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Influence of Ethanol Concentration on the Extraction of Color and Phenolic Compounds from the Skin and Seeds of Tempranillo Grapes at Different Stages of Ripening

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The aim of this paper is to study how grape ripeness and ethanol concentration affect the extraction of color and phenolic compounds from skins and seeds during the maceration/fermentation process. Simulated maceration assays were carried out with the grapes at three stages of berry development (*vitis vinifera* cv. Tempranillo) and different percentages of ethanol in the maceration media. Both ripeness and ethanol content have a considerable effect on the extraction of color and phenolic compounds. Of these two factors, ripeness increases the extractability most. The presence of ethanol in the medium facilitates anthocyanin and especially proanthocyanidin extraction, but it also decreases copigmentation phenomena, which can decrease the color intensity. The higher the ethanol concentration is in the maceration media, the higher the astringency of proanthocyanidins.

KEYWORDS: Anthocyanin; proanthocyanidins; ethanol; ripeness; extraction; HPLC; grapes; skins; seeds

INTRODUCTION

The color of red wine is mainly due to anthocyanins (*1*), which are present in the grape skin (*2*) and dissolve in the red wine during the maceration/fermentation process.

Anthocyanin synthesis starts during veraison and remains active throughout grape ripening (*3*). For this reason, anthocyanins accumulate gradually in the skins during ripening (*4*, *5*). However, anthocyanin concentration may decrease slightly during over-maturing (*6*−*8*). It has been established that the most abundant anthocyanin in nearly all grape varieties is malvidin-3-glucoside (*9*), while the concentration of the other anthocyanins varies as a function of the grape variety. During ripening, malvidin-3-glucoside and peonidin-3-glucoside levels usually increase, while the other anthocyanidin monoglucosides tend to decrease at the end of ripening (*7*, *10*). This is probably due to the fact that malvidin-3-glucoside and peonidin-3-glucoside are the final products of anthocyanin pathway biosynthesis (*11*, *12*). Simultaneously, acylated anthocyanins tend to increase throughout ripening, although in some cases they decrease at the end of the process (*5*, *13*).

For all these reasons, ripeness is the major factor affecting anthocyanin accumulation in grape skin. However, anthocyanins are not always easily extracted from skins, and poor extraction can lead to poorly colored wines, even when the anthocyanin concentration in the original grapes is sufficient (*14*). Therefore, the extractability of anthocyanins is also one of the main factors affecting their future concentration in wine (*14*). Moreover, the extractability of anthocyanins increases throughout grape ripening (*15*). Theoretically, wines obtained from well-ripened grapes have more color and are richer in anthocyanins than wines from unripened grapes.

Grape skins and seeds also contain many other phenolic compounds that are incorporated into the wine during the maceration process. Among these, proanthocyanidins, also known as condensed tannins, have a major role in wine quality. Unlike proanthocyanidins from seeds, proanthocyanidins from skins contain prodelphinidins and have a higher degree of polymerization and a lower proportion of galloylated subunits (*16*). Proanthocyanidins contribute to long-term color stability by combining with anthocyanins (*15*). Besides, proanthocyanidins are also associated with such texture sensations as body and astringency (*17*).

Several authors (*18*−*23*) have studied proanthocyanidins in grapes and their changes during ripening. In general terms, they have found that proanthocyanidin concentration is highest at veraison. Later, proanthocyanidin concentration decreases until some time just before complete ripeness, when it remains relatively constant. Simultaneously, the mean degree of polymerization (mDP) increases throughout ripening (*16*, *18*, *19*, *21*).

Nowadays, deeply colored and full-bodied red wines are highly valued by the market. For this reason, winemakers usually apply procedures to improve color extraction. The use of pectinolytic enzymes (*24*, *25*) or dry ice (*26*, *27*), the application of high temperature (*28*, *29*), an increase in maceration time, an increase in the volume and the frequency of pumping over, and the application of pigeage or delestage are the most common strategies (*15*). Nevertheless, these procedures may sometimes extract an excess of phenolic compounds, making the wine more astringent and affecting its quality (*30*), especially with unripened grapes (*14*, *15*).

