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Maceration Enzymes and Mannoproteins: A Possible Strategy To Increase Colloidal Stability and Color Extraction in Red Wines

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Different strategies were adopted to achieve increases in color stability in Tempranillo wines: (i) addition of maceration enzymes directly to the must, (ii) addition of commercial mannoproteins to the must, and (iii) inoculation of must with yeast overexpressed of mannoproteins. The addition of enzymes favored color extraction, and the wines obtained presented higher values of wine color, color intensity, bisulfite-stable color, and visually enhanced color intensity. The enzyme hydrolytic activity produced an increase in the acid polysaccharide content and polyphenol index and yielded to wines with more astringency, tannin, and length. Added mannoproteins had clearer effects on the analyzed parameters than yeast. Contrary to what may be thought, mannoproteins did not maintain the extracted polyphenols in colloidal dispersion and neither ensured color stability. These compounds clearly modified the gustative structure of the wines, enhancing the sweetness and roundness.

KEYWORDS: Red wine; maceration enzymes; mannoproteins; polysaccharides; polyphenol index; wine color; bisulfite-stable color; copigmentation color; sensory analysis

INTRODUCTION

The color of wines, especially that of red wines, is one of the most important properties used for the commercial evaluation and exerts a considerable influence on the marketability of the products. The color in young red wine is due to phenolics, especially anthocyanins, which are unstable molecules and highly susceptible to degradation (*1*). The color stability of anthocyanins is influenced by diverse factors (*2*−*4*).

The reactions that lead to the formation of stable pigments in wines have been mentioned by several authors (*5*−*8*). The reaction between anthocyanins and tannins is known as "polymeric pigments", which are primarily responsible for stable wine color and the main pigments in the aged wines (*9*). Copigmentation, the formation of complexes between anthocyanins themselves (self-association) or with other colorless cofactors, is another phenomenon that stabilizes color in young red wines (*10*−*15*). These interactions result in an increase in absorbance intensity (hyperchromism) and a positive shift in the visible wavelength (bathochromism) (*16*).

Color stability is directly related to wine quality, so the application of enological techniques that improve this factor is of major interest. The enrichment of the medium with polysaccharides could be a way to increase color stability. These compounds, described as protective colloids, are able to interact with tannins and anthocyanins in wines and reduce their reactivities and also lead to an increase in color stability (*17*−*19*). Recently mannoproteins have been shown to play a role as protective colloids by limiting self-aggregation of tannins (*20*), increasing the roundness and body of red wines (*21*, *22*) and reducing astringency and bitterness (*22*, *23*).

The addition of commercial macerating enzymes directly to the must is a common practice usually employed to facilitate the extraction of phenolic compounds during the vinification process (*24*−*26*). The enzyme preparations used contain pectolytic activities (polygalacturonase and pectin-lyase) in addition to hemicellulases, cellulases, and occasionally glycosidase activities. These preparations degrade the structural polysaccharides of grape cells and facilitate the liberation and solubilization of the phenolics bound to the cells of the skin, flesh, and seeds. Thus, the content of polyphenols would be increased since the first stages of the alcoholic fermentation, compensating the partial destruction of copigmentation anthocyanins due to the ethanol effect and temperature increase occurring during fermentation (*27*, *28*). Besides, the polymerization of tannins with anthocyanins would be favored, thereby avoiding their degradation and loss and, hence, color instability during storage of wine (*29*).

Different enological treatments are proposed in the present study in order to improve color extraction and stability during the winemaking process. The strategy adopted to achieve increases in color is the addition of maceration enzymes directly to the must and to increase the stability of the extracted color, the enrichment of the medium with protective colloids like mannoproteins. In order to do so, both yeast strains overexpressed of mannoproteins and industrial mannoproteins will be

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Table 1. Treatments Applied in the Study[a]

treatment	*S. cerevisiae* strain[b]	industrial man addition[c]	macerating enzyme addition[c]
212C: control 212	RC212	no	no
212E: 212 + enz	RC212	no	yes
212M: 212 + man	RC212	yes	no
BM45C: control BM45	BM45	no	no
BM45E: BM45 + enz	BM45	no	yes
BM45M: BM45 + man	BM45	yes	no

[a] Vinifications with each treatment were done in triplicate. [b] *S. cerevisiae* strain used for fermentation. RC212: underexpresser of polysaccharides. BM45: overexpresser of polysaccharides. [c] Addition in the prefermentative stage.

used. This paper reports the results of the strategies adopted during the vinification and aging of the Tempranillo red wines in the colloid content and color extraction and stability, as well as in the organoleptic qualities of the final wines. To the best of our knowledge, no reports of the effect of mannoproteins on wine color stability have been published.

MATERIALS AND METHODS

Reagents and Samples. All reagents were analytical grade unless otherwise stated. D-(+)-Galactose, D-(+)-glucose, and D-(+)-mannose were supplied by Sigma (St. Louis, MO), and myo-inositol and D-α-galacturonic acid were supplied by Fluka via Sigma.

Ethanol 96% v/v, acetyl chloride, sulfuric acid, sodium metabisulphite, tartaric acid, and phenol reagent were supplied by Scharlab (Barcelona, Spain), sodium hydroxide and hydrochloric acid 37% were supplied by Carlo Erba (Rodano, MI, Italy), acetaldehyde, dried methanol, and *o*-hydroxydiphenyl (biphenyl-2-ol) were supplied by Merk (Darmstadt, Germany), sodium tetraborate was supplied by Sigma, and the trimethylsilylation reagent (TriSil) was supplied by Pierce (Rockford, IL). Hexane supplied by Scharlab and Milli-Q water (Millipore, Molsheim, France) were used.

Wine samples were produced from *Vitis vinífera* Tempranillo grapes of the qualified origin denomination Rioja (D.O.Ca. Rioja). *Saccharomyces cerevisiae* var. *cerevisiae* RC212 and *Saccharomyces cerevisiae* var. *cerevisiae* BM45 were commercial yeast from Lallemand (Lallemad-Inc., Montreal, Canada). Industrial mannoproteins (Optired), maceration enzymes (Lallzyme EXV), and the bacterial strain *Oenococcus oeni* were also purchased from Lallemand.

