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Impact of 5-year bottle aging under controlled oxygen exposure on sulfur dioxide and phenolic composition of tannin-rich red wines

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ABSTRACT

Aim: This study aims at understanding the impact of the initial phenolic composition on the evolution of red wines after long bottle aging.
Materials and results: three different red wines rich in tannins, Aglianico, Casavecchia and Pallagrello, bottled with the same amount of total sulfur dioxide and different amounts of free sulfur dioxide, were analysed after 5 years of bottle aging under controlled exposure to oxygen passing through the closure. Acetaldehyde and monomeric anthocyanins were determined by HPLC, the chromatic characteristics and the main phenolic classes by spectrophotometry, the saliva precipitation index (SPI) by CHIP electrophoresis, and the astringency subqualities by sensory analysis. The results confirmed that during aging there is an increase in polymerisation reactions. A higher amount of acetaldehyde was detected in wines which were bottled with a lower content of free SO_2 and were less rich in anthocyanins and tannins; a significant closure effect was observed for these wines. Regarding the influence of closure on tannins, significant slight differences in vanilline reactive flavans and SPI content were observed for Pallagrello wines only, which were characterised by higher values for tannins at bottling. Astringency subqualities differed with closures for each wine.
Conclusion: this study indicates that the amount of initial free and combined sulfur dioxide, as well as that of anthocyanins and tannins, are key factors in driving polymerisation reactions and the aging of red wines. After five years of bottle aging the influence of closure could still be observed.
Significance of the study: this study provides new insights into the parameters that need to be evaluated before bottling in order to avoid the wrong evolution of red wines after long bottle aging.

KEYWORDS

red wine, long bottle aging, acetaldehyde, tannins, astringency subqualities

INTRODUCTION

Older vintages of some red wines are often more expensive than younger ones (Carew and Florkowski, 2010). However, not all red wines age well, and therefore it is important to assess the way in which a wine will age in order to correctly select wines suitable for long-term aging. Regarding the pre-requisites that a red wine will need if it has to be aged for a long time, several researchers have shown that red wines with aging potential are characterised by a high astringency level and colour intensity (Langlois *et al.*, 2010; Jaffré *et al.*, 2009). More recent studies have also shown that wines with long aging potential and premium prices have higher phenol and tannin content and, among phenolic structures, a higher proportion of polymeric tannins (Gómez-Plaza *et al.*, 2016) and pyranoanthocyanic compounds (Sáenz-Navajas *et al.*, 2011). Part of the polymerisation reactions involving wine tannins and pigments is due to the action of oxygen; therefore, the regulation of oxygen uptake during bottle aging is fundamental to managing the sensory and commercial quality of long-aged wine.

Over the past decades, numerous studies have dealt with the role of closure in the management of wine aging and oxidation (Ugliano, 2013), and most of them have considered the effect of closure on the evolution of sulfur dioxide (SO_2) over time. In wine, sulfur dioxide exists as both "free SO_2" (given by the sum of molecular SO_2 and HSO_3^- and responsible for the protective action of this additive) and "bound SO_2" (comprising covalent adducts between HSO_3^- and compounds susceptible to electrophilic attack in wine, including carbonyl compounds, sugars, and anthocyanins (Waterhouse *et al.*, 2016). The use of "total SO_2" (the sum of free and bound SO_2) in red wine is subject to regulation due to its negative health effects (Waterhouse *et al.*, 2016). Free SO_2 is known to regulate all reactions taking place during the micro-oxygenation of red wine (Gambuti *et al.*, 2015), with long-lasting effects even after bottling (Gambuti *et al.*, 2019). However, the addition of SO_2 to wines raises health concerns as sulfite-sensitive people can have serious allergic reactions to it (Waterhouse *et al.*, 2016). As a consequence, one of the main objectives of enologists is to ensure that wines contain the maximum free/total SO_2 ratio when bottling. As the concentration of bound sulfur dioxide in wine depends on i) the concentration of the HSO_3^- ion, ii) the amount of compounds susceptible to electrophilic attack in wine (Jackowetz and de Orduña, 2012; Ferreira *et al.*, 2015), and iii) the reversibility of the equilibrium between covalent adducts and their original active components (Boulton *et al.*, 1996), it is of interest to evaluate how the various forms of sulfur dioxide (free and bound) change over time. Within this context, regulating the reactions resulting from the complex mixture of phenolic compounds in red wine during bottle aging is quite difficult and requires controlled storage conditions and knowledge of the factors involved in oxidative processes. In a previous study, the effect of the oxygen transfer rate (OTR) of closures on the polyphenolic composition of three Southern Italy red wines with high total tannin content (Aglianico, Casavecchia and Pallagrello) was determined after 15 months of bottle aging, and SO_2 protection was found to have a strong influence (Gambuti *et al.*, 2017).

In the present study, the changes in free and total SO_2 during bottle aging under controlled oxygen exposure of wines with the same initial total SO_2 and different free SO_2 and phenolic composition were evaluated to determine which free/total SO_2 ratios are critical for wine antioxidant protection over time. Because long-aged wines are usually those with a high concentration of tannins, three red wines rich in tannins already studied during the first phases of bottle aging (Gambuti *et al.*, 2017) were analysed.

The nano-oxygenation trial was performed on Aglianico, Casavecchia, and Pallagrello wines spiked with different amounts of free SO_2 and similar amounts of total SO_2. A The wines were aged for five years in bottles tapped with closures of different oxygen permeability for a total oxygen uptake varying from 5.1 to 27.6 mg/L.

MATERIAL AND METHODS

1. Experimental wines

1.1. Nano-oxygenation (NOx) trial:

Monovarietal wines from the 2012 vintage were produced from Aglianico (AGL), Casavecchia (CAS) and Pallagrello (PALL) grapes from the Campania region (Italy) applying a standard industrial process. The base parameters (mean ± standard deviation) of wines (determined by using official methods of analysis) at bottling are shown in Table 1.

Two amounts of nano-oxygenation NOx were applied to each wine: 3.2 and 4.5 mg O_2/L year, through Nomacorc Select 300 (300) and Select 700 (700) closures respectively

TABLE 1. The base parameters (mean ± standard deviation) of wines at bottling.

	Ethanol content (%v/V)	pH	Residual sugars (g/L)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	Total anthocyanins (mg/L)	Total tannins (mg/L)	Ratio SO_2 Total/Free
AGL	12.48±0.07b	3.49±0.02b	2.95±0.05a	28.8±0.0c	108.8±0.6	198.89±4.28c	3813±61c	3.76
CAS	13.30±0.08a	3.71±0.03a	1.86±0.09c	60.8±0.6a	121.6±0.6	674.83±3.37a	4025±35b	2
PALL	13.40±0.10a	3.62±0.06a	2.19±0.06b	48±0.6b	109.0±0.3	591.82±4.50b	4187±18a	2.27

All the data are expressed as means ± standard deviation of four replicates (two experimental replicates x two analytical replicates). The different letters (a, b,c) indicate statistical differences ($p < 0.05$).