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In recent years, several studies have been published about the evolution of anthocyanins (*4*, *5*, *10*, *31*) and proanthocyanidins (*18*−*20*) during ripening (*21*−*23*). Nevertheless, most of them use much more aggressive methods of extraction than those used during winemaking, so the results may be overestimated (*16*). Moreover, these aggressive methods can extract proanthocyanidins with a greater degree of polymerization than those that are really found in wine.

The length of maceration is usually considered to be one of the major factors that determine phenolic extraction (*15*). However, during the making of red wine, skins and seeds are in a medium in which the ethanol concentration progressively increases. Evidently, the ethanol content of the medium must have a nonnegligible role in phenolic compound extraction. Therefore, the evolution of the ethanol concentration in the must/wine may also be one of the main factors affecting the quality and quantity of phenolic extraction.

Some publications have studied the influence of ethanol concentration on the extraction of phenolic compounds (*2*, *32*−*35*). However, to our knowledge, none of them have studied this effect throughout grape ripening. The aim of this study was to study how grape ripeness and ethanol concentration influence color and phenolic compound extraction in skins and seeds. We have selected the *Vitis vinifera* variety Tempranillo because it is the one that is most often used to produce quality red wines in Spain.

MATERIALS AND METHODS

Chemicals. Methanol, acetonitrile, and formic acid were HPLC grade and were purchased from Merck. Epicatechin, ovalbumin, and tannic acid were purchased from Sigma. The rest of the chemicals were of high purity and were purchased from Panreac.

Grapes. This study was carried out with the *V. vinifera* cv Tempranillo. All samples were produced and collected at the experimental fields and cellar belonging to the Tarragona Enology Faculty (Rovira i Virgili University) at Constantí (Tarragona) in 2003.

About 2000 grapes were randomly collected on August 7, August 22, and September 5. The first stage (August 7) corresponds to 10 days after complete veraison, and the last stage (September 5) corresponds to complete ripeness. To obtain random samples and avoid picking grapes from the same vine at the different sampling times, every third vine in the vineyard was marked. The first sample was collected only from the marked vines. The second sample was collected from the vine immediately next to the marked vine. The third sample was collected from the remaining vines. Grapes were also randomly selected within the vine to ensure a homogeneous distribution between grapes that had been exposed more or less to sunshine. Three grapes were collected from each bunch. They were randomly sampled: one was taken from the top, one from the bottom, and one from the middle of the cluster. Special care was taken to obtain a good distribution between berries from the inside and the outside of the bunch.

The weight of 100 berries, the sugar content, the titratable acidity, and the pH were determined in triplicate using the analytical methods recommended by the OIV (*36*).

Maceration Conditions. All experiments were carried out in triplicate, and 100 berries were randomly selected for each experimental condition. Skins and seeds were manually separated from the pulp and placed separately in flasks containing 125 mL of different synthetic solutions, all of which were made with 4 g/L of tartaric acid and different ethanol concentrations (0.0, 6.5, and 13.0%) in order to reproduce three points of alcoholic fermentation. They were all adjusted with sodium hydroxide to pH = 3.5. Potassium metabisulfite (80 mg/L) was added to all synthetic solutions to inhibit sugar fermentation and to reproduce real winemaking conditions. All flasks were protected from oxidation by carbon dioxide addition and were kept at 28 °C in darkness throughout the experiment. Each day, the flasks were shaken slightly to homogenize the medium. After 7 days of maceration, the contents of the different flasks were centrifuged and conserved at 4 °C in vials that were hermetically sealed until the moment of analysis (January, 2004).

Table 1. Evolution of Grape Parameters throughout Ripening

	stage of ripening		
	1	2	3
weight of 100 berries (g)	161 ± 1 A	173 ± 8 B	221 ± 2 C
sugar content (g/L)	160 ± 7 A	180 ± 5 B	192 ± 3 C
sugar content (Brix)	14.9 ± 0.6 A	16.7 ± 0.5 B	17.7 ± 0.3 C
titratable acidity (g/L)[a]	9.5 ± 0.5 A	6.3 ± 0.5 B	4.2 ± 0.3 C
pH	3.18 ± 0.04 A	3.42 ± 0.04 B	3.56 ± 0.03 C

[a] Titratable acidities are expressed as tartaric acid. All data are expressed as the average values of three replicates ± standard deviation. Statistical analysis: one-factor ANOVA and Scheffe's test (both, $p = 0.05$). Different letters indicate statistical differences.

