Effect of the prefermentative addition of copigments on the polyphenolic composition of Tempranillo wines after malolactic fermentation

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Abstract: The influence of the prefermentative addition of copigments and different winemaking technologies on the polyphenolic composition of Tempranillo red wines after malolactic fermentation, was studied. Six experiments dealing with the prefermentative addition of caffeic acid, rutin, (+) catechin, white grape skin tannin, white grape seed tannin and control wines were realised. Three different winemaking technologies (traditional vinification, prefermentative cold maceration at 6-8 ºC and cold soak at 0-2 ºC with dry ice) were studied. Prefermentative addition of copigments increases anthocyanin copigmentation reactions and produces wines with a greater colour, a higher anthocyanin concentration, a superior contribution of anthocyanins to the colour of the wine, a superior percentage of tannins polymerized with polysaccharides and less astringency. Cold prefermentative maceration increases the extraction of polyphenols, the anthocyanin copigmentation reactions and the polymerization reactions between tannins and polysaccharides. The effectiveness of the combination of copigments and prefermentative maceration treatments was demonstrated by the increase of the concentration of the polyphenolic compounds.

Key words: Copigmentation · Prefermentative maceration · Red wine · Polyphenolic compounds

Introduction

Polyphenolic composition is very important in the organoleptic properties of red wines. Grape colour is an important part of red wine quality. It is generally accepted that an increase in grape colour coincides with an improvement in phenol structure and an increase in wine quality [1, 2, 3].

The colour in young red wine is due to a number of factors including the type and concentration of anthocyanins and the ionization index according to the forms of flavilium cation [4]. Anthocyanins present in red grape skin dissolve in the wine during the maceration/fermentation process [5, 6]. During and after fermentation, the anthocyanins content decreases by degradation, bleaching with sulphur dioxide, oxidation, complexation, precipitation and combination [7, 8]. Decreasing the loss of colour is important to conserve the quality of the wine. It is difficult to predict the intensity and the tone of the colour considering only the anthocyanin composition since monomeric anthocyanins are implicated in copigmentation complexes, chemical reactions (oxidation, covalent linking) and polymerisation reactions of that transform the monomeric anthocyanins into polymeric pigments. The monomeric forms of anthocyanins are responsible for most of the red colour of young wines. According Versari et *al*. [9], the total colour of wine is an aggregate number of three components: copigmented anthocyanins (8-30%), non-copigmented free anthocyanins (24-35%), and polymeric pigments (35-63%). The formation of polymeric pigments between anthocyanins and flavanols permit the preservation of wine colour [10, 11, 12, 13, 14]. Some authors suggest that the copigmentation reactions of anthocyanins are the first phase in the formation of stable polymeric pigments during wine aging [15, 16, 17, 18, 19]. This conversion from monomeric to polymeric anthocyanins produces a loss in the red colour intensity and a change in tone from purple-red to red, and cause an increase in the colour stability [17, 18, 19, 20].

Copigmentation is a hydrophobic interaction between monomeric anthocyanins and other phenolic compounds present in red wine. The molecules that can act with cofactors require a planar disposition because they need to stack with the flavilium forms of monomeric anthocyanins [16]. According to several authors, flavonols are the best cofactors [17, 18, 19], but other compounds such as flavanols [20], hydroxycinnamoyltartaric acids and hydroxycinnamic acids can also act as copigments when they are in higher concentrations in musts and wines [21, 22, 23, 24]. There are only a few studies in the literature about copigmentation in red wine, and these suggest that supplying the winemaking process with copigments allows the increase of copigmentation reactions contributing to the improvement of the colour stability of the wines [25, 26, 27, 28, 29, 30, 31, 32].

In order to prolong the contact between the polyphenolic compounds in the must and to favour copigmentation reactions it is interesting to increase the prefermentative maceration phase. The purpose is to increase the extraction and stabilization of the polyphenolic compounds in the liquid phase in the absence of alcohol [33, 34, 35, 36, 37, 38]. The addition of cofactors to copigmentation in the pre-fermentative step of the winemaking process enhances the intermolecular copigmentation in red must and wines [39]. If the prefermentation skin contact is carried out with dry ice, the start of the fermentation process is retarded and the skin cells are broken and disorganized through freezing. Freezing increases the volume of the intracellular liquids thus disrupting the membranes and providing an easy exit for the aromatic and phenolic compounds [40, 41].

In this work, Tempranillo grapes from Valencia (Spain) were processed with the prefermentative addition of copigments and different prefermentative maceration technologies. The wines were analyzed with spectrophotometic techniques after concluding the malolactic fermentation. The phenolic parameters studied were selected on the basis of their significance in red wines [42, 43]. These parameters have been used by many authors to describe the evolution of colour, anthocyanins, polymeric and copigmented pigments, tannins and astringency perception [44, 45, 46, 47, 48]. The contribution of copigmentation to wine colour was evaluated, and the relationship between the degree of copigmentation and polymerization of the phenolic composition of the wines was measured.