Vinification and Sample Collection. Tempranillo grapes were sourced from Autol, La Rioja, Spain and harvested at 21.9 °Brix, pH 3.56 and 6.02 g tartaric acid/L of titratable acidity. Experimental vinifications were carried out in the experimental wine cellar of the University of La Rioja using stainless steel tanks of 100 L. Destemmed−crushed grapes were homogenized and distributed into the tanks, and 4 g/HL SO_2 was added. The different treatments were then applied, and three vinifications were made for each treatment (**Table 1**).

The first three treatments were inoculated with 25 g/HL *S. cerevisiae* yeast strain RC212 previously rehydrated. This yeast strain is a commercial active dry wine yeast selected by our laboratory because it is a strain low-expressed of polysaccharides (*17*). The rest of the deposits were inoculated with 25 g/HL *S. cerevisiae* yeast strain BM45 previously rehydrated, selected because it is a strain overexpressed of mannoproteins (*17*). Thereafter, 13.5 g/HL of industrial mannoproteins was added to tanks 212M and BM45M, and 2 g/HL of macerating enzymes was added to tanks 212E and BM45E. These enzymes, added as a liquid formulation to grapes, showed standard activities higher than 4000 uPG/g (polygalacturonase units), 1000 uPE/g (pectin-esterase units), and 120 uPL/g (pectin-lyase units) and other secondary activities such as acid protease, galacturonase, and cellulase/hemicellulase.

The prefermentation process went on for 6 h at 18 ± 1 °C; the fermentation−maceration process was carried out at a maximum temperature of 28 ± 2 °C and went on for 10 days. The postfermentative maceration went on for 4 days at 24 ± 1 °C. The cap was punched down during the maceration−fermentation and postfermentative maceration. Pumping-over extractions were also done during active fermentation. Wines were then runoff and inoculated with a commercial preparation of *O. oeni* (1 g/HL) to induce the malolactic fermentation, carried out at 18.5 ± 1 °C. After malolactic fermentation, all the wines were racked, corrected to 3.5 g/HL of free SO_2, and clarified by settling for 25 days at 10 °C. Wine aging was performed in new American oak barrels of 13 L, which are of higher area/volume than the traditional ones of 225 L. Due to this, and on the basis of the organoleptic analysis, the oak aging process went out for only 45 days. After this, wines were bottled and stored at 4 °C until analysis.

Samples were taken at the beginning of the prefermentative maceration (PM), during the alcoholic maceration−fermentation (beginning, 25−30% of sugars consumed, and 55−60% of sugars consumed, namely, 0AF, 30AF and 60AF, respectively), and at the beginning of the malolactic fermentation (BMF). Sample bottles were filled completely to minimize oxygen contact and immediately frozen at −18 °C. Samples were also taken at the beginning and end of wine aging (BA, EA).

Determination of Usual Enological Parameters. L-Malic acid was determined by an enzymatic method according to AOAC official methods of analysis (*30*). Conventional enological parameters (density, ethanol concentration, pH values, reducing sugars, titratable and volatile acidities) were determined according to official OIV practices (*31*). Color intensity (CI) was calculated as the sum of absorbances at 420, 520, and 620 nm, and the hue of wine was calculated as the ratio of absorbance at 420 and absorbance at 520 nm. These values were determined at wine pH (*31*). All these measurements were done in triplicate prior to freezing or cooling.

Microbiological Analysis. To ascertain the dominance of the inoculated strains, isolates were selected at random and subjected to molecular biology techniques (PCR and EPC) as described by Ness et al. (*32*). Samples were taken at the middle and final stages of alcoholic and malolactic fermentation. Analyses were carried out in the Laboratory of Microorganism Genetic Identification (Sigmo Vertou, France).

Exocellular Polysaccharide Production in a Synthetic Medium. After rehydration yeast were added to a synthetic medium without polysaccharides as described by Escot et al. (*17*) in order to obtain the polysaccharides released by the strains employed. Yeast was grown with stirring at 28 °C.

Isolation of Polysaccharides. Polysaccharides were isolated from the synthetic medium at the end of alcoholic fermentation. Samples were homogenized, and 5 mL was taken and centrifuged (9500*g*, 20 min, 4 °C). Polysaccharides were precipitated by addition of four volumes of cold ethanol containing 0.3 M HCl and kept 18−20 h at 4 °C (*33*). Thereafter, the samples were centrifuged (9000*g*, 20 min, 4 °C), the supernatants were discarded, and the pellets were washed four times with ethanol 96%. The precipitates were finally dissolved in ultrapure water and freeze-dried.

For the isolation of must and wine polysaccharides, samples were homogenized, and 50 mL was taken with a peristaltic pump and centrifuged (9500*g*, 20 min, 4 °C). The supernatants were then concentrated (five times for wines and three times for musts) under reduced pressure at 34 °C. Polysaccharides were precipitated by addition of four volumes of cold ethanol containing 0.3 M HCl and kept 18−20 h at 4 °C (*33*). Thereafter, the samples were centrifuged (9000*g*, 20 min, 4 °C), the supernatants were discarded, and the pellets were washed four times with ethanol 96%. The precipitates were finally dissolved in ultrapure water and freeze-dried.

Polysaccharide concentration was determined by a colorimetric method, and monosaccharide composition was determined by gas liquid chromatography as further described. Protein concentration was determined by the procedure of Lowry et al. (*34*).

Quantification of Polysaccharide Concentration. Soluble polysaccharides were first isolated as described previously and dissolved in water. The *o*-hydroxydiphenyl and the phenol methods were followed as described by Segarra et al. (*35*), but instead of 5% (w/v) phenol reagent, a solution of 3% was used. This concentration was found to be optimal to determine the 100% of the neutral polysaccharides (NPS) present in the sample but only 50% of the acid polysaccharides (APS), which were determined by the *o*-hydroxydiphenyl method. NPS concentration was calculated using a galactose calibration curve ($r = 0.999$ for the range 0−100 mg/L), whereas a galacturonic acid

calibration curve ($r = 0.999$ for the range 0−100 mg/L) was used for APS. All the analyses were conducted in triplicate on samples after polysaccharide isolation.

Determination of Monosaccharide Composition by GC. Sugars were determined in the polysaccharide extracts isolated from the synthetic medium by gas−liquid chromatography of their trimethylsilyl-ester *O*-methyl glycosyl residues obtained after acidic methanolysis and derivatization (*33*). The composition of the commercial mannoproteins used was also determined by this procedure. All analyses were performed in triplicate.

