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# The Extraction of Anthocyanins and Proanthocyanidins from Grapes to Wine during Fermentative Maceration Is Affected by the Enological Technique

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**ABSTRACT:** The effect of three enological techniques (low temperature prefermentative maceration, must freezing with dry ice, and the use of a maceration enzyme) on the extraction of anthocyanins and proanthocyanidins from must to wine during fermentative maceration was studied to determine the extent to which these compounds are extracted and to assess the changes on their qualitative composition due to enological technique applied. The results showed that the dry ice treatment led to wines with high color intensity and high anthocyanin content, the maximum rate of extraction being observed the first 6 days of fermentative maceration. Regarding the effect of the different techniques on the quantitative and qualitative composition of proanthocyanidins, only the dry ice treatment seemed to favor the extraction of high molecular weight skin proanthocyanidins. The low temperature prefermentative maceration treatment led to the highest concentration of proanthocyanidins at the moment of pressing; however, this treatment, contrary to expectations, led to wines with the highest content of seed-derived proanthocyanidins. The use of the maceration enzyme also increased the concentration of proanthocyanidins during all of the fermentative process, as compared to a control wine, although the increase was not only due to skin proanthocyanidins but also seed proanthocyanidins. We have demonstrated in this study that maceration enzymes also facilitate seed phenolic extraction.

**KEYWORDS:** Anthocyanins, proanthocyanidins, wine, fermentative maceration

## INTRODUCTION

Phenolic compounds, especially anthocyanins and proanthocyanidins, are important for wine chromatic and sensory characteristics through their effect on wine color, astringency, and bitterness.[1−3] Both compounds are found in berry skins, in the cell vacuoles, although proanthocyanidins are also found in the seeds. The composition of skin and seed proanthocyanidins is different. Seeds contain a higher proportion of galloylated procyanidins, whereas prodelphinidins are only found in skins.[4,5] In addition, skin proanthocyanidins have been shown to have a higher mean degree of polymerization (mDP) than seed proanthocyanidins.[6,7] These differences have a practical importance since they allow the percentage of seed and skin proanthocyanidins extracted into the wine to be estimated.[8]

The extraction of skin anthocyanins and proanthocyanidins during fermentative maceration is essentially a diffusion process, and the rate and extent of extraction are influenced by the skin phenolic concentration, the composition of berry cell walls, which clearly affects the extractability,[9] and the technological process applied at the winery. On the other hand, it is generally accepted that the extraction of proanthocyanidins from seeds needs the presence of ethanol to help eliminate the protective layers of the seed before tannin extraction really occurs.

Given the importance of anthocyanins and proanthocyanidins for wine color and the fact that it is assumed that skin proanthocyanidins confer a softening effect on the wine mouthfeel,[3,10] the final objective of some enological techniques is to promote skin cell walls degradation (that might facilitate polyphenol diffusion) while limiting seed phenolic extraction.

In this way, the effect of maceration enzymes has been widely studied for their effect on skin degradation and wine anthocyanins and color characteristics, although with conflicting results.[11−15] Less attention has been paid to the effect of these macerating enzymes on wine proanthocyanidins. The studies of Ducasse et al.[16] described the concentration and composition of proanthocyanidins in Merlot wines treated with maceration enzymes, reporting an increase in total proanthocyanidins but, surprisingly, not in the percentage of skin-derived proanthocyanidins (estimated from the percentage of epigallocatechin present in the wine proanthocyanidins), with the increase more related to an increase of seed proanthocyanidins. Similar results were reported by Busse-Valverde,[17] suggesting that the enzyme also modifies seed cell structures, facilitating seed proanthocyanidins extraction, although the effect of maceration enzymes on seed phenolics has not been demonstrated.

With regard to the use of low temperatures before fermentation (cold soak or must freezing), their positive effect on wine anthocyanins and color characteristics has been informed.[18,19] Moreover, Alvarez et al.[20] also studied the effect of must freezing with dry ice on the concentration of proanthocyanidins of

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Monastrell wines, finding that proanthocyanidin concentrations slightly increased with this technique. In a previous study on the proanthocyanidin composition of finished wines where these low temperature enological treatments (must freezing with dry ice and 10 days of prefermentative low temperature maceration) were used,[17] we found that although the prefermentative low temperature treatment increased wine proanthocyanidin concentration as compared with a control wine, the lack of any increase in the percentage of the prodelphinidin (−)-epigallocatechin in the wine proanthocyanindins and the increased percentage of galloylation suggested that the increase in proanthocyanidin concentration might be mainly due to an increase in seed proanthocyanidins and not skin proanthocyanidins, contrary to the expected results.