Color Parameters. Color intensity (IC) and hue (H) were estimated using the methodology described by Glories (*35*). The percentage of color due to copigmentation was calculated according to Boulton (*37*).

Anthocyanin Analysis. Total anthocyanin content was determined using the methodology described by Ribéreau-Gayon and Stonestreet (*38*). The content of anthocyanins combined with proanthocyanidins was calculated using the PVPP index (*35*). The anthocyanins contributing to wine color were calculated using the ionization index (*15*). HPLC analyses of anthocyanins were carried out with an Agilent (1100 series) liquid chromatograph and a Waters Spherisorb column (ODS2) in accordance with the methodology described by González-SanJosé (*39*).

Other Phenolic Compounds. The content of phenolic compounds was determined by measuring the absorbance value at 280 nm (*15*). Total proanthocyanidin content was estimated according to Ribéreau-Gayon and Stonestreet (*40*). The content of proanthocyanidins combined with polysaccharides was calculated using the ethanol index (*15*).

Astringency. Astringency was estimated using ovoalbumin as a precipitation agent and tannic acid solutions as standards in accordance with the previously described method (*41*).

Statistics. All the data are expressed as the arithmetic average ± standard deviation of three replicates. One- and two-factor ANOVA and Scheffe's test were carried out with SPSS software.

RESULTS AND DISCUSSION

Table 1 shows the physical and chemical parameters of grapes throughout ripening. The weight of 100 berries, the sugar content, the titratable acidity, and the pH show the usual behavior during ripening and confirm that the degrees of ripeness of the samples selected were significantly different.

Figure 1 shows how the ethanol content of the maceration medium influences the kinetics of extracting total anthocyanins at different stages of grape ripening. In all the experimental conditions, the total anthocyanin concentration increases during the first 3 days of maceration and stabilizes subsequently. The results also show that much fewer anthocyanins were extracted in the first stage of ripening. Nevertheless, no differences are observed between the second and third stages of ripening.

On the other hand, the extraction of anthocyanins is clearly influenced by ethanol. The higher the ethanol concentration is in the maceration media, the higher the anthocyanin extraction in all the stages of ripening.

Figure 2 shows how the ethanol content of the maceration medium influences the kinetics of extracting total phenolic compounds from skins (A280) at different stages of grape ripening. In general terms, the kinetics of extracting total phenolic compounds is similar to kinetics for anthocyanins. Most phenolic compounds are extracted from skins in the first days of maceration and tend to stabilize subsequently. Much fewer

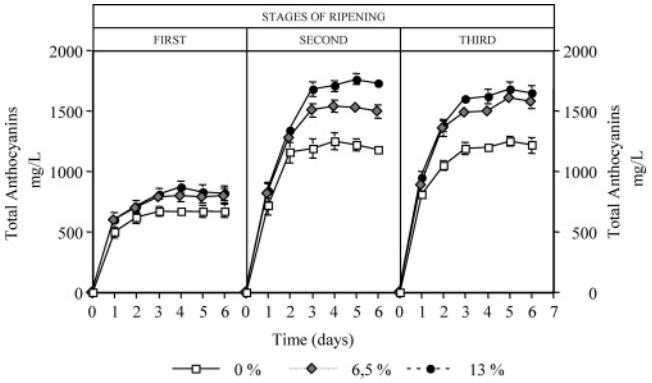

Figure 1. Influence of the ethanol content of the maceration medium on the kinetics of extraction of total anthocyanins at different stages of grape ripening. All data are expressed as the average values of three replicates ± standard deviation. Quantified by Ribéreau-Gayon and Stonestreet (*38*).