The GC system consisted of an HP5890 Series II gas chromatograph (Hewlett-Packard, U.S.A.) coupled to a FID. The GC system was equipped with a capillary split/splitless inlet and a fused-silica capillary column (30 m × 0.25 mm × 0.25 μm, Tecnokroma). The carrier gas was helium at a flow rate of 1.3 mL/min. Samples were injected in the pulsed split mode with a split ratio of 20:1. The injector and the FID were operated at 250 °C. The chromatograph was operated with temperature programming (120−145 °C at 1 °C/min, 145−180 at 0.9 °C/min, and 180−230 at 50 °C/min).

Color Composition and Total Polyphenol Index Measurements. The spectrophotometric measurements were performed on a Cary 300 Scan UV−vis spectrophotometer (Varian Inc., Madrid, Spain) using 2 mm and 10 mm path length quartz cells. All the samples were analyzed in triplicate, and all absorbance values were corrected to 10 mm path length.

Wine red color (WC), copigmentation color (CC), and bisulfite-stable color (BSC) were determined by the method proposed by Levengood and Boulton (*36*). Wine samples were adjusted to pH 3.6 to eliminate color differences due to pH and ultra centrifuged at 20 000*g* for 10 min. Wine color (WC) was measured by the addition of 20 μL of 10% acetaldehyde to a 2 mL of wine sample. The sample was kept for 45 min and measured at 520 nm in a 2 mm path length cell. Bisulfite-stable color (BSC), the result of the addition of 160 μL of a 5% SO_2 solution to 2 mL of wine sample, was measured at 520 nm after 50 min in a 2 mm path length cell. Copigmentation was measured by comparing diluted and undiluted samples. Samples were diluted 20-fold with synthetic wine (12% alcohol, 5 g/L of tartaric acid in water, pH 3.6) and measured at 520 nm after 20 min in a 10 mm path length cell ($A_{diluted}$). The CC is the difference between the WC value and the $A_{diluted}$ value.

The total polyphenol index (TPI) was determined by absorbance at 280 nm of diluted wine (1/20, v/v). The diluted samples used to measure the value of $A_{diluted}$ were also used to determine this parameter. Spectrophotometric measurements were made using 10 mm path length cells, and absorbance values were maintained along the time of reaction.

Sensory Analysis. Aged wines were analyzed by a panel of expert tasters for sensory profiling. Wines were judged on three levels: visual (color), olfactory (volatile fraction), and gustatory (taste and mouth-feel sensations).

A panel of six tasters (three males and three females), wine professionals from the D.O.Ca. Rioja, was convened. All wine tasters had participated on previous aroma and mouth-feel sensory descriptive panels and had regularly participated in quality scoring Tempranillo wine sensory panels. The wines were presented at 18 °C in coded standard wine-tasting glasses according to standard 3591 (ISO 3591, 1997). Assessment took place in a standard sensory analysis chamber (ISO 8589, 1998) equipped with separate booths. Wines were presented in two stages. In a first session, the panelists were asked to describe the olfactory and gustative attributes in their own words. Descriptive terms and their definitions were debated among the assessors, and a common consensus vocabulary was then compiled and discussed further with panelists. Tasters selected 12 attributes for the olfactory phase and 10 for the gustative, which were agreed upon as best for describing the sensory characteristics of the wines. All the generated terms were usual wine-tasting terms for describing red wines. In following sessions, assessors used the consensus vocabulary, scoring the intensity of each attribute on an interval scale with 10 levels of intensity (0 = no aroma or no taste; 1 = weak aroma or weak taste; 5 = intense aroma or intense taste; 9 = strong aroma or strong taste; intermediate values did not bear description). The color was also judged, and blue-red color was rated according to its intensity on an anchored scale with four levels of intensity (0 = no blue-red color; 1 = weak blue-red color; 2 = intense blue-red color; 3 = extremely strong blue-red color). Wine samples were assessed in triplicate.

Statistical Procedures. All the treatments were vinificated in triplicate, and all the analysis were performed in triplicate. Significant differences between samples were performed with the SPSS 12.0 program for Microsoft Windows (SPSS Inc., Chicago, IL). The values of color composition, TPI, and polysaccharide content were analyzed by a two-way analysis of variance (ANOVA) with repeated measurements in one factor, to test the effects of the treatments and vinification stage (repeated measurements), if the data met assumptions of normality. If this assumption was not satisfied, a Kruskal−Wallis test was used for each factor. The Excel program for Microsoft Windows was used, when necessary, to calculate the *P* value and the contrast statistical value by means of the conventional hypothesis tests. In the present paper, always we refer to differences between samples, either with different time or treatment; we will be referring to significant differences with at least $p < 0.05$.

Sensory data were subjected to ANOVA using the SPSS 12.0 program to determine reproducibility of attribute scores. Separate principal component analyses (PCA) were carried out on the mean ratings for olfactory and for gustatory attributes. The PCA was conducted using the covariance matrix with no rotation (XLSTAT 2007 program for Microsoft Windows). Average configuration plot dimensions were interpreted taking into account the descriptors used by each of the assessors, which were most highly correlated with each dimension. Dimension 1 explained 45% (aroma) and 56% (mouth), and dimension 2 explained 28% (aroma) and 31% (mouth) of the original variance. In this study, there were no significant differences in the scores given by the tasters for each attribute ($p < 0.05$), indicating that all the panelists used all attributes reproducibly.

RESULTS AND DISCUSSION

Microvinifications and Microbiological Analysis. Eighteen microvinifications were carried out as detailed previously. All alcoholic fermentations finished within 10 days, yielding final reducing sugar contents of less than 2 g/L. No differences in the fermentative progress were detected. Genetic analysis showed that the yeast strains *S. cerevisiae* BM45 and RC212 were successfully implanted in all the vinifications (data not shown).

The bacterial strain *O. oeni* was also successfully implanted in all the malolactic fermentations (data not shown). This fermentation was finished when the L-malic acid concentration was below 0.1 g/L, and the required time was different depending on the yeast strain inoculated in the alcoholic fermentation. Thus, the malolactic fermentation went on for 20 days in 212 wines, whereas it took longer in BM45 wines, between 18 and 30 days more. This fact does not agree with bibliography as liberated mannoproteins are described as stimulators of malolactic fermentation (*22*).

