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Modification of the organic acid profile of grapes due to climate changes alters the stability of red wine phenolics during controlled oxidation

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Summary

The effect of the main grape organic acids (tartaric, malic and citric) on the degradative oxidation of red wine was investigated by NMR, HPLC and spectrophotometry. Wines featuring the same pH value of 3.2 with different combinations of organic acids were prepared. Results showed that tartaric acid preserved native anthocyanins from oxidative degradation more than malic and citric acids, with malic acid being the one favoring oxidations the most and, consequently, acetaldehyde production. Wines richer in malic acids showed the highest reactivity towards saliva proteins and a potential higher astringency. Given the wide changes in tartaric/malic acid ratio with climate, these results can help to act in vineyard, as well as in winery, to manage the malic/tartaric acid ratio with the aim of improving red wine longevity.

K e y w o r d s : tartaric acid; malic acid; red wine; anthocyanins and tannins; oxidation.

Introduction

One of most important sensory attributes of wine is sourness. This important sensation is mainly due to a mixture of organic acids transferred from grape pulp to wine during winemaking. Among them, tartaric, malic and, to a lesser extent, citric acids are the most abundant. The equilibrium among them is strongly affected by climate that determines significant changes in terms of grape biochemistry. As early as 1964, Amerine (1964) pointed out that the tartrate/malate ratio at maturity for a given variety in a given region is relatively constant, unless unusual climatic conditions occur during the harvest. Warmer temperatures can in fact disrupt the physiological life cycle of grapevine by determining an early onset of flowering and fruit ripening. As a consequence, grapes will be characterized by low acidity (especially malic acid). A lower content of organic acids, on one hand, decreases the wine stability against microbes as well as oxidation processes, while, on the other, it irreversibly alters the wine gustative equilibrium. In colder seasons, higher malic/tartaric acid ratios are instead expected with an increase of the wine buffer capacity and perceptible sourness and astringency (Sowalsky and Noble 1998). Tartaric acid is a stronger acid than either citric or malic acids, thus implying that, for the same molar concentration, the wine pH level will result lower with tartaric acid because of its intrinsic higher tendency to release protons. Tartaric acid is also less susceptible to climatic conditions during ripening (Duchêne 2016). Varieties with a high tartaric acid content are consequently more resistant to climate changes (Poni *et al.* 2018). Hence, the consequences of climate change for the organic acid composition of grapevines can be enormous in terms of human productive activities, especially in countries where the wine industry constitutes an important pillar of the national economy. This is the case for Southern Italy where, following the dramatic climate change impacts of 2017, great changes in terms of titratable acidity of must were detected in the face of limited variations of pH values.

Apart from the fundamental contribution to sourness, these organic acids are also metal chelants and could affect all the redox reactions catalyzed by metals (Danilewicz 2014) that are responsible for wine aging. In particular, such oxidation reactions are responsible for modifications of the red wine color and of tannin structures entwined with wine astringency. Chromatic variations are due to the involvement of wine pigments and anthocyanins in numerous reactions with other compounds present in solution and with compounds deriving from the oxygen action. The shift of wine color from red to yellow hues is an indication of aging but also of oxidative spoilage of red wine and it is essentially due to the action of oxygen causing a loss of native anthocyanins not balanced by the formation of more stable red pigments. Chemically, the oxidation of wine is the result of a complex chain of redox reactions including the oxidation of polyphenols to quinones – that are reduced again by any nucleophile present in solution- and the reduction of oxygen to hydrogen peroxide, in presence of trace catalyst metals such as iron and copper (Waterhouse and Laurie 2006).Wine polyphenols involved in this starting phase contain at least two vicinal hydroxyls. Because wine native red pigments, such as malvidin 3-*O*-monoglucoside, contain isolated phenolic hydroxyl groups that require higher potential to be oxidized, they are not involved in the first steps of wine oxidation. Their loss is due to the action of hydrogen peroxide that, in the second step of wine oxidation, in presence of ferrous or cuprous species, reacts