To throw light on this subject, we have followed the phenolic composition of Monastrell musts/wines during the fermentative maceration of grapes vinified with the three different techniques mentioned above: Using a maceration enzyme, freezing the grapes at the moment of crushing with dry ice, or using a low temperature prefermentative maceration for 10 days before the alcoholic fermentation started. The aim was to determine how the enological treatment affects the rate of skin and seed phenolic diffusion from grapes to wine during fermentative maceration and their qualitative composition.

## MATERIALS AND METHODS

Grapes from *Vitis vinifera* L. cv. Monastrell were harvested in 2009 from a commercial vineyard in Jumilla (SE Spain). For the vinifications, grapes were carefully harvested in 20 kg boxes and transported to the winery.

**Vinifications.** Two low temperature prefermentation treatments were studied as follows: cold soak at 10 °C and must freezing with dry ice. Also, a control vinification was carried out together with another vinification in which a commercial enzyme was used. All vinifications were made by triplicate in 100 L stainless steel tanks using 90 kg of grapes. For all vinifications, after the grapes had been crushed and destemmed, sodium metabisulfite was added (8 g of $SO_2$/100 kg of grapes).

*Treatment 1: Must Freezing with Dry Ice (DIW).* The dry ice was added directly to the tank, mixing it with the crushed grapes. One hundred kilograms of dry ice was used for each tank, which kept the must frozen for 2 days.

*Treatment 2: Low Temperature Prefermentative Maceration (LTPW).* Tanks containing the crushed grapes were introduced into a refrigeration camera at 10 °C for 10 days, after which the tanks were returned to the winery.

*Treatment 3: Vinification with a Commercial Maceration Enzyme (EW).* The enzyme (Enozym Vintage, Agrovin, Spain) was added to the crushed grapes at a dose of 5 g/100 kg of grape.

*Treatment 4: Control Vinification (CW).* In all of the treatments, before alcoholic fermentation started, total acidity was corrected to 5.5 g/L, and selected yeasts were added (LevulineGALA, Oenofrane, France, 10 g of dry yeast/100 kg of grapes). All of the vinifications were conducted at 25 ± 1 °C. Throughout the fermentative pomace contact period (10 days in all cases), the cap was punched down twice a day, and samples were taken the day after yeasts were added (day 2), on day 6 and at the moment of pressing (day 10).

**Seed Assay.** The seeds of 200 berries were manually separated from skins and pulp and rinsed with distilled−deionized water. A 0.5 g amount of seeds was placed in flasks with 15 mL of different synthetic solutions at room temperature for 48 h, under gentle stirring. Three solutions were tested as follows: a control solution (aqueous solution containing 5 g/L of tartaric acid and pH 3.6) and two solutions where a commercial maceration enzyme was added, one of them identical to the control solution, and the other one containing 12% ethanol. The enzyme (Enozym Vintage, Agrovin, Spain) was added at a dose of 10 mg/L.

To minimize tannin oxidation, solutions were sparged with nitrogen, and the extraction was carried out in the dark. Following extraction, the extract was concentrated under reduced pressure at 35 °C and then lyophilized to a dry powder. This powder was redissolved in methanol in a volumetric flask and analyzed for proanthocyanidins.

**Determination of Anthocyanins.** This analysis was performed by direct injection of wine samples on a Waters 2695 liquid chromatograph (Waters, Milford, MA), equipped with a Waters 2996 diode array detector and a Licrochart RP-18 column (Merck, Darmstadt, Germany), 25 cm × 0.4 cm, 5 μm particle size, using as solvents water plus 4.5% formic acid (solvent A) and high-performance liquid chromatography (HPLC) grade acetonitrile (solvent B) at a flow rate of 0.8 mL/min. Elution began with a linear gradient from 5 to 10% B in 10 min, then a gradient from 10 to 14.5% B in 20 min, isocratic for 4 min, gradient from 14.5 to 15.2% in 9 min, again a gradient from 15.2 to 18% B in 15 min, and a gradient from 18 to 25 in 40 min, followed by washing and re-equilibration of the column. The anthocyanins and vitisins were quantified at 520 nm as malvidin-3-glucoside, using malvidin-3-glucoside chloride as an external standard (Extrasynthèse, Genay, France).