Conventional Enological Analysis in Wines. Values of pH, titratable acidity, ethanol concentration, and volatile acidity were in the range usually found in wines of Tempranillo variety (**Table 2**). The values obtained for pH, titratable acidity, and volatile acidity confirmed the absence of microbial alterations. Analyses performed on the different wines were in accordance with a good conservation of wines during oak aging. As expected, the aging was accompanied by a decrease in color intensity and an increase in hue (**Table 2**).

The ethanol production was similar in all tests (12.3−12.7% v/v) regardless of enzyme addition, showing that the amount of enzymes added did not affect the cell wall stability. On the other hand, titratable and volatile acidity and pH were not modified by grape enzyme treatment. These results confirmed the results of Lao et al. (*37*), who report that the use of pectic enzymes does not modify these parameters. Mannoproteins and

Table 2. Conventional Enological Parameters of Wines[a]

stage[b]	wine	% v/v[c]	pH	TA[d]	VA[e]	CI[f]	hue[g]
BMF	212C	12.6 ± 0.2	3.79 ± 0.007	5.2 ± 0.1	0.23 ± 0.05	6.79 ± 0.03	0.53
	212E	12.5 ± 0.3	3.75 ± 0.002	5.36 ± 0.05	0.19 ± 0.07	7.316 ± 0.002	0.49
	212M	12.6 ± 0.3	3.81 ± 0.01	5.6 ± 0.3	0.2 ± 0.1	6.099 ± 0.005	0.59
	BM45C	12.4 ± 0.2	3.74 ± 0.01	5.59 ± 0.05	0.22 ± 0.06	6.96 ± 0.01	0.58
	BM45E	12.6 ± 0.2	3.76 ± 0.007	5.87 ± 0.03	0.18 ± 0.05	7.491 ± 0.008	0.50
	BM45M	12.4 ± 0.2	3.81 ± 0.02	5.7 ± 0.1	0.2 ± 0.03	6.556 ± 0.001	
							0.54
BA	212C	12.5 ± 0.2	3.97 ± 0.02	3.71 ± 0.05	0.29 ± 0.05	5.82 ± 0.01	0.59
	212E	12.5 ± 0.3	3.91 ± 0.02	3.94 ± 0.03	0.30 ± 0.08	6.195 ± 0.02	0.50
	212M	12.5 ± 0.3	3.94 ± 0.007	3.86 ± 0.05	0.31 ± 0.02	5.478 ± 0.004	0.60
	BM45C	12.5 ± 0.2	3.92 ± 0.007	4.03 ± 0.03	0.37 ± 0.08	5.679 ± 0.005	0.57
	BM45E	12.7 ± 0.2	3.96 ± 0.007	3.96 ± 0.08	0.4 ± 0.02	6.453 ± 0.006	0.49
	BM45M	12.3 ± 0.2	3.93 ± 0.002	4.24 ± 0.05	0.35 ± 0.03	5.061 ± 0.003	
							0.53
EA	212C	12.6 ± 0.2	3.98 ± 0.01	3.98 ± 0.05	0.33 ± 0.5	5.008 ± 0.004	0.62
	212E	12.3 ± 0.3	3.91 ± 0.01	3.9 ± 0.05	0.34 ± 0.01	5.756 ± 0.003	0.55
	212M	12.7 ± 0.3	3.95 ± 0.01	3.79 ± 0.05	0.34 ± 0.03	4.891 ± 0.005	0.68
	BM45C	12.6 ± 0.2	3.94 ± 0.005	3.92 ± 0.03	0.39 ± 0.08	5.372 ± 0.001	0.60
	BM45E	12.4 ± 0.2	3.98 ± 0.01	4.22 ± 0.03	0.42 ± 0.06	5.732 ± 0.009	0.55
	BM45M	12.6 ± 0.2	3.93 ± 0.01	3.77 ± 0.08	0.42 ± 0.08	4.991 ± 0.002	0.58

[a] Mean ± SD ($n = 3$). [b] Vinification stage: BMF, beginning of the malolactic fermentation; BA, beginning of the oak aging; EA, end of the oak aging. [c] Milliliters of ethanol for 100 mL of wines at 20 °C. [d] Titratable acidity as g of tartaric acid per L. [e] Volatile acidity as g of acetic acid per L. [f] Color intensity as sum of absorbances at 420, 520, and 620 nm. [g] A_{420nm}/A_{520nm}.

yeast neither modified the ethanol concentration nor pH in the wines. Wine pH increased 0.1−0.2 units at the end of the malolactic fermentation in all the cases, and titratable acidity decreased by 25−31%. After malolactic fermentation, BM45 wines presented higher values of volatile acidity than 212 wines, which coincided with the longer duration of the malolactic fermentation.

The addition of enzymes increased color intensity values after both fermentations. Parley et al. (*38*) also observed that Pinot Noir wines produced with macerating enzymes presented higher color intensities at the end of the alcoholic fermentation. Enzyme-treated wines had the highest values of color intensity at the end of the aging process. However, the addition of mannoproteins had the opposite effect, and the color intensity of wines 212M and BM45M was significantly lower than in the rest of wines at the end of the aging process.

Evolution of the Polysaccharide and Polyphenol Content and Color Composition during the Different Vinification Stages. Polysaccharides were analyzed during the maceration−fermentation, postmaceration, malolactic fermentation, and oak aging. **Figures 1** and **2** show the concentration of NPS and APS in all the musts and wines studied. The ANOVA analysis verified that the polysaccharide content depended significantly on the time when the sample was taken and enological treatment applied. In all the cases, the NPS content was higher than that of APS, the APS being only a small percentage of total polysaccharides (4−18%), in agreement with what was observed by other authors (*35*). The values found ranged from 421 to 1026 mg/L for NPS and from 31 to 139 mg/L for APS. These values were in the range described in other studies for red varieties (*33*, *39*).

