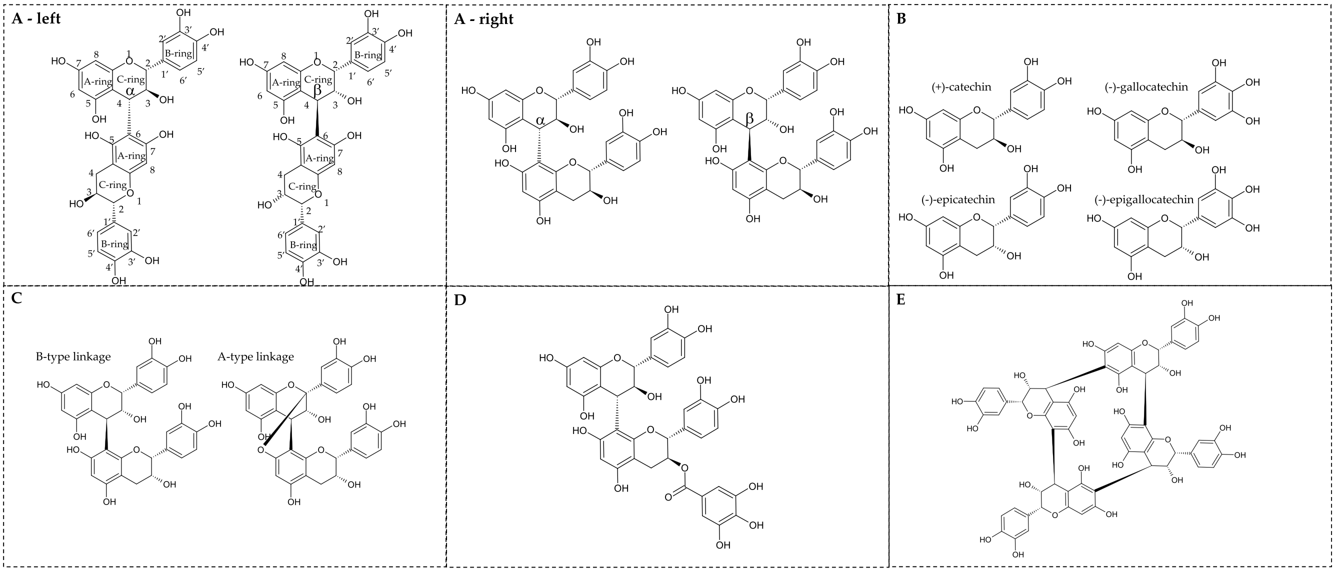

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Fig. 1. Elements of structural variability in condensed tannins (PAC). A-left) 4α-6 versus 4β-6 inter-flavan-3-ols B-type linkage (4α-6: procyanidin dimer B6, 4β-6: procyanidin dimer B5); A-right) 4α-8 versus 4β-8 inter-flavan-3-ols B-type linkage (4α-8: procyanidin dimer B3, 4β-8: procyanidin dimer B1); B) some of the possible monomers presents in PAC; C) A B-type dimer (e.g. procyanidin dimer B2 - left) versus an A-type dimer (e.g. procyanidin dimer A2 - right); D) Example of esterification at position C3 (galloylated dimer); E) NMR-resolved structure of the cyclic tetrameric procyanidin (adapted from Zeng et al. 2019.

sample. Finally, a subset of these samples was prepared by adding dissolved oxygen, to also study the effect of this parameter on the PAC profile. The dissolved oxygen content, free SO_2, and total SO_2 (free + combined) parameters were analysed in these commercial Schiava wines.

2. Materials and methods

2.1. Materials

All chemical reagents, solvents, and HPLC-phase organic modifiers used for the HPLC-MS analyses were at MS grade and were purchased from Merk Life Science S.r.l. (Milano, Italy). Ultrapure water was generated *in-house* using an Arium Mini generator (Sartorius Italy S.r.l., Varedo, Monza Brianza, Italy). *Schiava grossa* cv. grapes were purchased from a local producer (Hartmann Donà winery, Lana, Bolzano, Italy).

2.2. Sampling and winemaking

The laboratory-scale vinification method applied in this work was conducted according to Ribereau-Gayon, P. et al., 2006 and common winemaking procedures. *Schiava grossa* (120 kg) grapes were harvested at technological maturity on October 11th, 2021 in Appiano (Bolzano, Italy), in a vineyard exposed northwards. 60 Kg (theses F) were immediately put in a freezer at - 20 °C and stored for 14 days.

2.2.1. Winemaking of non-frozen (NF) grapes

Of the remaining 60 Kg (theses NF), 33 Kg (theses R_NF) were kept at + 4 °C for one night, while another 27 Kg (theses W_NF) were immediately destemmed and crushed in a bench crusher, and pressed in a pneumatic press in two stages: 0.2–0.5 bar, followed by a second stage at 0.5–2.0 bar. A small aliquot (1.2 L) of this juice was removed and divided 1:1 into two sterilized 2.5-L Duran borosilicate bottles, and then inoculated with 400 $mg{\cdot}L^{-1}$ of *Saccharomyces cerevisiae* yeast (Fermol DAVIS 522, AEB) – **P1**, or 400 $mg{\cdot}L^{-1}$ of yeast plus 40 $g{\cdot}L^{-1}$ of *Oenococcus oeni* malolactic bacteria preparation (EnartisML Silver, Enartis) – **P2**. Both the yeast and the bacteria were prepared according to the producer's specifications. **P1** and **P2** were the two *pied de cuve* used for inoculating the different theses (**P1** for AF theses = only alcoholic fermentation, **P2** for AMF theses = alcoholic + malolactic fermentations).

Meanwhile, the main 16.4 L volume of prepared must was placed in two demijohn glass containers previously sterilized with 5% NaOH solution followed by 5% citric acid solution and thorough hot water rinsing; then, the must was added with 30 $mg{\cdot}L^{-1}$ potassium metabisulphite, 50 $mg{\cdot}L^{-1}$ of potato protein extract (Vegepure Omnia, HTS Enologia, C.da Amabilina, 218/A, 91025 Marsala, TP, Italy), 30 $mg{\cdot}L^{-1}$ of the pectolytic enzyme (Enartizym RS(P), Enatic, via San Cassiano 99, 28069 San Martino, Trecate, NO, Italy), followed by 300 $mg{\cdot}L^{-1}$ of activated decolouring charcoal (Carbon 100 Plus, HTS Enologia, C/da Amabilina, 218/A – 91025 Marsala, TP, Italy) according to the specifications from the producer. The must was then left one night at + 4 °C.

The next day the clarified grape juice was divided equally into six sterilized 2.5-L Duran borosilicate bottles (2 L must per Duran). Meanwhile, the remaining non-frozen grapes (R_NF) were destemmed and pressed, as done for W_NF. Their juice was immediately placed in six 2.5 L Duran sterilized borosilicate bottles (1.8 L must per Duran) and each one was also added with 100 $g{\cdot}L^{-1}$ of the removed grape pomace. Finally, the 12 bottles were inoculated with either **P1** or **P2** (50 mL per Duran), so that W_NF and R_NF were inoculated either only with yeasts (W_NF_AF and R_NF_AF) in triplicates, or inoculated with yeasts and bacteria (W_NF_AMF and R_NF_AMF) also in triplicates. Each prepared Duran without added grape pomace (W_NF) was then added with 300 $mg{\cdot}L^{-1}$ of yeast activating agent containing thiamine, ammonium sulfate, and dibasic ammonium phosphate (Enovit, AEB, Brescia, Italy), and 50 $mg{\cdot}L^{-1}$ of enological gallotannins (Gallovin, AEB, Brescia, Italy) according to the producer's specifications. Each prepared Duran with added grape pomace (R_NF) was parallelly added with 300 $mg{\cdot}L^{-1}$ of a yeast activating agent containing thiamine, ammonium sulfate, dibasic ammonium phosphate (Enovit, AEB, Brescia, Italy), and 100 $mg{\cdot}L^{-1}$ of enological gallotannins (Gallovin, AEB, Brescia, Italy) according to the producer's specifications. Finally, all Duran bottles were added with 30 $mg{\cdot}L^{-1}$ of potassium metabisulfite, also according to the producer's guidelines. Each W_NF and R_NF containing bottle was then closed with a perforated cap inserted with a silicon air-stopper closure filled with water, to monitor the production of CO_2. All W_NF bottles were then placed into an incubator at 18 °C and all R_NF bottles were kept at 25 °C. After 3 days and 8 days, 30 $mg{\cdot}L^{-1}$ of potassium metabisulphite was added to all Duran bottles according to the producer's specifications. Diammonium phosphate (HTS Enologia) was added to all samples after 3 days and 4 days from the inoculum at the final concentration of 200 $mg{\cdot}L^{-1}$. On the fifth day, 300 $mg{\cdot}L^{-1}$ of the yeast activating agent (Enovit) was also added according to the producer's specifications. The fermentation ended after 10 days for R_NF samples. All R_NF samples were racked and added with 15 $mg{\cdot}L^{-1}$ of potassium metabisulfite. After another five days, all R_NF samples were brought to +6 °C, added with one Antiflor agent (Enartis), 40 $mg{\cdot}L^{-1}$ of potassium metabisulfite, and then with 250 $mg{\cdot}L^{-1}$ of enological tannins (Fermotan SG, AEB). The fermentation of W_NF samples ended after 20 days from the inoculum. All W_NF samples were added with Antiflor (Enartis) and 120 $mg{\cdot}L^{-1}$ potassium metabisulfite according to the producer's specifications.

The fermentations were daily monitored as reported below (enzymatic analysis on a Miura One multiparametric analyser). The monitored parameters were: 1) content of glucose and fructose ($g{\cdot}L^{-1}$), 2) content of YAN ($mg{\cdot}L^{-1}$, expressed as α-amine nitrogen and free ammonium), and 3) content of free and total SO_2 ($mg{\cdot}L^{-1}$).

2.2.2. Winemaking of frozen (F) grapes

After the 2 weeks at – 20 °C, the frozen-grapes samples (F) were left at + 4 °C for 2 days to defrost, then were processed exactly as for the (NF) samples.

