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Effect of Micro-oxygenation on Color and Anthocyanin-Related Compounds of Wines with Different Phenolic Contents

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Several factors may affect the results obtained when micro-oxygenation is applied to red wines, the most important being the moment of application, the doses of oxygen, and the wine phenolic characteristics. In this study, three red wines, made from *Vitis vinifera* var. Monastrell (2005 vintage) and with different phenolic characteristics, were micro-oxygenated to determine as to how this technique affected the formation of new pigments in the wines and their chromatic characteristics. The results indicated that the different wines were differently affected by micro-oxygenation. In general, the micro-oxygenated wines had a higher percentage of new anthocyanin-derived pigments, being that this formation is more favored in the wines with the highest total phenol content. These compounds, in turn, significantly increased the wine color intensity. The wine with the lowest phenolic content was less influenced by micro-oxygenation, and the observed evolution in the degree of polymerization of tannins suggested that it might have suffered overoxygenation.

KEYWORDS: Wine; color; anthocyanins; anthocyanin-derived compounds; micro-oxygenation

INTRODUCTION

Phenolic compounds are responsible for many of the organoleptic characteristics of wines. Among them, anthocyanins are responsible for the color of red wine, while their interactions with other compounds largely determine the color changes observed in maturing wines. The wine phenolic content depends on the grape characteristics and on the winemaking process. Factors such as phenolic maturity of the grapes, length of maceration, frequency of pumping over, etc. determine the final wine phenolic content (*1–3*).

The importance of oxygen in the evolution of wine color has been studied for many years, both as regards its role in polyphenol oxidation (*4–7*) and as a promoter of the formation on new anthocyanin-derived compounds (*5, 8–11*). Indeed, one way of manipulating the phenolic structure of a wine is to use the micro-oxygenation (MO) technique, which relies on the formation of microbubbles through the injection of gaseous oxygen into the wine using a microdiffuser (*12*). These bubbles rise through the wine, dissolving as they travel to the surface. Empirical results have indicated that MO benefits include the stabilization of wine color, the softening of tannins, and the lessening of vegetative aromas (*13, 14*). Reactions involving wine polyphenols are the key to these processes, which include changes in proanthocyanin chain length and the consequent effect on wine astringency and the linking of anthocyanins and tannins to form more stably colored forms. Dissolved oxygen leads to the formation of acetaldehyde that, in turn, reacts with flavanols and anthocyanins to induce the formation of a very reactive carbocation that quickly reacts with another flavanol molecule or with anthocyanin, producing ethyl bridge-linked compounds (*15, 16*). They are unstable (*17*) and undergo reactions that lead to the formation of new compounds such as flavanyl pyranoanthocyanins. These end products are more stable and colored than the original compounds (*11*). Moreover, incorporation of anthocyanin into tannin structures also may lead to a decrease in astringency (*18*).

Since its commercial adoption, MO has become common practice and is now used worldwide, although most of the available information is from empirical results. Only a few scientific publications can be found that are related to the effects of MO (*8, 19–23*). Several factors may affect the final results obtained by MO, the most important being the moment of application, doses of oxygen, and wine characteristics. Wines have marked differences in their respective abilities to consume oxygen. As a general rule, this facility is directly related to their relative concentration of polyphenols since phenolic compounds

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Table 1. Evolution of Some Physicochemical Parameters during MO of Monastrell Wines[a]

	t_0	t_1		t_2		t_3	
	W1	W1 (C)	W1 (MO)	W1 (C)	W1 (MO)	W1 (C)	W1 (MO)
pH	3.72	3.78	3.76	3.85	3.83	3.83	3.79*
titratable acidity[b]	5.87	5.76	5.35*	5.38	5.17*	5.06	4.90*
volatile acidity[c]	0.39	0.40	0.41	0.43	0.47	0.42	0.34
free SO_2 (mg/L)	25	26	22*	37	37	22	14*
total SO_2 (mg/L)	39	32	28*	50	49	48	46
acetaldehyde (mg/L)	22.2	13.5	12.8	15.1	16.6	21.3	32.6*
	W2	W2 (C)	W2 (MO)	W2 (C)	W2 (MO)	W2 (C)	W2 (MO)
pH	3.66	3.71	3.67	4.00	4.00	3.83	3.80
titratable acidity[b]	6.31	5.83	5.96	5.60	5.73*	5.08	4.90
volatile acidity[c]	0.33	0.33	0.34	0.29	0.32	0.37	0.33
free SO_2 (mg/L)	25	31	22*	38	33	16	11*
total SO_2 (mg/L)	41	35	33	61	61	51	35*
acetaldehyde (mg/L)	29.1	23.0	24.7	32.2	23.4*	41.4	45.2
	W3	W3 (C)	W3 (MO)	W3 (C)	W3 (MO)	W3 (C)	W3 (MO)
pH	3.63	3.66	3.66	4.01	4.02	3.77	3.78
titratable acidity[b]	6.45	5.70	5.56	5.66	5.27*	5.09	4.89*
volatile acidity[c]	0.40	0.37	0.38	0.33	0.37	0.37	0.39*
free SO_2 (mg/L)	27	32	25*	26	29	16	11*
total SO_2 (mg/L)	48	44	35	64	64	61	38*
acetaldehyde (mg/L)	32.3	25.6	23.2	28.7	28.4	22.3	35.0*

[a] An asterisk indicates significant differences between control and micro-oxygenated wines for each time considered. [b] Expressed as g/L of tartaric acid. [c] Expressed as g/L of acetic acid.

are the main consumers of oxygen (60%) together with ethanol (20%) and SO_2 (12%) (*22*). In our first studies, we tested the use of different doses of oxygen and the effect of the moment of application on wine chromatic characteristics (*23, 24*). Here, we report the results of a commercial scale MO experiment where the influence of applying oxygen to three wines made from Monastrell grapes of different phenolic contents was investigated.