**Chromatic Parameters.** The color intensity was calculated as the sum of absorbance at 620, 520, and 420 nm (Glories, 1984). Absorbance measurements were made in a Helios Alpha spectrophotometer (Thermospectronic, Whaltam, MA) with 0.2 cm path length glass cells.

**Determination of Proanthocyanidins.** Seed proanthocyanidins were determined according to the method described by Kennedy and Jones[21] with some modifications, as follows. A solution of 0.2 N HCl in methanol, containing 100 g/L phloroglucinol and 20 g/L ascorbic acid, was prepared (phloroglucinolysis reagent). One hundred microliters of methanolic extract was reacted with 100 μL of phloroglucinolysis reagent (1:1) in a water bath for 20 min at 50 °C and then combined with 2 volumes of 200 mM aqueous sodium acetate to stop the reaction.

For the wines, the samples were prepared by an optimization of the method described by Pastor del Río et al.[22] Five milliliters of wine was evaporated in a centrivap concentrator (Labconco, United States), redissolved in 3 mL of water and then passed through a C18-SPE column (1 g, Waters), previously activated with 10 mL of methanol followed by 20 mL of water. The cartridge was washed with 20 mL of water, and compounds of interest were eluted with 10 mL of methanol, evaporated, and then dissolved in 1 mL of methanol. Phloroglucinolysis was then carried out as described above.

HPLC analysis followed the conditions described by Ducasse et al.[16] The HPLC apparatus was a Waters 2695 system (Waters) equipped with an autosampler system and a Waters 2996 photodiode array detector. Samples (10 μL injection volume) were injected on an Atlantis dC18 column (250 mm × 4.6 mm, 5 μm packing) protected with a guard column of the same material (20 mm × 4.6 mm, 5 μm packing) (Waters). The elution conditions were as follows: 0.8 mL/min flow rate; oven

Table 1. Anthocyanin Composition during Fermentative Maceration[a]

| | nonacylated anthocyanins | acylated anthocyanins | vitisins | total anthocyanins | color intensity |
|---|---|---|---|---|---|
| | | day 2 | | | |
| CW | 245.41 a | 28.75 a | 0 | 274.16 a | 3.18 a |
| DIW | 440.15 c | 28.92 a | 0 | 469.07 b | 3.58 a |
| LTPW | 377.99 bc | 60.10 b | 0 | 438.09 b | 14.63 b |
| EW | 298.22 ab | 27.27 a | 0 | 325.49 a | 3.50 a |
| | | day 6 | | | |
| CW | 458.40 a | 102.76 a | 4.56 a | 561.17 a | 17.68 a |
| DIW | 579.53 c | 136.10 c | 5.43 b | 715.63 c | 23.90 c |
| LTPW | 524.27 b | 127.25 b | 6.33 c | 651.53 b | 21.65 bc |
| EW | 450.94 a | 110.78 a | 4.99 a | 561.38 a | 19.62 ab |
| | | day 10 | | | |
| CW | 593.89 a | 127.22 a | 4.92 a | 721.11 a | 18.89 a |
| DIW | 659.34 c | 143.56 b | 6.45 c | 802.0 b | 20.56 b |
| LTPW | 644.28 bc | 145.28 b | 5.58 b | 789.56 ab | 18.87 a |
| EW | 628.28 b | 127.22 a | 5.28 b | 755.5 ab | 19.63 ab |

[a] Different letters within the same column and for each studied time indicate significant differences ($p < 0.05$).

temperature, 30 °C; solvent A, water/formic acid (98:2, v/v); and solvent B, acetonitrile/solvent A (80:20 v/v). Elution began with 0% B for 5 min, linear gradient from 0 to 10% B in 30 min and gradient from 10 to 20% in 30 min, followed by washing and re-equilibration of the column. Proanthocyanidin cleavage products were estimated using their response factors relative to (+)-catechin, which was used as the quantitative standard. These analyses allowed determination of the total proanthocyanidin content, the apparent mDP, and the percentage of each constitutive unit. The mDP was calculated as the sum of all subunits (flavan-3-ol monomer and phloroglucinol adducts, in mol) divided by the sum of all flavan-3-ol monomers (in mol).

**Statistical Data Treatment.** Significant differences among wines and for each variable were assessed by analysis of variance (ANOVA). The least-squares difference test was used to separate the means ($P < 0.05$) when the ANOVA test was significant using the statistical package Statgraphics 5.0 Plus.