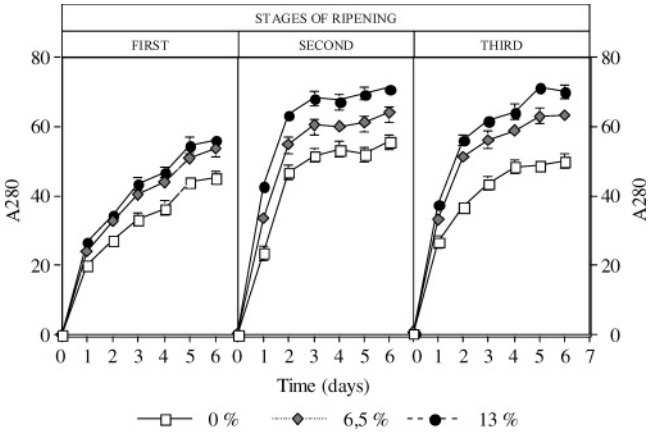

Figure 2. Macerations of skins. Influence of the ethanol content of the maceration medium on the kinetics of extraction of total phenolic compounds (A280) at different stages of grape ripening. All data are expressed as the average values of three replicates ± standard deviation.

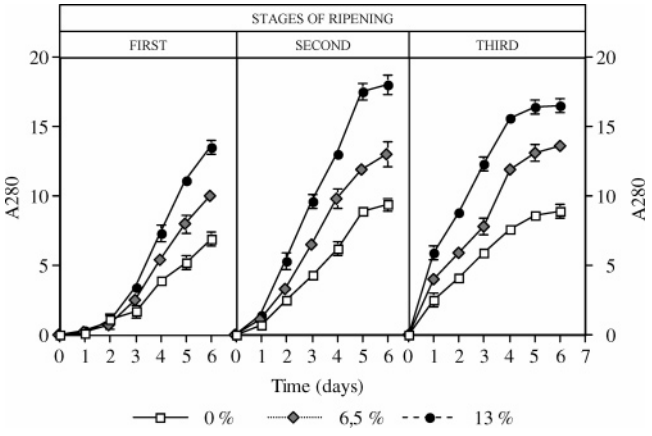

Figure 3. Macerations of seeds. Influence of the ethanol content of the maceration medium on the kinetics of extraction of total phenolic compounds (A280) at different stages of grape ripening. All data are expressed as the average values of three replicates ± standard deviation.

compounds are extracted in the first stage, and no differences are observed between the second and third stages of ripening. The presence of ethanol increases the extraction of total phenolic compounds in all the stages of ripening.

Figure 3 shows how the ethanol content of the maceration medium influences the kinetics of extracting total phenolic compounds from seeds (A280) at different stages of grape

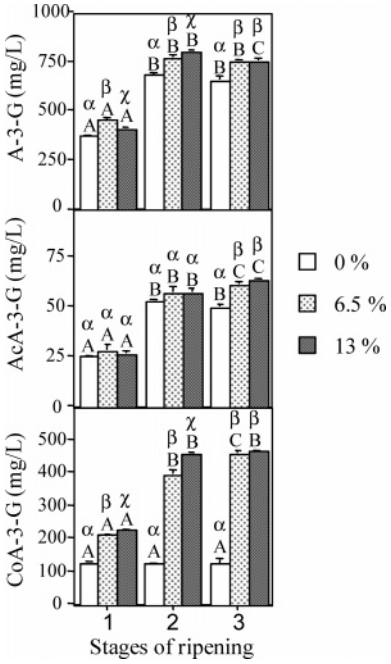

Figure 4. Influence of the ethanol content of the maceration medium on total anthocyanidin-3-monoglycosides (A-3-G), total acetylated anthocyanins (AcA-3-G), and total *p*-coumaroyl anthocyanins (CoA-3-G) at different stages of grape ripening. All data are expressed as the average values of three replicates ± standard deviation ($n = 3$). Statistical analysis: two-factor ANOVA and Scheffe's test (both, $p = 0.05$). Different letters indicate statistical differences. Greek letters (α, β, χ) are used to compare the ethanol influence. Latin letters (A, B, C) are used to compare the ripening influence. All data were determined by HPLC (*39*).

ripening. In this case, the kinetics of extraction is quite different from the kinetics of extraction from skins. Extracting total phenolic compounds from seeds seems to be slower and more progressive. These results are in agreement with González-Manzano et al. (*16*). When unripened grapes are used, the extraction of total phenolic compounds is not stabilized, whereas when the grapes are well-ripened, a certain tendency toward stabilization is observed at the end of the maceration process.

The higher the ethanol concentration is, the higher the extraction of total phenolic compounds from seeds in all the stages of maturity. Its influence is greatest for skins.

