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Convenience of Applying of Viticulture Technique as a Function of the Water Status of the Vine-Stock

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Abstract

This study determined the effect that the irrigation dosage and cluster thinning showed over the antioxidant activities and total phenols in grapes, and phenolic profile and chromatic characteristics of cv. Tempranillo wines. The experiment was carried out over two consecutive seasons at an experimental vineyard in Extremadura (Spain). The plants were subjected to two post-*véraison* irrigation treatments, one at 100% (R100) and the other at 25% (R25) of crop evapotranspiration (ETc). Two crop loads (C and T) were additionally established for each irrigation treatment by cluster thinning at *véraison*. The winemaking process involved separate use of the grapes from each of the four resulting treatment groups (R100C, R100T, R25C and R25T) and followed a common protocol. In grapes, the effect of thinning enhanced when combined with water deficit, resulting in increases phenylpropanoids and flavonoids at the harvest, while leaving the polyphenol oxidase's activity unaffected. In wines, the higher post-*véraison* water stress of the R25 treatments resulted in higher values of dimeric flavonol and flavanol concentrations. The wine hue was affected by water status. The cluster thinning caused additional increases in the concentrations of monomeric flavanols and anthocyanins, as well as resulting in stronger wine colour intensity.

Keywords: irrigation, crop load level, antioxidant activity, phenolic composition, wine, grapes

1. Introduction

Because of the high dependence of fruit quality on various environmental and endogenous factors [1, 2], several agronomic techniques have been used to improve the quality of the berries. Appropriate viticultural practices that enhance ripening, by creating favourable mesoclimates,

or by achieving adequate but not excessive vine vigour, will improve wine quality [3]. Soil water availability is a critical factor for vine performance and wine composition. Reports show that severe water stress can influence various physiological and metabolic plant processes, including growth, photosynthesis, and respiration and might be detrimental to fruit quality because of a poor canopy development and reduced leaf assimilation rate thus leading to an inadequate vine capacity to ripen the crop [4–6], particularly under high yield levels [7]. These, in turn, can affect the production, composition and characteristics of the must [8] and, in consequence, the quality of the resulting wine [9]. However, controversy remains as to whether there is any direct effect on berry metabolism other than inhibition of growth [10]. In semi-arid regions, irrigation is often applied as a technique to increase yield and in occasions to achieve a proper supply-demand vine balance. Regulated deficit irrigation (RDI) consists of applying short episodes of water restriction, typically starting after bloom (anthesis), whereby irrigation water is supplied at amounts below those lost to vineyard or crop evapotranspiration (ETc) [11]. Several studies have reported that water stress during the growing season has some beneficial effects (increasing anthocyanin and polyphenol concentrations and soluble solids content) on grape berry quality [12–15]. Water stress applied during the period from fruit set to *véraison*, heavily reduces fruit size [16] and in the late season water restriction may reduce fruit cell enlargement and water accumulation [17]. The timing and intensity of water deficits influence the extent of alterations occurring in berry metabolism and therefore in wine colour and flavour [18–20]. Irrigation is now a commonly used technique in vine-growing in Spain, where cv. Tempranillo is the most widespread cultivar for red wine production. Previous studies have been conducted to analyse the effect of irrigation in phenolic profile of grapes [9, 21–23]. In another hand, it is widely accepted that very high crop yields delay ripening and reduce fruit and wine quality [24]. This leads to increases in the required substrate levels for the synthesis and accumulation in thinned vine berries of, amongst others, phenolic substances [25–29]. Water restriction in Tempranillo grapes led to alterations in the levels of peroxidation and peroxidase activity [30].

In another hand, it is widely accepted that very high crop yields delay ripening and reduce fruit and wine quality [24]. The crop level is a determining factor in berry quality and one of the goals of modern viticulture is to establish field practices which are able to limit vineyard yield and improve grapevine composition [31, 32]. The cluster thinning is a common practice designed to control yield and ripening under adverse conditions of climate. This practice influences the sink-source ratio, restricting part of the yield without lowering leaf surface area. Thinning allows the plant to concentrate its activity on the remaining clusters, making it an effective method of regulating production and modifying the composition of the berries [31, 33, 34]. Several authors have found that cluster thinning enhances berry ripening, affecting the contents of sugars, acids, polyphenols and aromas of the harvested grape and therefore positively affecting the quality of the wine [34, 35]. Cluster thinning is in cases applied to regulate the yield levels and to help ripening the crop under poor climatic conditions or excessive crop demand. However, results presented in literature have reported contrasting results, with cluster thinning leading to better fruit quality in some cases [36, 37], but with no clear effect in some others [33, 38]. Studies on cluster thinning have also shown the importance of the time when it is performed. When thinning is performed at *véraison*, the effect on berry weight and cluster size is lower than when performed immediately after fruit set, since it is at *véraison* that potential berry size and the number of berries per cluster are determined, as well

as some compounds, such as the organic acids [39]. In addition, the result of cluster thinning can depend to a large extent on the climatological conditions during the season, with contrasting and even opposite results being obtained depending on the varieties and yields [36, 37].

The overall effect of irrigation might change according to other cultural practices particularly those affecting the crop level [24, 40]. Vines with higher crop level seem to benefit more of a higher amount of irrigation both in terms of yield [41] and of fruit composition [42]. Numerous studies exist in the effects of water deficit and control of yield by eliminating fruits (thinning) on berry composition, but less research has been developed to the combination of both techniques in its composition of wines. Determination of such relationships between composition and chromatic characteristics allow greater understanding of how interactions between cultural practices and chemical composition are reflected in the perception of colour. This type of study is particularly important in vineyards with resource limitations, as would be the case in deficit irrigated grapevines with high crop loads. Such studies are also relevant in relation to red grapevine cultivars, since the repercussions of these practices on the phenolic and chromatic characteristics of the wines obtained need to be analysed. Up to now, few studies have analysed how the combined practice of these techniques affects wine phenolic composition, and the results are not conclusive as to how the water status of the vine impacts on the effect of cluster thinning or vice-versa [18, 19, 39, 43, 44].