Red winemaking increased the polysaccharide content, the maceration−fermentation being the main process affecting this content (**Figures 1** and **2**). Concentration of total polysaccharides (NPS + APS) increased progressively by 91−142% between zero time and 4 days (60AF) reaching 871−1117 mg/L. NPS were doubled during this period, and APS, which were liberated later (between 30 and 60AF), were tripled or quadrupled. The evolution of polysaccharides could be interpreted, in addition to the liberation of yeast mannoproteins during fermentation,

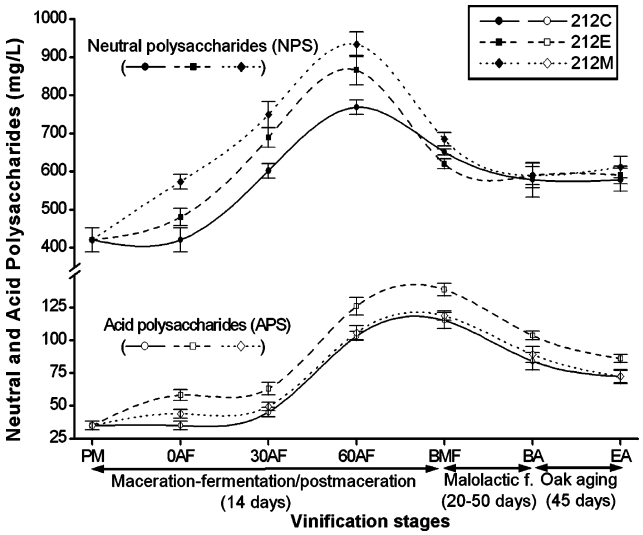

Figure 1. Neutral (mg galactose/L) and acid polysaccharides (mg galacturonic acid/L) of the samples vinificated with the yeast strain *S. cerevisiae* RC212. See text for conditions and calculations.

as the progressive release of carbohydrate polymers from skin and pulp cell walls. This interpretation is well sustained by the fact that in white wines (no pomace contact during fermentation), the fermentation process decreases the polysaccharide content (*35*, *37*). In all the cases, the content of NPS decreased substantially (15−28%) at the end of the maceration−fermentation process and during postmaceration, while the APS content remained constant or slightly increased. This different behavior, previously observed by our workgroup (*33*), could be due to the fact that APS are smaller molecules than NPS and their precipitation due to the ethanol formed during the fermentation process (*18*, *33*) is later than in the case of NPS. Both APS and NPS decreased again during the malolactic fermentation. After this stage, wine stopped giving off CO_2 and the temperature changed from 18.5 ± 1 to 15 ± 1 °C. Both effects would produce the precipitation of all the unstable material that was suspended while the temperature was high enough and there were convection currents formed by the fermentative processes. During the oak aging, the polysaccharide values were maintained

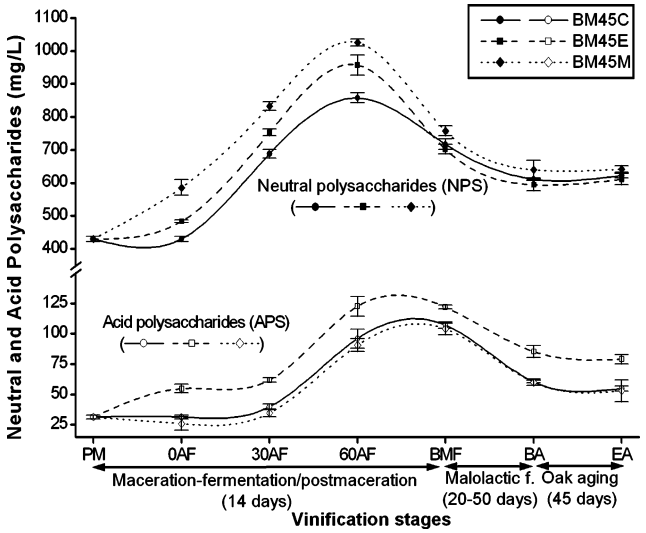

Figure 2. Neutral (mg galactose/L) and acid polysaccharides (mg galacturonic acid/L) of the samples vinificated with the yeast strain *S. cerevisiae* BM45. See text for conditions and calculations.

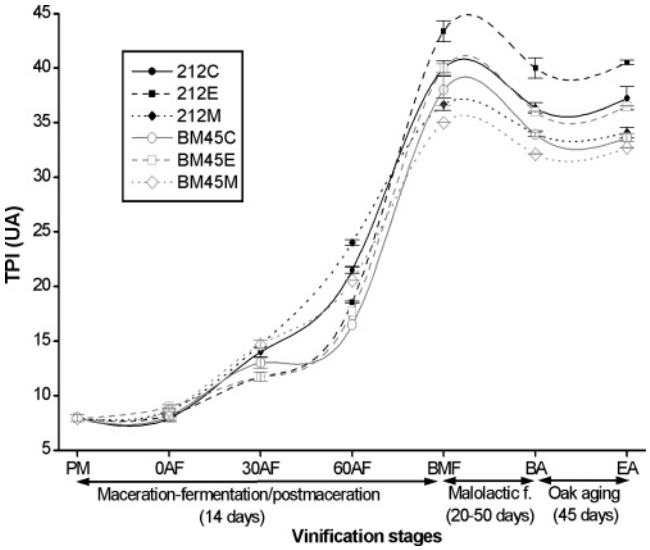

Figure 3. Total polyphenol index (TPI) of the must and wine samples.

as they would be low molecular weight molecules and, thus, maintained in solution.

Figure 3 shows the evolution of the TPI of the samples during the different vinification stages. **Figure 4a**−**c** shows, respectively, the evolution of WC, CC, and BSC, of the samples during the different stages. Bisulfite-stable compounds are usually termed "polymeric pigments", which is erroneous as they include compounds that are not polymeric in nature (i.e., pyranoanthocyanins), and not all anthocyanin-derived polymers are colored or bisulfite-stable pigments (i.e., the so-called T-A polymeric pigments). However, the practice of measuring BSC continues to be very useful (*40*). As in the case of polysaccharides, red winemaking increased the value of TPI, WC, CC, and BSC, the maceration being the main process affecting these values. The values of TPI, WC, BSC, and CC increased significantly in all the samples during maceration−fermentation and postmaceration, being more accused in WC (1890−2408%) and CC (1245−1918%) than in TPI and BSC (305−405% and 600−966%, respectively). Spranger et al. (*41*) observed that the evolution of the TPI during these stages is positively correlated with the content of tannins or condensed proanthocyanidins, which are major wine polymeric compounds. As in