2.2.3. Stabilization and bottling

The cold stabilization was performed at + 4 °C for all samples. When the samples were already at + 4 °C, and exactly at the 48th day after the inoculum, all samples were added with 120 mg of sodic bentonite (Bent Gold, HTS Enologia), after its pre-activation according to the producer's specification. A set of 50-mL sterile plastic tubes were used to collect each sample before adding the bentonite, and these other samples were kept in the same storage environment as the Duran bottles. Each sample was left in contact with bentonite for two weeks, then removed and transferred to another clean and sterilized Duran bottle. The samples were left to stabilize for another 76 days at + 4 °C, before being transferred to another sterilized Duran bottle and added with 50 $mg{\cdot}L^{-1}$ of potassium metabisulfite. Considering all transfers, the final volumes for all (W) samples were about 1.8 L, and for all R samples were about 1.6 L. Finally, the samples were filtered under gravity over a large-sized sterile filter cloth and bottled in N_2-pre saturated sterilized dark-brown 750-mL wine bottles. Then, they were sealed with a cork stopper under a gentle N_2 flow; the corks were externally further sealed with heated sealing wax. Two bottles *per* each Duran container (i.e., each replicate of each thesis) were obtained, producing 48 bottles overall (8 theses x 3 replicates x 2 samples). All samples were labelled and stored in darkness at + 4 °C.

2.3. Condensed tannins (PAC) profile by UHPLC-ESI(+)/SIM-MS

PAC profiles were analysed on an UHPLC-QqQ/MS instrument (Agilent LC/TQ 6465 system) equipped with a 1260 Infinity II UHPLC with a quaternary pumps system, a 1260 Infinity II WR PDA detector, in series to an AJS ESI QqQ mass analyser. The LC-MS method was adapted with slight modifications from previously reported studies (Dupas de

Matos et al., 2020; Poggesi et al., 2021). The chromatographic separation was carried out at + 30 °C on a Vertex Plus Eurosphere II (KNAUER, Berlin, Germany) column 4.6 mm × 250 mm x 5 μm with a pre-column. The separations were carried out at 0.7 mL min^{-1} flow rate. The mobile phase consisted of A) 0.1% formic acid in degassed ultrapure water, and B) 0.1% formic acid (MS grade) in acetonitrile. The gradient separation program was as follows: 1 %B from 0 to 2.5 min, 1 to 25 %B from 2.5 to 50 min, 25 to 99 %B from 50 to 51 min, 99 %B from 51 to 55 min, 99 to 1 %B from 55 to 56 min, and 1 %B from 56 to 62 min. The injection volume was 5 μL. The mass spectrometer was operated in ESI + ionization mode. The detection of the proanthocyanidins was performed in single-ion-monitoring (SIM) mode. The SIM parameters are listed in Supporting Information (SI) File 1 Supplementary Table 1. The parameters for the mass spectrometric detection were the following: fragmentor potential = 135 V, cell acceleration = 5 V, N_2 gas temperature = 230 °C, N_2 gas flow = 8 L min^{-1}, nebulizer pressure = 20 psi, sheath gas heater = 300 °C, sheath gas flow = 10 L min^{-1}, capillary voltage = + 3000 V, Nozzle Voltage = 2000 V. After the analysis, the SIM traces were extracted in MassHunter and exported as.MzData files. These were then aligned in target mode in MzMine3 (Pluskal et al., 2010). Targeted features were selected manually by direct inspection of the entire dataset. In-source produced fragments and adducts were eventually carefully filtered out by ordering of the obtained features by increasing retention times, and then calculating the correlation matrix for the retention time-aligned peak table. All multiplets (groups of co-eluting peaks with different *m/z* values) characterized by very close retention times (± 0.1 min) and significant strong positive correlations (at least $R^2 > 0.8$) were inspected. Only one mass was eventually assigned for each one of the multiplets; the other ones were filtered out. The criteria applied to filtering the fragments and adducts features were: 1) Fragments – presence of in-source fragmentation products, e.g. loss/losses of monomeric unit/units from a higher-molecular weight species, and 2) Adducts - + 2 Da peaks in low resolution spectra (as in our case) are also due to the second isotopic peak (in the isotopic pattern), which is a common feature confusing the assignments of c-PAC with that of their direct non-cyclic analogues (e.g. *m/z* 1153 – cyclic tetrameric procyanidin – shows a perfectly coeluting and very strongly correlated *m/z* 1155, which is not, in this case, one of its non-cyclic pentameric procyanidin analogues, but its instead a second isotopic peak). Filtered PAC features observed in the LC-MS analysis reported in SI File 1 Supplementary Table 2. The results, expressed as intensity, are reported in Supporting Information File 2 (SI 1, SI 4, SI 7, and SI 10).

2.4. *Determination of free and total sulfur dioxide, pH, and dissolved oxygen*

Free and total sulfur dioxide were determined on a MIURA One automatic analyser (Exacta + Optech Labcenter SpA, San Prospero, Modena, Italy). Each method was calibrated and validated using sets of provided reference standards. pH was determined on a pH 60 DHS equipped with a W/201T pH probe (XS instruments, Carpi, Modena, Italy). Dissolved oxygen was determined on an ORBISPHERE 3650 Portable Analyzer (Hach Lange Austria, Hütteldorferstraße 299/6, 1140 Wien, Austria) equipped with a 29971-72 separated piercing head operating with a ~1.5 bar N_2 flow and a GA2X00 O_2 EC sensor.

2.5. *Statistical analysis*

Principal component analysis (PCA) and related graphical representations were performed with XLStat (Addinsoft, 40, rue Damrémont, 75018 PARIS). For PCA, all variables were auto-scaled (mean-centering, followed by scaling to unit standard deviation calculated using the n-1 denominator) beforehand and the results are reported in Supporting Information File 2 (SI 2,5,8, and 11). One-way, two-way, three-way, and four-way ANOVA models with Tukey's HSD *post-hoc* tests were performed on R software version 4.2.1 (R Core Team, 2018). The results are reported in SI File 1 (Supplementary Tables 3–8). Agglomerative hierarchical clustering results and the heat map were plotted (clustered PAC features against clustered log10-transformed ANOVA F-ratios) using "pheatmap" R package (version 1.0.12) to graphically and synthetically display the results of the multi-way ANOVA tests; to ease the visualization, the F-ratios (representing the significance of the effect of each single-factor, of each two-factors interaction, of each three-factors interaction, and four-factors interaction) were extracted then log10-transformed.

For example, in a four-way ANOVA model with factors = A, B, C, and D, each chemical feature (a PAC species) was tested by ANOVA for the significance of four different linear terms (A, B, C, and D), six two-factors interactions (A*B, A*C, A*D, B*C, B*D, C*D), four three-factors interactions (A*B*C, A*B*D, A*C*D, B*C*D), and one four-factors interaction (A*B*C*D). These F-ratio terms were used as variables in the AHC, as follows.

The table including the log10-transformed F-ratios was submitted to agglomerative hierarchical clustering (AHC), using the PAC chemical features as the observations and the log10-transformed F-ratios as the variables. AHC provided a re-shuffling of the rows and columns of the data matrix, according to the respective groups' averages. The observations (PAC) were eventually grouped according to how similar their F-ratios were. Simultaneously, a colour scheme (heat map) was created for easing the visualization. The clustering distances were calculated using the Euclidean distance, and Ward.D2 linkage was used as the linkage method (Kolde, 2019). The results were presented in Fig. 7.

Trend plots in Figs. 4, 6, 8 and 10 and in SI File 1 Fig. 2 were generated to graphically report Tukey's HSD *post-hoc* test results using "ggplot2" R package (Wickham, H., 2016). The significant differences were accepted with a *p*-val. < 0.05, and are represented by different letters. For this purpose, the "agricolae" R package was used (de Mendiburu, F., 2010).

3. Results

3.1. *Musts from Schiava grapes*

3.1.1. *Effect of grape freezing and overnight clarification*

The PAC profiles in musts have been analysed by PCA and are reported in Fig. 3. The observed PAC profiles are presented in SI File 1 Supplementary Table 2. The factors investigated were: 1) application of grape freezing (samples F vs NF) before crushing, and 2) the musts that have been afterwards used for vinifications with fermentative maceration (samples R) or without fermentative maceration (samples W); the samples W also underwent an overnight clarification step, between the destemming/pressing and the inoculum steps, as described in the Material and Methods section. In the PCA Score plot (Fig. 3A) there was a clear separation between the musts obtained from non-frozen and frozen grapes, along PC1 and PC2 diagonally from the top-left corner to the down-right corner. PC1 and PC2 together accounted for 87.37% of explained variance. No apparent effect appeared for the overnight clarification of the musts used for the preparation of white wines. Indeed, no separation between W and R samples was observed, even considering further principal components.

The loadings of PC1 and PC2 represented as a bar plot are shown in Fig. 3B. The contribution of the variables (procyanidins and prodelphinidins) to each principal component can be easily observed from the bar graphs. In addition to them, the chemical indexes (percentage ratios (%)) of the cyclic tetrameric procyanidin (%C-4), cyclic tetrameric prodelphinidin having one (epi)gallocatechin and three (epi)catechins (%C-4-OH), cyclic pentameric procyanidin (%C-5) and cyclic hexameric procyanidins (%C-6)) were calculated. These concentration-independent internally normalized parameters have been previously proposed for authenticity assessments of wine from different grape varieties (Longo et al., 2018a, 2019), and have also been tested as varietal markers in a study including grapes from different geographical origins