The phenolic compounds from the grape are responsible for some of the main organoleptic characteristics of the resulting wine, including its colour, astringency and aroma. In the red wine making process, the phenolic compounds of the skin and seeds are transferred to the must during the fermentation and maceration process [45]. Phenolic compounds can be divided into flavonoid (anthocyanins, flavan-3-ols and flavonols) and non-flavonoid (stilbenes and *hydroxybenzoic and hydroxycinnamic* acids). Given the importance of colour in the quality of red wines, phenolic compound concentration can be used as a parameter for the evaluation of grape, must and wine quality [46]. Besides the variety, other factors such as location, climate, soil type, berry maturity and the vine-growing practices affect the phenolic composition of the grape berries [47]. Improved vine management techniques can therefore be used to modify phenolic content and enhance wine quality [48]. The question of grape berry size and its effect on wine composition has produced inconclusive results, and the relationship between grape berry size and the phenolic composition of wine remains unclear [49]. Wine colour is linked to the accumulation of anthocyanins in the grape berries and particularly in the skin. However, it is not only the anthocyanin concentration and profile that is responsible for wine colour: copigmentation can account for 30–50% of colour in young wines [50]. Copigmentation in wine results from molecular interactions between anthocyanic pigments and other organic molecules, called cofactors, which form molecular associations or complexes. For copigmentation in young wines to increase, there must be sufficient quantities of substances that can act as copigments. The most common cofactors are compounds such as phenolic acids, flavonoids, and in particular, flavonol and flavone derivatives [51]. Good quality grapes should have high concentrations of both stable pigments and substances that can act as cofactors.

In plants, increased synthesis of phenols is a common response to stress [52] and the same environmental conditions that cause oxidative stress are associated with the induction of phenylpropanoid metabolism. Phenylpropanoids and flavonoids are involved in the

protection against oxidative stress [53]. The level of lipid peroxidation and how the total antioxidant capacity and the phenolic compound content evolve can both be used as indicators of stress and the response to it. One response to stressors during ripening is an increase in the amount of phenolic compounds. These include flavonoids and phenylpropanoid glycosides, compounds that are part of the cellular antioxidant system, and which are involved in the elimination of ROS [52–55].

ROS have been implicated in plant growth and stress responses. The production and detoxification of ROS are both highly regulated processes, and ROS levels are kept under tight control. The antioxidant system plays an important part in ROS homeostasis [56, 57]. It includes such enzymes as peroxidase (POX), catalase (CAT) and superoxide dismutase (SOD). Stresses and physiological processes in plants both induce the development of a complex network of oxidant/antioxidant reactions that modulate the resulting oxidative response [58, 59]. Thus, a key role in the response to stresses is played by the activities involved in ROS production and elimination, and they are also involved in such physiological processes as ripening [60]. In this regard, while the participation of peroxidases, catalases and superoxide dismutase is crucial for the control of the production or elimination of ROS, their activities are affected by water stress and defoliation [30].

Tempranillo is the most common variety of red grape in Spain comprising a total of 205,975 ha (20% of Spain's total vineyard area), though most of these are cultivated under rainfed conditions with typically low yields ($\leq 6500 \text{ kg ha}^{-1}$). This cultivar is originally from northern, cool regions of Spain and today is the most widely cultivated for production of red wines all over Spain. It is reputedly to be sensitive to water stress and prone to early leaf senescence [61].

This chapter analyses and studies the effects of the combination of water deficit and thinning on the constitution and composition of the Tempranillo grape, namely on the lipid peroxidation, oxidant and antioxidant enzymes activities and their interactions on phenolic profile and chromatic characteristic from the wines. Also, relations between wine chromatic parameters and phenolic substances have been investigated.

2. Materials and methods

2.1. Vineyard site and experimental design

The experiment was conducted in a *Vitis vinifera* L. cv. Tempranillo vineyard in Finca La Orden (owned by the Regional Government of Extremadura) in Extremadura, Spain, over the 2007 and 2008 vintages. The vineyard was planted in 2001 on Richter 110 rootstock at a spacing of 2.5 by 1.2 m ($3333 \text{ vines ha}^{-1}$). Row orientation was north-south and vines were trained to a bilateral cordon system and vertical trellis.

The experimental design was a complete randomised block with 16 experimental plots, 4 replicates by 4 treatments (irrigation and cluster thinning). Experimental plots consisted of

48 vines across 6 rows. The experiment comprised 768 vines in total. The irrigation regimes, applied during *véraison* and maturation, were as follows:

- Full irrigation (R100), corresponding to 100% of crop evapotranspiration (ETc).
- Deficit irrigation (R25) corresponding to 25% of ETc

ETc was calculated using the equation $ETc = ET_o \times Kc$, where ETc was estimated as the product of reference evapotranspiration (ET_o), measured by a weather station at the site, and crop coefficient (Kc), following the methodology of Allen et al. [62]. Irrigation was initiated when stem water potential (Ψ_s) reached -0.5 MPa and stopped after harvest, in mid-September. Irrigation was applied with pressure-compensated emitters supplying 4 L h^{-1} and spaced 120 cm apart. Two cluster load levels were established for each irrigation regime:

- Control treatment (C) without cluster thinning (7–9 clusters m^{-2} of planting area)
- Cluster-thinning treatments (T), in which the load was adjusted to 4–5 clusters m^{-2} of planting area by removing clusters at *véraison*.

Table 1 represents the ET_o, ETc and Kc, as well as the irrigation amount applied in the R100 and R25 treatments for each of the study years. Also shown is the water status of the plant during the growing period in the two campaigns (stem water potential during the flowering-*véraison* and *véraison*-harvest periods) [18].

2.2. Physico-chemical composition of grapes and wine chromatic characteristics

Table 2 represents the physico-chemical characteristics of the grapes at harvest and the chromatic characteristics of the wines. The winemaking protocol and analytic methodology can be consulted in Gamero et al. [19].

2.3. Biochemical assay and enzymatic activities of the grapes

Phenols, flavonoids and phenylpropanoid glycosides were assayed colourimetrically. First, grapes were homogenised with methanol, chloroform and 1% NaCl (1:1:0.5). The homogenate was filtered and centrifuged at $3200 \times g$ for 10 min. Total phenols (expressed as μg caffeic acid g^{-1} FW) were determined at 765 nm with Folin:Ciocalteu reagent according to the method of singleton et al. [63]. Total flavonoids (expressed as μg rutin g^{-1} FW) were determined according to the method of Kim et al. [64], calculating the content on the basis of the rutin standard curve. Phenylpropanoids (expressed as μg verbascoside g^{-1} FW) were determined at 525 nm based on estimating an *O*-dihydroxycinnamic derivative using the Arnou reagent as described in Gálvez et al. [65], calculating the content on the basis of the 3,4-dihydroxyphenylalanine standard curve.