Materials and methods

Grape cultivar and winemaking technologies Grapes of *Vitis vinifera* cv Tempranillo grown in Chozas Carrascal winery in Valencia (Spain) with the Certification of Origin Utiel-Requena, were used to make red wines using various winemaking technologies combining the prefermentative addition of copigments and prefermentative maceration. Grapes with a total sugar content of 217 g/L, total acidity of 6.1 g/L (tartaric acid equivalent) and potential alcohol of 12.8 % were harvested manually. After destalking and crushing, the mash was transferred to closed 50 litre stainless steel tanks and supplied with 50 mg/kg potassium metabisulphite. All the vinifications were performed in triplicate. A total of 54 tanks were used. Nine as a control wines with no addition of copigments. Five groups of 9 tanks were produced each with 90 mg/l of copigments (caffeic acid (Fluka ≥ 95% HPLC), rutin (Sigma ≥ 95% HPLC), (+) catechin (Fluka ≥ 95% HPLC), white grape skin tannin (Protan Raisin of AEB) and white grape seed tannin (Tanéthyl of AEB). Each group of 9 was further divided into 3 groups of 3 tanks. Traditional winemaking methods were applied to the first group of 3 tanks, 3 with cold soak at 6-8 ºC for four days, and 3 with cold soak at 0-2 ºC with dry ice for four days. After prefermentative maceration, all the tanks were processed using traditional winemaking methods with inoculation of 30 g/hl commercial yeast (*Saccharomyces cerevisiae* strain Lalvil C 1108, Lallemand). The temperature was maintained below 28 ºC for 10 days; temperature and density were measuring daily. Manual punching down was carried out twice a day by “pigeage”. Then the wines were strained off and the mash was pressed which a CAREZZA water press at 1.5 kg/cm^2 until to get 5 litres. Press wine were transferred and mixed with 25 litrers of non-pressed wine in all the tanks. Subsequently, the malolactic fermentation was induced by inoculation with *Oenococcus oeni* lactic acid bacteria (Uvaferm Alpha. Lallemand). When the second fermentation was finished, the wines were supplied with 25 mg/L sulphur dioxide and analysed after two weeks.

Analytical methods. The common parameters (density, ethanol, pH, sugar, total and volatile acidity, total and free sulphur dioxide content) were determined according to the Official Regulation Methods established by the UE [49].

The phenolic composition was determined by spectrophotometic assays which a JASCO V-630 spectrophotometer according to the methods described in the literature. Colour intensity and hue were estimated using the analytical methods described by Glories [47, 48]. Total anthocyanin concentration was determined with the methodology described by Ribéreau-Gayon and Stonestreet [50]. The contribution of anthocyanins to wine colour was calculated using the ionization index [51]. The contribution of the copigmented anthocyanins, non copigmented free anthocyanins and polymeric anthocyanins to the total wine colour was determined following the method proposed by Boulton [52]. Total polyphenolic content was evaluated on the Folin-Ciocalteu index using the method described by Singleton and Rossi [46]. Total tannin concentration was estimated according to Sommers and Evans [53]. The content of anthocyanins combined with tannins was calculated using the PVPP index [48]. The polymerization degree of tannins was calculated using the Dmach index [44, 54]. The content of proantocyanidins combined with polysaccharides was estimated using the ethanol index [48]. Astringency was estimated by the method referenced by Llaudy *et al*. [55]. The wines were previously adjusted to pH 3.6 for measuring the parameters related to the colour. All analyses were realized in triplicate and the average and standard deviation values calculated.

Sensory evaluation. The sensory panel was composed of 30 trained official judges from the Polytechnic University of Valencia. They were asked to rank the wines according to their colour, quality and quantity of flavour, bitterness, astringency, structure and equilibrium. Replicate sensory analysis was performed in order to control the reliability of the panel. The reliability of the panel was evaluated on the basis of the calculation of the Pearson correlation coefficient from the multi-judge correlation matrix.

Statistical analysis. The data for each variable was analyzed with a multifactor analysis of variance (ANOVA), considering the interactions between factors. The effects of the prefermentative addition of copigments and prefermentative maceration were the factors for this analysis. The statistical significance of each factor under consideration was calculated using the LSD test. The data was statistically analyzed using Statgraphic Plus 5.1 software.

Results and discussion

Composition of the wines. The composition of the wines after malolactic fermentation was studied using a complete factorial model, considering the addition of prefermentative copigments and the winemaking technologies assayed. Eight conventional parameters and fourteen polyphenolic parameters were quantified due to their significance in red wines [42, 43]. To study the influence of the two factors, a multifactor analysis of variance (ANOVA) was carried out taking into consideration the addition of prefermentative copigments and winemaking technologies applied in each case, as well as the interactions between these factors.

No significant differences are appreciated in the conventional parameters determined in the 54 wines analysed two weeks after the malolactic fermentation. Prefermentative addition of copigments and prefermentative maceration do not produce significant differences in the parameters: density (994 ± 3 g/L), ethanol (12.74 ± 0.05 % vol), pH (3.62 ± 0.07) and total acidity (5.66 ± 0.23 g/L equivalent tartaric acid). The residual sugar (1.57 ± 0.43 g/L), volatile acidity (0.29 ± 0.11 g/L equivalent acetic acid), total sulphur dioxide (87 ± 16 mg/L) and free sulphur dioxide (28 ± 4.5 mg/L) do not show significant differences in the elaborated wines and are in accordance with the values of normal young dry wines.