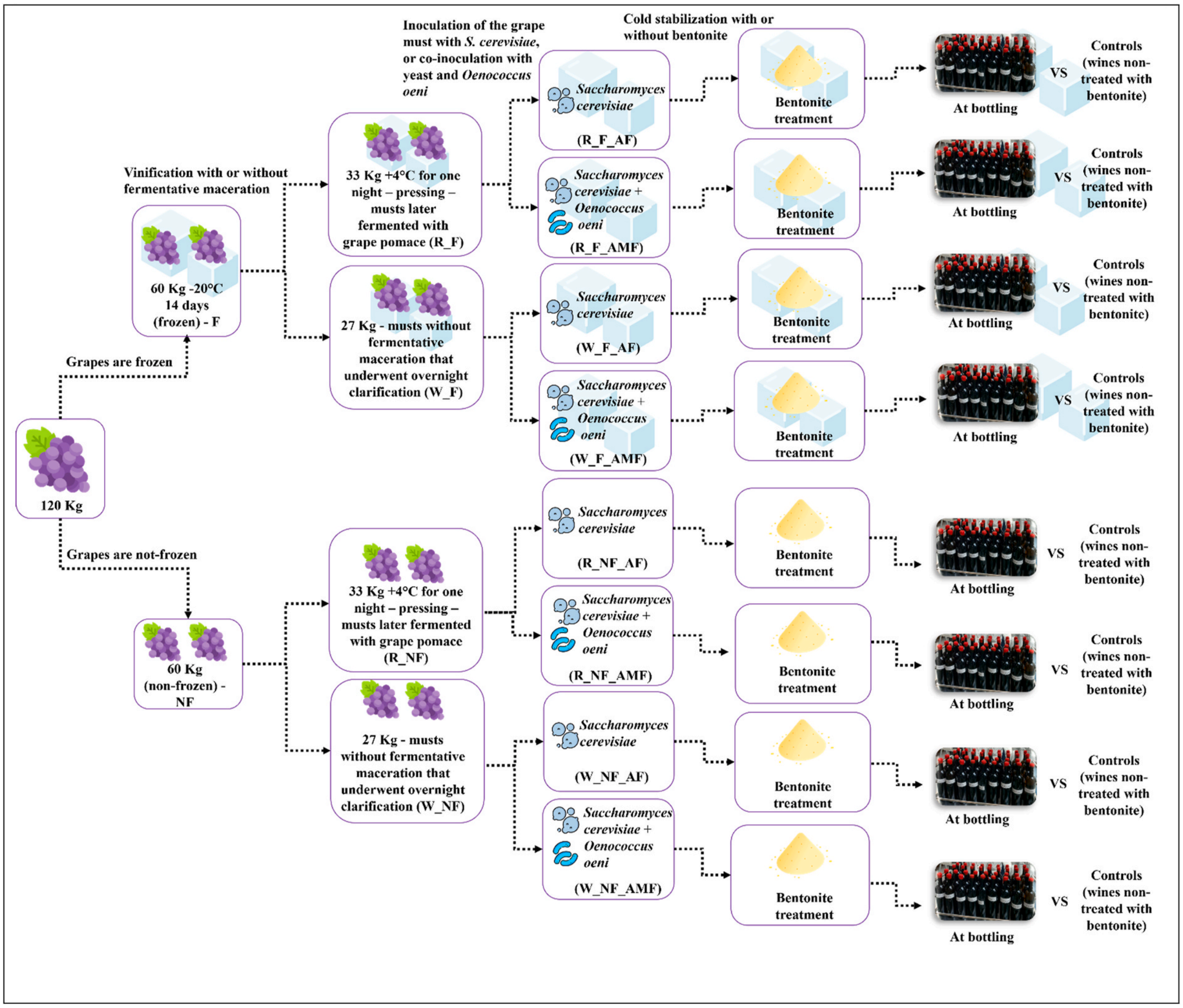

Fig. 2. Scheme of the sampling procedure. R = musts later fermented with grape pomace, W = musts without fermentative maceration that underwent overnight clarification; F, NF = frozen and non-frozen grapes, respectively; AF = alcoholic fermentation, AMF = alcoholic + malolactic fermentation.

(Poggesi et al., 2022). These (%) ratios were projected as supplementary variables in our PCA, because they are derived parameters.

Almost all of the observed PAC with degrees of polymerization ranging from dimeric to hexameric, showed a high contribution along PC1, hence mostly positively correlating with the separation between NF and F samples. Thus, leading to a higher concentration ranging from dimeric to hexameric PAC in the F samples than NF samples. Interestingly, in the case of c-PAC, most of the cyclic procyanidins, namely cyclic tetramer (c-tet), cyclic pentamer (c-pent), and one cyclic hexamer (c-hex (2)) showed very low contributions in the separation of F and NF samples along PC1, whereas they showed a high contribution along PC2. Additionally, c-tet, c-pent, and c-hex (2) were positively correlated with each other. Differently, the c-prodelphinidin (c–OH–tet) showed a similar trend to that of the rest of the non-cyclic PAC. The percent ratios corresponded to three different behaviours: 1) %C-4, %C-5, and %C-6(2) showed a strong negative correlation with PC1 having higher values in NF samples; 2) %C-4-OH representing the ratio for the only identified cyclic prodelphinidin, showed an almost completely anti-correlated trend with the former ones, thus having a trend more similar to the major non-cyclic PAC in musts; 3) %C-6(1) displaying an almost uncorrelated trend with respect to the separation between NF and F samples and the other ratios. Since %C-4, %C-5, and %C-6(2) represents the percentage of tetramers, pentamers, and one of the hexamers of cyclic procyanidins over the sum of cyclic and non–cyclic procyanidins respectively, they were higher in NF. Indeed, since the % ratios represent an inverse proportion, a higher concentration of non-cyclic procyanidins in F samples was found. A 2-way ANOVA (single factor and two-factors interactions) test was performed on the must samples to study the effect of grape freezing and overnight clarification and that of their interactions on the PAC. The Least Squares (LS) means and the groups obtained (indicated by alphabetical letters) by 2-way ANOVA and Tukey's HSD *post-hoc* (α = 0.05) for all PAC and their indexes (% ratios), are reported in SI File 1 Supplementary Table 3. There was no significant effect on the non-cyclic PAC in must samples that underwent overnight clarification. However, a significant effect of grape freezing application could be observed in non-cyclic procyanidins (dimers, trimers, and tetramers) and linear prodelphinidins (dimers-OH, trimers-OH, and tetramers-OH). They were significantly higher in the must samples from frozen grapes (F) in both red and white wines. Non-cyclic pentamers and some non-cyclic hexamers (in samples R_F and R_NF) were the only procyanidins unaffected by the application of grape freezing among the must samples.

The result for the observed c-PAC and their % ratios are represented as a trend plot shown in Fig. 4. No significant differences in the cyclic procyanidins can be observed in must due to the grape freezing and the samples R versus samples W types of processing of the grapes. The cyclic tetrameric prodelphinidin (c–OH–tet) was instead significantly lower in both R_NF and W_NF samples. In the case of % ratios, there was no significant difference due to overnight clarification (W_NF vs R_NF, and W_F_ vs R_F) among all of the indexes. %C-4 and %C-6(2) were

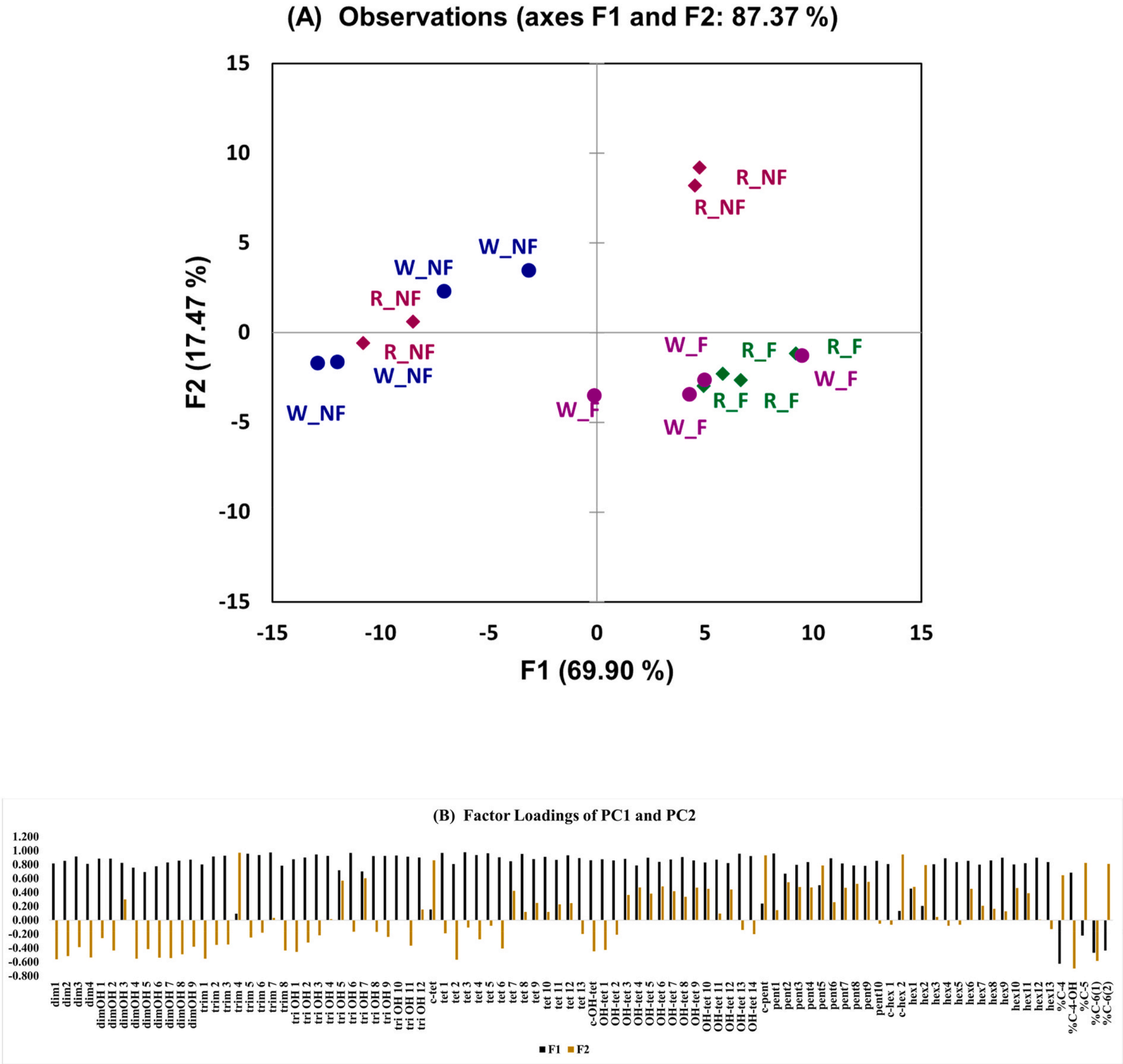

Fig. 3. Principal Component Analysis (PCA) of the Schiava musts, built on the observed PAC. A) Scores plot, B) Loadings plot represented as a bar chart. R = musts later fermented with grape pomace, W = musts without fermentative maceration that underwent overnight clarification; F, NF = frozen and non-frozen grapes, respectively.

significantly higher in both R_NF and W_NF samples than in R_F and W_F, respectively, while on the contrary %C-4-OH was lower in the R_F and W_F samples than in R_F and W_F, respectively.