Figure 4 shows how the ethanol content of the maceration medium influences the final concentration of anthocyanidin-3-glucosides (A-3-Gs) and acetylated and *p*-coumarylated anthocyanidin-3-glucosides (AcA-3-Gs and CoA-3-Gs, respectively) at different stages of grape ripening.

These results confirm that A-3-Gs are the predominant anthocyanins. The concentration of both acylated anthocyanins is lower than that of their corresponding monoglucosides. In particular, AcA-3-Gs are present between 4 and 5%, CoA-3-Gs between 15 and 35%, and A-3-Gs between 60 and 81% of total anthocyanins.

The total A-3-G concentration increased between the first and second ripening steps. This behavior is observed, in general terms, for all A-3-Gs (data not shown). However, Pa-3-G and Mv-3-G levels stabilized, while Dp-3-G and Pt-3-G decreased slightly in the third ripening step. This decrease probably occurs because they are transformed into Mv-3-G (*4*, *11*, *12*, *31*).

On the other hand, the presence of ethanol in the medium seems to facilitate the extraction of total A-3-G in all samples. When ethanol concentration was increased to 6.5%, the total A-3-G extraction was significantly higher. The increases

Table 2. Macerations of Skins: Evolution of Total, Free, and Combined Anthocyanin Content throughout Grape Ripening and Influence of the Ethanol Content of the Maceration Medium[a]

		stage of ripening		
	ethanol	1	2	3
total anthocyanins by HPLC (mg/L)	0%	529 ± 8 α,A	862 ± 11 α,B	835 ± 34 α,B
	6.5%	695 ± 15 β,A	1224 ± 30 β,B	1271 ± 17 β,B
	13%	665 ± 11 β,A	1316 ± 20 χ,B	1286 ± 22 β,C
		two-factor ANOVA: ripening $p < 0.0001$; ethanol $p < 0.0001$		
total anthocyanins SO_2 (mg/L)	0%	647 ± 16 α,A	1103 ± 38 α,B	1135 ± 45 α,B
	6.5%	786 ± 19 β,A	1419 ± 6 β,B	1513 ± 30 β,C
	13%	781 ± 8 β,a	1658 ± 40 χ,B	1543 ± 35 β,C
		two-factor ANOVA: ripening $p < 0.0001$; ethanol $p < 0.0001$		
free anthocyanins (mg/L)	0%	533 ± 18 α,A	862 ± 20 α,B	829 ± 16 α,B
	6.5%	645 ± 22 β,A	1125 ± 16 β,B	1186 ± 19 β,C
	13%	615 ± 12 β,A	1267 ± 32 χ,B	1189 ± 20 β,C
		two-factor ANOVA: ripening $p < 0.0001$; ethanol $p < 0.0001$		
combined anthocyanins (mg/L)	0%	114 ± 8 α,A	241 ± 32 α,B	306 ± 19 α,C
	6.5%	141 ± 29 α,A	294 ± 14 β,B	327 ± 15 α,C
	13%	166 ± 6 α,A	391 ± 23 β,B	354 ± 20 α,B
		two-factor ANOVA: ripening $p < 0.0001$; ethanol $p < 0.0001$		

[a] All data are expressed as the average values of three replicates ± standard deviation ($n = 3$). Statistical analysis: two-factor ANOVA and Scheffe's test (both, $p = 0.05$). Different letters indicate statistical differences. Greek letters (α, β, χ) are used to compare the ethanol influence. Latin letters (A, B, C) are used to compare the ripening influence.

observed in total A-3-G were 22% in the first ripening step, 13% in the second step, and 15% in the third step. Therefore, this effect seems to be higher when grapes are less ripe.

The total AcA-3-G concentration also increased throughout the ripening process, especially between the first and second ripening steps, and behaved in a similar way to A-3-G. The presence of ethanol in the extraction medium, however, does not seem to have a clear effect.

The total CoA-3-G concentration also increases throughout the ripening process, although only significantly in the presence of ethanol. Indeed, the presence of ethanol seems to have a greater effect on this pigment family than on A-3-G or AcA-3-G.