Table 1 shows the values of the phenolic parameters determined in the wines after the malolactic fermentation. All the winemaking protocols were realized in triplicate. All analyses were determined in triplicate and the average and standard deviation values calculated.

Table 2 shows the F-ratio obtained in this statistical analysis for the 14 polyphenolic parameters quantified according to the copigmented treatments and the different winemaking technologies used in the wines. The F-ratio represents the quotient between variability due to the effect considered and the residual variance. The F-ratio values are also comparable in each column, because the number of observations was the same in all cases. A higher value of F-ratio means a more marked effect of that factor on a variable. According to this, variables in general were affected by the copigmented treatments and the winemaking technologies used. The interactions between the two factors took place in most of the cases, which indicates that the polyphenolic parameter values in the wines supplied with copigments were different depending on the winemaking technology applied.

Effect of addition of copigments. Mean and standard deviation values of the phenolic parameters that show significant differences according to prefermentative addition of different copigments appear in table 3. The statistical analysis was realized considering 6 groups of wines: control wines without the addition of copigments, wines with prefermentative addition of caffeic acid, rutin, (+) catechin, white grape skin tannin and white grape seed tannin. Independently of winemaking technology used, the average values for wines of the control group whether they were produced with traditional winemaking or with prefermentativa maceration, and the same for the other groups. In these wines, the effect of adding copigments produces a significant increase in colour intensity, and the concentration and ionization of anthocyanins in wines. The most significant increase is produced with rutin, catechin, white grape skin and white grape seed tannin. The addition of copigments significantly increases the percentage of colour due to copigmented anthocyanins while slightly reducing the percentage of colour due to non copigmented free anthocyanins and the percentage due to polymerised anthocyanins, but these decreases are not significant. The increase observed in the concentration of anthocyanins could be due to an increase during extraction in vinification because of the effects of copigments [32], but it is more likely that this increase is due a greater proportion of anthocyanins in copigmented form, these being more stable during winemaking and having remained in the medium without precipitating [56]. The decrease appreciate in the hue of the wines with copigments also indicates a greater protection of the anthocyanins against of oxidation.

Caffeic acid is a hydroxycinnamic acid with a great capacity for copigmentation, as it has been observed by other authors in model solutions and wines [27, 28, 31, 57]. Caffeic acid is a product of the hydrolysis of caftaric acid [58], which may be induced by exposing the grapes to sunlight [59]. Caftaric acid is found in high concentrations, close to 30 mg/L [56], in the Tempranillo grapes. The high level of sunlight exposure during while grapes mature, leads us to consider a natural copigmentation effect in these grapes. It is possible for this reason, that supplementation has not produced the high increases as observed by the previously cited authors.

The copigmentation effect of rutin was more significant that of caffeic acid. Rutin belongs to the group of flavonols, the group of polyphenols considered to be the best copigments [16]. The concentration of rutin in grapes is very low, about 3-5 mg/L in the Tempranillo grapes [56], and additions as high as those realized in this study have given rise to an increase in copigmentation and a greater protection of colour and anthocyanins concentration [60].

Catechin concentration increases in copigmented wines with the addition of catechin, skin and seed tannins. Grape skin and seed tannins are polymeric flavanols that could have been hydrolysed to monomeric and dimeric derivates on addition to the must and wine, producing an increase in catechins and epicatechins. Catechin is a molecule with copigmenting properties as Darias *et al.* [27] have demonstrated. The levels of epicatechin are inferior to those of catechin in grapes and wines, but epicatechin is considered to be the flavanol with greater copigmentation capacity [16] and this could explain the better copigmenting behaviour on the addition of white grape tannins.

Total polyphenolic concentration increased by a small amount with the addition of copigments. Copigments do not have an effect on the concentration of total tannins nor on tannins in combination with anthocyanins. The addition of white grape skin and seed tannins increases the polymerization of tannin and polysaccharides.

The astringency found in the wines produced by the addition of copigments should be lower than that found in control wines with no copigment addition. There is no clear evidence concerning the relative roles of monomeric and polymeric phenols in the astringency in red wines, and there is growing evidence that the role of monomeric forms is more important. Many of the flavanols appear to be present at levels close to their flavour threshold concentration [61], and the rates of binding to receptors and proteins would be expected to be related to their free concentrations rather than to their total concentration. Copigmentation reduces the free pool of anthocyanins lowering the number of monomeric components and the astringency. The reduction of astringency could be due to the fact that some astringent monomeric components are included in the copigmentation stacks [16].

Effect of prefermentative maceration. Table 4 shows the mean and standard deviation values of the phenolic parameters that show significant differences according to the winemaking technology. Therefore, the control wines and the ones that have added copigments produced with traditional winemaking are included in the first group, all the wines produced with cold prefermentativa maceration are included in the second group, and the wines macerates with dry ice in the third. Prefermentative maceration has a positive effect on the total concentration of anthocyanins, percentage of anthocyanins that contribute to wine colour valuated by the ionization index, and percentage of colour due to copigmented anthocyanins. Increasing these parameters should increase the colour intensity, but this increase is not significant. The colour intensity of wines produced by traditional methods is mainly due to the greater contribution to colour of non copigmented free anthocyanins. These play an important part in the colour of young red wines although they are very unstable in the presence of oxygen and are very sensitive to pH and sulphur dioxide [31].