Lipid peroxidation was determined by measuring malondialdehyde (MDA) formation using the thiobarbituric acid (TBA) method as described by Madhava Rao and Sresty [66]. The MDA

Year	Seasonal measurements				Irrigation (mm)		Ψ_s flowering- <i>véraison</i> (MPa)		Ψ_s <i>véraison</i> -harvest (MPa)	
	Rainfall (mm)	ET _o (mm)	ET _c (mm)	K _c	R100 ¹	R25	R100	R25	R100	R25
2007	182	975	508	0.52	172	62	-0.54	-0.58	-0.56	-0.77
							-0.81	-0.51		
2008	129	1026	755	0.74	611	153	-0.52	-0.76	-0.49	-0.89

Effective seasonal rain, reference evapotranspiration (ET_o), crop evapotranspiration (ET_c), crop coefficient (K_c) from April to September and mean values in each season of stem water potential (Ψ_s). Irrigation: Volumes of water applied in the 2007 and 2008 treatments. In 2007 and 2008, the ET_c values were obtained by means of a lysimeter installed in the vineyard.¹R100 = Full irrigation (100% ET_c); R25 = Water deficit (25% ET_c)

Table 1. Water balance variables in Tempranillo vineyard, Extremadura (Spain).

concentration (expressed as nmol MDA g⁻¹ FW) was calculated using an extinction coefficient of $\epsilon = 155 \text{ mM}^{-1} \text{ cm}^{-1}$.

Enzymatic activities were determined on a crude extract of the grapes. The grapes (2 g mL⁻¹) were homogenised at 4°C in 50 mM phosphate buffer, pH 6.0. The homogenate was filtered and centrifuged at 39,000 × g for 30 min at 4°C. The pellet was discarded, and the supernatant filtered and collected for the enzyme assays. The protein content was determined by the method of Bradford [67].

Peroxidase (EC 1.11.1.7) activity, POX, was measured at 590 nm ($\epsilon = 47.6 \text{ mM}^{-1} \text{ cm}^{-1}$) [68], and the coniferyl alcohol (CA) peroxidase activity, CA-POX, was determined by measuring the decrease in absorbance at 265 nm of a reaction medium consisting of the enzyme extract and 0.1 mM CA in 25 mM acetate buffer pH 5.0 ($\epsilon = 7.5 \text{ mM}^{-1} \text{ cm}^{-1}$). A unit of CA-POX is defined as the amount of enzyme required to cause the oxidation of 1 nmol CA per minute at 25°C, pH 5.0. The superoxide dismutase (SOD) (EC 1.15.1.1) activity was determined from the absorbance at 560 nm according to [69]. A unit of SOD is defined as the amount of enzyme required to cause 50% inhibition of NBT reduction. Polyphenol oxidase (PPO) (EC 1.14.18.1) activity was determined from the absorbance at 390 nm [70]. A unit of PPO is defined as the amount of enzyme required to cause a decrease in absorption of 0.001 units min⁻¹.

2.4. Individual phenolic composition of the grapes analysed by HPLC-DAD-FLD

Low molecular weight phenols were analysed by HPLC-DAD, following the method described in Gómez-Alonso [71], though with some slight modifications to enhance the resolution. After filtering (0.25 m diameter Chromafil filters, Düren, Germany), 10 µl of wine were directly injected into a LC-Agilent 1200 (Agilent Technologies, Palo Alto, CA, USA) chromatographic system. Separation was performed in an Ace® 5 C18 250 × 4.6 mm (Advanced Chromatography Technologies, Aberdeen, Scotland) column used as stationary phase.

Parameters	Treatment	Irrigation dosage ^{1,3}		Significance level ²		
		R100	R25	R	T	R × T
Yield (kg ha ⁻¹)	C	21403.75 ^a	18749.58 ^a	ns	**	ns
	T	14987.03 ^b	14704.16 ^b			
Berry weight (g)	C	2.17 ^a	2.00 ^b	ns	**	ns
	T	2.03 ^b	1.90 ^b			
Total soluble solids (°Brix)	C	22.75 ^{ab}	21.93 ^b	ns	***	*
	T	23.87 ^{ab}	24.37 ^a			
Titratable acidity (g tartaric acid L ⁻¹)	C	6.43 ^a	5.51 ^{ab}	***	***	ns
	T	5.12 ^{ab}	4.32 ^b			
Maturity index ⁴	C	3.60 ^b	4.08 ^b	*	**	*
	T	4.66 ^{ab}	5.81 ^a			
Colour intensity (AU)	C	6.69 ^b	6.71 ^b	ns	***	***
	T	9.51 ^a	9.70 ^a			
Colour hue	C	0.65 ^a	0.62 ^b	*	ns	ns
	T	0.67 ^a	0.60 ^b			
% red	C	52.81 ^b	54.00 ^a	***	ns	ns
	T	52.06 ^b	54.88 ^a			
% blue	C	13.00 ^a	12.31 ^b	*	ns	ns
	T	13.20 ^a	12.08 ^b			
% yellow	C	34.18 ^a	33.67 ^b	*	ns	ns
	T	34.73 ^a	33.02 ^b			

¹R100C = Full irrigation (R100) without cluster thinning (C); R100A = Full irrigation with cluster thinning; R25C = Water deficit without cluster thinning and R25A = Water deficit with cluster thinning

²Significance level: ns = not significant; * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$

³Values followed by different letters indicate significant differences between them ($p \leq 0.5$)

⁴Maturity index = Total soluble solids/titratable acidity

Table 2. Mean values of 2007 and 2008 seasons combined for yield, berry composition and wine chromatic characteristics of cv. Tempranillo wines, subjected to different irrigation dosage levels with cluster-thinning treatment (T) and without cluster-thinning treatment (C) in Extremadura (Spain).

The various phenolic compounds were identified by order of elution and the retention times of their respective standards. A total of 42 phenolic compounds were identified, distributed between simple and conjugated anthocyanins and monomeric and dimeric flavonols and flavanols. The anthocyanins were identified in the monoglucoside, acetyl-glucoside and *p*-coumaroyl-glucoside forms of delphinidin, cyanidin, petunidin, peonidin and malvidin. The quantified flavanols were (+)-catechin, (-)-epicatechin and the procyanidins (B1, B2 and B3). The analysed flavonols were myricetin, quercetin, kaempferol and isorhamnetin in free form

and their respective 3-glucosides. Also quantified were the quercetin and kaempferol rutinosides, quercetin-3-glucuronide and quercetin-3-galactoside.