MATERIALS AND METHODS

Wine Samples. Three different red wines, made from *Vitis vinifera* var. Monastrell (2005 vintage), differing in their total phenol content, were used for the experiment (W1, W2, and W3). Each wine was distributed into four 17 500 L tanks, and the MO experiment was carried out in triplicate, with a control (C) and three MO tanks for each type of wine. The height of each tank was 4.5 m, enough for the complete dissolution of oxygen microbubbles into the wine. MO began just after alcoholic fermentation had finished (November 7, 2005), applying a oxygen dose of 10 mL/L/month. Malolactic fermentation (MLF) occurred spontaneously. When MLF started (detected by the decrease in malic acid), the MO system was stopped (November 30 for W1 and December 20 for W2 and W3). During MLF, the bacteria consume the acetaldehyde produced. They are capable of metabolizing acetaldehyde, even the acetaldehyde bound to sulfur dioxide (*25*); therefore, any excess of acetaldehyde produced during the MO process will disappear. When MLF was complete for all wines, SO_2 was added to leave the wines with a free SO_2 content close to 30 mg/L, and MO was resumed (January 19, 2006). At this moment, the oxygen supply was reduced to avoid accumulation of acetaldehyde (3 mL/L/month). After 2 months, the oxygen supply was reduced again (1.5 mL/L/month) before it was completely stopped. The wine was analyzed immediately before the MO system began (t_0), at the beginning of MLF (t_1), when MO began again after MLF (t_2), and 15 days after the MO system was stopped (t_3) since wines take 8−10 days to absorb the oxygen, depending on temperature and phenolic composition of the wines (*26*). The MO system (Agrovin S.A., Alcazar de San Juan, Spain) was comprised of an oxygen cylinder (food grade) and a dosing chamber that allowed the doses of oxygen flowing through nine different microdiffusers to be programmed.

General Determinations. pH and titratable acidity were measured using an automatic titrator (Metrohm, Herisau, Switzerland). Volatile acidity was determined according to the OIV Official Methods (*27*). Acetaldehyde and malic acid concentration were determined using enzymatic test kits from Roche Boehringer, Mannheim, Germany. The SO_2 concentration (free and total) was measured iodometrically by the Ripper procedure (*28*) modified to detect the end point potentiometrically with an automatic titrator (Metrohm, Herisau, Switzerland).

Identification and Quantification of Anthocyanins. Wines were analyzed by direct injection of the samples previously filtered through a 45 μm nylon filter (Teknokroma, Barcelona, Spain). The HPLC analyses were performed on a Waters 2690 liquid chromatograph (Waters, Milford, MA), equipped with a Waters 996 diode array detector and a 250 mm × 4 mm i.d., 5 μm particle size Lichrocart RP-18 column (Merck, Darmstadt, Germany), using as solvents 4.5% aqueous formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.8 mL/min. Elution was performed with a gradient starting with 10% B to reach 14.5% B at 30 min, 15.2% B at 45 min, 18% B at 60 min, 25% B at 100 min, and 25−100% B in 30 min. Chromatograms were recorded at 520 nm.

Compounds were identified by comparing their UV spectra recorded with the diode array detector and those reported in the literature (*11, 29*). In addition, an HPLC-MS analysis was conducted to confirm each peak identity. An LC-MSD-Trap VL-01036 liquid chromatograph-ion trap mass detector (Agilent Technologies, Waldbronn, Germany) equipped with an electrospray ionization (ESI) system was used. Elution was performed with the HPLC analysis conditions detailed previously. The heated capillary and voltage were maintained at 350 °C and 4 kV, respectively. Mass scans (MS) were measured from m/z 300−1100. Mass spectrometry data were acquired in the positive ionization mode. Anthocyanins and anthocyanin-derived compounds were quantified at 520 nm as malvidin-3-glucoside, using malvidin-3-glucoside chloride as an external standard (Extrasynthèse, Genay, France).

Color Determinations in Wines. Absorbance measurements were made in a Helios Alpha spectrophotometer (Thermo Electron Corp., Waltham, MA) with 0.1, 0.2, or 1 cm path length glass cells. Samples were previously centrifuged, and the pH was adjusted to 3.6. CIELab parameters were determined by measuring the transmittance of the wine every 10 nm from 380 to 770 nm, using a D_{65} illuminant and a 10° observer. The L^* (measure of lightness), C^* (measure of chroma), and H^* (hue angle) parameters were determined. Color intensity (CI), calculated as the sum of absorbance at 620, 520, and 420 nm, and percentages of red (%R), yellow (%Y), and blue (%B) color were determined according to Glories (*30*); the hue was calculated as the ratio between absorbance at 420 nm and absorbance at 520 nm (*31*). Total phenols (abs_{280}) were calculated according to Ribéreau Gayon et al. (*32*). Wine color (WC), total color of pigments (WCP), and color due to derivatives resistant to SO_2 bleaching (CDR_{SO2}) were determined using a method adapted from that described by Levengood and Boulton (*33*), with all measurements made at 520 nm. These parameters were calculated as follows.

WC. Twenty microliters of 10% acetaldehyde solution was added to 2 mL of a wine sample in a 10 mm plastic cuvette to release any anthocyanins involved in bisulfite adducts. After 45 min, the sample was placed in a 1 mm cuvette. The reading was corrected to 10 mm path length by multiplying by 10.

WCP. A total of 0.5 mL of wine was diluted in 20 mL of 0.1 N HCl. Absorbance was measured in a 10 mm cuvette after 30 min to ensure complete conversion of anthocyanins into the flavylium form. The reading was corrected for dilution.

CDR_{SO_2}. A total of 160 μL of a 5% SO_2 solution (10% potassium metabisulfite solution) was added to 2 mL of the wine sample. After 1 min, the sample was placed in a 2 mm cuvette. The reading was corrected to 10 mm path length by multiplying by 5.