3.2. Wines at bottling

3.2.1. Effect of grape freezing, type of co-inoculum, and fermentative maceration

The effects of grape freezing (F vs NF), fermentative maceration (R vs W), and co-inoculum (AF vs AMF) were analysed in the wines at bottling using PCA. The control samples without bentonite treatment were not considered for this analysis, and will be discussed in the next paragraph. The score plots of the first four components in Fig. 5(A, B&C) accounted for 95.3% of the total variance. Looking at the first two components (total variance 86.9%), a clear separation between the R (wines made with fermentative maceration) and W (wines obtained without fermentative maceration) samples can be observed along PC1, which explains the greatest variation in the data. The separation takes place diagonally (top-left to down-right). The replicates are well-grouped in clusters with some exceptions (outliers). In the case of PC2 vs PC3, 15.8% of the total variance was explained. The separation between F and NF samples was evident along PC3. The samples were neatly grouped according to their types, according to W_R and NF_F factors. Furthermore, a certain but slight separation between AF and AMF samples appeared only for red (R) samples along PC4, although not so clear as the effect shown by the other factors. The variability accounted for by PC4 was extremely low (2.6%), suggesting it could be a minor effect.

The loadings plots of PC1, PC2, PC3, and PC4 are shown in Fig. 5 (D&E). Most of the observed PAC, both procyanidins and prodelphinidins, showed a high contribution in separating the R and W wines along the PC1 (Fig. 5D). Again, the percentage ratios were projected as supplementary variables for the PCA model built for the wine samples. From the bar chart, it can be observed that %C-4 and %C-6(1) were strongly negatively correlated with the separation along PC1, whereas a positive correlation along PC2 was attributed to %C-4-OH and %C-6(1). Furthermore, %C-4, %C-5, and %C-6(2) were positively correlated with each other. Again, the % for the only identified cyclic prodelphinidin (%C-4-OH) displayed a negative correlation between the former mentioned % ratios. Another peculiar trend that can be observed is the contribution

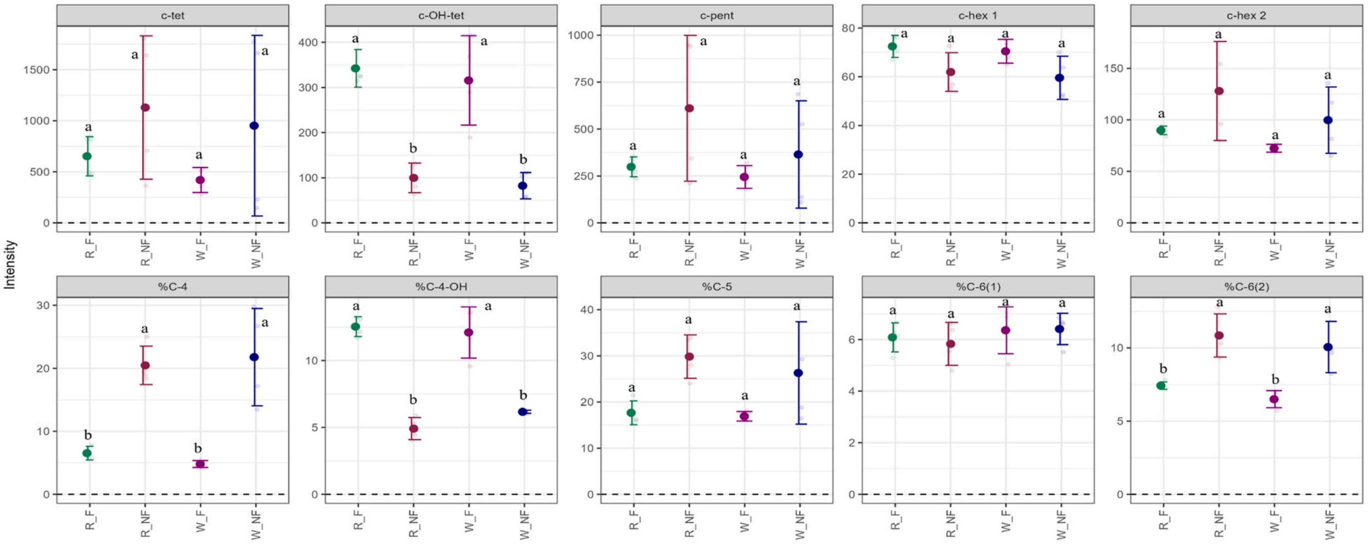

Fig. 4. Trend plot for c-PAC and their percentage ratio in must samples according to two-way ANOVA, with the Tukey HSD *post-hoc* results. The points indicate the average between the four replicates (data shown as transparent points) for must samples also reporting the standard deviations represented by error bars. Letters a–b indicate the groupings found with Tukey's *post-hoc* analysis. R = musts later fermented with grape pomace, W = musts without fermentative maceration that underwent overnight clarification; F, NF = frozen and non-frozen grapes, respectively.

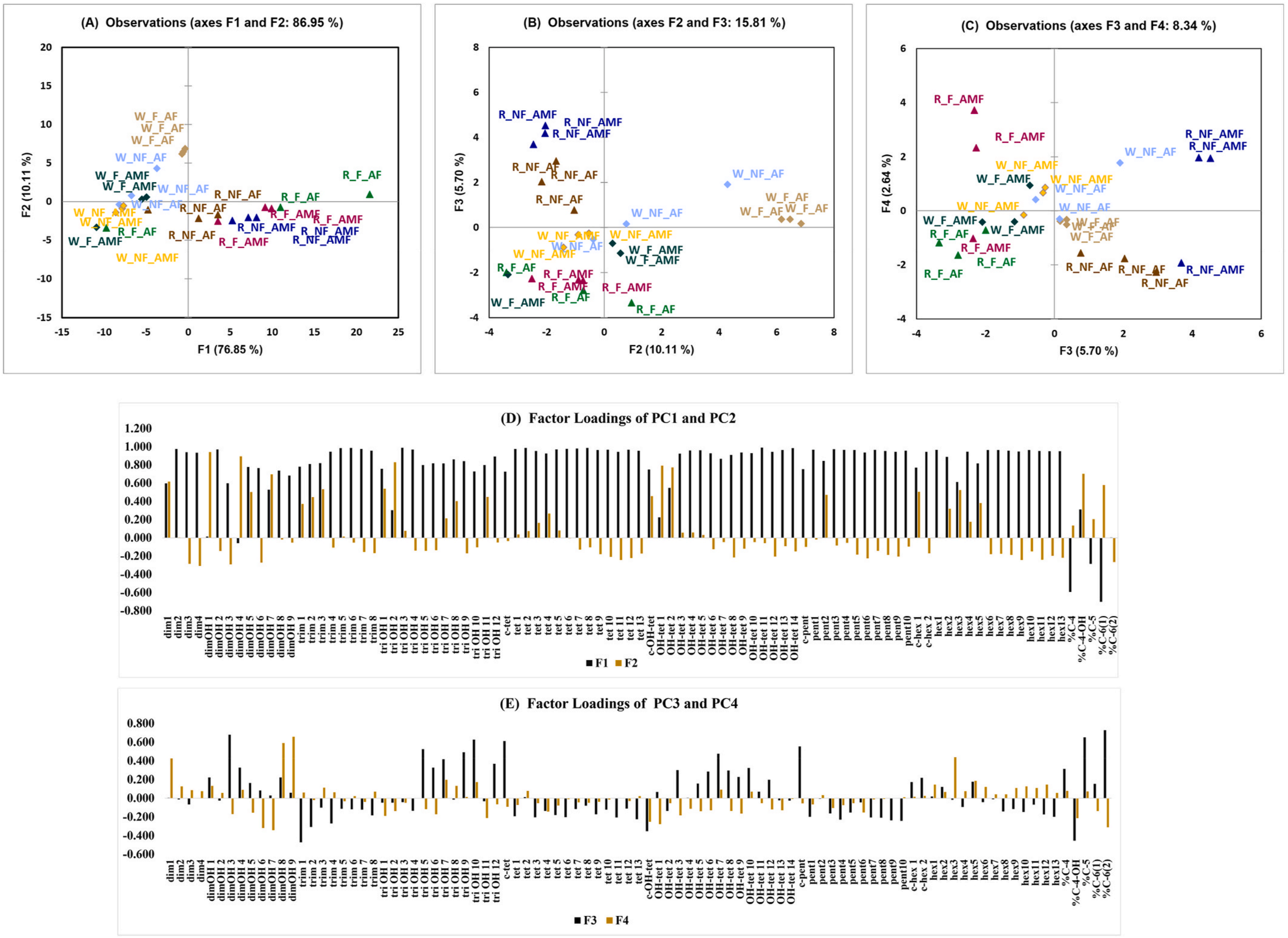

Fig. 5. Principal Component Analysis (PCA): Scores plot (A) PC1 vs PC2 (B) PC2 vs PC3 and (C) PC3 vs PC4 of the Schiava wines at bottling built on the observed PAC. (D) PC1&PC2 Loadings plot, and (E) PC3&PC4 Loadings plot. R = musts later fermented with grape pomace, W = musts without fermentative maceration that underwent overnight clarification; F, NF = frozen and non-frozen grapes, respectively, AF = alcoholic fermentation, AMF = alcoholic + malolactic fermentation.