The concentration was calculated in mg L^{-1} of malvidin-3-glucoside, myricetin-3-glucoside and catechin for anthocyanins, flavonols and flavanols, respectively.

2.5. Wine colour composition

Wine colour was evaluated using the indices proposed by Glories [72]: colour intensity (CI) was calculated by adding the absorbance readings at 420, 520 and 620 nm, while hue (CT) was determined as the ratio of absorbance readings at 420 and 520 nm, and percentages of red, blue and yellow (red %, blue % and yellow %). The wines were centrifuged for 3 min at $1100 \times g$ before spectrophotometric analysis. Measurements were taken using a Shimadzu UV-1700 spectrophotometer (Shimadzu, Japan).

2.6. Statistical analysis

Data were analysed using a general linear model, with water status (two levels), cluster thinning (two levels) and their interactions as factors. Differences between treatments were obtained using Tukey's test for significant differences ($p \leq 0.05$).

A principal component analysis (PCA) was carried out to observe the distribution of the wines of the different treatments as a function of their phenolic composition. A correlation analysis was performed to determine the relationships between phenolic compounds and agronomic factors and Tukey's test was used to evaluate significant correlations ($p < 0.05$).

The partial least squares (PLS) approach was used to analyse the relationships between wine colour and HPLC-quantified phenolic compounds. Collinearity between X variables was discarded as significant correlations were not found between them. The statistical analyses were performed with the XLSTAT-Pro (Addinsoft 2009, Paris, France) statistical software package.

3. Results

3.1. Effect of treatments on the phenolic content and oxidant/antioxidant activities in grapes

Table 3 shows the different values obtained for oxidant and antioxidant activities, and phenolic content in grapes at harvest. With thinning, all these phenolic components were found in higher or equal concentrations, but not lower. The greater value of lipid peroxidation corresponds to CT (rainfed and thinned). The SOD activity was always lower than in the unthinned treatments. It stands out that the lowest values of SOD activity corresponded to CT. Regarding the activity of PPO, an enzyme which catalyses the formation of *o*-quinones, at harvest, the PPO activity levels were similar in all treatments, although showing a minimum value in CT. The lowest POX values correspond to the thinning treatments (CT and R100T). The activity levels of CA-POX, an enzyme which is involved in processes of lignification, were slightly higher with thinning than without thinning.

Treatment*	Lipid peroxidation nmol MDA g ⁻¹ FW	Superoxide dismutase (SOD) U mg ⁻¹ prot	Peroxidase (POX) U mg ⁻¹ prot	Conyferilalcohol peroxidase (CA-POX) U mg ⁻¹ prot	Polyphenoloxidase (PPO) U mg ⁻¹ prot	Total Phenols µg caffeic acid g ⁻¹ FW	Total Phenylpropanoid glycosides µg verbascoside g ⁻¹ FW	Total flavonoids µg rutin g ⁻¹ FW
C	94.6 ± 20.5 ^b	89.3 ± 21.7 ^b	4.9 ± 1.2 ^b	53.6 ± 16.5 ^b	1415.5 ± 615.4 ^b	988.6 ± 142.2 ^a	1689.9 ± 290.5 ^b	1122.3 ± 211.0 ^b
CT	130.1 ± 23.0 ^a	71.6 ± 18.0 ^b	4.2 ± 1.5 ^b	66.2 ± 19.3 ^{ab}	900.3 ± 189.0 ^a	1108.9 ± 158.0 ^a	2013.5 ± 188.3 ^b	1589.7 ± 132.4 ^a
R100C	88.3 ± 18.4 ^b	158.7 ± 22.8 ^a	5.2 ± 1.2 ^b	78.2 ± 20.8 ^a	1510–3 ± 312.5 ^b	1045.6 ± 100.1 ^a	1865.0 ± 366.0 ^{ab}	1325.6 ± 126.1 ^{ab}
R100T	91.4 ± 24.3 ^b	81.5 ± 20.1 ^b	2.6 ± 1.0 ^a	79.0 ± 22.1 ^a	1389.0 ± 340.0 ^b	966.0 ± 144.5 ^a	1924.4 ± 171.3 ^b	1159.0 ± 206.6 ^b

Data were the means ± SD of at least 10 replicates obtained from 5 different experiments. The values were subjected to the Tukey test (different letters within each column indicate significant difference, $p \leq 0.05$)¹C = Rainfed without cluster thinning; R100C = Full irrigation without cluster thinning; CT = Rainfed with cluster thinning; R100T = Full irrigation with cluster thinning

Table 3. Average values (2007 and 2008 vintage) of lipid peroxidation, antioxidant activities, total phenols, phenylpropanoid glycosides and flavonoids on grapes cv. Tempranillo at harvest.

3.2. Effect of treatments on the phenolic profile of wines

Table 4A and **B** shows the mean concentration values over the 2 year study period of anthocyanin and non-anthocyanin flavonoid substances (flavanols and flavonols) of the R100C, R100T, R25C and R25T wines, as well as the results of the statistical analysis.

Table 4A shows the amount of monoglucosides (G), acetates (A) and coumaroyl derivatives (C) and the amounts of delphinidin (Dp), cyanidin (Cy), petunidin (Pt), peonidin (Pn) and malvidin (Mv) compounds.

In the case of the flavanols (**Table 4B**), as well as the mean concentration values of (+)-catechin (Cat) and (-)-epicatechin (Ep), also shown are the global mean values of these monomers (Mon) and, as well as the individual mean of (-)-epicatechin-(4 β -8)-(+)-catechin (PB1), (-)-epicatechin-(4 β -8)-(-)-epicatechin (PB2) and (+)-catechin-(4 β -8)-(+)-catechin (PB3) values, the combined mean values of these dimers (Dim). The flavonols are grouped together by the flavonol molecule present, such that myricetin (My), quercetin (Qc), kaempferol (Kp) and isorhamnetin (Ir) glucosides represent the total values, respectively.