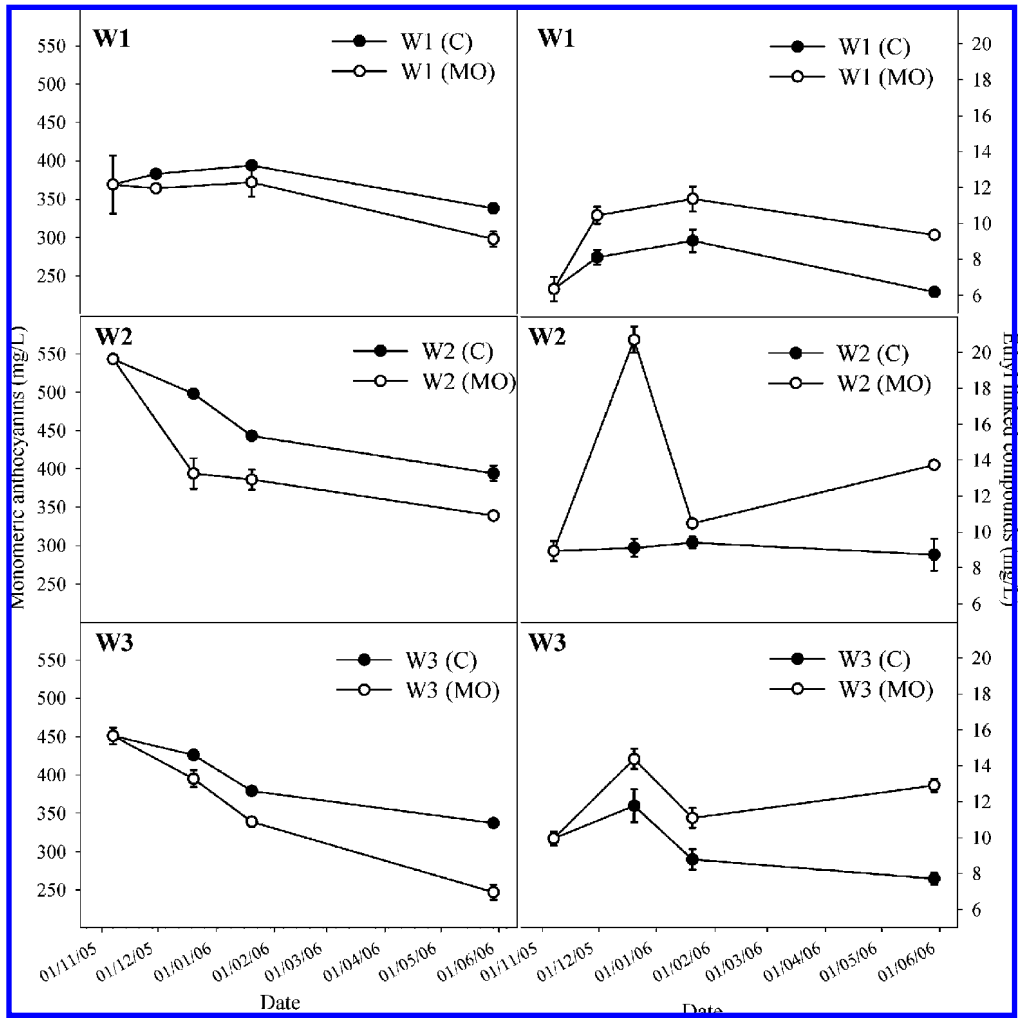

Figure 1. Development of monomeric anthocyanins and ethyl-linked compounds in W1, W2, and W3 wines (±SD). Each point corresponds to t_0, t_1, t_2, and t_3.

Determination of Total Tannins. The wine tannin concentration was evaluated using a protein precipitation assay, with bovine serum albumin (BSA). Sample preparation and a protein precipitation assay were conducted according to methods described by Harbertson et al. (*34*). For quantification, results were compared with a (+)-catechin standard and reported as mg/L (+)-catechin equivalents.

Analysis of Proanthocyanidins. Proanthocyanidin composition was determinated by HPLC-DAD-MS after purification and concentration of the wine extract and acid catalysis in the presence of excess phloroglucinol. The results of phloroglucinolysis provided information on the proanthocyanidin subunit composition (terminal and extension unit concentrations) and the mean degree of polymerization (mDP). The analyses were carried out in triplicate.

The phloroglucinolysis protocol, described by Drinkine et al. (*35*), included a purification step using C_{18} solid-phase extraction (SPE). Water (15 mL) was added to 5 mL of wine, and the sample was passed through a preconditioned cartridge. The retained compounds were eluted with 50 mL of methanol with a flow rate of 2 mL/min. This fraction was dried under reduced pressure and then dissolved in 2 mL of methanol.

The second step was the phloroglucinolysis reaction. A solution of 0.2 N HCl in methanol, containing 100 g/L phloroglucinol and 20 g/L ascorbic acid, was prepared. A total of 100 μL of the wine sample was reacted with 100 μL of the phloroglucinol reagent at 50 °C for 20 min, and then 1 mL of 80 mM aqueous sodium acetate was added to stop the reaction.

The extension and terminal units resulting from phloroglucinolysis (catechin, epicatechin, (epi)catechin gallate, and (epi)gallocatechin) were determined by LC-MS. LC-MS analyses were performed on a Micromass Platform II simple quadruple mass spectrometer (Micromass-Beckman, Roissy Charles-de-Gaulle, France) equipped with an electrospray ion source. The mass spectrometer was operated in negative-ion mode. The source temperature was 120 °C, the capillary voltage was set at 3.5 kV, and the cone voltage was −30 V. HPLC separations were performed on a Hewlett-Packard 1100 series (Agilent, Massy, France) instrument including a pump module and a UV detector. Both systems were operated using Masslynx 3.4 software. The absorbance was recorded at 280 nm, and mass spectra were recorded from 200 to 1200 amu.

The elution conditions were wateras follows: acetic acid (99:1; v/v) as solvent A, methyl alcohol as solvent B, and the following elution gradient: from 5 to 15% B in 5 min, 15% B for 2 min, from 15 to 20% B in 3 min, from 20 to 50% B in 10 min, from 50 to 100% B in 2 min, and from 100 to 5% B in 2 min, with a flow of 1 mL/min. The column used was a reversed-phase 4.6 mm × 100 mm i.d., 3.5 μm packing C_{18} Waters Xterra protected with a guard column of the same material (Agilent, Saint Quentin-en-Yvelines, France).

Statistical Analysis. Significant differences between wines and for each variable were assessed with analysis of variance (ANOVA). This statistical analysis, together with a cluster analysis and a principal components analysis, was performed using Statgraphics 5.1 (Statistical Graphics Corporation, Rockville, MD).