## RESULTS AND DISCUSSION

**Anthocyanin Concentration and Wine Color.** Table 1 shows the evolution of anthocyanins and color intensity during the fermentative maceration of the studied wines. Nonacylated anthocyanins predominated in Monastrell must and wines and vitisin-like compounds (vitisin A and vitisin B) were only detected after 6 days of maceration. Anthocyanin monoglucoside derivatives predominate throughout maceration process, and their levels significantly increased with the use of low temperature treatments. Monastrell grapes are characterized by low content of acylated anthocyanins; however, they also increased along maceration time, the highest increases being found in DIW and LTPW. The day following yeast addition (day 2), there was a substantial difference between the wines, the LTPW and DIW presenting very high values of total anthocyanins as compared with the other musts, which was attributed to the low temperature prefermentative treatments, which favored anthocyanin extraction. Gómez-Míguez et al.[23] and Gordillo et al.[24] reported a similar anthocyanin concentration after 7 days of low temperature prefermentation maceration of Syrah and Tempranillo musts. CW and EW showed lower and similar values at days 2 and 6. At pressing (day 10), the highest concentration of anthocyanins was found in DIW, although with no significant differences from LTPW or EW. Gil-Muñoz et al.[19] found that the two low temperature techniques led to a high anthocyanin content at the end of alcoholic fermentation in Cabernet Sauvignon wines, while only dry ice resulted in a significant higher anthocyanin content in Syrah wines.

The color intensity showed very a high value on day 2 in LTPW, due both to its high anthocyanin content but also to a low $SO_2$ content in this wine after 10 days of prefermentative maceration, as compared with the other wines (data not shown). This gave as a result that a large proportion of these anthocyanins were in their colored form, increasing the color intensity in this wine. All wines became more colored as the fermentative maceration progressed and specially when dry ice was used, the DIW being the only wine that presented significantly higher color intensity at pressing as compared with CW. It seems that the use of dry ice improved the extraction of anthocyanins from skins, probably because freezing increases the volume of the intracellular liquids, disrupting the membranes, and providing an easy exit for the phenolic compounds. Moreover, it has the additional advantage that as it freezes the berries, it sublimates to $CO_2$ gas, protecting the berries from oxygen before fermentation.[25]

**Proanthocyanidins.** Figure 1 shows the concentration of proanthocyanidins and their composition during the 10 days of fermentative maceration. On day two, the differences between the four musts were quite pronounced. Figure 1A shows that the 10 days of low temperature prefermentative maceration undergone by LTPW led to a significant extraction of proanthocyanidins. Other authors also reported proanthocyanidin extraction after low temperature prefermentative maceration,[8,26] although they found a lower proanthocyanidin concentration than those reported in our experiment. In the LTPW vinification, the quantity of proanthocyanidins continued to increase until day 6, and then, a stabilization was observed.

The other vinifications also showed a substantial increase in proanthocyanidins during the first part of the fermentative