Results of the general linear model analysis only found statistical significance of the irrigation and thinning interaction in Cy, PB1, PB3, Dim and total flavonoid substances. A statistical analysis was then performed of the overall effect of irrigation dosage and its individual effect on each crop load, as well as of the overall effect of cluster thinning and its effect on each water status.

3.2.1. Effect of irrigation

As can be seen in **Table 4A**, the different irrigation dosages had almost no effect on wine anthocyanin concentration values. Slightly lower concentrations of these substances were found in the wine from R25 treatment compared to the wine from R100 treatment, but the differences were non-significant ($p > 0.05$). This trend was also observed when studying separately the effect of irrigation dosage on the different crop loads (R100T vs. R25T and R100C vs. R25C).

However, the opposite trend was observed for the rest of the flavonoid families (flavanols and flavonols). The R25 wines contained significantly higher concentrations of Cat, the flavonol compounds My, Qc, Kp and Ir, Mon and total flavanols (**Table 4B**). An analysis of the effect of irrigation dosage on the different crop loads revealed significant differences only in the wines from the cluster-thinned treatments (R25T vs. R100T).

3.2.2. Effect of cluster thinning

In terms of wine anthocyanin composition, the effect of thinning was greater than of irrigation, with the A-treatment wines having significantly higher values of all the substances of this family, except for the acetyl-acetate derivatives. The biggest differences were found in the G (18%), Mv (15%) and Dp (29%). **Table 4A** also shows the separate analysis made of the effect of thinning on the R25 and R100 wines, with a greater and more significant response in R100. These results confirm the importance of vine water status in the effect of cluster thinning on

Compound	Load	Water status ²		Significance level ¹						
		R100	R25	R	T	Irrigation		Thinning		R and T
						in C	in T	in R25	in R100	
Σ Glucosides	C	90.21 ^{ab}	83.35 ^b	ns	*	ns	ns	ns	**	*
	T	111.08 ^a	100.19 ^a							
Σ Acetates	C	11.96 ^a	10.26 ^a	ns	ns	ns	ns	ns	ns	ns
	T	11.28 ^a	11.2 ^a							
Σ Cumarates	C	10.18 ^b	11.65 ^b	ns	**	ns	ns	ns	ns	*
	T	14.45 ^a	14.32 ^{ab}							
Σ Malvidins	C	82.88 ^a	79.59 ^a	ns	**	ns	ns	ns	**	ns
	T	100.35 ^a	92.06 ^a							
Σ Petunidins	C	15.04 ^{ab}	13.23 ^b	ns	***	ns	ns	ns	**	*
	T	18.73 ^a	17.58 ^{ab}							
Σ Delphinids	C	7.97 ^b	8.11 ^{ab}	ns	***	ns	ns	ns	***	**
	T	11.80 ^a	11.06 ^{ab}							
Σ Peonidins	C	3.32 ^a	2.62 ^a	ns	*	ns	ns	ns	ns	ns
	T	3.93 ^a	3.49 ^a							
Σ Cyanidins	C	3.15 ^a	1.70 ^b	Significant interaction						*
	T	2.01 ^b	1.53 ^b							
Total Anthocyanins	C	112.36 ^a	105.25 ^a	ns	**	ns	ns	ns	*	ns
	T	136.82 ^a	125.67 ^a							

¹ANOVA test significance level. R: irrigation; A: cluster thinning; R and A: combination of both treatments. ns = not significant; * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$

²For the same compound, values followed by different letters indicate significant differences between treatments ($p \leq 0.5$)

Table 4A. Average values (2007 and 2008 vintage) of anthocyanins (mg L^{-1}) in Tempranillo wines from vines subjected to different irrigation dosages and with different crop level loads, cluster thinned (T) and non-thinned (C), in Extremadura (Spain).

the concentration of anthocyanin substances present in the wines. In addition, when compared to the C-treatment wines, the T-treatment wines had lower concentrations ($p < 0.001$) of Cat, and higher concentrations of My and Qc (**Table 4B**).

3.2.3. Effect of treatments

Table 4A and **B** also shows the results of the combined statistical analysis of the four treatments. In concordance with the results described above, the highest and lowest values for anthocyanin substances were found in the 100T and 25C wines, respectively. Of these substances, Cy (the sum total of cyanidin compounds) is a special case as both the lower irrigation

Flavanols										
Compound	Load	Water status ²		Significance level ¹						
		R100	R25	R	T	Irrigation		Thinning		R and T
				in C	in T	in R25	in R100			
(+)-catechin	C	25.67 ^b	31.89 ^a	**	***	ns	***	*	***	***
	T	16.35 ^c	23.08 ^b							
(-)-epicatechin	C	4.35 ^a	4.01 ^a	ns	ns	ns	*	ns	ns	ns
	T	5.42 ^a	4.31 ^a							
Procyanidin 1 (PB1)	C	34.54 ^c	49.44 ^b	Significant interaction						***
	T	40.95 ^{ab}	71.18 ^a							
Procyanidin 2 (PB2)	C	6.64 ^b	6.00 ^b	ns	***	ns	ns	**	***	**
	T	6.00 ^b	8.24 ^a							
Procyanidin 3 (PB3)	C	17.08 ^{bc}	16.14 ^c	Significant interaction						***
	T	21.96 ^a	18.27 ^b							
Monomers	C	30.03 ^b	35.91 ^a	***	***	ns	**	*	**	***
	T	21.77 ^c	27.38 ^b							
Dimers	C	58.26 ^c	71.59 ^b	Significant interaction						***
	T	71.04 ^b	97.69 ^a							
Total Flavanols	C	88.29 ^c	107.49 ^b	***	**	***	***	**	ns	***
	T	92.81 ^c	125.07 ^a							
Σ Myricetins	C	8.06 ^c	9.64 ^b	***	***	ns	*	***	***	***
	A	11.46 ^b	14.06 ^a							
Σ Quercetins	C	6.52 ^c	8.20 ^b	***	***	ns	**	**	**	***
	A	7.84 ^{bc}	10.5 ^a							
Σ Kaempferols	C	1.40 ^c	1.74 ^b	*	ns	***	ns	***	***	***
	A	1.55 ^{bc}	2.17 ^a							
Σ Isorhamnetins	C	0.96 ^c	1.28 ^b	***	***	*	*	**	**	***
	A	1.24 ^b	1.62 ^a							
Total Flavonols	C	16.94 ^c	20.85 ^b	***	***	ns	**	***	***	***
	A	22.09 ^b	28.37 ^a							

¹ANOVA test significance level. R: irrigation; A: cluster thinning; R and A: combination of both treatments. ns = not significant; * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$

²For the same compound, values followed by different letters indicate significant differences between treatments ($p \leq 0.5$)

Table 4B. Average values (2007 and 2008 vintage) of flavanols, flavonols, (mg L⁻¹) in cv. Tempranillo wines from vines subjected to different irrigation dosages and with different crop load levels, cluster thinned (T) and non-thinned (C) in Extremadura (Spain).

dosage as well as cluster thinning resulted in lower concentration values, with the consequent concentration value for the R100C wines being significantly higher than for the other wines.