RESULTS AND DISCUSSION

When comparing the initial composition of the studied wines (Table S1 in the Supporting Information and initial point in all figures), W1 and W2 showed a very similar phenolic content (abs_{280} of 52 and 58, respectively), while the highest phenolic

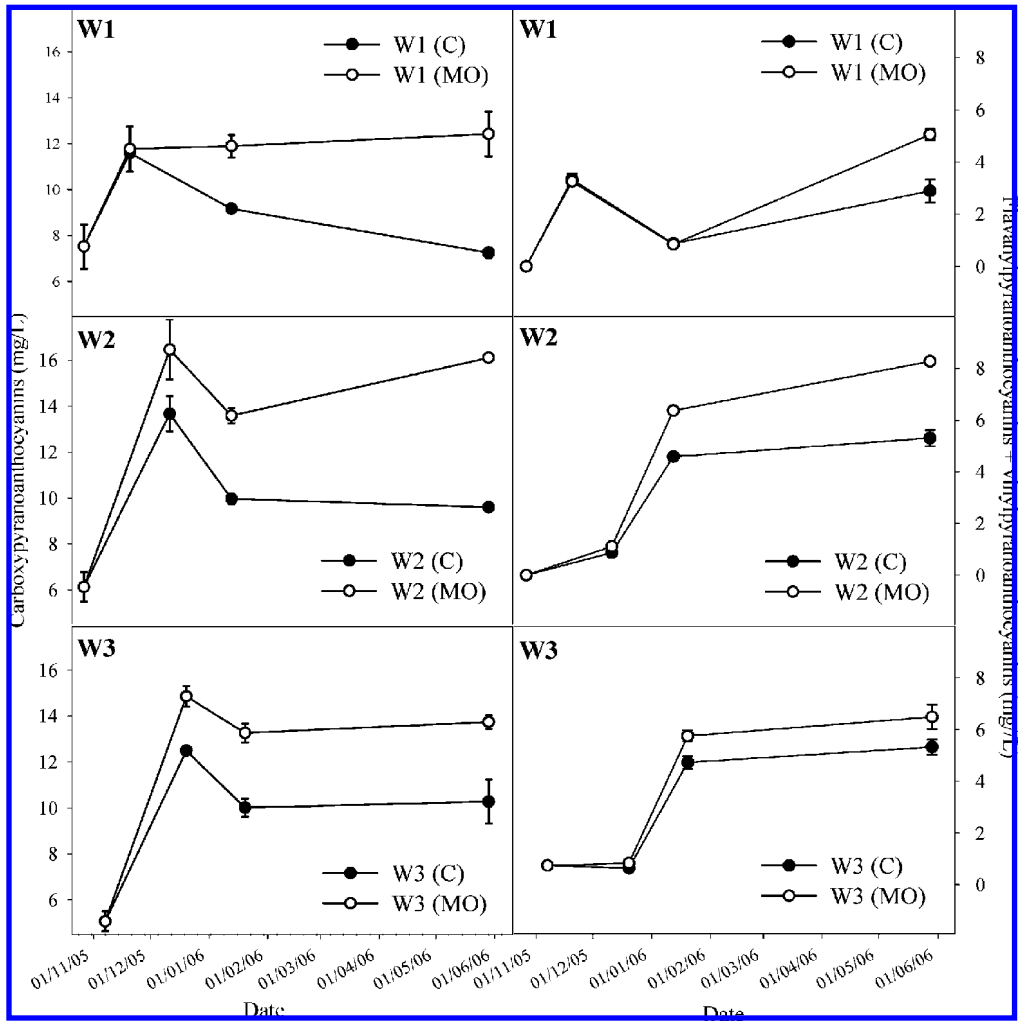

Figure 2. Development of carboxypyranoanthocyanins and sum of flavanyl- and vinylpyranoanthocyanins in W1, W2, and W3 wines (±SD). Each point corresponds to t_0, t_1, t_2, and t_3.

content was found in W3 (abs_{280} of 76), mainly due to its high tannin content. Although tannins and color density were higher in W3, monomeric anthocyanins and WCP showed higher values in W2, a wine that showed a low degree of anthocyanin polymerization (CRD_{SO_2}). Color percentages also differed between the wines. W1 presented a higher yellow percentage and lower red percentage. W3 was the darkest wine, with the highest blue percentage. The tannin characterization showed that the percentages of the different flavanol units also differed. The profile of W1, with the highest percentage of epicatechin and epigallocatechin, indicated a high percentage of skin tannins (*18, 36*), whereas the lowest percentage of epigallocatechin and relatively high percentage of epicatechin gallate in W3 were according to the longer maceration period used during the elaboration of W3, with seed tannins contributing significantly to the tannin fraction. The profile found in W2 indicates an intermediate situation, the maceration time being intermediate between W1 and W3.

As seen in **Table 1**, there was little effect of MO on pH, and small differences were observed in titratable acidity after MLF (t_2) and at the end of the experiment (t_3). With regard to concerns about potential microbial spoilage, the volatile acidity did not increase during the studied period. When the MO application was finished, a higher concentration of acetaldehyde was detected in the W1 and W3 MO wines, as compared to their control counterparts, and similar quantities of acetaldehyde were found in W2 (C) and W2 (MO). Excessive oxidation may result in increased levels of acetaldehyde, a compound that at sensory threshold levels adversely affects wine flavor and aroma. Sensory detection limits for red wines are typically in the range of 40−100 ppm (*37, 38*). Our results showed that, even in wines containing the highest content of acetaldehyde, its sensory detection limit barely was reached.

MO promoted a slightly lower content of free SO_2 in the wines, but differences were small. Total SO_2 was also lower in W2 and W3 MO wines. Boulet and Moutounet (*12*) reported no effect of MO on SO_2 content, whereas Pérez-Magariño et al. (*39*) reported small decreases due to MO, results that are similar to our findings. The results of Tao et al. (*21*) showed that SO_2 had a moderating effect on the interaction of oxygen with wine polyphenols since it has the ability to reduce oxidized polyphenols and to remove peroxide. The levels of SO_2 in MO wine affect the rate of development of wine polyphenol chemistry, including the formation of polymeric pigments and changes in tannin structure, affecting wine astringency.