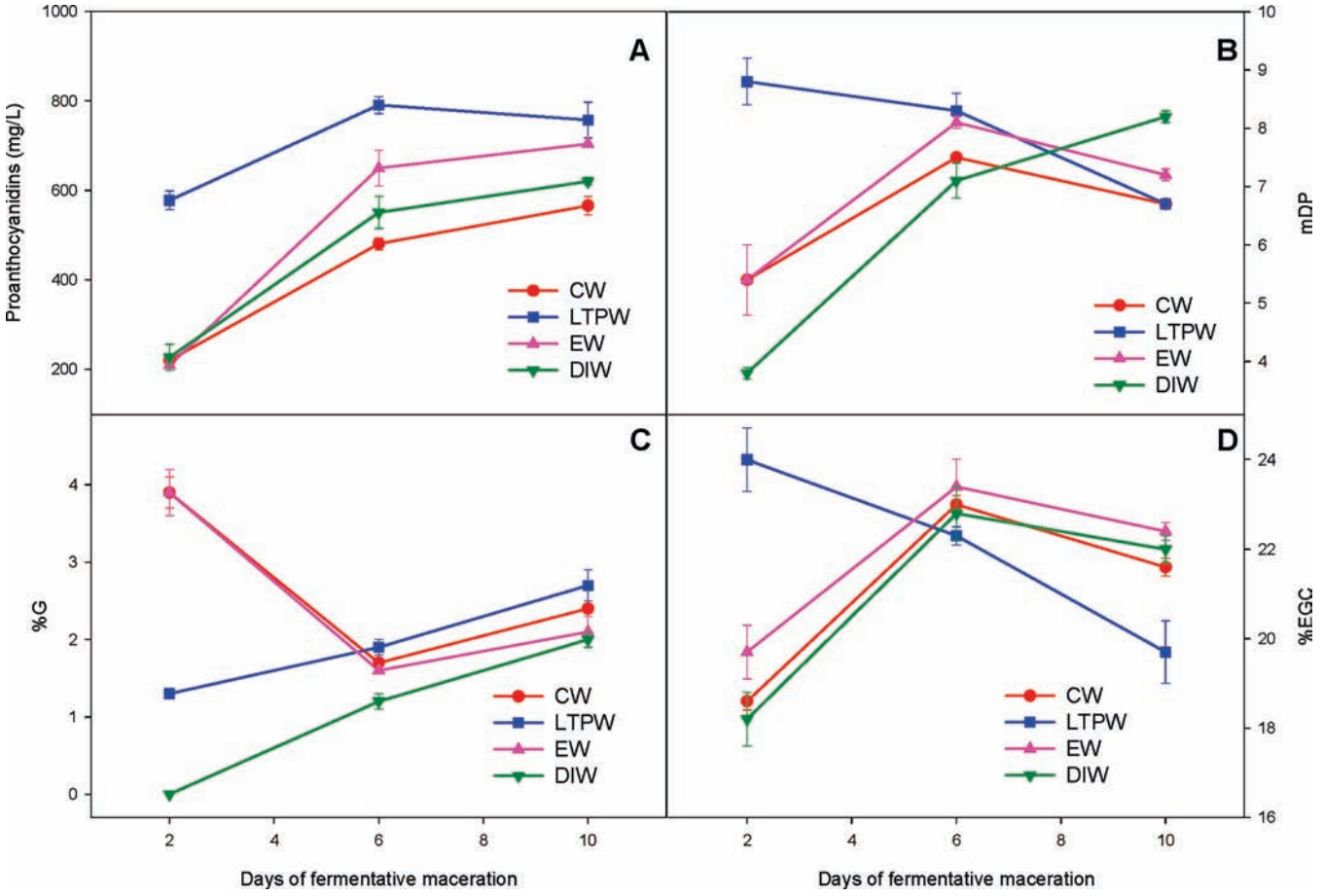


**Figure 1.** Evolution of (A) proanthocyanidins (mg/L), (B) proanthocyanidin mDP, (C) percentage of galloylation of the wine proanthocyanidins (% G), and (D) percentage of extension epigallocatechin (%EGC) during the fermentative maceration of Monastrell wines.

**Table 2. Proanthocyanin Concentration of the Solutions Containing Seeds**[a]

| | control | enzyme (aq) | enzyme (EtOH) |
|---|---|---|---|
| proanthocyanidin (mg/L) | 1126 a | 4622 b | 7727 c |
| mDP | 8.9 a | 9.0 a | 8.3 a |

[a] Different letters within the same row indicate significant differences ($p < 0.05$). Aq, aqueous solution; EtOH, ethanolic solution.

process, decreasing the rate of extraction from day 6 to day 10; however, EW reached, at day 10, the same values as those observed in LTPW. It is interesting to note this large extraction of proanthocyanidins when the maceration enzyme is used. Several studies using maceration enzymes have reported a large extraction of tannins when enzymes are used,[15,19,27] but this has normally been attributed to skin proanthocyanidins. In this study, we wanted to test whether a maceration enzyme also affected the extraction of seed proanthocyanidins, and so, we soaked seeds from Monastrell grapes in three different solutions: a control solution (aqueous solution containing 5 g/L of tartaric acid and pH 3.6) and two solutions where a commercial maceration enzyme was added, one of them identical to the control solution and the other one containing 12% ethanol. It can be clearly seen how the enzyme largely increased the extraction of proanthocyanidins from seeds (Table 2), an effect that was even more evident when ethanol was present, although we could not find differences in the mDP of the extracted proanthocyanidins. The seed cell walls are composed of cellulose, hemicelluloses, pectins, proteins, lignin, mucilage, and gums;[28,29] therefore, the use of a maceration enzyme, in which several activities are present, may be able to disrupt the cellular and subcellular organization of the seed tissues, increasing the release of seed tannins. Such an effect could justify the results of Passos et al.,[30] who improved grape seed oil extraction with an enzymatic pretreatment of the seeds.