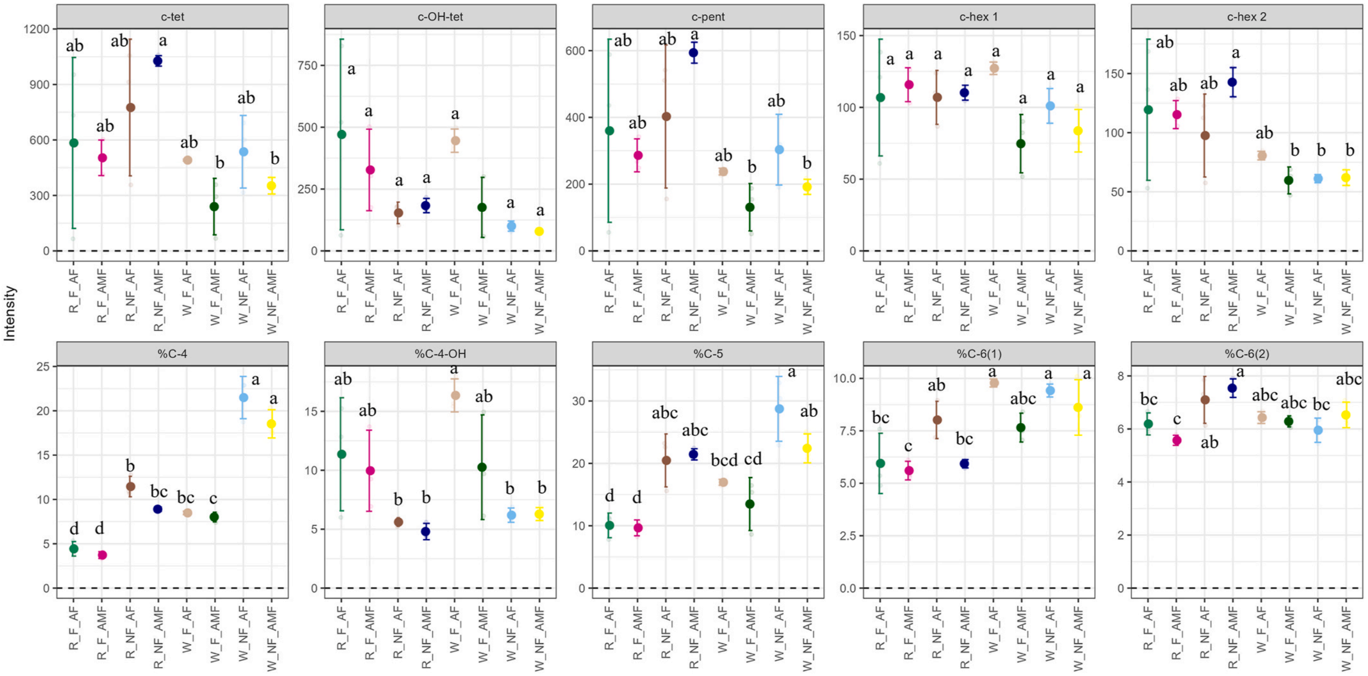

Fig. 6. Trend plot for c-PAC and their percentage ratio in wine samples according to three-way ANOVA, with the Tukey HSD *post-hoc* results. The points indicate the average between the three replicates (data shown as transparent points) for wine samples also reporting the standard deviations represented by error bars. Letters a–d indicate the groupings found with Tukey's *post-hoc* analysis. R = musts later fermented with grape pomace, W = musts without fermentative maceration that underwent overnight clarification; F, NF = frozen and non-frozen grapes, respectively; AF = alcoholic fermentation, AMF = alcoholic + malolactic fermentation.

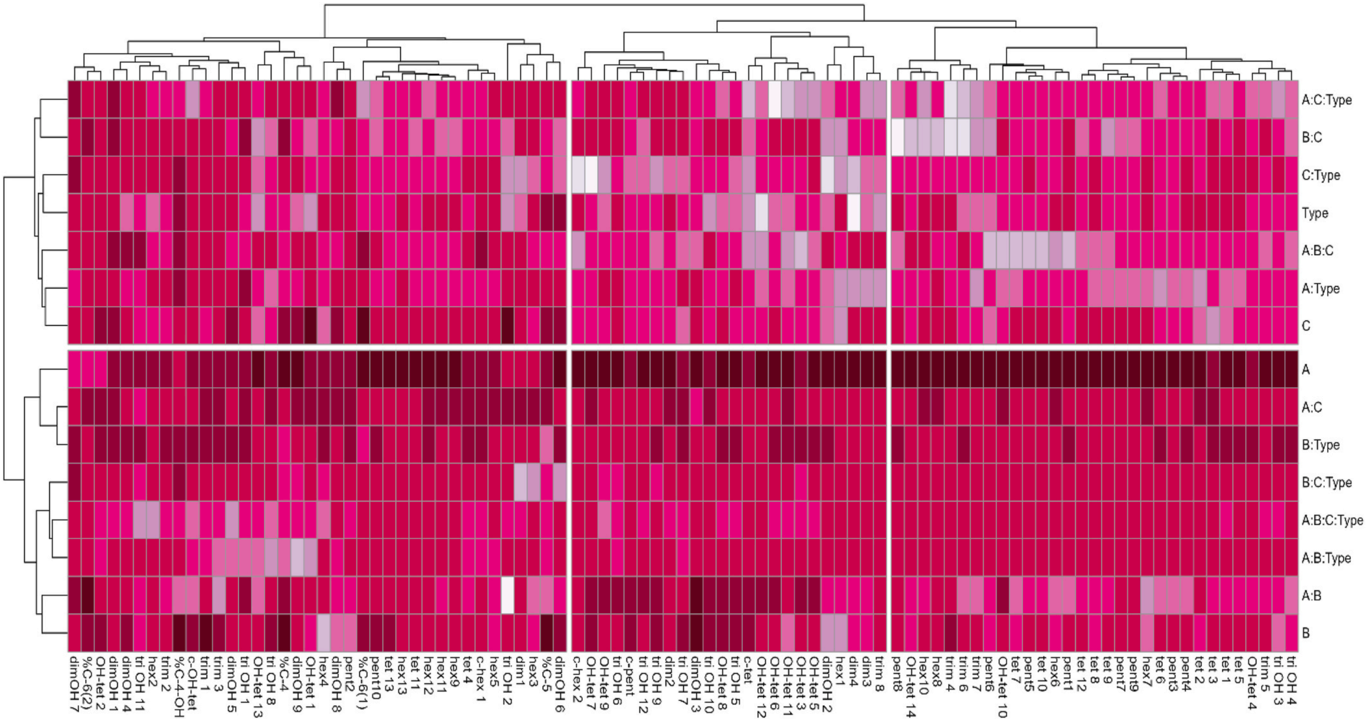

Fig. 7. Heat map analysis of all the observed PAC after ANOVA test. Each column represents the proanthocyanidins and each row represents the interaction between the factors. A: musts later fermented with grape pomace (R), musts without fermentative maceration that underwent overnight clarification (W); B: frozen (F) and non-frozen (NF) grapes, respectively; C: alcoholic fermentation (AF) and alcoholic + malolactic fermentation (AMF); Type: samples (with and without bentonite treatment). The rows and columns are clustered based on Euclidean distance using ward.D2 as the linkage method. The log10-transformed F-values are displayed to test the overall significance (F-value = variation between sample means/variation within the samples). The darker color indicates larger values that represent greater scattering from the mean, hence greater statistical significance. The observations (proanthocyanidins) are grouped according to their similarities. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

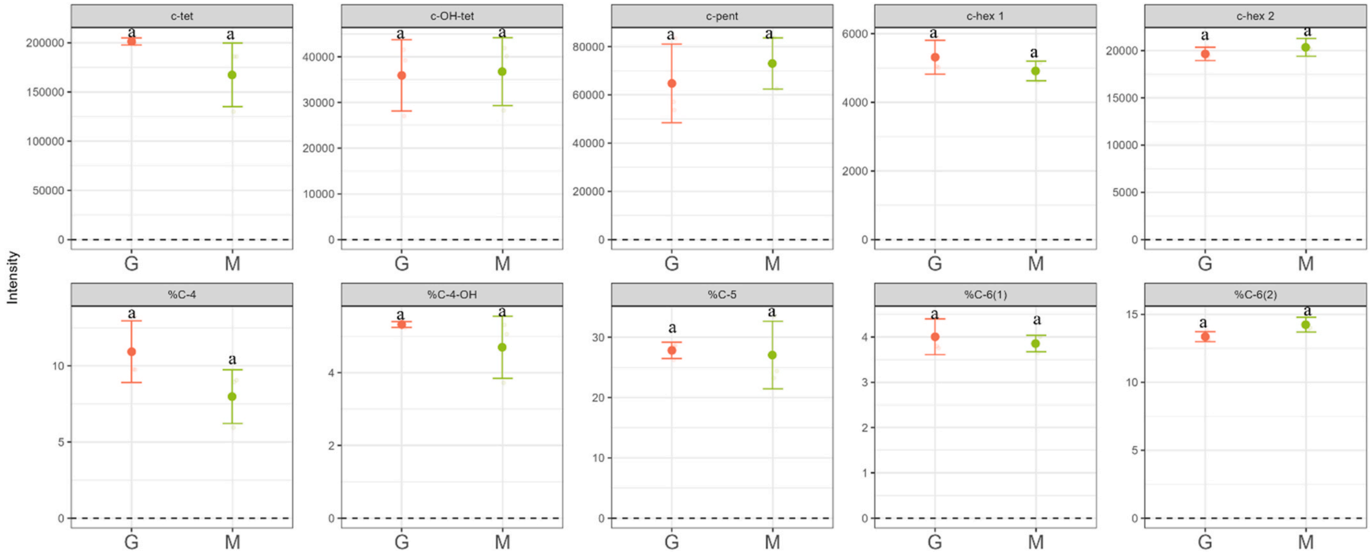

Fig. 8. Trend plot for c-PAC and their percentage ratio in commercial Schiava samples (T0) according to one-way ANOVA, with the Tukey HSD *post-hoc* results. The points indicate the average between the three replicates (data shown as transparent points) for commercial Schiava samples (T0) also reporting the standard deviations represented by error bars. Letter a indicates the grouping found with Tukey's *post-hoc* analysis. G, M = different wineries.

of most of the non-cyclic prodelphinidins along PC3. They are positively correlated with PC3, showing an influence in the separation between F and NF samples. The cyclic tetramer and cyclic pentamer were also positively correlated with PC3, while the cyclic prodelphinidin was negatively correlated with the separation of the F and NF sample. It is evident from the bar plot for PC4, that the influence of the variables was very weak. However, the slight separation of the AF and AMF samples was due mostly to some dimeric prodelphinidins.A 3-way ANOVA (single factor, two-factors interactions, three-factors interactions) test was performed to study the effect of grape freezing (F vs NF), fermentative maceration (R vs W), and co-inoculum (AF vs AMF) and their interactions on the PAC profile. The LS mean and the groups obtained (indicated by alphabetical letters) using 3-way ANOVA and Tukey's HSD *post-hoc* ($\alpha = 0.05$) for all PAC and their indexes (% ratios) are reported in SI File 1 Supplementary Table 4. The dimers were significantly higher in the red wines that underwent both alcoholic and malolactic fermentation and from frozen grapes (R_F_AMF) than their analogue white wines (W_F_AMF). The dimeric prodelphinidin on the other hand was significantly affected by all three factors. Different trends were observed where interestingly these prodelphinidins were higher in white wines produced from frozen grapes that underwent alcoholic fermentation (W_F_AF) than the ones that underwent both alcoholic and malolactic fermentation from non-frozen grapes (W_NF_AMF) and higher in the NF red wines that underwent both alcoholic and malolactic fermentation (R_NF_AMF) than the F red wines undergoing only alcoholic fermentation (R_F_AF). For trimers, no significant differences were observed. Trimeric prodelphinidins showed similar trends to dimeric prodelphinidins. In the case of tetramers, only a few were significantly different. They were higher in the red wines that underwent both alcoholic and malolactic fermentation made from frozen grapes (R_F_AMF). Again, for the tetrameric prodelphinidins, in both F and NF white wines that underwent only alcoholic fermentation, they were higher. Also, they were significantly higher in the F red wines that underwent both alcoholic and malolactic fermentation. Regarding pentamers and hexamers, only a few were significantly higher in the F red wines that underwent both alcoholic and malolactic fermentation. Other than that, there were no significant effects of these applied factors on the pentamers and the hexamers.