Anthocyanins and Derived Compounds. The predominant compounds detected in HPLC analyses were the monoglucosides of malvidin, petunidin, delphinidin, peonidin, and cyanidin and their respective acetyl and coumaryl derivatives. The combined concentration of all these compounds diminished with time (**Figure 1**) the decrease being larger in the MO wines, as also found by Atanasova et al. (*8*), a decrease that might be explained by the various reactions where anthocyanins are involved, including degradation or polymerization reactions. The most

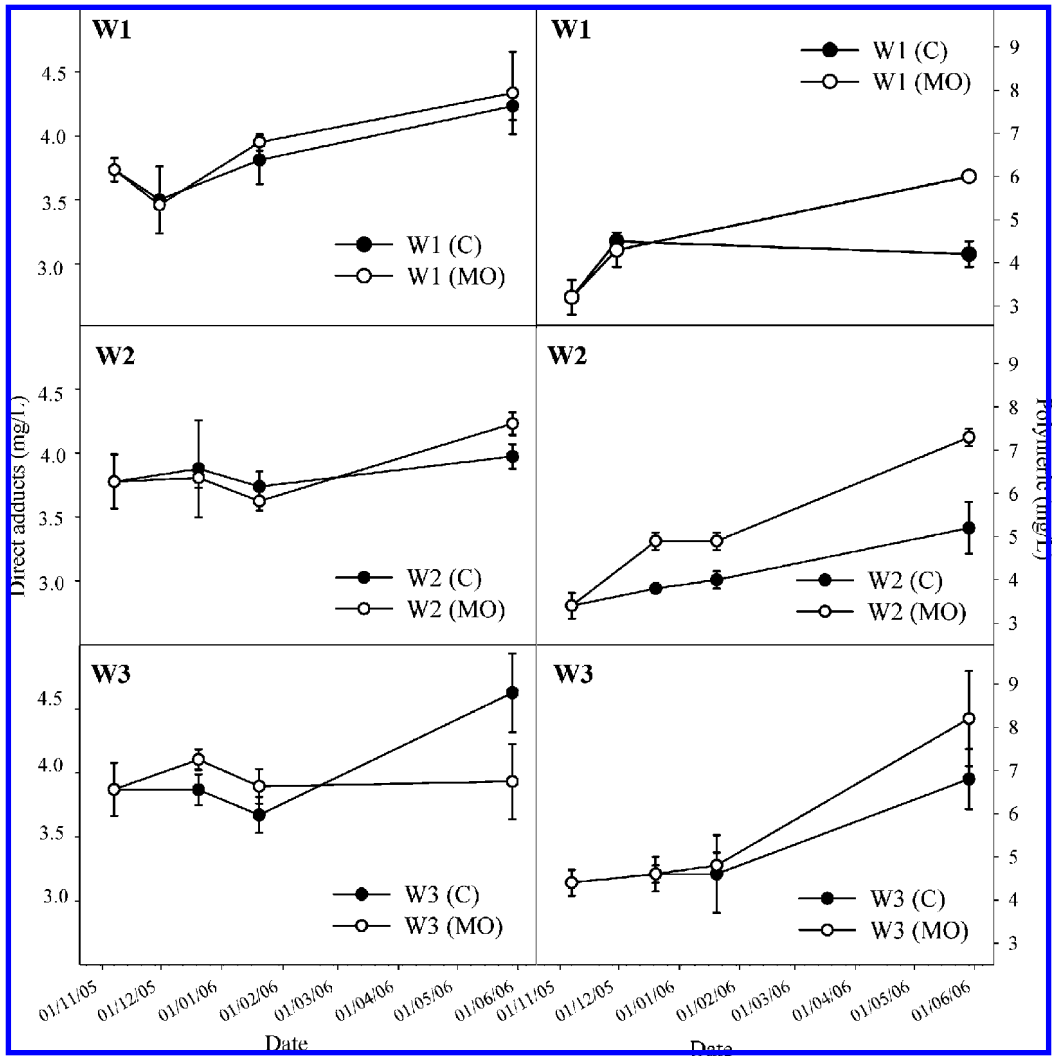

Figure 3. Development of direct anthocyanin-flavanol adducts and polymeric compounds in W1, W2, and W3 wines (±SD). Each point corresponds to t_0, t_1, t_2, and t_3.

significant decrease was detected between t_0 and t_2, except in W1, whose concentrations barely changed during this period. This was probably due to the fact that W1 wines were MO for 17 days before MLF started, whereas W2 and W3 were MO for 44 days since the starting of MLF was delayed in these wines.

Acetaldehyde-mediated condensation between anthocyanin-3-glucoside and (epi)catechin leads to ethyl bridge-linked compounds. Three possible isomers were elucidated as well as another compound in which the flavanyl moiety is a dimer. Throughout the experiment, the concentration of ethyl-linked compounds was higher in the MO wines (**Figure 1**). These are compounds with a purple color, less sensitive to bleaching by SO_2 and pH than monomeric anthocyanins, and their formation is favored by oxygen (*8, 40*). A very large increase was observed in W2 (MO) from t_0 to t_1, after which it fell sharply. These compounds are unstable and have a tendency to increase in size in the presence of available acetaldehyde. The behavior is in accordance with their reactivity; they are rapidly formed, but also, they can be rapidly broken down, releasing ethyl-flavanol units that, in turn, may react again with anthocyanins or dimers, giving more condensed products or even polymers (*11*).

Pyroanthocyanins are compounds formed when a pyran ring is introduced between the C4 and the hydroxyl group attached to C5 in the anthocyanin molecule. Some compounds result from the addition of anthocyanin and pyruvic acid (carboxypyranoanthocyanins). Several of these compounds were detected in our samples: petunidin 3-glucoside pyruvate, vitisin A (malvidin 3-(glucoside)pyruvate), acetyl vitisin A (malvidin 3-(acetylglucoside)pyruvate), and coumaryl vitisin A (malvidin 3-(coumarylglucoside)pyruvate). Another pyranoanthocyanin resulting from the cycloaddition of acetaldehyde to malvidin 3-glucoside, and referred to as vitisin B, also was found, and its quantification was included with the carboxypyranoanthocyanins. Pyranoanthocyanins are considered to be important compounds concerning the color of red wine since the cycloaddition process seems to strongly increase the stability of the products, and, in this way, vitisin A has been reported as being more stable than malvidin 3-glucoside or ethyl-linked compounds and more resistant to oxidation (*41*). The sum of carboxypyranoanthocyanins and vitisin B increased from t_0 to t_1 (**Figure 2**), with MO wines increasing more (except for W1). At the end of the experiment, carboxypyranoanthocyanins showed lower concentrations in the control wines than in the MO wines, the greatest increases being detected in W2. Other authors found a strong decrease in vitisin A and related compounds during the first year of wine storage, after which the concentration remained relatively constant (*42–44*). Such a decrease, observed mainly in our study between t_1 and t_2, was ascribed to their incorporation into polymeric compounds (*42*). The concentration of carboxypyranoanthocyanins in wines is a result of a balance between the formation reactions and their incorporation in the polymeric

compounds. The formation of vitisin A and related compounds requires the presence of free monoglucosides and pyruvic acid. Oxygen or reactive oxygen species are also necessary for the reaction to proceed since all cycloaddition pathways require an oxidation step to recover the flavylium moiety within the final structures (*45–47*). Therefore, the MO process seemed to enhance the formation of vitisin-type compounds by providing oxygen. This result is contrary to that of Atasanova et al. (*8*), who stated that the addition of pyruvic acid to anthocyanins is not influenced by oxygen. W2, with a higher anthocyanin monoglucoside content (as quantified by HPLC), was the wine showing the highest final concentration of carboxypyranoanthocyanins.