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by the Fenton reaction to give the destructive oxidant radicals. A direct reaction of anthocyanin-3-*O*-monoglucoside with H_2O_2 has been reported (Brouillard and Delaporte 1977) but the main reason of anthocyanin loss is linked to their involvement in complex reactions. These reactions are triggered by carbonyls produced by the radicals deriving from the Fenton reaction such as acetaldehyde, produced by the oxidation of ethanol, and glyoxylic acid by the oxidation of tartaric acid (Es Safi *et al.* 1999, Elias *et al.* 2009). Tannins, polymerized structures responsible for wine astringency and bitterness, are even involved in numerous reactions triggered by small amount of oxygen during wine aging thus improving the quality of red wines by contributing to soften the tannin harshness (Atanasova *et al.* 2002, Gómez-Plaza *et al.* 2011). Numerous and complex intra- and intermolecular reactions are responsible for the modification of tannin structures (Mouls and Fulcrand 2012) and, as a consequence, for the variations in their reactivity towards saliva proteins (Gambuti *et al.* 2017). Reactions stabilizing color such as the formation of anthocyanin-derived pigments (e.g. A-type and B-type vitisins or flavanol-anthocyanin acetaldehyde-mediated condensation products) were even observed in wines supplied with ellagitannins (García-Estévez *et al.* 2017). Furthermore, the same authors showed that the addition of commercial ellagitannins protects anthocyanins against oxidation.

A large number of these reactions affecting wine color stability and astringency are due to acetaldehyde. This molecule is produced by ethanol oxidation due to the Fenton reaction and reacts readily with flavan-3-ols by increasing their polymerization (Drinkine *et al.* 2005, Peterson and Waterhouse 2016). The same high reactive aldehyde is involved in numerous reactions stabilizing wine color (Timberlake and Bridle 1976). However, an excessive production of acetaldehyde can result in the appearance of oxidation off-flavors (Carlton *et al.* 2007).

Although the effects of pH on anthocyanin equilibria and tannin reactivity and astringency (Heredia *et al.* 1998, Sowalsky and Noble 1998, Rinaldi *et al.* 2012) are known, few studies on the role of the nature of organic acids have been published. In addition, information on their impact on pigments and tannin evolution during oxidative aging is not exhaustive. With the purpose of furnishing data useful for enologists to address urgent issues deriving from climate changes (in this study the effect of pH and of the main grape organic acids (tartaric, malic and citric)) on acetaldehyde, on red pigments and tannins evolution during degradative oxidation of red wine was investigated.

Material and Methods

Wine trial: A 2016 'Aglianico' wine stored for 18 months in stainless steel tank with a pH level of 4.0 was used. Base parameters of red wine were: ethanol 12.6 % v/V, color intensity 5.56 Abs Units, hue 0.52, total phenolics 943 $mg \cdot L^{-1}$, total anthocyanins 242 $mg \cdot L^{-1}$ and BSA (bovine serum albumin) precipitable tannins 205 $mg \cdot L^{-1}$, free sulfur dioxide was not detectable, total sulfur dioxide 42 $mg \cdot L^{-1}$, iron 0.521 $mg \cdot L^{-1}$, copper 0.194 $mg \cdot L^{-1}$. Oxidation reactions were performed in 33 mL reagent bottles adjusted to pH 3.2 with tartaric acid (H2T sample, final concentration of tartaric acid of 2.6 ± 0.2 $g \cdot L^{-1}$), malic acid (H2M sample, final concentration of malic acid of 10.4 ± 0.2 $g \cdot L^{-1}$) and citric acid (H3C sample, final concentration of citric acid of 9.6 ± 0.3 $g \cdot L^{-1}$). Pure acids were added to wines; treated wines were then stirred and pH was controlled for two days to make sure that the desired pH value had been reached. Values of titratable acidity and buffer capacity of experimental samples are shown in the Table. The bottles were purged with nitrogen and stored in the darkness at 20 °C. The oxidation was performed by adding hydrogen peroxide (1.25 mM) at a concentration of 20 $mg \cdot L^{-1}$ of O_2eq to trigger the Fenton reaction (Elias and Waterhouzse 2010). Because this reaction requires hydrogen peroxide and metal ions at concentrations (< 0.2 mm) much lower than expected in all commercial wines (Clark *et al.* 2007) the occurrence of the Fenton reaction under our experimental conditions was guaranteed. Four oxidized (ox) samples were obtained by adding hydrogen peroxide to the Aglianico wine with the initial pH of 4.0 and to the H2T, H2M and H3C wines. Samples were monitored after 3, 7 and 18 days of incubation at 20 °C. All samples (model solutions and wines) were prepared in duplicate. On each replicate two analyses were performed as to have a datum from the mean of four values.