(Nomacorc SA, Thimister Clermont, Belgium). Because AGL wines contained less anthocyanins than the other wines (Table 1), and because they are strongly influenced by closure OTR (Gambuti *et al.*, 2012), a closure with lower oxygen ingress (1.6 mg O_2/L/year) was also tested: Nomacorc Select 100 (100). Total package oxygen (TPO) is the sum of dissolved and headspace oxygen after bottling. TPO at bottling was measured by means of oxo-luminescence, using a Nomasense oxygen analyser (Nomacorc SA, Thimister Clermont, Belgium). Measurements were taken approximately 30 min after bottling (Table 2). Wines were analysed after 0, 7, 15, and 60 months of aging in bottles in controlled conditions (10-12 °C, 60 to 70 % humidity). At each sampling time, two bottles for each treatment were analysed.

TABLE 2. Wine codes, total package oxygen (TPO) at bottling, closure contribution, and total oxygen exposure (TOE) for the three wines and the closures used.

	TPO bottling (mg/L)	Closure Contribution (mg/L)	TOE (mg/L)
AGL100	1.0	8.0	9.0
AGL300	1.0	16.0	17.0
AGL700	1.0	22.5	23.5
CAS300	5.0	16.0	21.5
CAS700	5.1	22.5	27.6
PALL300	1.1	16.0	17.2
PALL700	2.0	22.5	24.5

2. Method of analysis

2.1. Main compositional parameters of the wines

The main compositional parameters of the grape juice and wines (residual sugar, titratable acidity, volatile acidity, and alcohol) were determined following the official method of analysis (OIV-MA-F1-07, RESOLUTION OIV-OENO 419A / 2011, www.oiv.int).

2.2. Determination of sulfur dioxide

Both the free and combined sulfur dioxide were determined by the aeration-oxidation (A-O) official method of analysis (OIV-MA-F1-07, RESOLUTION OIV-OENO 419A / 2011, www.oiv.int).

2.3. Determination of acetaldehyde

The analysis of acetaldehyde was carried out by direct injection of the wine sample into HPLC-DAD devices (RESOLUTION OENO-SCMA 16-597/2016, www.oiv.int). Analyses were performed by employing a HPLC SHIMADZU LC10 ADVP apparatus (Shimadzu Italy, Milan), consisting of a SCL-10AVP system controller, two LC-10ADVP pumps, a SPD-M 10 AVP detector, and an injection system full rheodyne model 7725 (Rheodyne, Cotati, CA) equipped with a 50 µL loop. Briefly, wine sample aliquots (100 µL) were poured into a vial, followed by 20 µL of freshly prepared 1120 mg/L SO_2 solution. Next, 20 µL of 25 % sulfuric acid (Carlo Erba reagent 96 %) was added, which was followed by 140 µL of 2 g/L 2,4-dinitrophenylhydrazine reagent (Aldrich chemistry). After mixing, the solution was left to react for 15 min at 65 °C and then promptly cooled to room temperature. Analysis of carbonyl hydrazones was conducted by using the HPLC system described above. A Waters Spherisorb column (250 x 4.6 mm, 4µm particles diameter) was used for separation. The chromatographic conditions were: sample injection volume, 50 µL; flow rate, 0.75 mL/min; column temperature, 35 °C; mobile phase solvents, (A) 0.5 % formic acid (Sigma Aldrich ≥ 95 %) in water milli-Q (Sigma Aldrich) and (B) acetonitrile (Sigma Aldrich ≥ 99,9 %); gradient elution protocol, 35 % B to 60 % B (t = 8 min), 60 % B to 90 % B (t = 13 min), 90 % B to 95 % B (t = 15 min, 2-min hold), 95 % B to 35 % B (t = 17 min, 4-min

hold), total run time: 21 min. Eluted peaks were compared with derivatised acetaldehyde standard. All analyses were conducted on two experimental replicas and two analytical replicas.

2.4. Analysis of the chromatic characteristics and phenolic compounds of the wine by spectrophotometry.

The colour and phenolic compounds of the wines were determined, as they are responsible for the fundamental organoleptic properties of wines, such as visual properties, astringency, and bitterness. The wine colour (intensity and hue) analysis was performed using spectrophotometric techniques. The analysis of the phenolic compounds was carried out by means of spectrophotometric techniques. Colour intensity CI, abs 420, abs 520, abs 620 nm, and hue were evaluated according to the OIV compendium of international methods of analysis of wine and musts (www.oiv.int). Total anthocyanins, short polymeric pigments (SPP), large polymeric pigments (LPP) and BSA reactive tannins were determined by the Harbertson–Adams assay (Harbertson *et al.*, 2002). The method described by Gambuti *et al.* (2015) was used to determine vanillin reactive flavans (VRF).

2.5. Analysis of monomeric anthocyanins.

The analysis of anthocyanins was carried out by high performance liquid chromatography analysis HPLC-DAD as previously reported (www.oiv.int). The HPLC system described above and a Waters Spherisorb column (250 x 4.6 mm, 4µm particles diameter) with pre-column were used. Twenty µl of wine or calibration standards were injected into the column. Detection was performed by monitoring the absorbance signals at 518 nm. All the samples were filtered through 0.45 micron, durapore membrane filters (Sigma Aldrich, Milan, Italy) into glass vials and immediately injected into the HPLC system. The HPLC solvents used were: a) water/formic acid/acetonitrile (87:10:3) v/v, and b) water/formic acid/acetonitrile (40:10:50) v/v. The gradients used for a and b at zero time conditions were 94 and 6 % respectively. After 15 min the pumps were adjusted to 70 and 30 % for a and b respectively, then at 30 min to 50 and 50 %, at 35 min to 40 and 60 %, and at 41 min (end of analysis) to 94 and 6 %. After a 10-min equilibrium period, the next sample was injected. The flow rate was 0.80 mL/min. For calibration, the external standard method was used: the calibration curve was plotted for the malvidin-3-*O*-monoglucoside (Extrasynthese, Lyon, France) on the basis of peak area and the concentration was expressed as mg/L of malvidin-3- *O*-monoglucoside. All the analyses were conducted in duplicate on each experimental replicate.