Another group of anthocyanins, constituted by hydroxyphenyl-pyranoanthocyanins, results from the reactions of anthocyanins and vinyl derivatives (*48, 49*). Malvidin 3-glucoside-4-vinylphenol, pinotin A (malvidin 3-glucoside-4-vinylcatechol), and malvidin 3-glucoside-4-vinylguaiacol were detected in our samples. The presence of vinyl derivatives in wine was attributed to enzymatic decarboxylation of phenolic acids by yeast enzymes (*48*). However, Schwarz and Winterhalter (*50*) demonstrated that pinotin A also could be formed as a result of the direct addition of caffeic acid to malvidin-3-glucoside, without the need for prior decarboxylation of cinnamic acid derivatives by wine yeasts (*51*). Also, flavanylpyranoanthocyanin was detected in our samples. We found that the concentration of vinyl and flavanylpyranoanthocyanins increased with time (**Figure 2**), the increases being larger in the MO wines, especially from t_2 to t_3.

We also detected compounds formed by direct reactions between anthocyanins and flavanols (**Figure 3**). These may result from the addition of flavanols to anthocyanins (*47, 52*). Little differences were detected in the direct adducts between control and MO wines.

A broad peak at the end of the chromatogram was observed (polymeric peak). It absorbed at around 540, indicating that it contained flavylium units. Its concentration increased with time while its λ_{max} value decreased, perhaps because anthocyanin-ethyl-flavanol adducts would first have been formed during winemaking and then transformed into flavanyl-pyranoanthocyanins during wine aging, compounds that present lower λ_{max} values. The contribution of these polymeric pigments to the overall color of aged red wines may be superior to the contribution of either genuine monomeric anthocyanins or pyranoanthocyanins (*50*). The area of this peak, as shown in **Figure 3**, is larger, in general, in MO wines, although no significant differences were observed between W3 (C) and W3 (MO) at the end of the experiment.

Chromatic Characteristics of Wine. Figure 4 reflects the evolution of color intensity and hue. The wines behaved in a similar way, independently of their phenolic content. An increase from t_0 to t_1 was observed in all wines and was higher in MO wines, these wines also being darker in color (see Table S2 in the Supporting Information). A decrease during MLF was then detected, probably due to an increase in pH and the degradation of pigmented compounds by lactic bacteria. After MLF, CI increased, although MO wines maintained a statistically higher

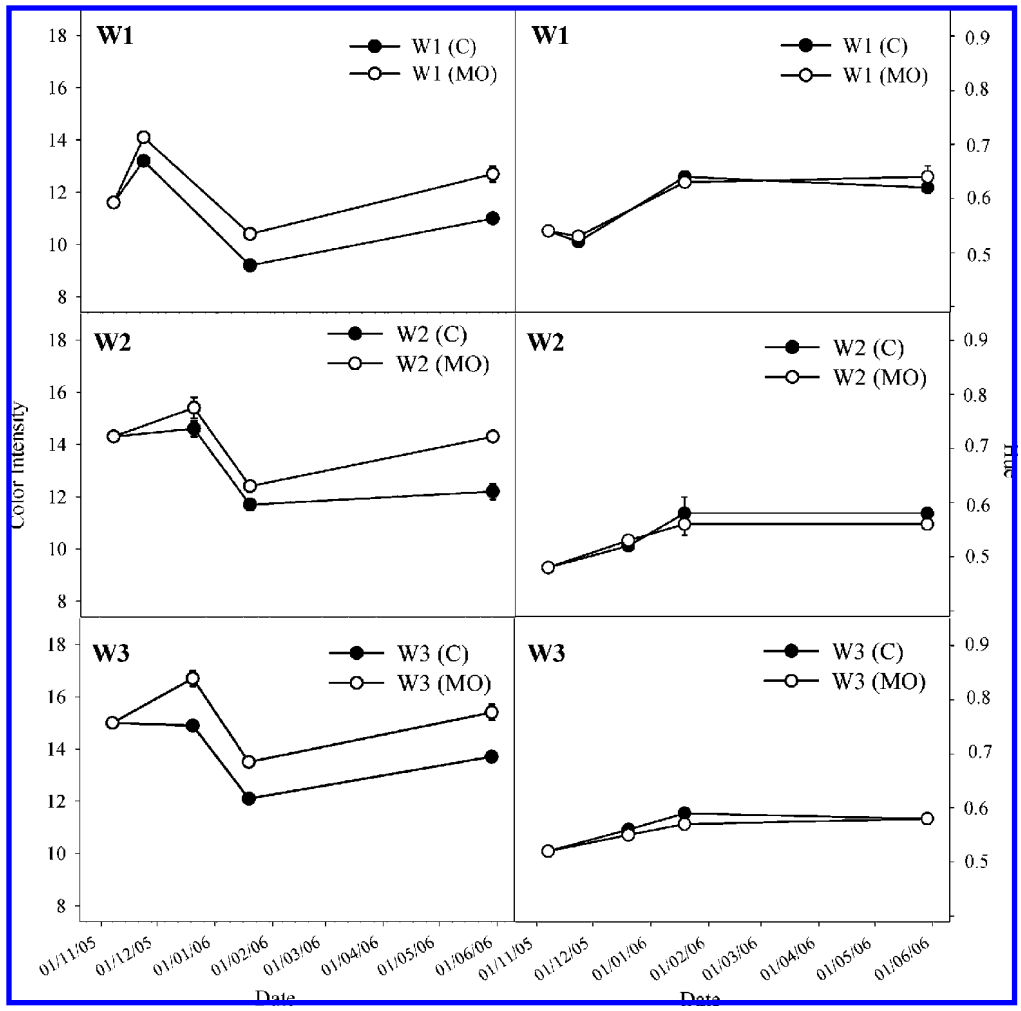

Figure 4. Development of color intensity and hue in W1, W2, and W3 wines (±SD). Each point corresponds to t_0, t_1, t_2, and t_3.