Table

Values of titratable acidity and buffer capacity of experimental wines

	titratable acidity ($g \cdot L^{-1}$ tartaric acid)	buffer capacity ($meqL \cdot pH^{-1}$)
pH 4.0	4.4 ± 0.0 C	56.3 ± 8.8 B
pH 3.2 H2T	8.7 ± 0.0 B	63.5 ± 11.2 B
pH 3.2 H3C	19.9 ± 0.1 A	165.0 ± 66.1 AB
pH3.2 H2M	20.0 ± 0.2 A	208.3 ± 58.9 A

Wines differ by the type of added acid: pH 4.0, pH 3.2 H2T, pH 3.2 H3Cand pH3.2 H2M. Different letters indicate statistical differences ($p < 0.05$). Capital letters are used to compare treated wines.

Reagents and standards: Solvents of HPLC-gradient grade and all other chemicals of analytical reagent grade were purchased from Sigma (Milan, Italy). The solutions were prepared in deionized water produced by a pure lab classic system (ElgaLabwater, Marlow, UK). For calibration curves malvidin-3-*O*-glucoside chloride was supplied by Extrasynthèse (Genay, France). For identification purposes, anthocyanin standards (delphinidin-3-*O*-glucoside chloride, malvidin-3-*O*-glucoside chloride, petunidin-3-*O*-glucoside chloride, peonidin-3-*O*-glucoside chloride, and cyaniding-3-*O*-glucoside chloride) were purchased from Extrasynthèse.

Methods. Base parameters: Base parameters were measured according to the OIV Compendium of International Methods of Wine and Must Analysis (2007). Buffering capacities were measured by adding NaOH 1.0 M into 100 mL of wine till the pH value increased by 1 unit.

High-performance liquid chromatography determination of anthocyanins: HPLC separation of anthocyanins was carried out according to the OIV compendium of international methods of analysis of wine and musts with some modifications (OIV 2007). 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. A Waters Spherisorb column (250 x 4.6 mm, 4 µm particles diameter) with pre-column was 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 mm, durapore membrane filters (Sigma Aldrich, Milan, Italy) into glass vials and immediately injected into the HPLC system. The HPLC solvents were: solvent a: water/formic acid/acetonitrile (87:10:3) v/v; solvent b: water/formic acid/acetonitrile (40:10:50) v/v. The gradient used was: zero-time conditions 94 % a and 6 % b, after 15 min the pumps were adjusted to 70 % a and 30 % b, at 30 min to 50 % a and 50 % b, at 35 min to 40 % a and 60 % b, at 41 min, end of analysis, to 94 % a and 6 % b. After a 10-min equilibrium period the next sample was injected. The flow rate was 0.80 mL·min^{-1}. 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^{-1} of malvidin-3-*O*-monoglucoside. All the analyses were conducted in duplicate on each experimental replicate.

High-performance liquid chromatography determination of acetaldehyde: Acetaldehyde was determined by using the method of Han *et al.* (2015). Briefly, wine sample aliquots (100 µL) were dispensed to a vial, followed by addition of 20 µL of freshly prepared 1120 mg·L^{-1} 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^{-1} 2,4-dinitrophenylhydrazine reagent (Aldrich chemistry). After mixing, the solution was allowed 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, 4 µm particles diameter) was used for separation. The chromatographic conditions were: sample injection volume, 50 µL; flow rate, 0.75 mL·min^{-1}; 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 derivatized acetaldehyde standard. All analyses were conducted through two experimental replicas and two analytical replicas.

Spectrophotometric analyses: Chromatic characteristics and spectrophotometric measures were determined by using a spectrophotometer (Jenway 7305 Spectrophotometer). Color intensity C.I., abs420, abs520, abs620 nm and hue were evaluated according to the OIV compendium of international methods of analysis of wine and musts (OIV 2017). Total anthocyanins, short polymeric pigments (SPP) and large polymeric pigments (SPP) were determined by the Harbertson-Adams assay (Harbertson *et al.* 2002). Briefly, pH changes allowed the evaluation of total anthocyanins while the large polymeric pigments (LPP) were obtained by combining analysis of supernatant obtained after protein precipitation using bovine serum albumin (SIGMA Life Science USA) with the bisulfite bleaching of pigments in wine. All analyses were conducted through two experimental replicas and two analytical replicas. The CIELAB parameters (L*, a*, b*) were determined by using the software Panorama (PANORAMA SOFTWARE UPGRADE PATH), following the recommendations of the Commission Internationale de L'Eclariage (CIE). Color differences (DE⁄ab) were calculated as the Euclidean distance between two points in the 3D space defined by L*, a*, and b*.