The value of the mean degree of proanthocyanidin polymerization (mDP), together with the % of galloylation (%G) and the % of extension epigallocatechin (%EGC) (Figure 1B–D) can provide information on how the composition of must/wine proanthocyanidins changed during the fermentative maceration. As stated above, skin proanthocyanidins contain prodelphinidin units (which are not present in seed proanthocyanidinds) and a higher mDP than seed proanthocyanidins, the latter presenting a higher proportion of galloylated units. Therefore, the percentage of extension epigalocatechin could inform us on the percentage of skin-derived proanthocyanidins in the wine.

These three parameters evolved in totally different ways in the different vinifications. In LTPW, the initial value of mDP and the %EGC was high and the %G was low, suggesting that at this moment (the starting point of alcoholic fermentation), the must proanthocyanidins came mainly from skins. This was to be expected, since, during a low temperature maceration and in

the absence of ethanol, the extraction of skin proanthocyanidins would be favored over the extraction of seed proanthocyanidins.[31,32] On the basis of the compositional data of the original grapes, reported in 17, we could calculate, following the method proposed by Peyrot des Gayons and Kennedy,[8] that the percentage of skin-derived proanthocyanidins in this must at day 2 was 87.2%, very similar to the values reported by these authors in a similar experiment.[8] When alcoholic fermentation started, the composition changed rapidly, with an important decrease of the proanthocyanidins mDP and %EGC and an increase of %G. Moreover, at the moment of pressing, LTPW was the wine with the lowest %EGC and mDP and highest %G, indicating that this wine had the highest proportion of seed-derived proanthocyanidins (29% of seed-derived proanthocyanidinds, as compared with 21.5, 19, and 20% for CW, EW, and DIW, these three values being very similar to those reported by Cerpa-Calderon and Kennedy[33] after 9 days of fermentative maceration). These results also agree with those of the study of Busse-Valverde[17] in finished wines and indicate that although the extraction of skin proanthocyanidins was favored during the low prefermentative maceration, this step also prepares the seeds for an easier extraction of their proanthocyanidins the moment alcohol from the fermentation is present. This leads to a final wine very different from that which might be expected, with a high proportion of seed proanthocyanidins; therefore, it could present a high astringency.

The CW and EW evolved similarly as regards mDP, %G, and %EGC. It seems that the action of the enzyme facilitates a higher extraction of proanthocyanidins from both skin and seeds but without changing their proportion or composition, as compared to CW. Both musts experienced an increase in mDP and %EGC during the first part of fermentation, indicating a readily extraction of skin proanthocyanidins at the beginning of alcoholic fermentation and a decrease in the rate of extraction during the second part of fermentative maceration, when the extraction of seed proanthocyanidins seemed to predominate.

DIW behaved differently. Its final concentration of proanthocyaninds was lower than that of LTPW, but its mDP increased throughout the fermentative maceration, the %G and %EGC being similar to CW. It seems that the use of dry ice clearly facilitated the extraction of high molecular weight proanthocyanidins from skins by degrading the structures, a result concordant with that observed for the wine anthocyanins. In agreement with the results of Gil-Muñoz et al.,[19] dry ice seems to have a significant effect in varieties with rigid cell walls, as is the case of Syrah and Monastrell.[9] Sacchi et al.[25] stated that freezing may also break the tannin-containing cells of the seeds, increasing the extractability of proanthocyanidins, but we did not observe this effect.

In conclusion, the dry ice treatment allowed us to obtain the most colored wines, with high anthocyanin content, the greatest increase being observed during the first 6 days of fermentative maceration. With regard to the effect of the different techniques on proantocyanidins, low temperature prefermentative maceration, contrary to what was expected, produced the wines with the highest content of seed-derived proanthocyanidins. The use of maceration enzymes also promoted a large extraction of proanthocyanidins but without important changes in their composition with respect to control wine, indicating that the enzyme favored the extraction of both skin and seed phenolics as it has been demonstrated in this study. Only the dry ice treatment seemed to favor the extraction of high molecular weight skin proanthocyanidins. Given that it has been reported that differences in proanthocyanidin quantities and composition may lead to differences in the sensory characteristics of wines, especially the astringency, and that improved astringency quality could be obtained as a result of an increase in the proportion of skin proanthocyanidins, the use of dry ice could be considered an interesting technique for obtaining highly colored quality wines with low astringency perception.

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