2.6. The Saliva Precipitation Index (SPI)

Astringent tannins were measured by means of the Saliva Precipitation Index (SPI), due to the ability of tannins to precipitate salivary proteins. The SPI method was performed as described in Rinaldi *et al.* (2014), and the potential astringency of wines was expressed in g/L of gallic acid equivalent (GAE). The analyses were carried out in duplicate on each bottle (two bottles for each treatment) for a total of four replicates.

2.7. Sensory evaluation of the wines

The wine evaluation sessions comprised a panel of 13 judges from the Division of Sciences of Vine and Wine, Department of Agriculture, University of Naples Federico II, in Avellino (Italy), who were trained in astringency and mouthfeel sensations (Rinaldi and Moio, 2018). Wines were evaluated in duplicate. Two tasting evaluations of four unknown samples were performed in each session. They were presented in balanced random order at room temperature (18 ± 2 °C) in black tulip-shaped glasses coded with 3-digit random numbers. The mouthfeel sensations were evaluated as described in Rinaldi and Moio (2018). Judges used the CATA questionnaire with different terms describing astringency (s*ilk*, *velvet*, *dry*, *corduroy*, *adhesive*, *aggressive*, *hard*, *soft*, *mouthcoat*, *rich*, *full-body*, *green*, *grainy*, *satin*, *pucker)*, checked the subqualities if present, and then rated the intensity (RATA). The modified frequency of the astringency subquality was a mixture of intensity (RATA) and frequency of citation (CATA), which was calculated with the following formula (Tao *et al.*, 2009):

$$MF\% = \sqrt{F(\%)/I(\%)}$$

where F (%) is the detection frequency of an astringent attribute expressed as a percentage, and I (%) is the average intensity expressed as a percentage of the maximum intensity.

2.8. Data analysis

Fisher's least significant differences (LSD) procedure was used to discriminate between the means of the variables when they fulfilled the parametric conditions for chemical, OCR (oxygen consumption rate) and SPI data. When the variances were not homogeneous, data

were analysed using the Kruskall–Wallis test, and significant differences were established by using Notched Box Plots. Differences of $p < 0.05$ were considered significant. Partial least square (PLS) regression was carried out using the PLS module of the XLSTAT software (Addinsoft, 2009) to predict OCR from the set of data collected.

Sensory attributes were evaluated on two replicates using the Duncan test. Significant differences in MF % between samples were examined using the Fisher's exact test. Differences of $p < 0.05$ were considered significant. Elaborations were carried out by means of XLSTAT software (Addinsoft, XLSTAT 2017).

RESULTS AND DISCUSSION

The content and loss of free and total SO_2 of wines aged for 5 years with respect to the bottling data are shown in Table 3. During aging, the main form of SO_2 at wine pH, the bisulfite ion (HSO_3^-; which is in equilibrium with molecular SO_2), is consumed by reactions with hydrogen peroxide and several electrophilic wine components, such as those derived from the oxidation cascade, including quinones and acetaldehyde (Waterhouse *et al.*, 2016). Recently, new sulfonated products promoted by small amounts of oxygen derived from reactions with indolic (Arapitsas *et al.*, 2016) and phenolic (Arapitsas *et al.*, 2018) wine components have been added to this list as SO_2-binders. It has been shown that, after long bottle aging of red wines, the reactions of SO_2 with the flavanols (Arapiptas *et al.*, 2018; Ma *et al.*, 2018) are predominant. No proportionality between oxygen exposure and SO_2 consumption was detected when taking into account the TOEs listed in Table 2 for all of the wines, and the data on SO_2 loss shown in Table 3. The sulfonate adducts can be considered as bound SO_2, because the A-O method to determine total SO_2 requires a heating step that disrupts all sulfonate adducts (Waterhouse *et al.*, 2016); therefore, the loss of total sulfur dioxide should be exclusively related to the reactions with hydrogen peroxide and the loss of molecular SO_2 through the closure. However, to our knowledge, there is no evidence in the literature of HSO_3^- ions being released by all covalent adducts during the heating step. Thus, the lack of proportionality could be related to the presence of sulfonate adducts which cannot be evaluated via the A-O method, and/or to the influence of other wine antioxidants which consumed oxygen. Ferreira *et al.* (2015) also observed a lack of direct correlation between O_2 uptake and SO_2 consumption when fifteen Spanish red wines were subjected to five consecutive cycles of air saturation at 25 °C. In a recent study, a lot of variability in amounts of final total SO_2 was also detected after bottle aging of Cabernet Sauvignon wines with different initial content of acetaldehyde, phenolic compounds and glutathione (Gambuti *et al.*, 2019); this confirms the important role of the initial wine composition in SO_2 consumption.

After 5 years of aging, the highest amounts of total SO_2 were detected in AGL wines, while the lowest were detected in PALL wines. The AGL wines showed higher amounts of initial combined SO_2 and final free acetaldehyde, thus confirming previous findings of Ferreira *et al.* (2015) that, after oxidation, the remaining unreacted SO_2 of fifteen Spanish red wines was proportional to initial combined SO_2 and final free acetaldehyde.

Concerning the differences among wines, the same relationship among the free SO_2 contents at bottling (AGL < PALL < CAS) was detected even after 5 years of aging, while a significant difference in the values of free SO_2 content was still observed in CAS wines.

A comparison of amounts of acetaldehyde - the main product of wine oxidation detected at the end of storage time - is shown in Figure 1. AGL wines showed the highest amounts of acetaldehyde, probably because they contained less free SO_2 at bottling (Table 1). The significant lower values detected in AGL700 and AGL300 (which were both exposed to higher levels of oxygen) with respect to AGL100 could be due to two different reasons: 1) the involvement of acetaldehyde released from its sulfonate form in further reactions with other wine components, and 2) the oxidation of acetaldehyde by spoilage bacteria when no more SO_2 was in the bottle. The first hypothesis (which goes against widely held expectations) is not so surprising, because Sheridan and Elias (2015) found that, in systems containing SO_2, the rate of reaction of acetaldehyde with catechin was slowed down, but was not prevented until SO_2 was in great excess. Therefore, it is likely that in the wines which were more exposed to oxygen (AGL700 compared to AGL300 and AGL100) the reaction involving the dissociation of acetaldehyde from its SO_2-bound form was favoured, and the acetaldehyde released from the bound form was consumed in other reactions with wine nucleophiles (sulfites, thiols, alcohols, flavonoids). On the other hand, the presence of spoilage bacteria cannot be ruled out, although no differences in volatile acidity among AGL wines were detected.

TABLE 3. SO_2 values and loss of SO_2 in the wines after 5 years of bottle aging.