NMR spectroscopy: ^{1}H (700 MHz) and ^{13}C (175 MHz) NMR spectra were recorded on an Agilent INOVA spectrometer equipped with a cryoprobe. One-dimensional proton spectra of the samples solubilized in D_2O were recorded. Identification of analytes contained in wine samples was conducted by comparison with NMR data available in an online metabolome database.

Quantifications of analytes were carried out by adding 5 mL of methanol to each sample and by running ^{1}H NMR spectra with a d1 value of 7.0 sec in order to allow a complete relaxation of the observed nuclei to equilibrium. The area of the resonances of the metabolites to be quantified, selected on the basis of resolution, was measured by integration, divided by the number of the protons generating the signal, and finally converted into number of moles by comparison with the area of the methanol protons, for which the number of moles was known, according to the following equation:

$$m_x = \frac{MM_x}{MM_{std}} \times \frac{nH_{std}}{nH_x} \times \frac{A_x}{A_{std}} \times m_{std}$$

where m_x and m_{std} are the analyte and standard masses, respectively, expressed in grams; MM_x and MM_{std} are the analyte and standard molecular masses expressed as g·mol^{-1}; nH_x and nH_{std} are the analyte and standard number of protons generating the investigated NMR signals; A_x and A_{std} are the analyte and standard areas of the integrated NMR signals.

The Saliva Precipitation Index (SPI): Tannins reactive to salivary proteins, which represented astringent tannins, were measured by the Saliva Precipitation Index (SPI). The SPI method was performed as described in Rinaldi *et al.* (2014). Briefly, 50 µL of a sample of resting saliva, collected by different non-smoking volunteers, was mixed with 25 µL of wine solution (diluted 1:4 with HPLC grade water) and maintained at 37 °C for 5 min. Resting saliva after collection was centrifuged at 10,000 *g* for 10 min at 4 °C to remove any insoluble material. The

resulting supernatant was used as saliva for analysis, and was always kept in ice during manipulation to avoid enzymatic reactions. The binding between salivary proteins and tannins was stopped by centrifugation at 10,000 g for 10 min at 4 °C. The pellet represented the saliva-tannins bound fraction, while the supernatant contained the salivary proteins that had not reacted. Electrophoresis of salivary proteins in supernatant was performed with the commercial Experion Pro260 analysis kit (Biorad, Milano Italy). The SPI was calculated by the fluorescent signal of selected proteic bands of saliva before and after the binding reaction. The selected proteic bands are a protein at about 60 kDa that can be tentatively assigned to α-amylase, and a protein band at about 15 kDa which could be a basic PRP, according to their molecular weights. The mean percentage of bands reduction was quantified in $g \cdot L^{-1}$ of gallic acid equivalent (GAE). We used gallic acid because the percentage decrease of bands was correlated with the astringency associated to increasing concentration of tannin solutions, which in turn were quantified as gallic acid in Folin-Ciocalteau assay. As a consequence, the SPI (percentage of protein reduction) was expressed in gallic acid equivalent as previously reported (RINALDI et al., 2014).

Data analyses: All data are means of four values (2 experimental replicates x 2 analytical replicates). Analysis of variance was carried out on phenolic compound and sensory data. Fisher's Least Significant Differences (LSD) procedure was used to discriminate among the means of the variables. Differences of $p < 0.05$ were considered significant. The ANOVA analysis was performed using categorical variables on quantitative sensory data. Elaborations were carried out by means of xlstat software (addinsoft, xlstat 2017).

Results and Discussion

Wine aging is an oxidative process during which polyphenol oxidation depends on the Fe catalysis. The pH level affects this process, because the reduction potential of the oxygen and polyphenol couples is pH dependent, decreasing linearly by 59 mV per pH unit increase (Danilewicz 2014). As Fe(III) and Fe(II) form octahedral complexes with tartaric, malic and citric acid, and the reduction potential of the couple will depend on the relative affinity of ligands for the two oxidation states, it is clear that wine organic acids do not just provide an acidic environment but also determine the catalytic activity of the Fe redox couple. This is the reason why Danilewicz (2014) stated that not only should pH be considered, but metal ligands mimicking those present in the system being studied should also be taken into account. After the addition of hydrogen peroxide, a lower production of acetaldehyde was detected in H3C compared to all other wines (Fig. 1). This result confirmed previous studies suggesting that the best metal chelant among the investigated organic acids is citric acid (Timberlake 1964).