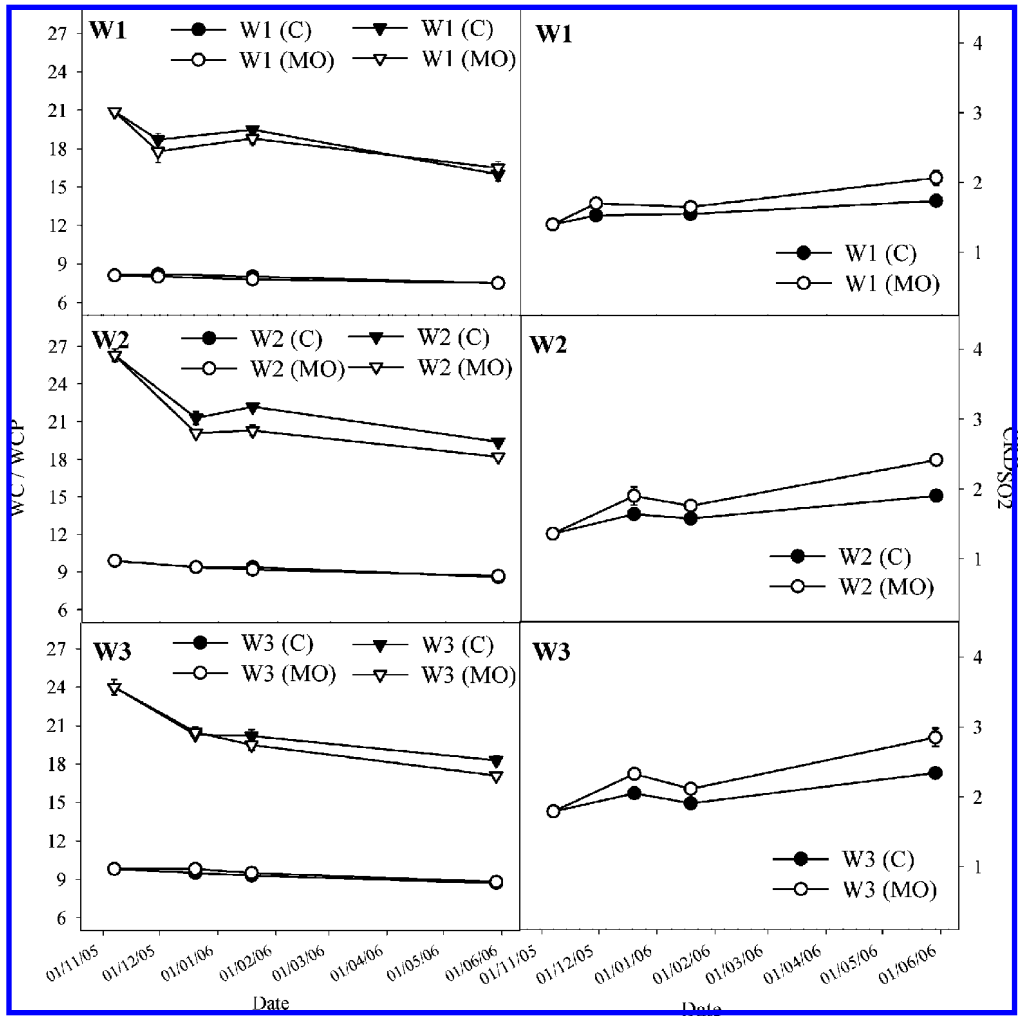

Figure 5. Development of WC, WCP, and CRD_{SO_2} in W1, W2, and W3 wines (±SD). Each point corresponds to t_0, t_1, t_2, and t_3.

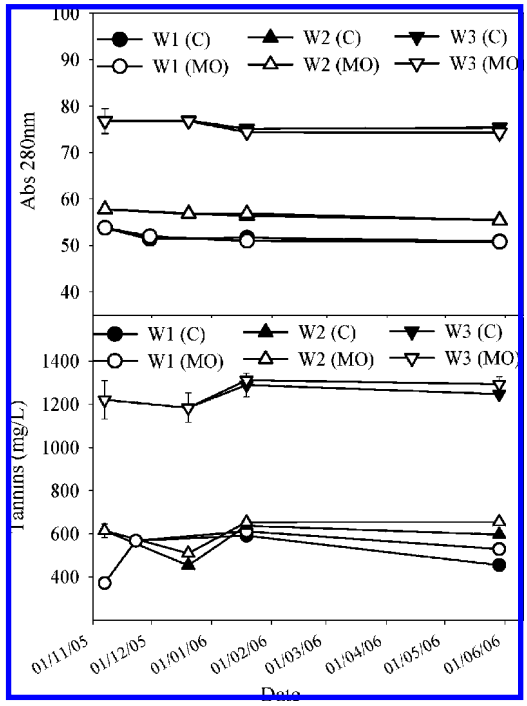

Figure 6. Development of abs_{280} and tannins in different wines (±SD). Each point corresponds to t_0, t_1, t_2, and t_3.

CI value. The higher CI in MO wines was probably due to the contribution of the newly formed pigments, for example, ethyl-linked pigments, since the absorbance of these molecules at 620 nm is higher than that of genuine anthocyanins (the percentage of blue color in MO wines was higher than in control wines). Pyranoanthocyanins also may have participated in the higher CI as they show both higher absorbance at 420 nm and contribute to the redness of wines. The hue evolved in a very similar way in all the wines, with a tendency to increase, but more so in control wine, demonstrating that no detectable browning occurred in the MO wines.

Figure 5 reflects the evolution of WC, WCP, and CRD_{SO_2}. A very small decrease in WC was observed in all wines with no differences due to MO. WCP decreased with time, and the decreases were concomitant with the loss of free anthocyanins usually observed during wine evolution, meaning that the formation of anthocyanin-derived pigments did not compensate for free anthocyanin degradation.

A continuous increase in pigments resistant to SO_2 bleaching was observed. The formation of pyranoanthocyanins, which are very stable compounds toward SO_2 and pH due to the substitution of C4 (*47*), was probably associated with this evolution. Ethyl-linked compounds also should be resistant to sulfite addition and may have contributed to the observed changes, explaining as to why the values were higher in MO wines.