	Free SO_2 (mg/L)				Total SO_2 (mg/L)				Free SO_2 loss (mg/L)	Tot SO_2 loss (mg/L)
AGLIANICO										
bottling	29.00	±	0.00	A	109.00	±	1.00	A		
7 months aging										
AGL100	18.00	±	2.00	B	73.40	±	5.65	B	11.00	35.40
AGL300	13.60	±	3.39	B	76.30	±	0.70	B	15.20	32.50
AGL700	15.20	±	1.13	B	70.20	±	5.65	B	13.60	38.60
15 months aging										
AGL100	14.72	±	0.28	Ba	67.20	±	0.97	B	14.08	41.60
AGL300	11.52	±	0.00	Bb	67.20	±	2.46	B	17.28	41.60
AGL700	12.16	±	0.90	Bb	67.20	±	0.00	B	16.64	41.60
5 years aging										
AGL100	2.72	±	1.31	C	39.52	±	1.09	Ca	26.28	69.48
AGL300	nd				29.88	±	3.17	Db	29.00	79.12
AGL700	nd				27.84	±	0.36	Db	29.00	81.16
CASAVECCHIA										
	Free SO_2 (mg/L)				Total SO_2 (mg/L)				Free SO_2 loss (mg/L)	Tot SO_2 loss (mg/L)
bottling	61.00	±	1.00	A	122.00	±	1.00	A		
7 months aging										
CAS300	44.80	±	0.00	B	73.80	±	2.54	B	16.00	47.88
CAS700	44.00	±	1.13	B	69.00	±	4.00	B	16.80	52.28
15 months aging										
CAS300	33.92	±	0.90	C	51.84	±	4.52	C	26.88	69.84
CAS700	32.00	±	0.00	C	56.96	±	2.71	C	28.80	64.72
5 years aging	8.00	±	0.36	D	22.08	±	0.82	D	53.00	99.92
CAS300	7.36	±	1.33	D	21.28	±	1.41	D	53.64	100.72
CAS700										
PALLAGRELLO										
	Free SO_2 (mg/L)				Total SO_2 (mg/L)				Free SO_2 loss (mg/L)	Tot SO_2 loss (mg/L)
bottling	48.00	±	1.00	A	109.00	±	0.00	A		
7 months aging										
PALL300	46.40	±	2.26	A	59.20	±	2.26	B	1.60	49.80
PALL700	45.60	±	3.39	A	58.40	±	3.39	B	2.40	50.60
15 months aging										
PALL300	28.20	±	0.84	B	59.80	±	1.41	B	19.80	49.20
PALL700	30.72	±	2.71	B	61.12	±	1.35	B	17.28	47.88
5 years aging										
PALL300	5.12	±	0.32	Ca	15.52	±	2.17	C	42.88	93.48
PALL700	3.84	±	0.45	Cb	14.40	±	2.26	C	44.16	94.60

All the data are expressed as means ± standard deviation of four replicates (two experimental replicates x two analytical replicates). The different letters indicate statistical differences ($p < 0.05$). Lower case letters (a, b, c) are used to compare wines aged with closures at different oxygen ingress at each time. Capital letters (A, B, C) indicate significant differences among the same kind of wine over time.

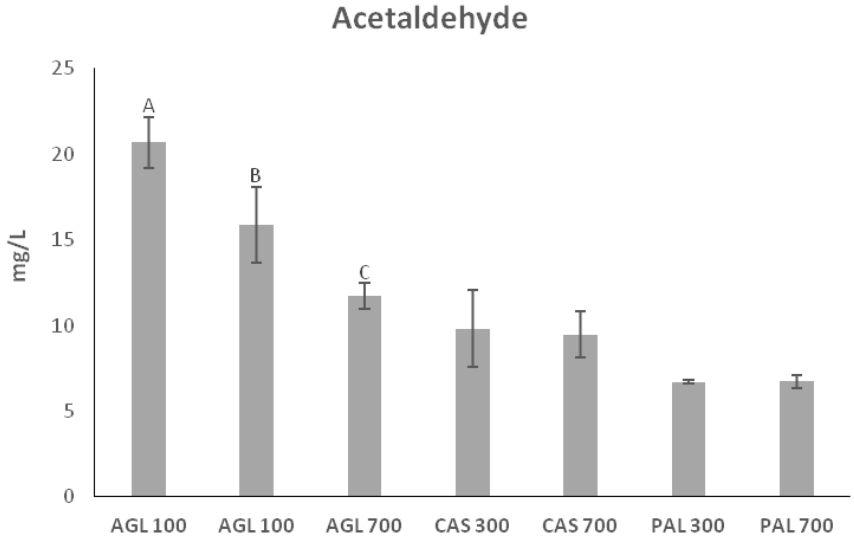

FIGURE 1. Acetaldehyde detected at the end of nano-oxygenation.

All the data are expressed as means ± standard deviation of four replicates (two experimental replicates x two analytical replicates). Letters indicate significant differences among the same kind of wine with $p < 0.05$.

Data on the evolution of monomeric anthocyanins confirmed the influential roles of SO_2 protection and the total/free ratio on the evolution of red wine (Table 4.). A dramatic loss of total and monomeric anthocyanins was detected in AGL wines, while a less important loss was observed in CAS and PALL. A significant influence of the closure on the preservation of Pn3glc for AGL and CAS, and on the preservation of Dp3glc for AGL and PALL, was detected. Mv3acglc was also higher for PALL300 with respect to PALL700. However, differences among wines stored in bottles with different closures were under 10 mg/L and thus, from a practical point of view, it would be more appropriate to claim that after 5 years of aging closure had no effect on the evolution of anthocyanins.

Hue increased for all wines over time, while the colour intensity decreased only for AGL, probably due to the lower content in monomeric and polymeric anthocyanins of these wines (Table 5). After 5 years of aging, when a higher exposure to oxygen occurred, a significant higher CI was detected for AGL and CAS. However, these differences can be considered as negligible from a quantitative point of view. A significant influence of closure permeability to oxygen on CI after long bottle aging has already been shown in a study on Cabernet Sauvignon wines analysed after 5 years of aging in bottles (Han *et al.*, 2015).

The fact that, for all the wines in our study, native anthocyanins collapsed over time during bottle aging has already been widely observed (Monagas *et al.*, 2006, Gambuti *et al.*, 2012) and is easily justified by the numerous reactions which these molecules undergo (He *et al.*, 2012); some are degradative (Gambuti *et al.*, 2017), and others involve the formation of new polymeric structures (Gambuti *et al.*, 2015).