The results for the c-PAC and their indexes (% ratios) were presented as a trend plot in Fig. 6. No sole influence of the grape freezing (F vs NF) and the type of inoculum (AF vs AMF) can be observed in the cyclic proanthocyanidins in the bottled wines. However, cyclic tetramer, cyclic pentamer, and cyclic hexamer (2) were significantly higher in NF red wines that were fermented using a co-inoculum with *Oenococcus oeni* (R_NF_AMF) than in the NF white wines with co-inoculum (W_NF_AMF). This was a similar finding as reported by Longo et al. (2018a), regardless of the grape freezing and the type of fermentation implied. In the case of the % ratios, %C-4 and %C-5 were significantly higher in the NF wines with the same vinification and the type of inoculum. The percentage ratio for the second isomeric form of the cyclic hexameric procyanidin (%C-6(2)) was only significantly higher in NF red wine than in the F red wine that underwent both alcoholic and malolactic fermentation. The ratio %C-4 was significantly lower in red wines than in white wines in all F_AF, NF_AF, F_AMF, and NF_AMF samples. The ratio %C-6(1) was significantly higher in white F_AF and NF_AMF samples than in the same red wine samples. No significant changes in the % ratios were observed due to the type of inoculum used.

3.2.2. *Effect of bentonite treatment*

The heat map in Fig. 7 represents the results of the 4-way ANOVA in a graphical and synthetic way in order to understand whether the treatment with bentonite had a significant effect on the PAC profile. The heat map was constructed on the log10-transformed F-ratios dataset, which was chosen in consideration of the great differences in their F-ratios values. ANOVA log10-transformed F-ratios were computed and extracted for one-factor, two-factors, three-factors, and four-factors - interaction terms. If the effect of bentonite treatment had not been included, the outcome would have been exactly the one shown in the previous paragraph. Therefore, presenting the complete ANOVA treatment would have been redundant to half of its extent. Hence, Least Squares (LS) means with Tukey's groupings was opted for further discussing these results, and introducing this new synthetic representation (Fig. 7), which allows overviewing comprehensively all significances for factors and interactions at the same time, while also grouping them according to the similarity in their significance for factors and interactions.

F-values represent the ratio between the variance between sample groups' means and the variances within the sample groups' replicates. The log10-transformed F-ratios were then submitted to hierarchical cluster analysis and the similarity between the observations (PAC) was calculated by Euclidean distance and Ward.D2 linkage criterion on the log10-transformed data. The results were presented as a heat map to simply represent the factors-dependencies of all grouped PAC. The heat map provides by color coding an intuitive overview of PAC clusters; clusters are characterized by increasingly similar effects from the applied factors and their interactions taken altogether. Darker colors indicate larger log10 transformed F-ratio values, representing greater dispersions from the overall dispersion from the mean, hence greater statistical significance. Two main clusters along the rows and three main clusters along the columns were identified. The observations (PAC) were grouped according to how similar they depend on the specific factors and interactions. The statistical significance of percentage ratios for the cyclic tetrameric procyanidin (%C-4) was higher in the A (R and W wines), B (F and NF wines), and B*C interactions, implying that %C-4 was statistically different for red wines and white wines in the F and NF samples. Since %C-4 represents the percentage of tetramers of cyclic procyanidins (c-tet) over the sum of cyclic and non–cyclic tetramers procyanidins (tet), the distances between all tetrameric procyanidins were observed. Most of the tetrameric procyanidins were clustered together, and only a few were far from this group. In contrast, the latter ones were close to %C-4. Moreover, the %C-4 and all tetrameric procyanidins were significantly higher in red and white wines. It is worth mentioning that the long distance between %C-4 and all tetrameric procyanidins confirms that the ratios are inversely proportional to the abundance of the latter ones. The statistical significance of %C-4-OH was higher in the B (F and NF wines). The non-cyclic tetrameric prodelphinidins (OH-tet) were found in the central hierarchical clustering and the statistical significance was higher in A and A*B interactions (R and W*F and NF). On the contrary, the log10-transformed F-ratio of the cyclic pentameric procyanidin (c–OH–tet) was similar to that of %C-4-OH, indeed the distance between them was comparatively low, thus suggesting that c–OH–tet and %C-4-OH had a higher statistical significance in the B (F and NF wines) factor. Additionally, the statistical significance of %C-5 was higher in the B (F and NF wines) and the Type (with and without bentonite treatment) factors. The distance between the %C-5 and non-cyclic pentameric procyanidins (pent) was substantial, thus meaning that the dissimilarities between the percentage ratio and the non-cyclic pentameric procyanidins were statistically relevant.

The statistical significance was also higher in A, C, and A*B*C factors for %C-6(1) and in A*B factors for %C-6(2). Therefore, several factors affected the cyclic hexameric procyanidins (c-hex 1 and c-hex 2), thus influencing the procyanidins fingerprint. The non–cyclic hexameric procyanidins (hex) were hardly clustered by the hierarchical clustering. This 4-way ANOVA test was performed to see the effect of bentonite treatment along with the previously tested factors and their interactions on the PAC profile. The Least Squares (LS) means and the groups obtained (indicated by alphabetical letters) by 4-way ANOVA and Tukey's HSD *post-hoc* ($\alpha = 0.05$) for all PAC and their indexes (% ratios) are reported in SI File 1 Supplementary Table 5. Only a few dimers present were affected by the treatment with bentonite as they were significantly higher in the F red wines that underwent both alcoholic and malolactic fermentations (R_F_AMF). After the bentonite treatment, significant increases in the dimeric prodelphidins were evident in only a few NF red wines with both alcoholic and malolactic fermentations (R_NF_AMF) compared to the white wines (W_NF_AMF). Furthermore, no significant changes in the trimers, trimeric prodelphinidins, tetramers, tetrameric prodelphinidins, pentamers, and hexamers were observed after the treatment with bentonite in the wine samples.

The results for the c-PAC and their indexes (% ratios) are presented as a trend plot in SI File 1 Supplementary Fig. 2 to see an additional effect of the bentonite treatment. No apparent influence of the treatment with bentonite can be seen in the c-PAC. A specific arrangement between the samples was observed in the %-C-4 and %-C-5 trend graphs, however, no statistical differences were found between the samples with and without bentonite treatment. Furthermore, the separation between all wine samples was determined by the type of vinification (R and W samples) and the application of freezing processes (F and NF samples). The type of inoculum within the same vinification hardly influenced the %C-4 and %C-5 ratios. The %C-4 was significantly lower in red wines than in white wines in all F_AF and F_AMF samples. On the contrary, %C-5 was significantly higher in NF_AF and NF_AMF whites. In the white F_AF sample, significant changes in the % C-4-OH were observed due to the artificial treatment with bentonite. No significant changes in the other % ratios can be observed after the treatment with bentonite.

3.3. Evolution of PAC in the bottle and effect of dissolved oxygen

The commercial wines from two different producers reported as G and M wineries, Italy, were analysed after six months (T0) and at eighteen months (T12) storage in bottle for the PAC profile, with additional analysis of dissolved oxygen (DO) content, free SO_2, and total SO_2 (free + combined) in these wines.

A PCA model was built on the observed PAC features to facilitate the identification of similarities or differences in the commercial Schiava wines from G and M at six months (T0). The score plot (PC1 vs PC2) in SI File 1 Supplementary Fig. 3A showed a total explained variance of 68.9%. There was a clear separation of the two producers along PC1.

The loadings plot (SI File 1 Supplementary Fig. 3B) showed that a high number of non-cyclic dimers, trimers, tetramers, pentamers, and a majority of dimeric, trimeric, and tetrameric prodelphinidins along with cyclic pentamer and cyclic hexamer 2 (c-hex 2) exhibit a strong negative correlation influencing the separation along PC1, thus leading to a high number of these observed PAC features that characterize the M samples. Only a few dimeric, trimeric, and tetrameric prodelphinidins, hexamers along with cyclic tetramer (c-tet) and cyclic hexamer 1 (c-hex 1) showed a positive correlation influencing this separation. %C-4 and %C-4-OH were strongly positively correlated with the separation along PC1. %C-5 and %C-6(1) were also positively correlated while %C-6(2) was the only % ratio that negatively correlated with the separation along PC1. Therefore, in the M samples, %C-6(2) was higher than in the G samples and it was the only % ratio that negatively correlated with %C-4, %C-4-OH, %C-5, %C-6(1). Indeed, these ratios were higher in the G samples.

A 1-way ANOVA (single factor) test was performed to see if there was a significant difference in the PAC profile between the two different producers: G and M. The Least Squares (LS) means and the groups obtained (indicated by alphabetical letters) and Tukey's HSD *post-hoc* ($\alpha = 0.05$) for all PAC and their indexes (% ratios) are reported in SI File 1 Supplementary Table 6. Only a small amount of non-cyclic procyanidins and prodelphinidins were significantly different in the two commercial Schiava wines. Hence, it can be stated that scarce differences in these PAC profiles were observed due to the winery. This is also a confirmation of the similarity of these two products even though they were from different producers.

The results from 1-way ANOVA for the c-PAC and their indexes (% ratios) are presented as a trend plot in Fig. 8. No significant influences between the two producers were observed likewise for the c-PAC and their % ratios. The dissolved oxygen was approximately 0.200 ± 0.005 ppm in wines from G and 0.331 ± 0.010 ppm in wines from M at six months (T0).

These wine samples were then analysed at eighteen months (T12) in the bottle. Half of these samples were subjected to mechanical stress (each bottle subjected to the mechanical stress was taken out of the container and mechanically turned three times upside down, once per week for 12 months) and the other half was not; herein, these bottles are referred to as Treatment (T – subjected to mechanical stress) and Control (C – not subjected to mechanical stress). Noteworthy, all other storage conditions were carefully checked in order to be identical for the two sets of bottles (same temperature and constant throughout the year – 21 °C, same low level of light exposure, same containers for the bottles, same relative humidity).