In this work the analysis was realised immediately following malolactic fermentation and at this time there are no significant differences in the colour of the wines produced by traditional technology and the cryomacerated wines. The fact that wines produced with traditional technology have a lower percentage of colour due to copigmented anthocyanins and a greater percentage of colour due to non copigmented free anthocyanins could cause instability and a greater loss in anthocyanin concentration and colour after a number of months. The significant increase of colour caused by copigmented anthocyanins due to the prefermentative maceration does not have a positive effect on colour intensity but it is hoped that it increases colour stability [62]. Other authors have found that prefermentative maceration increases the colour of wines when they are analysed during and after conservation [63, 64, 65, 66].

Prefermentative maceration increases the values of the total polyphenols; total tannins and the percentage of tannins in combination with polysaccharides measured on the ethanol index, and decreases the astringency of wines. In previous studies the increase of anthocyanins and low molecular weight tannin concentration in wines produced with prefermentative maceration was observed [63, 64, 65, 66]. Gómez-Míguez *et al*. [67] studying prefermentative maceration with different times and temperatures found significant differences according to colour and phenolic composition, they found important increases in caffeic acid and *p*-hydroxybenzoic acids in cryomacerated wines. This increase in copigments could be one of the causes of the increase in the colour fraction due to copigmented anthocyanins observed with prefermentative maceration. The increase of the polyphenol concentration could be due to the greater contact of the must with the skins in the absence of ethanol [62, 68] and above all when this contact is realised using cold soak with dry ice. This increased polyphenolic extraction is due to the cellular destructurization effect causing the breaking of cellular membranes by freezing the intracellular liquids with carbonic snow. This effect has been mentioned by other authors [62, 69, 70, 71]. At the same time as the polyphenolic extraction, a greater extraction of polysaccharides from grape skin cells could occur. These polysaccharides will complex with tannins as soon as they are released from the skin resulting in a wine with fuller structure, enhanced mouth feel, more softness and less astringency, in agreement with the results obtained by other authors [66, 72].

Combined effect of the addition of copigments and prefermentative maceration. As we have seen, table 2 shows the interactions produced between vinification with the addition of copigments and the different winemaking technologies studied. As we can see, there is a combined effect of these techniques employed in practically all the polyphenolic parameters analyzed. Figure 1 shows a graphic representation of colour intensity in the wines (the mean values used to make de figures appear in table 1). We can see that the addition of copigments slightly increases de colour, but this increase is significantly different (ANOVA data not shown) according to the winemaking technique used. The winemaking technologies applied do not produce differences in wines on a global level. By considering the effect of each of the copigments added we see that the prefermentative addition of rutin is more effective in maintaining colour when prefermentative maceration with dry ice is realized. Also, the addition of caffeic acid is more effective when prefermentative maceration is realized, maybe due to the increase in the extraction of this acid as observed by Gómez-Míguez *et al*. [67] studying the effect of cryomaceration in white wines of cv. Zalema.

We can also observe significant interaction between vinification with copigments and winemaking technologies in the anthocyanin concentration and in the ionization index. The addition of copigments produced a slight increase in anthocyanin concentration (figure 2) and in the percentage of anthocyanins that contribute to wine colour. This rise is more significant in wines with catechin, skin and seed tannin added, and produced with prefermentative maceration. This behaviour is the same in the two prefermentative maceration technologies applied, reaching similar values in wines treated with prefermentative maceration at 6-8 ºC and with prefermentative maceration with dry ice. This intensification of the red colour could be due to the reactions between polyphenols during prefermentative maceration, maintaining red hue and providing more stability to the molecules [20]. The effect of prefermentative maceration on colour seems to be less evident than that previously observed with total anthocyanin concentration. In fact not all anthocyanin compounds are responsible for colour. This could mean that the colour in slightly less intense, but could be more stable with time, achieving one of the most important objectives of winemaking technology.

Figure 3 shows the combined effect between the addition of copigments and prefermentative maceration on the percentage of colour due to copigmented anthocyanins. The addition of copigments and maceration prefermentative technology significantly increased the copigmented anthocyanins. This increase is high in wines produced with cold soak at 6-8 ºC, and even more so when the prefermentative maceration was realised with dry ice at 0-2 ºC. The copigmenting effect is more intense in the wines with added white grape skin and seed tannin. The percentage of colour due to free anthocyanins decreases when the percentage of copigmented anthocyanins increases. This decrease is significant in cryomacerated wines, independent of the maceration technique used. On the other hand, after malolactic fermentation, the wines with added copigments show a slight decrease in the percentage of colour due to polymerised anthocyanins, while winemaking techniques do not influence the behaviour of the polymerised anthocyanins. The prefermentative addition of copigments and the cryomaceration increase copigment reactions protecting the anthocyanin from oxidation and precipitation during fermentation and conservation [16, 31]. It is also hoped that they facilitate the posterior polymerization reactions which are responsible for colour stability in wines [15, 16, 17, 18, 19].