Values of the PB1 and PB3 dimers increased as irrigation dosage fell, with the 25T and 100C wines having the minimum and maximum values, respectively. Finally, and given the sensitivity of flavonols to both irrigation dosage and crop load, the R25T wines had significantly higher values than the R100C.

3.2.4. Correlation between agronomic parameters and grape berry ripeness indicators and concentration of wine phenolic substances

To determine the degree of correlation between the agronomic parameters (*véraison*-harvest Ψ_s and yield values of the different treatments), berry weight and berry ripeness indicators (TSS—total soluble solids and MI—maturity index) and the values of the different phenolic families of the vines, the mean values for the 2 years of the study were subjected to a Pearson's correlation and a PCA.

No significant correlation was found between *véraison*-harvest Ψ_s and wine phenolic values. However, significant negative correlations were found between yield and concentration

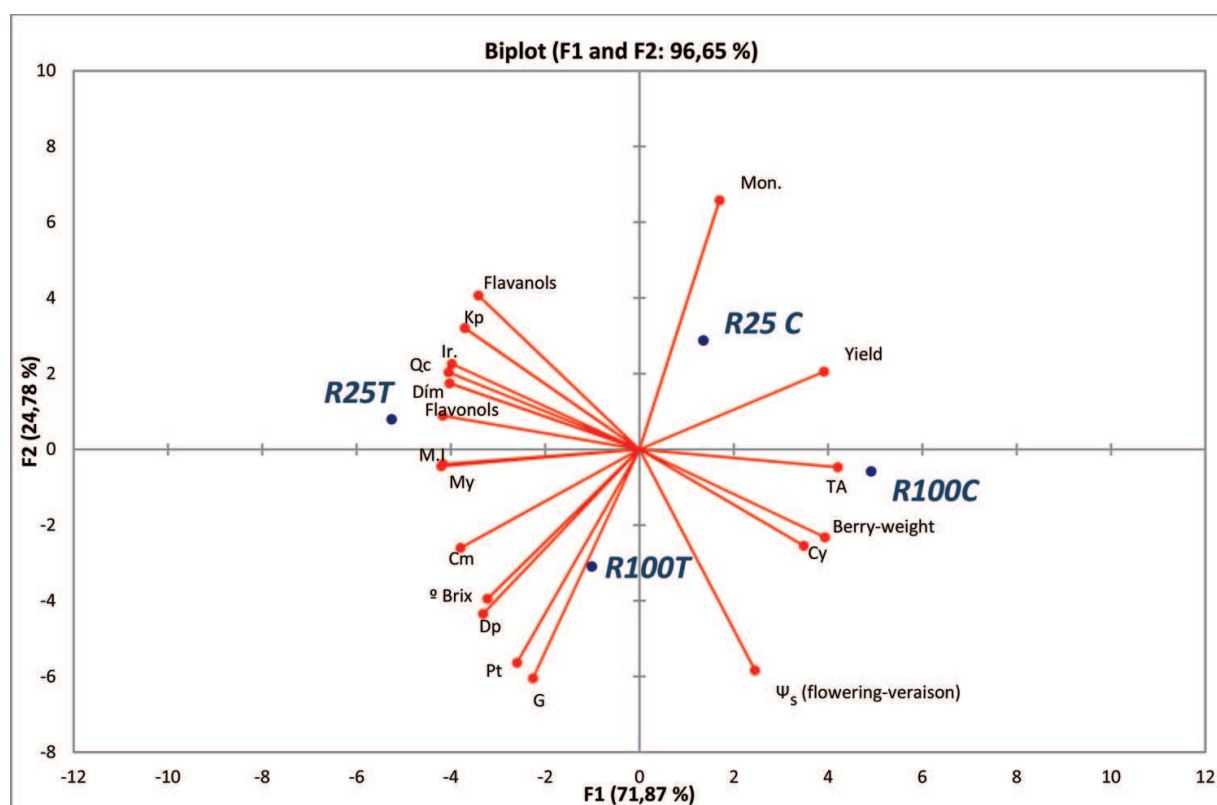


Figure 1. Principal component analysis (PCA) between the different parameters studied (stem water potential Ψ_s , yield, ° brix and maturity index (MI) and wine phenolic compounds. G: Σ Glycosides, Cm: Σ Coumarates, Pt: Σ Petunidins, Dp: Σ Delphinids, Cy: Σ Cyanidins, My: Σ Myricetins, Qc: Σ Quercetins; Kp: Σ Kaempferols; Ir: Σ Isorhamnetin; Mon: Σ Monomers; Dim: Σ Dimers, TA: total acidity.

of C (anthocyanin coumarates, $r = -0.997$) and between Qc and Ih ($r = -0.961$ and -0.984 , respectively) and berry weight. This latter parameter was also positively correlated with Cy ($r = 0.955$). Conversely, berry acidity was negatively correlated with dimer values ($r = -0.956$) and all the flavonols except Kp. Finally, berry ripeness as reflected by the MI was positively correlated with dimers and My ($r = 0.955$ and 0.997 respectively).

Figure 1 represents the projection of the variables and samples in the plane defined by the first two principal components. The two principal components explain 96.65% of total variance. Principal component 1, which explains 71.87% of the variance, allows to distinguish the T wines from the C wines. This component is characterised by titratable acidity (TA), yield and berry weight on the positive side of the axis and by practically all the phenolic substances except G and Pt on the negative side, such that as we shift from left to right the wines are poorer in phenolic substances. The second principal component explains 24.78% of the variance and allows to distinguish the R100 from the R25 wines and is characterised by high monomer concentrations on the positive side of the axis and by *véraison*-harvest Ψ s and G values on the negative side.