The PCA model was built on the observed PAC data, and with the % ratios as supplementary variables, including also the analysed dissolved oxygen (DO) and the pH projected as supplementary variables. Two samples (M_O_2 and G_O_2) were artificially added with an additional ~ 1

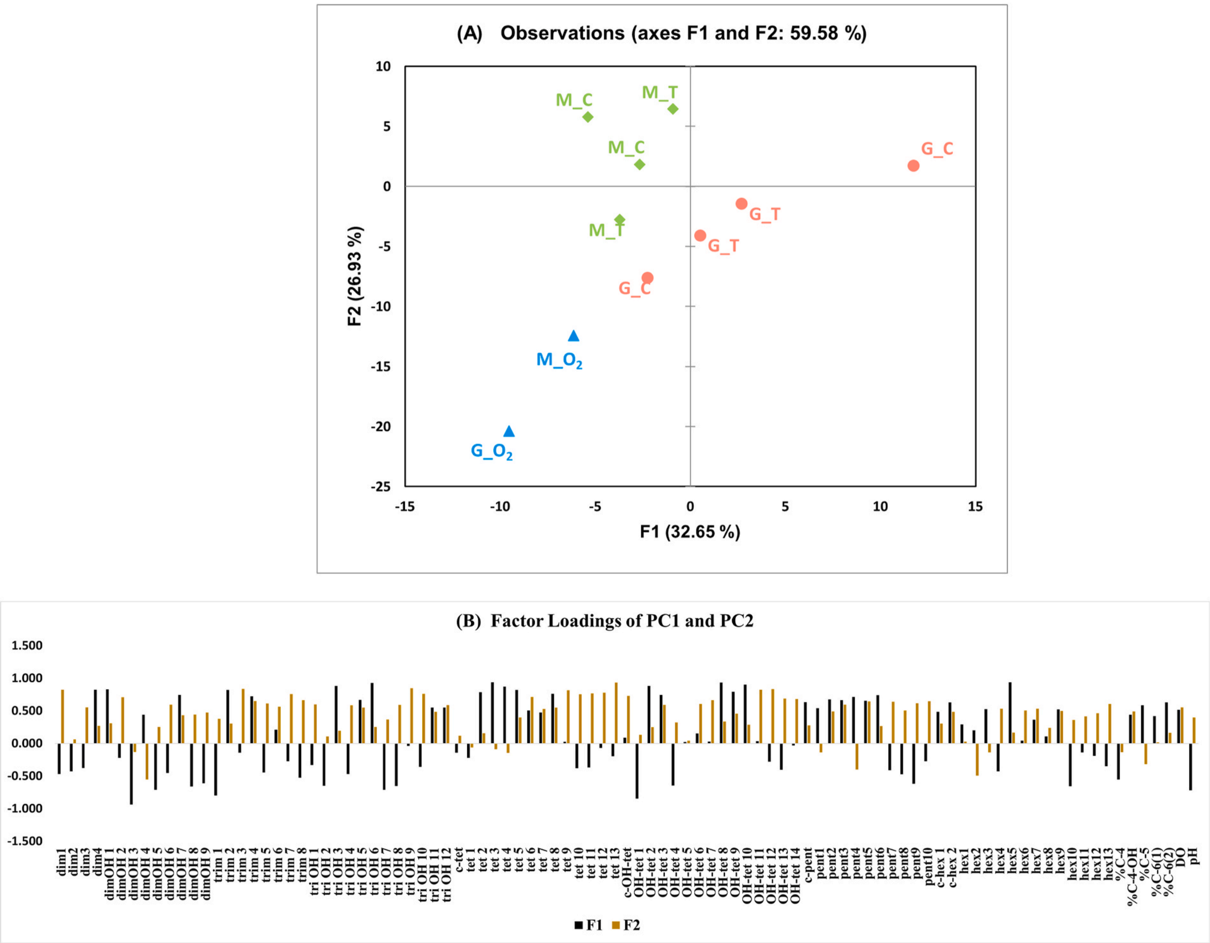

Fig. 9. Principal Component Analysis (PCA) of the commercial Schiava wines analysed at eighteen months (T12). The blue triangles represent the samples (M_O2 and G_O2) with artificially introduced DO projected as supplementary observation. A) Scores plot, B) Loadings plot. G, M = different wineries; C= Control, T = Treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

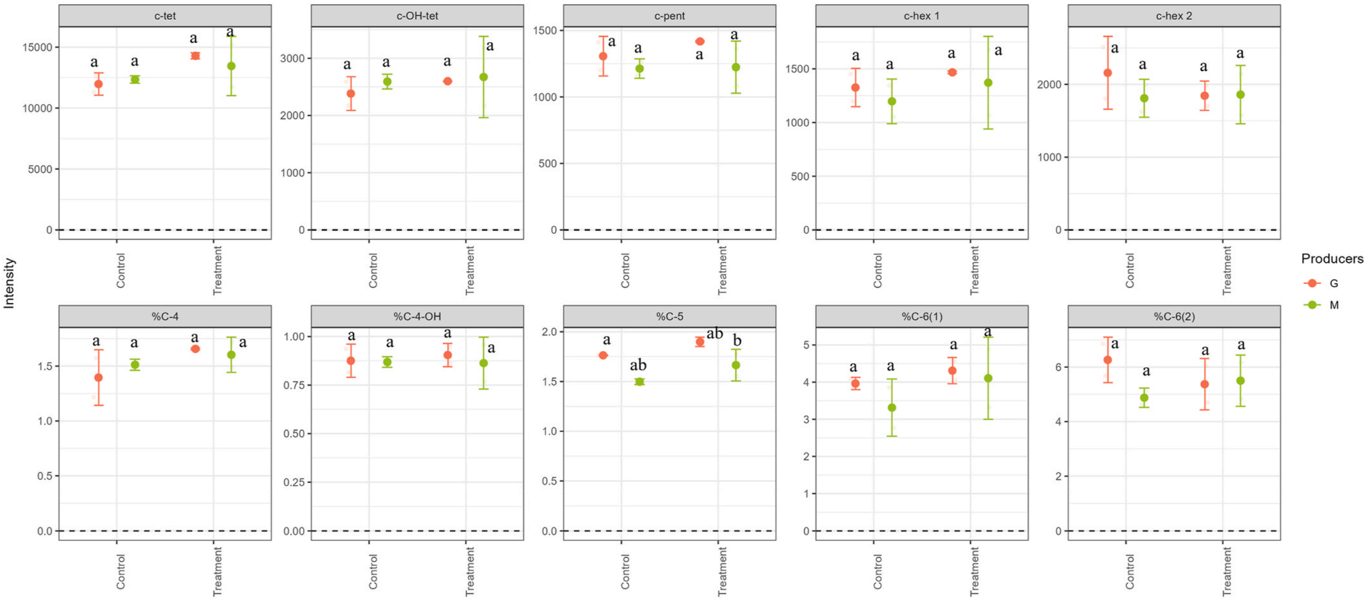

Fig. 10. Trend plot for c-PAC and their percentage ratio in the commercial Schiava samples (T12) according to two-way ANOVA, with the Tukey HSD *post-hoc* results. The points indicate the average between the two replicates (data shown as transparent points) for commercial Schiava samples (control and treatment) also reporting the standard deviations represented by error bars. Letters a–b indicate the groupings found with Tukey's *post-hoc* analysis. G, M = different wineries.

ppm of dissolved oxygen, to see if this would cause differences in the PAC profiles. Hence, their PAC profiles with their analysed DO and pH were analysed in the second stage. This dataset was projected only as supplementary observation in the following PCA (Fig. 9).

In Fig. 9A, the model accounted for 59.93% of the total variance. The two producers were neatly separated along PC1 and PC2; however, there was not a clear separation between the C (control) and the mechanically treated (T) samples. The samples with higher DO values can clearly be seen as outliers along the direction of PC2.

The loadings plot in Fig. 9B showed that almost all of the non-cyclic procyanidins (dimers to hexamers) and the prodelphinidins influence the separation of the samples from two different producers at T12. In the case of c-PAC, different trends can be observed. The cyclic tetrameric procyanidin showed a very low contribution to the separation along PC1 and PC2. On the other hand, the cyclic pentameric procyanidin (c-pent) and cyclic hexameric procyanidins (%C-6(1) and %C-6(2)) show a very high contribution in the separation along PC1 and PC2 and they are positively correlated. High contribution in the separation along PC2 was attributed to the cyclic tetrameric prodelphinidin (c–OH–tet). For the projected supplementary variable, %C-4 was the only parameter that was negatively correlated to the separation along both PC1 and PC2, whereas the other % ratios (%C-4-OH, %C-5, %C-6(1) and %C-6(2)) showed a strong positive correlation to the separation along PC1. Noteworthy, the %C-5 showed a negative correlation along the PC2. The %C-6(1) was found to be positively correlated with %C-6(2), whereas both were negatively correlated with %C-4. The pH showed a negative correlation to the separation along PC1 as well as a positive correlation with the separation of the samples along PC2, and was higher in the M wines. The DO was also positively correlated to the separation along both PC1 and PC2.

A 2-way ANOVA (single factor and two-factors interactions) test was performed to see the effect of mechanical stress on these wines from different producers and that of their interactions on the PAC profiles. The Least Squares (LS) means and the groups obtained (indicated by alphabetical letters) by 2-way ANOVA and Tukey's HSD *post-hoc* ($\alpha = 0.05$) for all PAC and their indexes (% ratios) are reported in SI File 1, Supplementary Table 7. No apparent significant differences in the linear PAC were observed between the control (C) and the treated (T) samples.

The results for the c-PAC and their indexes (% ratios) are presented as a trend plot in Fig. 10 to see the differences between control and treatment in two Schiava commercial wines. The c-PAC were also not affected by the mechanical stress applied. Only a significant influence in %C-5 between the two producers and the two-factor interactions (control and treatment) was found. %C-5 was significantly higher in the G_C than M_T wines.

A 2-way ANOVA test was also performed to evaluate the additional effect of the artificially introduced DO on the PAC profiles in the two Schiava wines. The LS mean and the groups obtained (indicated by alphabetical letters) by 2-way ANOVA and Tukey's HSD *post-hoc* ($\alpha = 0.05$) for all PAC and their indexes (% ratios) are reported in the SI File 1, Supplementary Table 8. There were no significant changes in the dimeric procyanidins and the prodelphinidins between the controls and the samples with added DO from both the producers. In the case of the trimers (procyanidins and prodelphinidins), some of them were significantly affected and were higher in the control samples. Tetrameric procyanidins and tetrameric prodelphinidins also showed the same trend, where some were higher in control samples and especially in G wines. There was no significant effect of dissolved oxygen addition for pentamers and hexamers. The c-PAC were also unaffected by dissolved oxygen. Instead, in the case of % ratios, %C-5 was significantly higher in G wines with DO addition, but not in M wines. %C-4-OH was significantly higher in both wines with DO addition. There was no effect of DO addition in the %C-4 and %C-6 ratios.