For all wines, polymeric pigments increased during aging, while BSA reactive tannins and VRF decreased (Table 6). This behaviour confirms that polymerization reactions occurred during aging. The loss of VRF was higher in CAS (more than 50 % of the original value) with respect to PALL (less than 28 %) and AGL (around 40 %) indicating that after 5 years of aging the CAS wine contained the lowest amount of tannins. This result confirms previous findings showing that CAS had the lowest T/A and VRF/A ratios, and it also confirms previous results showing the key role that these ratios (Gambuti *et al.*, 2018) and low molecular weight flavanols (Carrascón *et al.*, 2018) had in the oxidative reactions. In a similar way for all wines, SPP increased for up to 15 months and then decreased with time. LPP, however, continued to increase over time for CAS and PALL, while for AGL it started to stabilise after 15 months. SPP are therefore produced in the first phases of aging and then consumed as they are involved in the formation of larger molecules. A slightly significant influence of closure permeability was only observed on VRF for PALL. Because polymerisation reactions are inhibited by free SO_2 and the measurement of VRF may be considered an indirect, inverse measure of the oxidative polymerisation of flavanols

TABLE 4. Evolution of monomeric anthocyanins during nano-oxygenation of Aglianico, Casavecchia and Pallagrello wines.

AGLIANICO																							
	Dp3glc			Pt3glc			Pn3glc			Mv3glc			Mv3acglc			Mv3cmglc						
bottling	29.50	±	0.47	A	33.88	±	5.53	A	14.71	±	3.20	A	238.36	±	5.01	A	31.52	±	0.67	A	15	±	0.9	A
7 months aging																							
AGL100	13.03	±	1.27	Ba	14.27	±	0.51	Ba	4.25	±	0.62	B	99.21	±	1.26	Ba	12.80	±	1.27	Ba	4.4	±	0.6	Ba
AGL300	11.07	±	0.22	Bb	13.04	±	1.19	Ba	4.05	±	0.87	B	86.84	±	1.53	Bb	10.05	±	0.91	Bb	5	±	0.2	Ba
AGL700	10.65	±	0.85	Bb	11.63	±	0.37	Bb	4.23	±	0.66	B	81.76	±	1.16	Bc	9.94	±	0.56	Bb	2.5	±	0.4	Bb
15 months aging																							
AGL100	7.71	±	0.46	C	7.75	±	0.29	Ca	2.51	±	0.11	Ca	58.89	±	1.69	Ca	8.16	±	0.18	C	nd			
AGL300	7.06	±	0.85	C	7.62	±	0.30	Ca	2.52	±	0.16	Ca	60.43	±	2.06	Ca	8.02	±	0.76	C	nd			
AGL700	6.72	±	0.99	C	6.32	±	0.35	Cb	1.52	±	0.90	Cb	53.20	±	4.30	Cb	7.53	±	0.87	C	nd			
5 years aging																							
AGL100	2.04		0.40	Db	1.21	±	0.26	D	0.32	±	0.05	Db	13.54	±	1.10	D	0.63	±	0.21	D	nd			
AGL300	2.44		0.1	Dab	1.52	±	0.09	D	0.50	±	0.09	Da	14.34	±	0.55	D	0.72	±	0.01	D	nd			
AGL700	2.63		0.20	Da	1.44	±	0.12	D	0.49	±	0.08	Da	14.28	±	1.38	D	0.69	±	0.10	D	nd			

CASAVECCHIA																							
	Dp3glc			Pt3glc			Pn3glc			Mv3glc			Mv3acglc			Mv3cmglc						
bottling	75.80	±	0.50	A	125.08	±	2.81	A	79.54	±	0.85	A	1164.38	±	5.78	A	239.06	±	30.11	A	136	±	0	A
7 months aging																							
CAS300	64.9	±	3.56	B	98.21	±	1.15	B	47.20	±	2.04	B	895.95	±	11.93	B	188.85	±	2269	B	11	±	0.7	B
CAS700	63.67	±	2.63	B	97.11	±	1.67	B	45.97	±	0.64	B	888.47	±	8.75	B	189.30	±	2.14	B	12	±	1.3	B
15 months aging																							
CAS300	42.86	±	0.34	C	74.47	±	1.23	C	32.71	±	0.45	C	588.61	±	6.31	C	105.82	±	4.75	C	nd			
CAS700	43.45	±	0.64	C	75.85	±	0.83	C	30.62	±	5.00	C	597.80	±	6.80	C	111.47	±	5.82	C	nd			
5 years aging																							
CAS300	11.67	±	5.00	D	16.91	±	3.60	D	7.40	±	1.51	Da	150.03	±	27.61	D	25.40	±	0.97	D	nd			
CAS700	13.04	±	0.93	D	13.69	±	0.96	D	4.68	±	0.23	Eb	130.54	±	13.38	D	27.14	±	4.34	D	nd			

PALLAGRELLO																							
	Dp3glc			Pt3glc			Pn3glc			Mv3glc			Mv3acglc			Mv3cmglc						
bottling	97.25	±	2.10	A	141.51	±	3.08	A	50.12	±	2.06	A	1082.49	±	20.78	A	255.52	±	6.94	A	116	±	3.4	A
7 months aging																							
PALL300	74.81	±	1.53	B	107.44	±	1.89	B	33.24	±	0.70	B	806.30	±	6.94	B	175.19	±	2.57	B	11	±	0.8	B
PALL700	78.05	±	1.38	B	110.27	±	1.95	B	34.38	±	0.93	B	825.21	±	12.26	B	180.39	±	3.40	B	13	±	1.1	B
15 months aging																							
PALL300	53.53	±	0.53	C	76.52	±	7.17	C	23.24	±	0.50	C	546.46	±	9.27	C	97.80	±	0.54	C	tr			
PALL700	54.33	±	3.47	C	81.68	±	6.01	C	23.75	±	2.03	C	559.73	±	43.20	C	95.80	±	1.79	C	tr			
5 years aging																							
PALL300	16.76	±	0.96	Da	18.50	±	1.14	D	4.42	±	0.93	D	138.25	±	6.93	D	24.57	±	0.891	Da	nd			
PALL700	14.66	±	0.41	Db	18.51	±	0.55	D	3.52	±	0.83	D	132.00	±	2.91	D	23.00	±	0.54	Db	nd			

Dp3glc = delphinidin 3-glucoside. Cy3glc = cyanidin 3-monoglucoside. Pt3glc = petunidin 3-monoglucoside. Pn3glc = peonidin 3-monoglucoside. Mv3glc = malvidin 3-glucoside. Mv3acglc = malvidin 3-(6II-acetyl)-glucoside. Mv3cmglc = malvidin 3-(6II-coumaroyl)-glucoside.
All the data are expressed as means ± standard deviation of four replicates (two experimental replicates x two analytical replicates). Different letters indicate statistical differences ($p < 0.05$). Lower case letters (a, b, c) are used to compare wines aged with closures at different oxygen ingress at each time. Capital letters (A, B, C) indicate significant differences among the same kind of wine over time.