3.2.5. Correlation between wine phenolic composition and chromatic parameters

A PLS regression was performed to determine correlations between the chromatic parameters of the different wines, Colour intensity and Colour hue (CI, CT), percentage of red, blue and

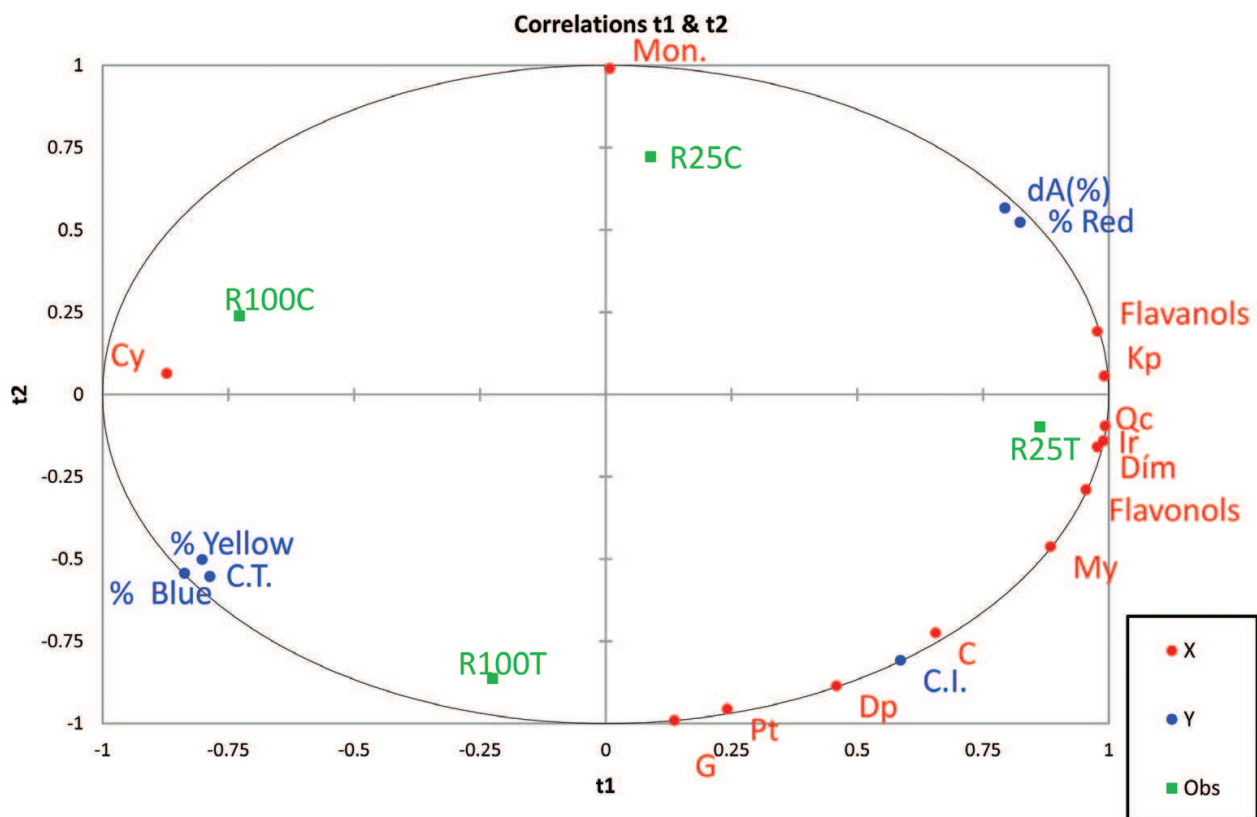


Figure 2. Partial least squares (PLS) regression between the colorimetric parameters and wine phenolic compounds. G: Σ Glycosides, Cm: Σ Coumarates, Pt: Σ Petunidins, Dp: Σ Delphinids, Cy: Σ Cyanidins, My: Σ Myricetins, Qc: Σ Quercetins; Kp: Σ Kaempferols; Ir: Σ Isorhamnetin; Mon: Σ Monomers; Dim: Σ Dimers.

yellow and dA%, and the phenolic compounds with significant inter-treatment differences. The chromatic data were taken as Y variables and phenolic substance concentrations as X variables. The data were also standardised to approximately the same scale to better reveal the correlations between the colour and chemical variables. Two new variables (axes) were obtained through this technique which explains together 97% of total variance.

Figure 2 shows how CI was high and positively correlated with the presence of G, C, Dp, Pt and My, with this correlation being higher and more intense in the R100T and R25A wines. Conversely, %dA and %red were positively correlated with Kp (sum total of kaempferol glucosides) and the overall amount of flavonol, with this correlation being higher in the R25T wines. These results allow to make wine colour predictions based on wine phenolic composition. Accordingly, colour intensity can be predicted by the amount of certain phenolic compounds in the wines: G and C, Dp and Pt, and My. In the same way, the %dA and %red of a wine can be predicted by the concentration of Kp and flavonols.

4. Discussion

The results obtained for phenols, flavonoids and PPGs in grapes at harvest are consistent with those obtained by Tardáguila et al. [73] who describe increased overall amounts of phenols and anthocyanins in grapes from thinned cv. Tempranillo vines. Likewise, Diago et al. [27] also describe an increase in these compounds caused by thinning in this variety, although the treatment was much less effective in the Garnacha variety. Esteban et al. [74] and Ojeda et al. [75] describe similar results in the content of total phenols, flavonoids, anthocyanins and flavonols as a result of water stress. Again, the combination of water deficit and thinning is the treatment with the greatest incidence on the evolution and content of these phenolic compounds. The SOD activity was always lower than in the unthinned treatments, an aspect which could have been the cause of the observed greater level of lipid peroxidation in the water deficit and thinned treatment. The nonspecific peroxidase activity was very low, especially in the thinned treatments. On the contrary, in the harvest the CA-POX activity's levels were slightly higher with thinning than without thinning. This enzyme might be involved in the production of H₂O₂ during the formation of lignins [76]. According to our previous results [30], it does not appear to be affected by either the stage of ripening or the different treatments (thinning and water deficit).