With respect to the total polyphenolic index, a slight increase was observed in wines subjected to different treatments. There is a positive interaction between the addition of copigments and cryomaceration with dry ice. The effect of disrupting the membranes caused by dry ice increased the polyphenolic extraction from the solid parts of the grapes [40, 41], and the copigmentation protected anthocyanins preventing their loss [14, 73].

The behaviour of the concentration of catechin and the level of polymerization of the tannins measured on the Dmach index is similar but opposite. The addition of the copigments catechin, white grape skin and seed tannin give rise to an increase of catechin in wine and a decrease in the level of polymerization of the tannins. There is a positive interaction between the addition of white grape skin and seed tannin and cryomaceration with dry ice. The wines subjected to these treatments were the ones that exhibited a lower level of tannin polymerization.

A synergistic effect between prefermentative maceration with dry ice and the addition of copigments was observed on the ethanol index. The polymerization of tannins with polysaccharides gives the wines more volume in mouth and more smoothness. It also makes the wines less astringent as it neutralizes the chemical groups that tend to join with proteins [14, 73]. The positive action of catechin and the skin and seed tannins could be due to an increase in the concentrations of these components in the wines which is produced at the same time as an increase of the polymerization with polysaccharides [4].

The decrease of astringency observed with the addition of copigments is greater when the wines are subjected to cryomaceration (figure 4). The lower astringency caused by the inclusion of some astringent monomeric components into copigmentation stacks [16] and the greater possible extraction of polysaccharides followed by a greater combination with tannins was observed in wines produced with prefermentative maceration, it gives rise to important reductions of astringency, contributing to an increase in wine quality in an important way.

Sensory evaluation of wines. The sensory panel did not observe significant differences in the wines after malolactic fermentation for sensorial colour, quality and quantity of flavour, and bitterness. The wines with added white grape tannins produced with prefermentative maceration achieved a greater score for the attribute “structure” (table 5). The greatest differences were found when we valued the “astringency” and “equilibrium” of the wines. The correlation between these two attributes leads us to think that the tasters considered the decrease of astringency found in the copigmented wines, and winemaking with prefermentative maceration more balanced and more acceptable.

Conclusions. Caffeic acid, rutin, (+) catechin, white grape skin tannin and white grape seed tannin added before fermentation can act with cofactors increasing the anthocyanin copigmentation reactions and produce wines with more intense colour, higher anthocyanin concentration, superior contribution of anthocyanins to the colour of the wine, superior percentage of tannins polymerized with polysaccharides and less astringency. The increase in polyphenolic parameters related to colour demonstrated the effectiveness of the combination of the addition of copigments and prefermentative maceration treatments when the wines were examined a short time after the conclusion of malolactic fermentation. These effects were more noticeable when white grape tannins were added and prefermentative maceration was realised with dry ice. Sensory analysis showed an increase in the structure of wines produced with prefermentative maceration and with added grape tannins; as well as less “astringency”, better “equilibrium” and a better global evaluation when the addition of copigments and prefermentative maceration treatments were combined.

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Table 1. Mean values and standard deviations of polyphenolic parameters in the wines produced