4. Discussion

This study sheds new light on the substantial differences in the behaviour of cyclic versus non-cyclic oligomeric PAC during the winemaking process. The first section (Section 1) of the results dealt with the must samples. As expected, almost all the non-cyclic PAC were extracted more from the frozen Schiava grapes compared to cyclic oligomeric PAC. Grape freezing is a well-known technique for causing a higher extraction of skin components in must (Unterkofler, J. et al., 2020). However, this trend was largely absent for cyclic procyanidins (c-PAC: c-tet, c-pent, and c-hex 2). It has already been reported by Longo et al., 2018b that these compounds exhibit very different physicochemical properties compared to their main non-cyclic analogues. Indeed, the higher polarity of c-PAC compared to linear PAC makes them easier to extract from grapes, so their proportions in musts are largely less affected by the grape freezing applied than their non-cyclic congeners. Greater differences were found in the related chemical indexes (% ratios), which is the ratio of the relative abundance for cyclic PAC to the sum of cyclic and non-cyclic PAC (taking into consideration analogues from tetramers to hexamers). The difference in these chemical indexes (higher % ratios in musts from non-frozen grape) could therefore be accounted to the higher abundance of non-cyclic PAC extracted in must samples from frozen grapes, rather than the lower c-PAC content.

The trend of the cyclic prodelphinidin c-tet-OH on the other hand appeared completely different from that of cyclic procyanidins, and more in line with the trends observed with the non-cyclic PAC. As a possible explanation, due to the presence of a higher level of hydroxylation on the B-ring than procyanidins, non-cyclic and cyclic prodelphinidins are generally more polar than procyanidins. Therefore, cyclic and non-cyclic prodelphinidins are less differentiated than procyanidins in the proportions extracted from frozen or unfrozen grapes. For instance, little information is available on the interaction of cyclic proanthocyanidins with other grape components, which could also explain the differences in trends observed between the different subclasses.

The relative abundance of proanthocyanidins (both linear and cyclic) in red wines was much higher (10-fold) than in white wines, as previously observed by Longo et al. (2018a). In the case of Schiava wines at bottling in section 2 of results, procyanidins were mostly affected by the vinification (W vs R samples) and the grape freezing (NF vs F samples) factors, compared to the type of inoculum applied (AF vs AMF samples). Procyanidins were significantly higher in the red wines from frozen grapes that underwent malolactic fermentation (R_F_AMF). Studies on the effect of grape freezing on proanthocyanidins are scarce; however, a related study (Unterkofler, J. et al., 2020) showed that freezing results in the release of more phenolic compounds, due to the improved extraction of pigments, proanthocyanidins, and aroma compounds, from the grape skins into the must/wine. This process has a strong effect on the volume of intracellular liquids and ruptures membranes, providing an accelerated release of phenolic compounds, breaking down skin and seed cells that contain tannins, thereby increasing their extractability (Moreno-Pérez et al., 2013). Prodelphinidins on the other hand showed different trends: 1) they were higher in white wines from frozen grapes that underwent alcoholic fermentation without co-inoculation; 2) they were also higher in red wines from non-frozen grapes subjected to co-inoculation. Since the non-cyclic and cyclic prodelphinidins are generally more polar than procyanidins, they were extracted in different proportions based on the aqueous environment.

The chemical indexes (% ratios) were significantly higher in R_NF samples in both musts and wines at bottling. These ratios depend on both the abundance of cyclic compounds and on the abundance of their non-cyclic congeners; indeed, the ratios are inversely proportional to the abundance of the latter, which is the reason for the higher values of these %-indexes in NF wines.

In winemaking, bentonite is widely used as a decanting aid to remove protein constituents. This reduces the risk of protein haze in wines which strongly influences the composition and final appearance of the wine (Catarino et al., 2008). Besides proteins, other positively charged molecules, such as anthocyanins, can also be removed (González-Neves et al., 2014). According to our results (section 2.2), neither non-cyclic nor cyclic PAC in wines at bottling showed to have been affected by the bentonite treatment. A study by Gonçalves and Jordão, 2009 also reported that for different bentonite concentrations no statistically significant difference was found between oligomeric flavanols in unaged and aged red wines. With regards to the % ratios in Schiava, significantly higher values of %-C-4 and %-C-5 were only observed in W_NF wines, although again, no effect of bentonite treatment was found.

Two bottles from two different producers (G and M) were also analysed for the PAC profile (section 3). No significant differences were observed, showing similarities in these wines even though they were from different producers, thus fueling the hypothesis of using cyclic PAC as varietal markers of wines (Longo et al., 2018a, 2019).

It is known that the dissolution of oxygen in wine occurs when the wine comes into contact with air. Oxygen introduced into wines is consumed by various chemical reactions that lead to important changes in chemical composition. Two of the Schiava wine samples (one from each producer) were artificially added with oxygen, resulting in a concentration approximately five times higher than the control wines. The two wine samples (M_O_2 and G_O_2) added with dissolved oxygen (DO) resulted in a detected loss of sulfur dioxide (both free and total), which is evident as seen in Table 1. A rapid decrease in sulfur dioxide after the introduction of dissolved oxygen at bottling was also reported by Dimkou et al., 2013).

In addition, ANOVA was performed to test whether there were significant changes in the PAC profile due to the additional introduction of DO. Only non-cyclic trimeric and tetrameric procyanidins and prodelphinidins were affected by the higher DO content and were lower in the wines due to the oxidation process. This means that the cyclic procyanidins showed a more stable oxidation-resistance tendency than the non-cyclic procyanidins. Our results were supported by a recent study (Jouin et al. (2022)) in which the stability of the tetramer of cyclic procyanidins compared to the tetramer of non-cyclic procyanidins was reported. However, our study also considered the changes in the abundance of different oligomeric non-cyclic, and cyclic PAC after the DO addition in two wines. No changes were found in the content of cyclic procyanidins in wine fortified with DO, suggesting that they were more resistant to oxidation. Furthermore, cyclic prodelphinidin was stable after the addition of DO. A possible hypothesis could be that, since the number of cyclic procyanidins and prodelphinidins was comparatively lower than that of non-cyclic congeners, the oxidation process affected the more concentrated non-cyclic PAC in the wines. Furthermore, the different proportions of epicatechin and epigallocatechin in the chemical structure of PAC could confer greater resistance to oxidation.

The different trends of both oligomeric non-cyclic and cyclic PAC have been summarised in Table 2. This type of studies could provide the winemakers a flexible approach to investigate winemaking variables, ensuring reliability of results and allowing to investigate many different conditions in a systematic way; for example, this approach could even be used to produce different winemaking lines from the grapes, for blending, helping the winemaker find the wine style that best fits the quality sought.

Furthermore, our study also highlights a potential approach for the development of new strategies in winemaking. The contribution that studies such as this one could provide to the winemakers would be in the flexibility of the approach, the reliability of the results, and finally in providing useful variability in different types of wines from the same grape: this approach can be used to produce experimental wine blends under controlled conditions and with a flexible experimental plan, helping the winemaker's task of finding the desired wine style, that best fits the sought quality.

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Aakriti Darnal: Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Simone Poggesi:** Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – review & editing, All authors read the final version of the manuscript and approved for final submission. **Adriana Teresa Ceci:** Methodology, Software, Validation, Formal analysis, Investigation, Writing – review & editing. **Tanja Mimmo:** Resources, Writing – review & editing, Funding acquisition, All authors read the final version of the manuscript and approved for final submission. **Emanuele Boselli:** Resources, Writing –

Table 1
Dissolved oxygen (DO), pH, Free and Total SO_2 measured for the two different commercial Schiava wines (two replicates A&B) at eighteen months (T12). G, M = different wineries, C= Control, T = Treatment.

Sample	DO (±0.002 ppm)	pH (±0.1)	Free SO_2 (±1 mg/L)	Total SO_2 (±1 mg/L)
G_C_A	0.062	3.6	20	89
G_C_B	0.116	3.6	15	92
G_T_A	0.085	3.6	13	83
G_T_B	0.086	3.6	14	88
M_C_A	0.089	3.7	17	68
M_C_B	0.100	3.8	14	60
M_T_A	0.090	3.8	15	68
M_T_B	0.082	3.8	16	59
G_O_2	1.560	3.6	6	29
M_O_2	1.820	3.8	5	14

*Rows highlighted in grey are samples with added dissolved oxygen (introduced artificially).

Table 2
Differences in the oligomeric PAC, cyclic PAC profiles and in the chemical indexes (% ratios) of Schiava wine samples (grape musts, wines at bottling, and commercial wines) based on the factors applied. R, W, red or white winemaking, respectively. F, NF, frozen and non-frozen grapes, respectively. ↑ and ↓ indicate samples with high or low values of the parameters reported in the column, respectively. C, Control; T, Treatment; G and M, Schiava bottled wines from two different wineries.

Factor/Factors	Schiava samples	Non-cyclic PAC	Cyclic PAC	Chemical Indexes (% ratios)
Grape freezing/Vinification	Grape musts	Dimeric, trimeric, tetrameric procyanidins and prodelphinidins: ↑R_F and ↑W_F	No significant changes in cyclic procyanidins. c–OH–tet: ↓ R_NF and ↓W_NF	%C-4 and %C-6(2): ↑R_F and ↑W_F %C-4-OH ↓ R_NF and ↓W_NF
Overnight clarification	Grape musts	No significant changes		
Grape Freezing/ Fermentative maceration/ co-inoculum	Wines at bottling	Dimeric procyanidin: ↑R_F_AMF dimeric prodelphinidin: ↑W_F_AF; ↑R_NF_AMF tetrameric procyanidin: ↑ R_F_AMF	c-tet, c-pent, and c-hex 2: ↑R_NF_AMF	%C-4 and %C-5: ↑NF samples
Bentonite Treatment	Wines at bottling	No significant changes		
Mechanical stress	Commercial wines	No significant changes		%C-5: ↑G_C and ↓ M_T
Dissolved Oxygen (DO)	Commercial wines	Trimeric procyanidin and prodelphinidin ↑G_C and ↑M_C tetrameric procyanidin and prodelphinidin ↑G_C	No significant changes	%C-5: ↑G %C-4-OH: ↑G and ↑M in treated samples

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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