TABLE 5. Evolution of chromatic characteristics and total monomeric anthocyanins of wines during nano-oxygenation.

AGLIANICO			
	Color Intensity	Hue	Tot anth (mg/L)
bottling	8.01 ± 0.07 B	0.78 ± 0.01 B	362.88 ± 6.08 A
7 months aging			
AGL100	7.78 ± 0.07 Bb	0.77 ± 0.00 Ba	147.93 ± 2.35 Ba
AGL300	7.85 ± 0.12 Bab	0.75 ± 0.01 Cb	130.10 ± 2.00 Cb
AGL700	7.93 ± 0.03 Bab	0.78 ± 0.00 Ba	120.78 ± 1.73 Dc
15 months aging			
AGL100	8.71 ± 0.37 A	0.77 ± 0.01 Bb	85.04 ± 1.77 Ea
AGL300	8.68 ± 0.24 A	0.77 ± 0.01 Bb	85.04 ± 2.36 Ea
AGL700	8.64 ± 0.19 A	0.78 ± 0.00 Ba	72.29 ± 4.59 Fb
5 years aging			
AGL100	6.99 0.14 Db	0.91 ± 0.00 A	17.13 ± 1.76 G
AGL300	6.92 0.03 Db	0.90 ± 0.00 A	18.80 ± 0.77 G
AGL700	7.17 0.03 Ca	0.90 ± 0.00 A	18.84 ± 1.67 G

CASAVECCHIA			
	Color Intensity	Hue	Tot anth (mg/L)
bottling	10.85 ± 0.11 B	0.70 ± 0.00 C	1819.60 ± 30.69 A
7 months aging			
CAS300	10.55 ± 0.07 C	0.71 ± 0.02 C	1306.10 ± 12.84 B
CAS700	10.67 ± 0.01 C	0.71 ± 0.00 C	1296.60 ± 9.50 B
15 months aging			
CAS300	11.00 ± 0.04 A	0.71 ± 0.00 C	844.49 ± 7.92 C
CAS700	11.00 ± 0.14 A	0.70 ± 0.01 C	859.20 ± 10.28 C
5 years aging			
CAS300	10.42 0.20 Cb	0.88 ± 0.00 A	186.02 ± 37.68 D
CAS700	10.71 0.11 Bca	0.85 ± 0.00 B	161.96 ± 15.50 D

PALLAGRELLO			
	Color Intensity	Hue	Tot anth (mg/L)
bottling	10.78 ± 0.07 A	0.69 ± 0.00 C	1742.90 ± 22.36 A
7 months aging			
PALL300	10.96 ± 0.19 A	0.73 ± 0.01 Ba	1208.50 ± 7.64 B
PALL700	10.71 ± 0.12 A	0.72 ± 0.01 Bb	1241.60 ± 12.89 B
15 months aging			
PALL300	10.70 ± 0.05 Ab	0.71 ± 0.00 C	795.55 ± 9.32 C
PALL700	10.79 ± 0.01 Aa	0.7 ± 0.01 C	815.31 ± 43.42 C
5 years aging			
PALL300	10.47 ± 0.27 B	0.90 ± 0.00 A	173.75 ± 8.36 D
PALL700	10.58 ± 0.13 B	0.90 ± 0.00 A	168.71 ± 4.31 D

All the data are expressed as means ± standard deviation of four replicates (two experimental replicates x two analytical replicates). Different letters indicate statistical differences ($p < 0.05$). Lower case letters (a, b, c) are used to compare wines aged with closures at different oxygen ingress at each time. Capital letters (A, B, C) indicate significant differences among the same kind of wine over time.

TABLE 6. Evolution of short SPP and large LPP polymeric pigments, BSA Reactive Tannins and vanilline vanilline reactive flavans (VRF) during nano-oxygenation of Aglianico, Casavecchia and Pallagrello wines.

AGLIANICO																			
	SPP				LPP				BSA Reactive Tannins				VRF (mg/L)						
bottling	0.69	±	0.00	A	0.27	±	0.00	D	934.26	±	14.63	A	1703.9	±	4.80	A			
7 months aging																			
AGL100	0.69	±	0.00	A	0.35	±	0.02	C	832.54	±	74.08	B	1579.53	±	18.74	B			
AGL300	0.7	±	0.01	A	0.36	±	0.03	C	854.25	±	17.86	B	1610.95	±	31.98	B			
AGL700	0.69	±	0.01	A	0.36	±	0.03	C	838.68	±	21.51	B	1567.74	±	32.76	B			
15 months aging																			
AGL100	0.68	±	0.01	Ab	0.44	±	0.01	B	785.85	±	12.03	C	1464.05	±	33.94	Ca			
AGL300	0.70	±	0.01	Aa	0.42	±	0.01	B	745.6	±	19.09	C	1402.77	±	30.41	Cab			
AGL700	0.70	±	0.02	Aa	0.46	±	0.01	A	772.64	±	12.37	C	1384.7	±	65.08	Cb			
5 years aging																			
AGL100	0.27	±	0.01	C	0.48	±	0.02	A	571.23	±	13.11	D	1007.63	±	15.69	D			
AGL300	0.28	±	0.01	C	0.46	±	0.02	A	570.75	±	23.26	D	1043.76	±	7.86	D			
AGL700	0.28	±	0.01	C	0.48	±	0.02	A	564.15	±	20.81	D	1009.2	±	37.83	D			
CASAVECCHIA																			
	SPP				LPP				BSA Reactive Tannins				VRF (mg/L)						
bottling	0.58	±	0.00	C	0.30	±	0.01	B	1142.44	±	54.64	A	1432.62	±	12.57	B			
7 months aging																			
CAS300	0.68	±	0.04	B	0.32	±	0.04	B	1022.64	±	79.7	B	1684.01	±	2.57	A			
CAS700	0.71	±	0.06	B	0.31	±	0.05	B	903.77	±	29.97	B	1606.5	±	39.29	A			
15 months aging																			
CAS300	0.84	±	0.01	Ab	0.29	±	0.00	Ca	720.44	±	4.75	C	1321.07	±	46.57	C			
CAS700	0.90	±	0.02	Aa	0.23	±	0.02	Db	694.03	±	14.41	C	1323.43	±	29.30	C			
5 years aging																			
CAS300	0.65	±	0.12	B	0.57	±	0.13	A	634.43	±	74.7	D	658.83	±	32.19	D			
CAS700	0.61	±	0.10	B	0.63	±	0.07	A	605.97	±	39.77	D	648.62	±	130.66	D			
PALLAGRELLO																			
	SPP				LPP				BSA Reactive Tannins				VRF (mg/L)						
bottling	0.60	±	0.02	C	0.30	±	0.02	B	990.34	±	22.33	A	1503.85	±	9.60	A			
7 months aging																			
PALL300	0.69	±	0.02	B	0.28	±	0.02	B	913.68	±	25.93	B	1639.23	±	91.66	A			
PALL700	0.67	±	0.01	B	0.31	±	0.03	B	893.4	±	45.1	B	1504.11	±	40.35	A			
15 months aging																			
PALL300	0.76	±	0.01	A	0.31	±	0	B	777.04	±	6.07	C	1371.35	±	33.01	B			
PALL700	0.74	±	0.01	A	0.31	±	0.02	B	752.36	±	25.1	C	1376.85	±	44.91	B			
5 years aging																			
PALL300	0.39	±	0.02	D	0.84	±	0.07	A	598.58	±	18.7	D	1160.81	±	51.67	Ca			
PALL700	0.42	±	0.02	D	0.72	±	0.11	A	581.13	±	18.39	D	1037.48	±	22.13	Db			