	Wine-making	Addition of copigments					
		Control	Caffeic acid	Rutin	Catechin	Skin tannin	Seed tannin
Color intensity	T	10.28±0.13	10.63±0.12	10.5±0.16	10.86±0.10	10.77±0.19	11.02±0.29
	Cold	10.54±0.08	10.73±0.12	11.19±0.09	10.89±0.10	10.70±0.16	11.12±0.08
	Dry ice	10.35±0.36	10.98±0.33	11.09±0.18	11.05±0.13	10.89±0.71	11.19±1.09
Hue (%)	T	67.74±0.31	67.38±0.80	68.72±0.33	66.93±0.14	67.66±0.74	63.92±3.25
	Cold	68.81±0.19	68.54±0.25	69.14±0.39	70.64±0.37	67.03±0.18	69.96±0.20
	Dry ice	70.79±0.11	69.15±0.48	70.60±0.15	70.03±0.22	69.50±0.14	68.95±0.23
Total anthocyanins (mg/L)	T	322.89±4.06	333.33±10.41	343.62±35.64	326.76±14.96	381.62±29.31	363.78±15.73
	Cold	426.51±1.30	421.65±0.51	404.59±0.50	466.21±0.24	465.92±1.97	481.24±1.18
	Dry ice	416.02±0.78	343±0.76	394.56±1.06	446.72±1.65	452.79±1.81	465.30±1.65
Ionization index (%)	T	26.89±0.92	29.78±0.22	27.41±5.88	26.21±1.31	29.46±2.16	29.57±3.14
	Cold	33.31±0.19	35.74±1.22	32.39±0.20	35.68±0.14	31.53±0.23	33.83±0.19
	Dry ice	32.54±0.29	37.19±1.11	32.33±0.16	34.42±0.55	36.36±0.40	37.53±0.44
% Copigmentatio anthocyanins	T	12.89±2.03	20.33±3.55	18.5±4.35	17.88±4.13	25.49±0.67	24.11±7.41
	Cold	22.30±1.32	23.71±0.68	26.14±1.35	25.63±1.65	27.25±0.82	31.55±0.92
	Dry ice	28.64±0.83	29.31±0.75	29.12±1.05	33.64±0.52	34.73±1.42	38.22±0.72
% Free anthocyanins	T	52.96±3.47	45.63±1.92	50.57±1.74	48.60±1.20	47.81±1.73	45.30±3.62
	Cold	45.77±0.87	41.79±1.59	42.32±0.96	42.69±0.18	40.26±0.50	41.78±0.97
	Dry ice	40.36±0.92	36.94±0.48	41.61±0.36	38.16±0.40	38.97±1.18	36.92±0.52
% Polymeritation anthocyanins	T	34.15±1.46	34.04±1.85	30.83±3.65	33.52±3.41	26.70±1.63	27.59±3.83
	Cold	31.88±0.33	34.52±0.97	31.29±0.32	31.71±1.51	32.23±0.27	26.67±0.16
	Dry ice	31.01±0.41	33.76±0.41	30.72±0.53	28.19±0.41	26.31±0.46	25.74±0.47
Catechin (mg/L)	T	159.13±16.74	167.76±5.21	163.08±23.77	190.32±10.16	186.97±17.11	168.41±3.86
	Cold	137.00±0.45	156.46±2.07	146.48±0.29	184.92±3.39	176.46±1.40	187.68±3.13
	Dry ice	150.43±1.82	142.39±1.63	159.49±0.95	164.45±0.98	155.49±2.94	165.24±2.98
Condensed tannins (g/L)	T	1.64±0.08	1.65±0.24	1.61±0.15	1.64±0.04	1.57±0.07	1.47±0.06
	Cold	1.70±0.21	1.34±0.14	1.48±0.14	1.77±0.06	1.76±0.07	1.92±0.05
	Dry ice	1.83±0.09	1.73±0.10	2.07±0.12	1.88±0.10	1.37±0.09	1.47±0.10
Total polyphenols index	T	29.05±0.24	31.56±0.04	30.43±1.29	30.10±0.22	31.33±1.12	30.95±1.85
	Cold	28.71±0.75	30.75±0.83	29..68±0.76	29.98±1.23	31.36±2.69	31.93±0.29
	Dry ice	31.63±0.04	32.45±0.07	33.52±0.06	33.46±0.05	33.41±0.03	32.16±0.11
PVPP index (%)	T	38.91±0.77	37.60±0.92	36.20±1.50	35.46±4.12	38.48±0.30	34.42±0.67
	Cold	33.59±0.21	38.21±0.26	36.53±4.16	36.50±0.34	36.22±0.16	36.20±0.20
	Dry ice	36.48±0.27	37.94±0.42	35.11±0.65	35.87±0.46	35.59±0.27	34.07±0.38
Dmach index (%)	T	58.07±3.48	56.32±0.15	58.44±0.21	55.87±1.31	58.95±2.07	59.90±1.59
	Cold	57.18±1.86	65.83±0.90	62.06±4.34	58.03±0.60	55.57±0.57	50.10±0.66
	Dry ice	49.02±0.69	49.54±0.89	44.71±1.56	59.80±1.61	70.90±0.62	64.91±1.47
Ethanol index (%)	T	28.74±0.25	28.95±2.32	32.82±6.87	27.55±1.31	36.99±2.53	33.12±4.75
	Cold	30.13±0.21	27.31±0.04	31.21±0.91	29.63±0.24	31.48±0.48	36.70±0.16
	Dry ice	33.83±0.11	33.64±0.10	37.48±0.03	38.50±0.10	33.78±0.73	34.74±0.25
Astringency index (%)	T	65.92±4.15	62.66±12.77	57.02±3.47	61.67±2.48	50.46±5.15	38.08±0.43
	Cold	60.95±1.26	43.80±2.39	46.28±2.88	43.92±2.29	32.70±2.53	35.12±1.46
	Dry ice	53.34±0.80	45.29±0.60	52.18±1.91	51.49±2.79	38.19±1.43	39.49±2.80

T. traditional winemaking; Cold. cold prefermentative maceration at 5-8 ºC; Dry ice. prefermentative maceration with dry ice at 0.2 ºC

Table 2. ANOVA F-ratio for the copigmented treatments (C). the different winemaking technologies (W) and their respective interaction (C x W) in the 14 polyphenolic parameters analyzed

	F-ratio		
	C	*W*	*C x W*
Colour intensity	6.07**	9.57	10.4*
Hue	87.4	10.67	8.05
Total anthocyanins (mg/L)	406.3***	69.18***	14.52***
Ionization index (%)	10.96*	6.86***	4.6***
% copigmentacion anthocyanins	151.72***	29.05***	14.60*
% Free anthocyanins	27.4	15.33***	3.08**
% polymerization anthocyanins	14.61	4.25	6.59
Catechin (mg/L)	37.78***	28.22	4.75***
Condensed tannins (g/L)	19.16	15.64*	23.04
Total Polyphenols index	61.77***	8.79***	6.97***
PVPP index (%)	4.91	2.92	4.31
Dmch index (%)	34.6***	35.49	82.68***
Ethanol index	6.53**	12.29**	7.88***
Astringency index	59.87***	64.93***	7.32***