As acetaldehyde is a high reactive compound responsible for numerous reactions stabilizing the wine color (Timberlake and Bridle 1976), it is likely that changes in

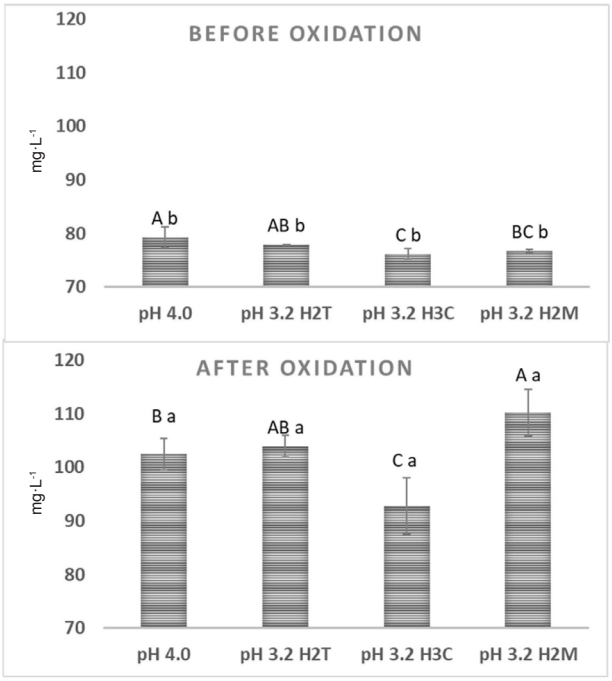

Fig. 1: Effect of organic acids and oxidation on acetaldehyde content of 'Aglianico' wine. Different letters indicate statistical differences ($p < 0.05$). Capital letters are used to compare treated wines at the same time and small letters are used to compare the same treated wine before and after oxidation.

global chromatic characteristics observed after oxidation of wines added with different organic acids are due to the involvement of this high reactive aldehyde (Fig. 2). In particular, a great variation in DE, a key parameter to understand the possible effect of a given treatment on human visual perception, was detected. DE has been calculated by considering the color coordinates of the wines before and after the addition of a strong oxidant. According to Mokrzycki and Tatol (2011) the following ΔE classification can be considered to understand if two samples show a visible difference: 0-0.5 (not noticeable), 0.5-1.5 (slightly noticeable), 1.5-3.0 (noticeable), 3.0-6.0 (well visible) and > 6.0 (great). In all wines with a pH level of 3.2, the DE values calculated after oxidation were superior to 1. This means that a standard observer can hardly notice a color difference that is more evident for the H3C sample after the addition of hydrogen peroxide. As expected, an increase of red color (Abs 520 nm) as the pH decreased was observed due to the dominance of the flavylium form of anthocyanins. The higher values of hue (CIELab coordinates) of H3C sample confirmed this trend (Fig. 2). This increase has been previously observed during wine aging and after treatment of red wine with hydrogen peroxide and it is due to the formation of orange-yellow pigments and orange pigments resulting from oxidation of compounds such as flavanols or from reactions of anthocyanin pigments (Gambuti *et al.* 2017, Picariello *et al.* 2018). The involvement of native pigments in numerous reactions changing their chromatic characteristics is consistent with the decrease of total and native anthocyanins detected after the oxidation (Figs 3 and 4), and with similar effects observed in red wines during micro oxygenation (Gambuti *et al.* 2015).