All the data are expressed as means ± standard deviation of four replicates (two experimental replicates x two analytical replicates). Different letters indicate statistical differences ($p < 0.05$). Lower case letters (a, b, c) are used to compare wines aged with closures at different oxygen ingress at each time. Capital letters (A, B, C) indicate significant differences among the same kind of wine over time.

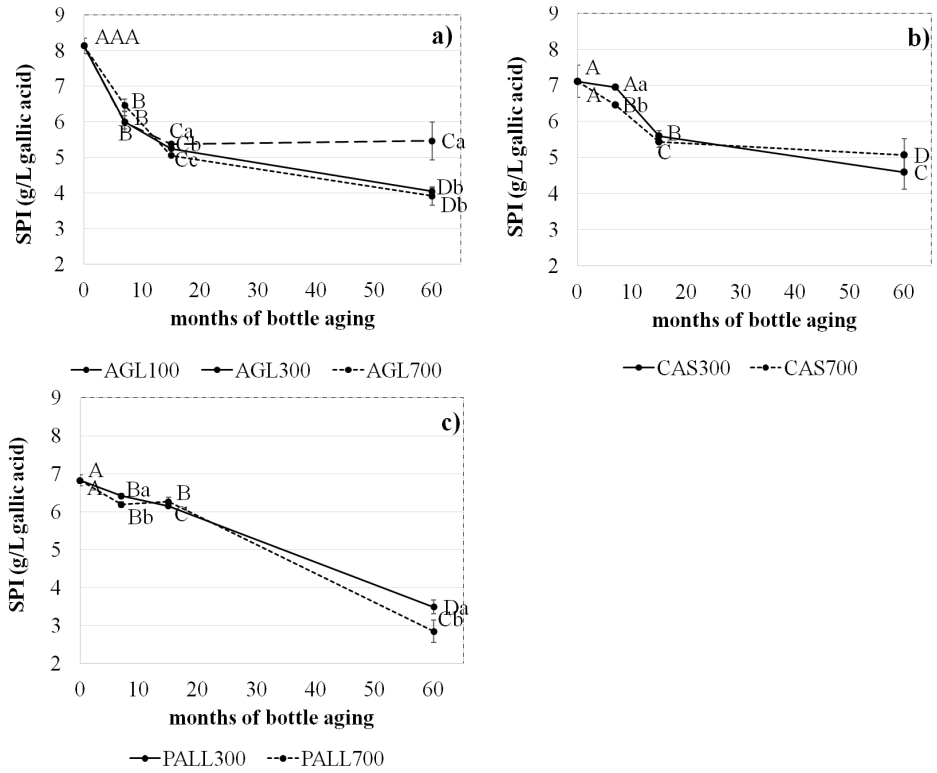

FIGURE 2. The Saliva Precipitation Index (SPI, g/L of gallic acid equivalent, GAE) of a) Aglianico, b), Casavecchia ,and c) Pallagrello wines during 5 years (60 months) of aging with different closures.

Different letters indicate statistical differences ($p < 0.05$). Lower case letters (a, b, c) are used to compare wines aged with closures at different oxygen ingress at each time. Capital letters (A, B, C) indicate significant differences among the same kind of wine over time.

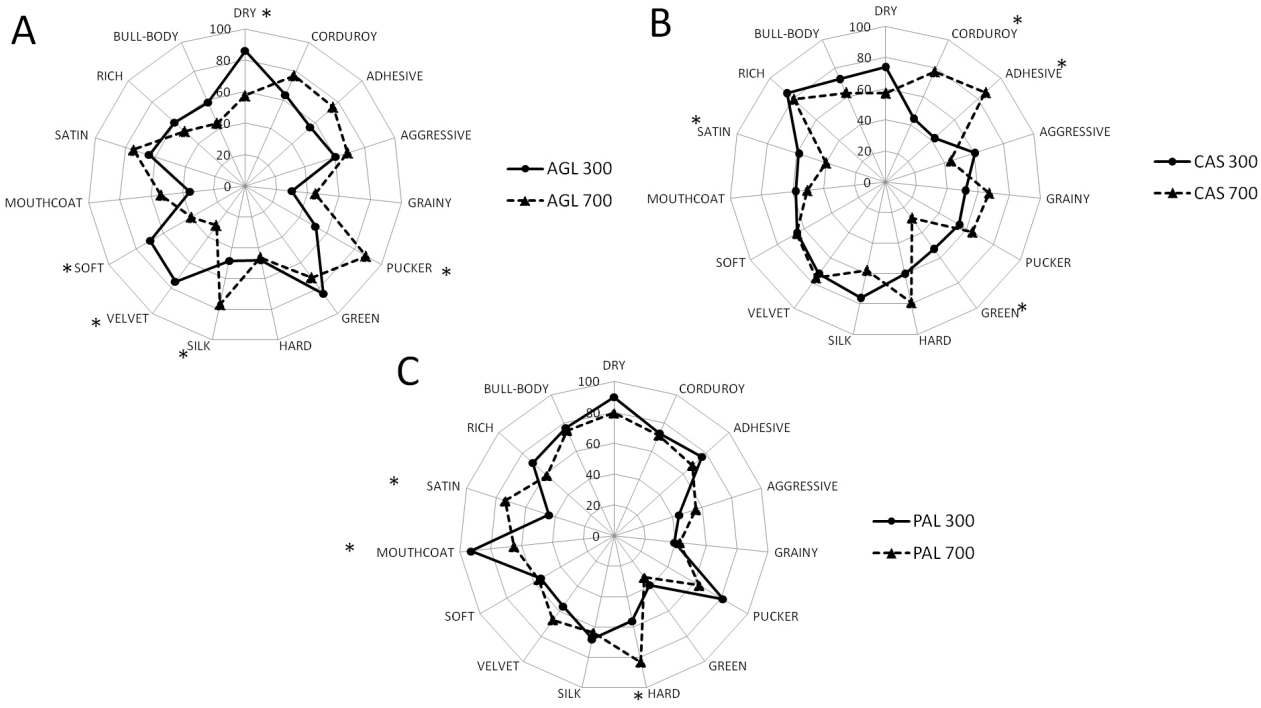

FIGURE 3. The astringency subqualities of Aglianico (AGL), Casavecchia (CAS) and Pallagrello (PAL) wines after 5 years of aging with different closures.