With regard to optical density and tannin content (**Figure 6**) and mean degree of polymerization (mDP) (**Figure 7**), no changes in abs_{280} were detected in any of the wines, indicating the absence of significant wine pigment precipitation. As regards tannins, their levels decreased during MLF in W2 and W3, but

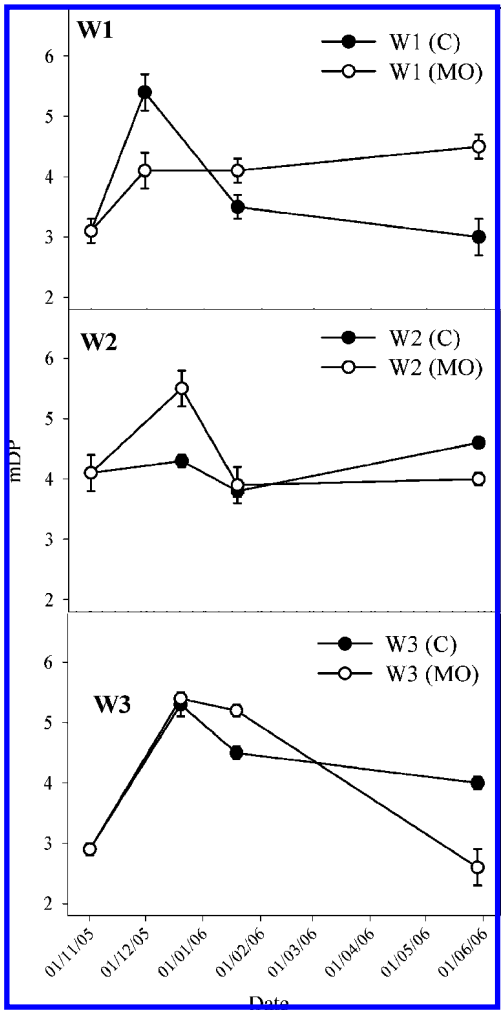

Figure 7. Development of mean degree of polymerization of tannins in W1, W2, and W3 wines (±SD). Each point corresponds to t_0, t_1, t_2, and t_3.

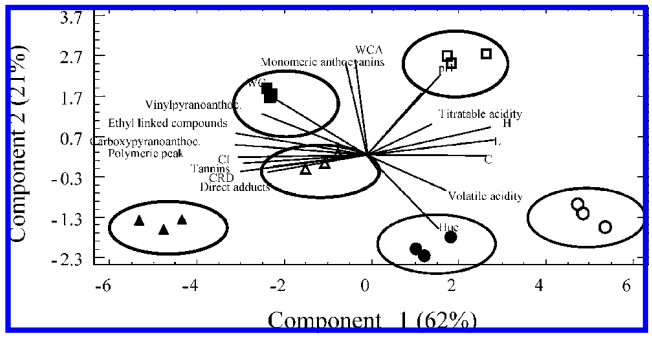

Figure 8. Graphical plot representing the distribution of the wine samples in the plane defined by principal components 1 and 2 as regards their chromatic characteristics (MO wines: solid symbols; control wines: open symbols; W1: circles; W2: squares; and W3: triangles). The variance explained by each principal component is shown in parentheses.

since no decrease in abs_{280} was detected, this phenomenon was probably due to tannins breaking down to give small structures that were not detected by the BSA method. Similar values for the control and MO wines were observed at t_2. From t_2 to t_3, the concentration barely changed, although MO wines showed a slightly higher tannin content.

Different behavior with regard to mDP was observed for the different wines. This parameter always increased in the first period (from t_0 to t_1) for all wines and then fell, except for W1. According to Nikfardjam and Dykes (*53*), this increase corresponds to the wine structuring phase described in empirical observations (*13*), and this phase appears to occur irrespective of whether oxygen is being dosed into the wine. The reduction of mDP observed afterward, mainly in W2 (MO) and W3 (MO), may be due to a possible structural rearrangement of the tannins to shorter forms, which would correlate with the small decreases observed in the tannins measured by the BSA method. This reduction in mDP may be responsible for a reduction in wine astringency (*54, 55*). The interaction of these newly formed intermediates with anthocyanins also may reduce astringency since they can form terminal units of these shorter forms, preventing further polymerization (*56*). The different behavior of W1 (MO) was similar to that described by Du Toit et al. (*57*) when MO was applied to a wine for too long a period of time. It seems that W1 could have suffered overoxygenation in the MO wine, and therefore, an increase of mDP was observed.

Multivariate statistical analysis was used in this study to check as to whether the wines could be grouped according to the variables studied. First, a cluster analysis was conducted using all the studied variables since this is a powerful visualization tool that enables groupings to be observed within the data. This is an unsupervised method for pattern recognition, where the samples were clustered without prior knowledge of their belonging to any wine type. The distance matrix was calculated using square Euclidean distances and an average linkage method algorithm. Distance measures the similarity or dissimilarity between the different samples. Two clearly differentiated clusters were found (data shown in Supporting Information). In one of them we found W1, both control and MO wines, and W2 (C), showing that W1 (C) and W2 (C) were quite close. W2 (MO) was classified as closer to the W3 wines. In the case of W2, it seems that MO originated the maximum differences in the wine characteristics. MO promoted changes in its phenolic structure, leading to a wine with chromatic characteristics closer to a wine with a higher phenolic content.

Having obtained this grouping, a principal components analysis was conducted with the same data and using the same variables to find as to which variables were mainly responsible for the grouping found (**Figure 8**). The first two principal components explained 83% of the variance. The representation of these two principal components showed that all MO wines had lower values in component 1 than their corresponding control wines mainly due to lower values of L^*, H^*, and hue and higher values of CI and anthocyanin-derived compounds, among others. The wines with the highest phenolic content showed negative values of PC1, while W2 differed from the other wines especially in the highest values of PC2, due to the high WC and anthocyanin monoglucoside values. W2 (MO) and W3 (C) were again very close.

MO favored reactions that led to the greater formation of new pigments, which, in turn, increased CI and CRD_{SO_2} in all the wines, regardless of their phenolic content. W1 wine was less influenced by MO and the observed increases in mDP for W1 (MO) suggested overoxygenation. This wine presented an anthocyanin content that was too low to promote any great level of condensation reactions. W3 was favored by MO, but the most evident results were found in W2 (MO), with its high anthocyanin content that favored the formation of more stable derived pigments and led to a wine close to W3 (C).

Supporting Information Available: Figure of differentiated clusters and tables of wine characteristics at the end of alcoholic

fermentation and evolution of CIELab and color. This material is available free of charge via the Internet at http://pubs.acs.org.

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