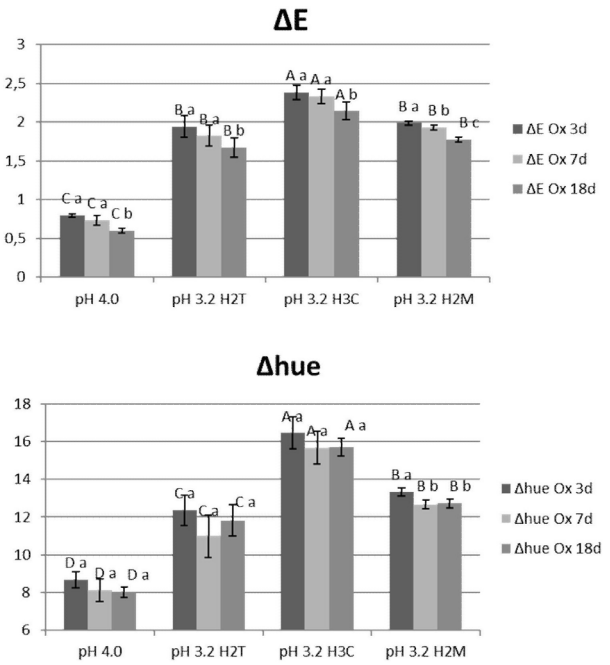

Fig. 2: Changes in global chromatic characteristics (CIElab coordinates) after treatment of samples with hydrogen peroxide (ox). ΔE is the three dimensional color distance between wines before and after oxidation. Different letters indicate statistical differences ($p < 0.05$). Capital letters are used to compare treated wines at the same time and small letters are used to compare the same treated wine before and after oxidation.

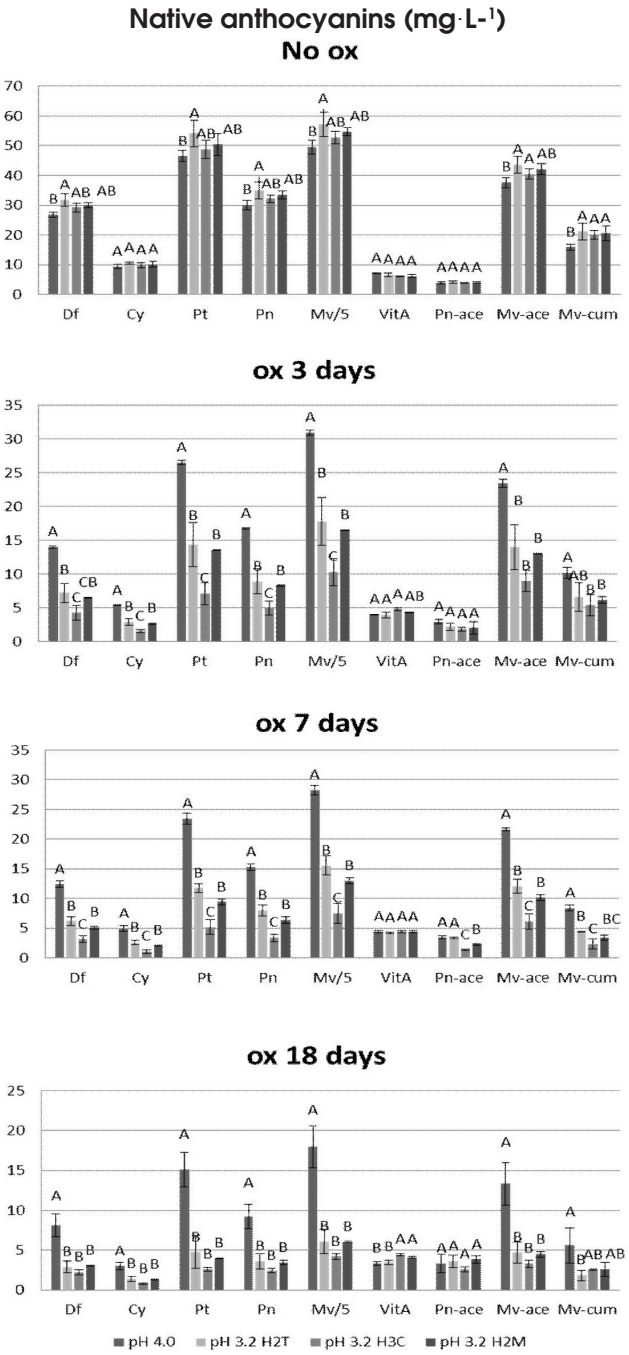

Fig. 3: Effect of organic acids and oxidation on individual anthocyanins of Aglianico wine. Df = delphinidin-3-*O*-glucoside, Cy = cyanidin-3-*O*-monoglucoside, Pt = petunidin-3-*O*-monoglucoside, Pn = peonidin-3-*O*-monoglucoside, Mv = malvidin-3-*O*-glucoside, VitA = vitisin A, Pn-ace = peonidin-3-*O*-(6^{II}-acetyl)-glucoside, Mv-ace = malvidin-3-*O*-(6^{II}-acetyl)-glucoside, Mv-cum = malvidin-3-*O*-(6^{II}-coumaroyl)-glucoside. Different letters indicate statistical differences ($p < 0.05$). Capital letters are used to compare treated wines at the same time and small letters are used to compare the same treated wine before and after oxidation.