Asterisks indicate significant differences among the same kind of wine with $p < 0.05$, according to the the Fisher test.

(Gambuti *et al.*, 2015), differences in amount of free SO_2 in PALL could be responsible for the lower presence of polymeric flavonoids in wines stored with 300.

An *in vitro* measurement of astringency using the Saliva Precipitation Index (SPI) was performed on Aglianico (AGL), Casavecchia (CAS) and Pallagrello (PAL) wines during 5 years of aging with the different closures, as shown in Figure 2.

For Aglianico, the data shows a decrease in the precipitation of salivary proteins at 15 months (Gambuti *et al.*, 2017), and wines aged in bottles with 300 and 700 closures were found to have a higher SPI value than those aged in bottles with 100 closures. AGL100 could be characterised by having more astringent tannins; the closures which were more permeable to oxygen (300 and 700) may have favoured the formation of new phenolic compounds unable to precipitate salivary proteins, thus reducing astringency. In the case of CAS, no differences were found between 300 and 700, like for AGL, but a significant decrease compared to previous data (Gambuti *et al.*, 2017) occurred. In these wines, the two closures did not affect the SPI after 5 years of aging; in PALL wine, however, there was a significant difference between 300 and 700. In the latter case, the precipitation of salivary proteins was more affected by the higher permeability to oxygen of 700, as indicated by the lower SPI value. VRF data showed the same trend, suggesting that the polymerisation reactions of low molecular weight flavonoids are responsible for the differences in SPI.

It is known that the aging of wines for 5 years can affect the sensory properties of red wines (Han *et al.*, 2015); however, until now, the particular effect of closures on wine astringency subqualities had not been studied. In the present study, a trained jury evaluated wines for their qualitative astringency by selecting the sensation they considered appropriate for each wine from a list of subqualities in the CATA questionnaire (s*ilk*, *velvet*, *dry*, *corduroy*, *adhesive*, *aggressive*, *hard*, *soft*, *mouthcoat*, *rich*, *full-body*, *green*, *grainy*, *satin*, *pucker)*. The astringency subqualities, expressed as modified frequencies (MF %) of AGL, CAS, and PALL wines, are shown in figure 3A, B and C respectively. The significant subqualities ($p < 0.05$) are indicated with an asterisk (*).

The astringency subqualities, dry, pucker, silk, velvet and soft, differed among the AGL wines. AGL300 was drier, softer and more velvet. Meanwhile, AGL700 was more pucker and silkier. The different permeability of the closures showed an effect on the mouthfeel of Aglianico wines (Figure 3A). The wine aged in bottles with 300 closures evolved towards positive astringency subqualities, like velvet and soft, even if there was still a sensation of dryness; AGL700, however, was characterized by satin and pucker subqualities, the latter sensation being associated with an excess of oxidation (Rinaldi *et al.*, 2020). In Figure 3B, the astringency subqualities of Casavecchia wines are compared. CAS 300 was characterised by green and satin attributes. The green attribute has been defined as a combined effect of excess acidity and astringency (Gawel *et al.*, 2001), and it can be also defined as unripe astringency, therefore our data indicates that the tannins evolved toward sourness and silkiness feeling. CAS 700 was more adhesive and corduroy, meaning that this wine was unbalanced in terms of mouthfeel sensations. Pallagrello wines differed in three astringency subqualities: hard, satin, and mouthcoat (Figure 3C). PAL 700 was characterised by a silky astringency (satin) and a higher sensation of bitterness (hard). It seems that the higher permeability of the 700 closure to oxygen allowed higher oxidation, which, as in the case of CAS 700, makes the wine less viscose and much more bitter in accordance with previous results (Rinaldi *et al.*, 2020). PAL 300, conversely, was judged by the panel to be mouthcoating. This subquality represents one of the main drivers of liking and contributes to astringency for many red wines, such as Sangiovese (Rinaldi *et al.*, 2020), Tannat (Vidal *et al.,* 2018), and Côtes du Rhône and Rioja appellations wines (Sáenz-Navajas *et al.*, 2011). The nano-oxygenation of PALL by 700 contributed to a higher satin subquality of the wines. This subquality seems to be due to the lower content of astringent tannins and to a higher formation of polymers, as indicated by the lower VRF value (Table 6) detected in these wines. Although the wines showed a similar composition of SPP, LPP, BSA tannins and VRF, they differed from a sensorial point of view; this may be because of the different evolution of the structural characteristics of tannin, as well as of changes in the profile of compounds that we did not analyse (e.g., proanthocyanidins or phenolic acids), which affect the qualitative perception of astringency.

Previous studies have also shown that more specific variations in molecular structure, like changes in mean degree of polymerisation of proanthocyanidins, are responsible for the soft and mellow sensations of aged wines (Chira *et al.*, 2011).

CONCLUSION

After 5 years of aging, the effect of closure was still significant for a few parameters (e.g., some monomeric anthocyanins); however, some differences were very small (a few mg/L), and therefore, the influence of closure on phenolic composition can be considered as negligible, especially when compared to that observed after shorter aging. Our results confirm the important role of free SO_2 in the aging of wines, and the necessity for winemakers to take into account the sulfur dioxide binding power of compounds reactive to HSO_3^- ions in their practices. The desired amount of free SO_2 could thus be added to a wine once the value for sulfur dioxide has been determined via preliminary laboratory tests: this is especially true before bottling. Our data also indicate that not only is the amount of consumed SO_2 related to the uptake of oxygen during red wine aging, but it is also proportional to the amount of free SO_2 in initial wines; this should be seriously considered before managing sulfur dioxide during wine production, and when bottling a red wine that is intended to be aged for a long time. In terms of the evolution of phenolic compounds during aging, differences in polymerization reactions were observed, which were a result of differences among the initial amounts of anthocyanins, T and VRF. This study provides new insights into the management of nano-oxygenation during the aging of red wines via sulfur dioxide addition and closures to ensure their sensory and commercial quality.

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