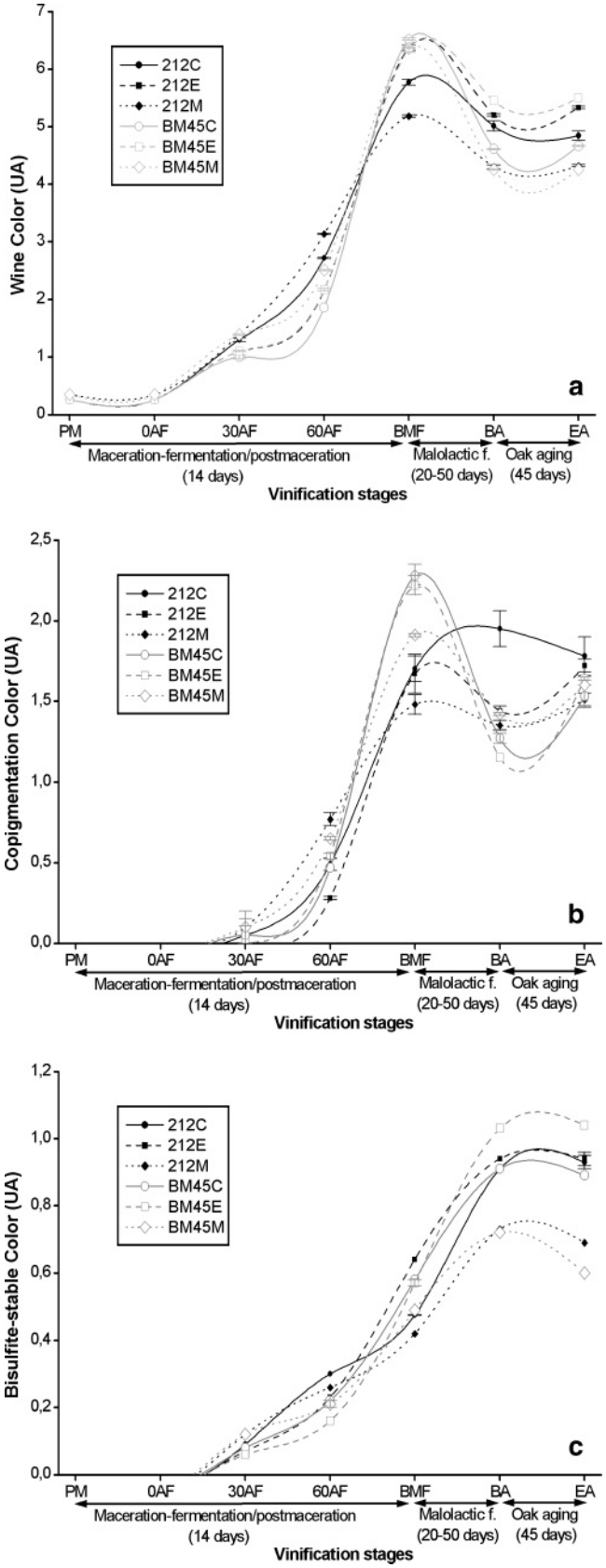

Figure 4. (**a**) Wine color (WC), (**b**) copigmentation color (CC), and (**c**) bisulfite-stable color (BSC) of the must and wine samples. See text for conditions and calculations.

the case of polysaccharides, the malolactic fermentation resulted in a considerable decrease in the content of total polyphenols (7.5−15%), WC (13−33.2%), and CC (5.8−48.2%), except in

the wine 212C, with an increase of 14%. However, the BSC increased significantly by 57% and 91.6% in all the wines. It is widely known that both malolactic fermentation and wine aging produce important loss of color due to the precipitation of the unstable colloidal material (*42*). However, new and stable pigments are also formed during these periods, with either high or low molecular weights. These new pigments are described to be resistant, not only to bisulfite addition, but also to the formation of the hemiketal through pH changes, and resistant to oxidation (*43*). Although polyphenols from the wood are extracted during wine aging, TPI values obtained before and after wine aging were not significantly different, indicating that polyphenol extraction was minimum during the short aging carried out. The values of WC and BSC were also maintained in this period, but CC increased by 11.8−42.6% in all the wines except for 212C, which had a decrease of 8.7%. Other authors point out that copigmentation in older red wines is negligible as this phenomenon has only been demonstrated to occur with monomeric anthocyanins (*40*). This fact would explain the decrease in CC occurring in the control wine 212C; the increase in this parameter in the rest of the wines could be due to the enological treatments applied.

Effect of Macerating Enzymes on the Polysaccharide and Polyphenol Content and Color Composition. Polysaccharide concentration was different in enzyme-treated samples and in controls, both in 212 and BM45 (**Figures 1** and **2**). From the beginning of the maceration−fermentation to 60% of sugars consumed (60AF), wines treated with commercial enzymes (212E and BM45E) had larger concentrations of NPS (10−14%) and APS (32−74%) than control wines. These findings indicated that solubilization of polysaccharides had occurred during the maceration−fermentation process due to the action of the commercial enzymes added. This solubilization affected to a greater extent to APS and therefore parietal polysaccharides, such as homogalacturonans and rhamnogalacturonans (RGs). Commercial enzymes have been traditionally used in wine technology in order to produce higher modifications in grape compounds than those produced by endogenous pectinases. As in the present study, Ducruet et al. (*44*) observed that the addition of commercial enzymes to must produced an increase in the amount of total acid (49%) and NPS (5%). Ayestarán et al. (*33*) also observed that after the maceration−fermentation process, wines treated with enzymes had larger concentrations of soluble monosaccharides, arabinogalactans, arabinogalactan proteins, and RG-IIs than control wines. However, very few reports on the evolution of these polysaccharides after the maceration−fermentation are available. As can be observed in **Figures 1** and **2**, most of the NPS liberated by macerating enzymes during the first stages of maceration−fermentation precipitated at the end of this period and during postmaceration (27−28%). Therefore, the polysaccharide content was similar in both control and enzyme-treated wines at the beginning of the malolactic fermentation. This precipitation phenomenon did not occur in the case of APS, and the differences were maintained, the enzyme-treated wines being the ones with the highest concentration of APS during all the vinification process and aging.