Concerning each anthocyanin it is remarkable that, apart from the VitA, all monomeric anthocyanins decreased (Fig. 3). The lower the pH, the higher the loss of native anthocyanins. This could be surprising considering that oxidation is favored at higher pH but, it is known that acidic conditions favor the protonated aldehyde, which is a powerful electrophile that reacting with the resorcinol-type A ring of the flavonoid structure (also of anthocyanins) ultimately leads to polymers. In addition, flavanol-anthocyanin polymers are produced in higher amounts in the most acidic solutions (Fulcrand *et al.* 2006). Therefore, more than oxidized, monomeric anthocyanins are preferentially involved in polymerization reactions at lower pH. The higher content of total anthocyanins, a parameter which includes polymeric pigments, detected at lower pH (Fig. 4) confirms this hypothesis. The decrease of all native anthocyanins was more significant for H3C sample thus revealing a dependence on the type of organic acids present in wines. Two reasons could explain the observed behavior: a different ability of tartaric, malic and citric acids to inhibit the Fenton reaction and/or the occurrence of specific reactions between organic acids degradation byproducts and anthocyanins. In a previous experiment conducted on model solution, Grant-Preece *et al.* (2017) showed that, when the oxidation is triggered by irradiation, dissolved oxygen was consumed at a rate that was dependent on the specific organic acid present (tartaric, malic or citric acid). Also Danilewicz (2014) showed that Fe (II) was rapidly oxidized in air saturated model wine with tartaric acid (33.3 mmol·L^{-1}) and Cu (II), while it was not oxidized in an equivalent solution containing acetic acid (66.6 mmol·L^{-1}) instead of tartaric acid. The author hypothesized a stronger interaction between tartrate anions and Fe(III) compared to that between acetate anions and Fe(III). The evidence that a lower production of acetaldehyde was obtained in H3C (Fig. 1) and the fact that this aldehyde is the main product of the Fenton reaction in an alcoholic solution suggested

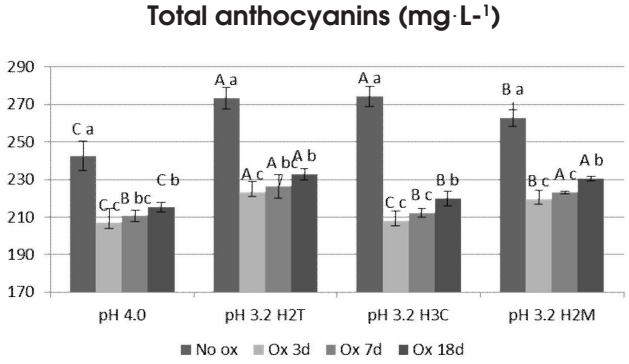

Fig. 4: Effect of organic acids and oxidation on total anthocyanins of 'Aglianico' wine. Different letters indicate statistical differences ($p < 0.05$). Capital letters are used to compare treated wines at the same time and small letters are used to compare the same treated wine before and after oxidation.

that the oxidation reaction was more inhibited in H3C. The NMR-based analyses of all wine samples brought to light the occurrence of syringic acid, among other wine metabolites. This is a key compound, since it is a degradation product of malvidin-3-*O*-monoglucoside (Lopes *et al.* 2007, Gambuti *et al.* 2017), and thus an insightful marker of the anthocyanin susceptibility to oxidation. Under our experimental conditions, after the addition of hydrogen peroxide and by means of quantitative NMR-based analysis, we found out that the quantity of syringic acid was lower in H3C (10.9 ± 0.2 mg·L^{-1}) with respect to either H2T (11.1 ± 0.5 mg·L^{-1}) or H2M (12.6 ± 0.2 mg·L^{-1}).

The higher decrease in H3C of native anthocyanins should be instead explained by considering that acetoacetic acid is one of the carbonyl degradation products of citric acid after oxidation (Grant-Preece *et al.* 2017) and that it could give pyranoanthocyanins by reacting with native anthocyanins.

In addition to and in agreement with our results on chromatic characteristics of H3C wines, this kind of pigment is supposed to contribute to the orange hues of red wines observed during maturation. However, no presence of this pyranoanthocyanin was detected by NMR analysis, but it is not ruled out its occurrence at concentrations below those detectable by this analytical technique. On the basis of the obtained results, it is clear that changes in chromatic characteristics and pigments concentrations after oxidation are the results of two phenomena: the presence of different concentrations of acetaldehyde and probably other reactants such as acetoacetic acid consuming native anthocyanins in polymerization reactions and the degradation of anthocyanins by high reactive radicals produced by the Fenton reaction. Under our experimental conditions, these last reactions were dominant.