* $p < 0.05$; ** $p < 0.01$. *** $p < 0.001$

Table 3. Effect of prefermentative addition of copigments in polyphenolic parameters with significant differences

Polyphenolic parameters	Addition of copigments					
	Control	Caffeic acid	Rutin	Catechin	Skin tannin	Seed tannin
Color intensity	10.36 ± 0.21 a	10.78 ± 0.35 ab	10.95 ± 0.71 b	10.93 ± 0.21 b	10.78 ± 0.31 b	11.11 ± 0.92 b
Total anthocyanins (mg/L)	368.47±48.70 a	365.99±41.67 a	387.26±31.5 b	413.23±41.46 b	397.2±41.46 ab	436.77±54.97 b
Ionization index (%)	31.91 ± 4.51 a	33.57 ± 3.28 b	32.04 ± 3.74 ab	32.10 ± 4.45 b	31.45 ± 3.23 a	33.64 ± 3.78 b
% copigmentacion anthocyanins	20.94±8.70 a	24.45±4.32 ab	24.62±5.23 ab	25.71±7.12 ab	29.15±4.28 b	31.29±7.18 b
% polymerization anthocyanins	33.68±3.42 ab	34.11±1.16 b	30.95±1.95 ab	31.14±3.03 ab	29.42±2.96 a	27.66±2.17 a
Total polyphenols index	29.79±1.47 a	30.92±0.69 ab	30.54±2.58 ab	31.18±1.78 ab	32.03±1.63 b	31.01±1.27 ab
Catechin (mg/L)	148.85±12.95 a	155.54±11.26 a	149.69±16.16 a	179.89±12.92 c	172.97±16.4 c	170.44±14.24 bc
Dmach index (%)	54.76±4.77 a	56.23±7.03 ab	55.07±8.09 a	57.90±4.76 b	61.81±6.98 b	58.30±6.53 b
Ethanol index (%)	32.23±4.26 a	29.30±2.23 a	33.83±4.09 ab	31.89±5.01 a	33.42±3.45 bc	35.51±3.01 c
Astringency index (%)	60.07±4.69 c	50.58±11.23 b	51.83±5.25 b	56.03±9.23b c	40.45±8.34 a	37.69±2.22 a

Different letters within the same file for each polyphenolic parameter mean significant differences ($p < 0.01$)

Table 4. Effect of winemaking technology in polyphenolic parameters of wines

Polyphenolic parameters	Winemaking technologies					
	Traditional winemaking		Cold soak 5-8 ºC		Cold soak dry ice	
Color intensity	10.67±0.24	a	10.87±0.58	a	10.92±0.60	a
Ionization index (%)	28.22±1.57	a	33.75±1.61	b	35.39±2.71	b
Total anthocyanins (mg/L)	345.33±23.03	a	442.52±33.75	b	419.56±45.54	b
% copigmentation anthocyanins	19.22±5.89	a	26.09±3.20	b	32.27±3.88	c
% free anthocyanins	49.31±2.54	c	42.43±1.83	b	38.83±1.89	a
Condensed tannins (g/L)	1.60±0.08	a	1.66±0.23	ab	1.77±0.31	b
Total polyphenols	29.73±0.92	a	31.29±1.35	b	32.44±1.25	b
Ethanol index (%)	30.86±3.50	a	32.08±1.73	a	35.15±3.17	b
Astringency index (%)	55.97±10.27	b	43.79±9.99	a	49.06±9.59	a

Different letters within the same file for each polyphenolic parameter mean significant differences ($p < 0.01$)

Table 5. Mean values and standard deviations of astringency. Structure and equilibrium valued in the sensory evaluation with a numeric scale 0-7

	Wine-making	Addition of copigments					
		Control	Caffeic acid	Rutin	Catechin	Skin tannin	Seed tannin
Structure	T	3.92 ± 1.20 a α	4.07 ± 0.35 a α	3.78 ± 0.89 a α	4.39 ± 0.67 b α	4.61 ± 0.38 b α	4.99 ± 1.45 b α
	Cold	4.32 ± 0.87 a β	4.41± 0.42 a β	4.19 ± 1.12 a β	4.73 ± 1.08 b β	4.79 ± 0.61 a α	5.24 ± 0.75 b β
	Dry ice	4.23 ± 0.64 a β	4.23 ± 0.81 a β	4.70 ± 0.67 b β	4.61 ± 0.38 b β	5.25 ± 0.45 b β	5.17 ± 0.83 b α
Astringency	T	4.88 ± 1.16 a α	4.50 ± 1.09 a α	4.35 ± 0.89 a α	4.15 ± 0.62 b α	3.71 ± 1.05 c α	3.58 ± 1.24 c α
	Cold	4.57 ± 1.03 a α	3.54 ± 1.03 b β	3.58 ± 1.24 b β	3.42 ± 0.99 b β	3.17 ± 1.11 b β	3.08 ± 0.90 c β
	Dry ice	4.05 ± 1.06 a α	3.12 ± 0.16 b β	3.26 ± 0.45 b β	3.92 ± 0.90 a αβ	3.38 ± 0.94 b β	2.84 ± 1.07 b α β
Equilibrium	T	4.45 ± 1.00 a α	4.33 ± 0.89 a α	4.50 ± 0.80 a α	5.11 ± 0.66 ab α	4.92 ± 0.90 ab α	5.25 ± 0.45 b α
	Cold	4.06 ± 0.55 a α	4.29 ± 0.81 a α	3.73 ± 1.28 a α	5.57 ± 0.43 b β	5.38 ± 0.58 b β	5.42 ± 0.83 b β
	Dry ice	4.12± 1.45 a α	4.83 ± 0.59 ab α	4.70 ± 0.38 a β	5.45 ± 0.43 b β	5.19 ± 0.45 b α	5.32 ± 0.45 b α β