Commercial macerating enzymes enhance the degradation of structural polysaccharides of grape skin cell walls during the maceration−fermentation process, thus enhancing the extraction of grape phenolics (*24*, *25*). Therefore, the TPI was higher in enzyme-treated wines than in controls at the end of maceration−fermentation, 7.8% in 212E and 5% in BM45E (**Figure 3**). The value of WC was also higher in the 212E wine in comparison with the control one (10.4%) (**Figure 4a**). Enzyme-treated wines presented also the highest value of color intensity and WC both before and after wine aging (**Figure 4a** and **Table 2**). The BSC was also higher in BM45E and 212E wines before aging, but this difference was not maintained in 212E wines after wine aging (**Figure 4c**).

Table 3. Composition of Mannoproteins Produced by Lallemand and Macromolecules Released in Synthetic Medium by Yeast at the End of the Alcoholic Fermentation

	mannoproteins from Lallemand	yeast strain	
		RC212	BM45
proteins (%) (nitrogen)[a]	2.1	10.9	11.8
polysaccharides (%)[b]	72.8	81.1	78.1
mannose (%)[c]	90.6	71	55
glucose (%)[c]	9.4	29	45

[a] Lowry method. [b] Colorimetric method. [c] Capillary GC.

Effect of Industrial Mannoproteins on the Polysaccharide and Polyphenol Content and Color Composition. Commercial mannoproteins were added to samples in the prefermentative stage. The commercial preparation of mannoproteins consisted of 73% polysaccharides and 2% proteins (**Table 3**). Mannose was the main sugar (91%) with glucose far behind (9%). The remaining 25% of the preparation was probably represented by other cellular components isolated during the extraction procedure.

From the beginning of the alcoholic fermentation, mannoprotein-added samples (212M and BM45M) had higher values of NPS than their respective controls (144−168 mg/L), but the content of APS was obviously similar (**Figures 1** and **2**). This fact indicated that the mannoproteins added in the prefermentative stage were maintained soluble during this period and did not precipitate. However, these differences were minimized due to the polysaccharide decrease occurring at the end of the maceration−fermentation, which was more pronounced in the samples 212M and BM45M, which reached the highest values of total polysaccharides during this period. Therefore, the polysaccharide values tended to be similar at the beginning of the malolactic fermentation in all the wines although there were still significant differences between the samples with mannoprotein addition and the controls.

Mannoprotein-added wines presented the lowest values of the TPI at the end of the maceration process (**Figure 3**), which could be a consequence of a higher extent of polyphenol precipitation due to the formation of extra mannoprotein−polyphenol colloidal complexes. These results do not agree with what is reported in bibliography, where polysaccharides are described to function as protective colloids that slow or prevent self-aggregation of tannins in synthetic media (*20*, *45*). However, the complex polysaccharide−polyphenol could be unstable and precipitate as described by Siebert et al. (*46*, *47*), who observed that certain polysaccharides interact with gliadin and catechin and precipitate. During malolactic fermentation, polyphenolic compounds precipitated in all the samples, being more important in the control wines (10% in 212C and 15% in BM45C) than in 212M and BM45M wines (∼7.5%). These data suggested that the precipitation of the complex "added mannoprotein−polyphenol" occurred mainly at the end of maceration−fermentation and during postmaceration, coinciding with the higher precipitation of the NPS. Wines produced with commercial mannoproteins presented the lowest levels of TPI at the end of wine aging.

In regard to color, samples 212M and BM45M showed lower values of WC, CC, and BSC than controls at the end of

maceration−fermentation (**Figure 4a**−**c**). WC and BSC differences increased during malolactic fermentation and aging, and these wines presented the lowest levels of these parameters both at the beginning and end of aging. Moreover, wines produced with mannoprotein addition had the lowest values of color intensity (**Table 2**). These results question the strategy of using polysaccharides as "color protection molecules", a hypothesis mentioned in general bibliography and research papers but not properly contrasted and studied. However, it is important to highlight that wine polysaccharides are very different both in origin and composition and thus in physical and chemical properties; the results of the present paper are referred to the effect of the mannoproteins previously described (**Table 3**).

Effect of Yeast Mannoproteins on the Polysaccharide and Polyphenol Content and Color Composition. Vinifications were also affected by the yeast strain selected, RC212 or BM45. Both strains differed in their capacity to secrete polysaccharides in the growth synthetic medium. The results showed that the yeast strain BM45 released twice the polysaccharides released by the strain RC212 at the end of the alcoholic fermentation (118 vs 62 mg/L). The analysis of macromolecules released by both strains revealed that they were polysaccharides (∼80%) and some protein (∼11%) (**Table 3**). Of the sugars present in the polysaccharidic fraction, mannose was the prevalent one followed, at some distance, by glucose (**Table 3**). This composition confirmed not only the parietal nature of the polysaccharidic fraction but also that the polysaccharides released into the medium were essentially mannoproteins with a low proteic fraction. The distribution of neutral sugars was different for both strains. For RC212, mannose was the main component and glucose represented only 29%. In macromolecules from strain BM45, mannose and glucose were present in the same proportion. The greater proportion of glucose in BM45 macromolecules has also been observed by other authors (*17*) and may be due to a different composition of its cell wall.

Polysaccharide content was higher in BM45 than in 212 samples (**Figures 1** and **2**). In both cases, polysaccharides were continuously released from yeast during the first stages of maceration−fermentation, coinciding with the yeast exponential phase of growth. From the beginning of the winemaking process (30AF), the amount of NPS was significantly higher (10−14%) in the samples vinificated with the overexpresser yeast strain in comparison with their respective controls. This difference was attributed to the mannoproteins released by the overexpresser yeast strain and was quite similar to that found by other authors when analyzing the same yeast strain (*17*). Although these differences were reduced at the end of maceration−fermentation, there were still significant differences between BM45 and 212 samples at the beginning of malolactic fermentation. In this stage, wines produced with the strain BM45 had higher levels of NPS than those produced with the strain RC212 (10−11% more). The NPS content of wines BM45 and 212 was not significantly different after malolactic fermentation, indicating that the precipitation of these compounds was greater in wines made with the BM45 strain. In relation with APS, the yeast strain also influenced on their release but with a contrary effect. The content of APS was always equal or even higher in the wines made with the RC212 strain.