Most studies on the effect of irrigation on grape berry and wine anthocyanin composition have found a decrease in the concentration of different anthocyanin derivatives and in their overall concentration as irrigation dosage is increased [77, 78]. Until a few years ago, these results were explained as being secondary effects of irrigation, including modification of berry size and consequently of skin/pulp ratio, which were responsible for the decrease in the concentration of these substances in the berry [75]. In this respect, sufficiently large increases in R100 berry size were not obtained in our study (**Table 2**) to generate, by dilution effect, a decrease in the concentration of these substances in the wines. However, recent studies have found that these substances are synthesised in the biosynthetic phenylpropanoid pathway, and it has been shown that it is the pre-*véraison* deficit which regulates the expression of genes involved in this biosynthesis [79]. Accordingly, the lack of response to irrigation in the G, and

T and C forms, as well as in the Mv, Pt, Df and Pn derived compounds can be explained based on the different irrigation dosages applied in the post-*véraison* period.

In a similar study to ours conducted in Requena (Spain) with Tempranillo vines, Intrigliolo et al. [9] found increased anthocyanin concentrations in wines from the most irrigated vines. They explained their results as the consequence of the greater water requirements and the inability of cv. Tempranillo, typically grown in colder regions, to adapt to edapho-climatic conditions. This could also be the case in our study, as the highest values were observed in the R100 wines. This result might also suggest a greater synchrony between sugar and anthocyanin accumulation in the berry of the higher irrigation treatments [18].

The significant differences that were found in the flavanol- and flavonol-type substances are not a decisive factor for these substances. Studies have shown that while water deficit has a moderate effect on flavonol synthesis, the effect of the irrigation application period is almost negligible [23, 80]. This would explain the significant increase in My, Qc, Kp and Ir in the R25 wines compared to the R100 wines. The synthesis of these substances is light dependent, so the lower leaf area observed in the R25 treatment may also have contributed to this increase. In this respect, Matthews et al. [81] reported that the decrease in berry weight was higher, and consequently the increase in flavonoid concentration was higher, when water stress was imposed to pre-*véraison*.

Given the higher content of anthocyanin substance in the A-treatment musts [18], the formation of copigments must have taken place in them in a greater proportion during fermentation, which would in turn have resulted in greater flavonol extraction. This may be one of the reasons why, when the effect of irrigation was analysed separately on the two load levels, significant differences were only found in My, Qc and Ir values in the R100T versus R25T comparison. This result is very important, as copigments stabilise the colour of young red wines [50].

The results shown in **Table 4A** and **B** concurs with those from previous studies which show that cluster thinning is a useful tool to increase the concentration of different flavonoid compounds in grapes and wines [29, 37, 82]. Oenologically speaking, and in full concurrence with the results observed by Peña-Neira et al. (29), the most important and notable effects of cluster thinning are those related to the increase in phenolic compound values via the phenylpropanoid pathway.

Among the anthocyanin substances, the increase in the coumarate forms is particularly important, as these are more stable than the monoglucosides [83, 84] and offer greater stability to wine colour. Also notable is the low significance of cluster thinning on the R25 wines. In a similar study performed with the Syrah grape [82], cluster thinning led to significant increases in the coumarate forms of malvidin and peonidin. In our study, the maximum anthocyanin content in the R25 berries may have been generated at lower TSS values than those at which the berries were harvested (24.37°Brix), so that anthocyanin material may have been lost in these berries.

The increase in flavonols is especially interesting, since these contribute to astringency and also play an important role in anthocyanin copigmentation and in the formation of more complex pigments during the ageing process [85, 86]. In the copigmentation phenomenon which contributes to the stabilisation of the colour of red wines [50, 87], Baranac et al. [88] reported that flavonol substances are amongst the best copigments, especially quercetin [89].

The increased concentrations of catechin and procyanidin B1 are also important given their relation to colour and body stability through these copigmentation and polymerisation reactions with other flavonols.

Similar to the results from other studies [19, 26, 39], wines from the cluster-thinned treatments had higher CI values. Colour hue variation between wines as a function of vine water status has also been described in other studies, though with contradictory results [21]. In our study, the greater presence of cyanidin due to the later ripening of the R100C grapes may have been responsible for the higher percentage of blue hues ($p < 0.05$) of these wines.

Many studies have used statistical techniques to find correlations between phenolic compounds and colour parameters during the maturation and ageing processes of red wine [90]. In one interesting work, Monagas et al. [91] showed that chromatic attributes of red wines could be predicted by their phenolic profile using polynomial regression techniques. The substances which provided the best fitting model in that study were the anthocyanin compounds, and in the specific case of cv. Tempranillo wines the pyruvic adducts. According to the PLS regression which we performed, the CI of Tempranillo wines can also be predicted as a function of anthocyanin compounds, specifically C, G, and Df and Pt compounds. Along the same line, our work also confirms the findings of Escudero-Gilete et al. [92]. They studied the correlations between colour and anthocyanin composition of wines made from a blend of cv. Tempranillo and cv. Graciano grapes and found that the petunidin compounds (monoglucoside, acetyl-acetate and acetyl-coumarate) were the ones most closely related to the chromaticity of these wines. Similarly, the negative correlation between the percentages of yellow and blue and the flavanols which were also found by the PLS regression concur with the findings of the previously cited work of Monagas et al. [91]. From all of the above, it can be concluded that the thinning treatment generated, most notable, an increase in wine colour intensity and that vine water stress modified, most notably, wine hue as a result of differences in flavonol concentrations.

5. Conclusion

This work contributes to our understanding of how vine-growing practices can affect the compounds responsible for the sensory attributes of wines. It is shown how modification of water status and crop load through deficit irrigation and cluster-thinning techniques can induce variations in the phenolic composition and consequently the chromatic characteristics of a wine. It can be concluded from the results that post-*véraison* water stress increased the concentration values of non-anthocyanin flavonoids, though the extent of the increase varied depending on the compound under consideration and, in the case of flavanols, on the crop load. Consequently, special care should be taken to adapt and control irrigation dosages in regions of very dry and hot summers where post-*véraison* irrigation is an absolute necessity. The results of our study also show an increase in the phenolic composition of wines as a result of the cluster thinning of irrigated vines and, above all, show how the extent and significance of this technique depends in the case of anthocyanins and flavanols on vine water status. Furthermore, our work demonstrates how these practices can affect wine colour. With the increase in anthocyanin content, thinning augmented wine colour intensity, while the different levels of vine

water status affected colour hue by modifying the concentration of flavonols which participate in the reactions that help to stabilise wine colour.

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