T: traditional winemaking; Cold: cold prefermentative maceration at 5-8 ºC; Dry ice: prefermentative maceration with dry ice at 0.2 ºC
Different letters indicate the existence of statistically significant differences. Latin letters (a, b, c) are used to compare copigments addition influence. Greek letters (α, β) are used to compare winemaking technology influence.

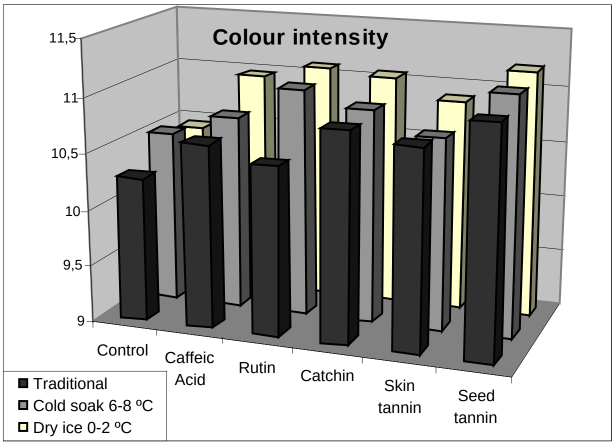

Figure 1. Representation of the colour intensity in the wines according to prefermentative addition of copigments and winemaking technologies

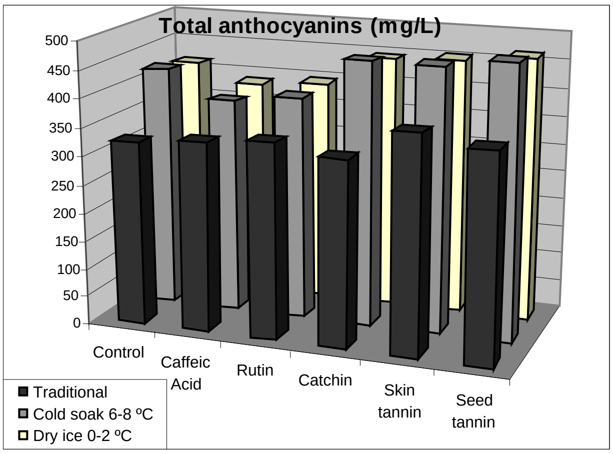

Figure 2. Representation of total anthocyanin concentration in the wines according to prefermentative addition of copigments and winemaking technologies

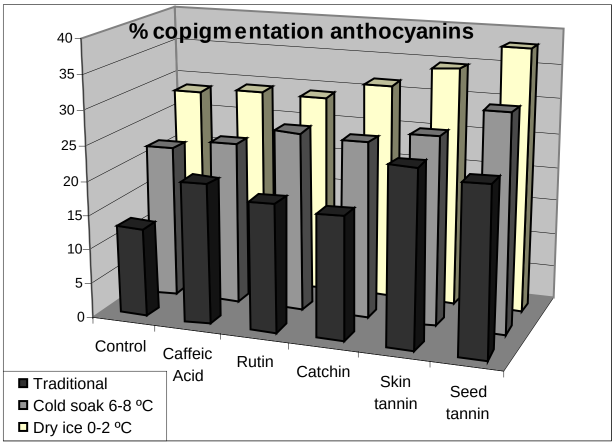

Figure 3. Representation of percentage of colour due to copigmented anthocyanins in the wines according to prefermentative addition of copigments and winemaking technologies

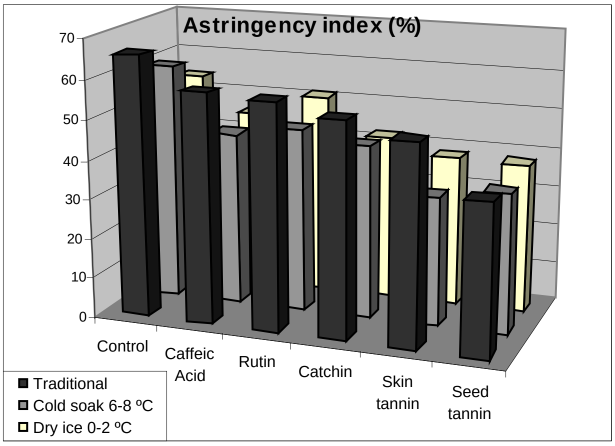

Figure 4. Representation of astringency index in the wines according to prefermentative addition of copigments and winemaking technologies