As in the case of industrial mannoproteins, the use of a strain overexpresser of mannoproteins produced wines with lower values of the TPI than 212 wines at the end of maceration−fermentation (**Figure 3**). This fact confirmed the observation previously done about the precipitation of the complex mannoprotein−polyphenol, although in this case the yeast mannoprotein is the one forming the unstable complex and not the commercial one. The precipitation of polyphenols occurring during malolactic fermentation was more accused in BM45C and BM45E wines (15% and 11%, respectively) than in 212C and 212E wines (10 and 8%, respectively) and 212M and BM45M (∼7.5%). These data suggested that the precipitation of the complex yeast mannoprotein−polyphenol began at the end of maceration−fermentation and postmaceration but went on in a marked way during malolactic fermentation, coinciding again with the higher precipitation of NPS occurring in the BM45 wines. Therefore, the precipitation of the unstable complex yeast mannoprotein−polyphenol occurred later than in the case of "industrial mannoprotein−polyphenol". This different behavior observed with both mannoproteins could be due not only to their different liberation moment but also to their different structure composition and thus reactivity (**Table 3**). During wine aging, BM45 wines presented lower values of TPIs than 212 wines, and among them, the one with enzyme addition had the highest value of this parameter during all the vinification stages (**Figure 3**). This indicated that the higher content of phenolics in wines due to the enzymatic treatment counteracted the effect of the precipitation of the complex yeast mannoprotein−polyphenol.

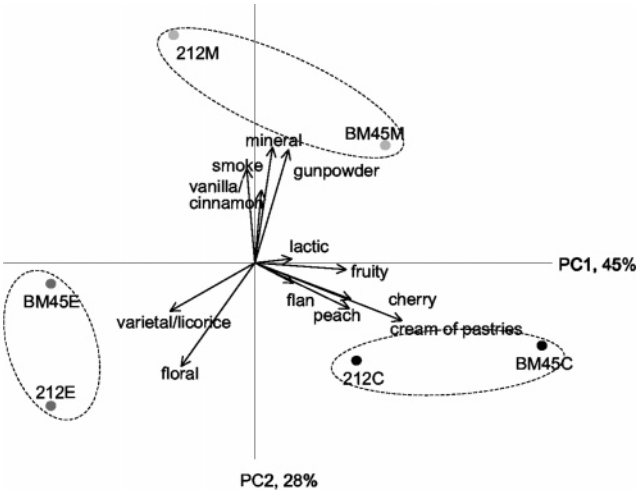

Figure 5. Biplot of principal components I and II of the olfactory attributes of the aged wines.

In relation to color, wines BM45 presented higher values of WC than 212 at the end of maceration−fermentation (13% in BM45C and 29.8% in BM45M) (**Figure 4a**), and the value of the copigmentation color (CC) was also higher (34%, 22.5%, and 24.7% in BM45C, BM45M, and BM45E) (**Figure 4b**). Note the different behavior of wines made with addition of industrial mannoproteins, which had lower values of WC, CC, and BSC than their respective controls. At the end of aging there were not clear differences in color composition parameters between wines produced with each of the strains.

Sensory Analysis of the Wines. Sensory evaluations of the aged wines were performed to verify the differences observed between wines on the organoleptic perception.

In the visual phase, enzyme-treated wines showed enhanced color intensity, obtaining mean punctuations between 2 and 3. Wines with mannoproteins, both commercial and from the yeast, had a color intensity similar to controls and even weaker, with mean punctuations close to 1.5. **Figures 5** and **6** provide a graphic representation of the relationship of the wines as determined by their olfactory and gustatory perceptions. Wines were properly located in the vectorial dimension defined by the first two factors, which accounted for 73% of the total variance

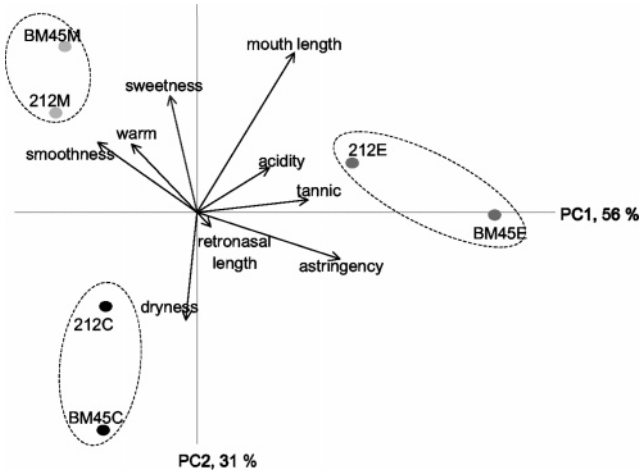

Figure 6. Biplot of principal components I and II of the gustatory attributes of the aged wines.

in the olfactory PCA space and for 87% in the gustatory PCA space (**Figures 5** and **6**, respectively). As can be observed, the technology applied in the wine making process (enzyme or industrial mannoprotein addition) was the most significant effect, being much more evident than the differences found with the different yeast strains. In the olfactory phase (**Figure 5**), control wines were highly related with the sweet and fruity descriptors. Enzyme-treated wines had high floral and varietal characters, with licorice aromas, which are characteristic of the Tempranillo variety (*48*). Oak aging and smoke and mineral aromas were predominant in the wines made with addition of industrial mannoproteins. This could be due to the fact that mannoproteins have been found to interact and reduce the volatility of fruity, floral, and green aromas (i.e., ethyl hexanoate, β-ionone, and hexanol) at the concentrations that these macromolecules occur in wine (*49*), which would cause an enhancement in the perception of the rest of the aromas. In the mouth (**Figure 6**), wines with mannoprotein addition obtained the highest scores in sweet perception and roundness sensation. The enzyme addition had a strong effect on the mouth-feel and length, enhancing acidity, tannin, astringency, and length. Control wines were described as quite dry.

All the data obtained in the wine tasting were in good agreement with the analytical data found in the present paper. The enzymatic treatment produced the wines with the strongest visual color intensity, tannin, and astringency, coinciding with the highest values of color intensity, WC, and TPI. The use of industrial mannoproteins produced a considerable decrease in the TPI, related with the roundness and low astringency of these wines, and did not maintain the stable color, which also was in good agreement with the sensory data.

Taking into account these findings, the combination of both industrial mannoproteins and maceration enzymes could be considered to be of potential application in red wine maceration process in order to improve the sensory characteristics of the wines. However, a more detailed analytical study should be performed in order to confirm and better understand the differences observed between treatments. Further investigations will examine the effect of these treatments on wine polysaccharide and polyphenolic composition.

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