As previously shown (Rinaldi *et al.* 2012), pH strongly affects the reactivity of tannins towards saliva with important implication for the wine astringency and also in this experiment passing from pH 4.0 to 3.2 an increase of SPI was detected (Fig. 5). A significant effect of organic acids in the medium on this important binding reaction was even detected. Sowalsky and Noble (1998) observed an effect of the nature of the organic acids on the astringency perception, but this is the first time that an effect on the reactivity towards the two saliva proteins used to determine SPI, glycosylated PRP protein and alpha-amylase was observed. Therefore, the binding seems to be affected by the nature of the organic acid. However, this is not surprising because saliva is a proteic mixture and buffers can alter protein conformation especially when metals are involved in the binding or act as catalysts in redox reactions (Arnold and Zhang 1994). About the possible consequences on astringency perception it is necessary to underline that Obreque-Slier *et al.* (2016) demonstrated that the pH level of the wine/saliva mixture in the mouth after drinking wine equals that of wine, suggesting that during the wine-tasting the buffering capacity of wine prevails over that of saliva. Thus, it is reasonable to hypothesize that the differences in terms of buffer capacity observed in samples added with different acids (Table) strongly affect wine astringency.

According to previous reports (Picariello *et al.* 2017), after the oxidation of all samples a decrease of reactivity towards saliva proteins was observed (Fig. 5). The decrease was higher in samples at higher pH. This can be

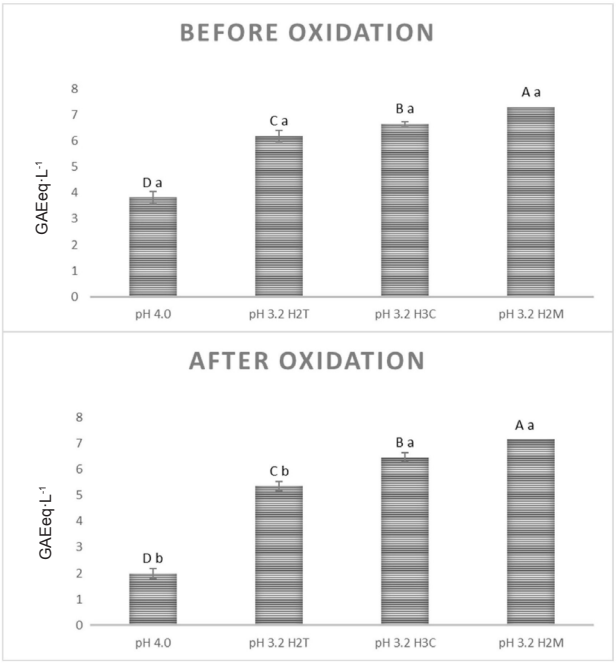

Fig. 5: Effect of organic acids and oxidation on SPI (saliva precipitation index) of 'Aglianico' wine. Different letters indicate statistical differences ($p < 0.05$). Capital letters are used to compare treated wines at the same time and small letters are used to compare the same treated wine before and after oxidation.

related to the higher formation of quinones occurring at higher pH. It is known that quinones are involved in the first step of chemical oxidation of wine, thus contributing to the formation of polymeric pigments and tannins (Gambuti *et al.* 2015). Results also indicate that in samples richer in tartaric acid, a greater variation in reactivity towards saliva proteins with oxidative aging occurred, suggesting that for these samples a greater decrease of astringency is to be expected.

Conclusion

Results obtained in this study highlight the important role of the nature of organic acid for the stability of anthocyanins and reactivity of tannins towards saliva during oxidation. Red wines with added citric acid are more subjected to color loss, but are more stable to oxidation, while malic acid determines the occurrence of the Fenton reaction to a higher extent with an increase of oxidative by-products. Wines at pH 3.2 containing mainly tartaric acid were the less reactive towards saliva proteins indicating that, especially for varieties rich in astringent tannins, it is important to shift grape and wine acidic profile toward this acid and limit the amount of malic acid. Given the wide changes in tartaric/malic acid ratio with climate, it is important to act in vineyard, as well as in winery, to decrease the malic/tartaric acid ratio before bottling, otherwise the production of a less long-lived wine will not be prevented. As white wines are more sensitive to oxidation, further studies on red wines are needed to consider the effect of these acids on oxidative by-products on white wines as well.

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