Table 2 shows the evolution of total, free, and combined anthocyanins in the skin macerations throughout ripening and the influence of ethanol concentration. **Table 2** shows the total anthocyanins analyzed by HPLC (*39*) and the total anthocyanins analyzed by spectrophotometry after bleaching with sulfur dioxide according to the classical method of Ribéreau-Gayon and Stonestreet (*38*). The comparison of these results indicates that the total anthocyanin content obtained by HPLC is clearly lower than that obtained by spectrophotometry. This is absolutely logical because the HPLC method detects only free anthocyanins and not the combined anthocyanins formed when they bond to flavanols. The spectrophotometric method, however, detects all pigments susceptible to bleaching by sulfur dioxide. Therefore, this method must detect all free anthocyanins and most combined ones.

The PVPP index makes it possible to quantify free anthocyanins and, by subtraction, the combined anthocyanins (*35*). It is certainly worth pointing out that the data about total anthocyanins obtained by HPLC and the PVPP index are very similar. These data agree with the results obtained by Rivas-Gonzalo et al. (*42*).

In general terms, total, free, and combined anthocyanin contents are similar to the information in previous tables. They increase throughout ripening, especially during the first period, and the presence of ethanol makes it easier for them to be extracted.

Table 3 shows the evolution of color parameters in the skin macerations throughout grape ripening and the influence of ethanol content. The color intensity shows a clear tendency to increase throughout ripening, which is especially significant during the first period. However, there is not a direct relationship between anthocyanin concentration and color intensity. Moreover, the effect of ethanol on color intensity seems to be related to its concentration but not in a linear way. In general terms, the color intensity increases between 0.0% and 6.5% of ethanol. A slight decrease is detected, however, at 13%.

The ionization and copigmentation indices may be able to explain this. The ionization index gives an idea of the percentage of anthocyanins that contribute to the wine color (*15*). The copigmentation index, on the other hand, indicates the percentage of color due to this phenomenon (*43*). Both indices are considerably decreased by ethanol. It has been described that ethanol can disrupt the associations between anthocyanins and copigments (*43*, *44*), so these results are completely logical. Although ethanol makes it easier for anthocyanins to be extracted, its presence decreases copigmentation phenomena. When the ethanol concentration increases from 0.0 to 6.5%, anthocyanin extraction increases significantly, which may compensate for the negative effect of ethanol on copigmentation phenomena. However, when the ethanol concentration increases from 6.5 to 13%, the slight increase in anthocyanin extraction is not sufficient to compensate for the considerable decrease in copigmentation, which causes the decrease in color intensity. On the other hand, the hue seems to be increased by ripening and also by greater ethanol concentrations.

Table 4 shows the evolution of total phenolic compounds and proanthocyanidins throughout grape ripening and the influence of ethanol content. These results show that the extraction of proanthocyanidins and total phenolic compounds (A280) is higher in skins than in seeds, at least in our maceration conditions. It has been reported that seeds contain many more proanthocyanidins than skins (*16*, *33*, *45*). However, these studies aim to determine the real proanthocyanidin concentration of seeds and skins and not to determine how many proanthocyanidins can be extracted in real winemaking conditions. For this reason, these studies have used much more aggressive methods of extraction than those used during winemaking, so they may lead to overestimation (*16*). Our results have been obtained in conditions that are similar to winemaking and must

Table 3. Macerations of Skins: Evolution of Color Parameters throughout Grape Ripening and Influence of the Ethanol Content of the Maceration Medium[a]

		stage of ripening		
	ethanol	1	2	3
IC	0%	9.5 ± 0.4 α,A	15.7 ± 0.6 α,B	16.4 ± 1.1 α,B
	6.5%	9.9 ± 0.3 α,A	18.7 ± 0.4 β,B	21.8 ± 0.5 β,C
	13%	8.3 ± 0.3 β,A	16.9 ± 0.8 α,B	18.2 ± 0.9 α,B
		two-factor ANOVA: ripening $p < 0.0001$; ethanol $p < 0.0001$		
ionization index (%)	0%	29.0 ± 1.2 α,A	27.9 ± 0.3 α,AB	26.7 ± 0.3 α,B
	6.5%	23.1 ± 0.6 β,A	24.9 ± 0.6 β,B	26.1 ± 0.3 α,B
	13%	19.2 ± 1.0 χ,A	17.9 ± 0.3 χ,A	21.3 ± 0.8 β,B
		two-factor ANOVA: ripening $p = 0.005$; ethanol $p < 0.0001$		
copigmentation index (%)	0%	54.8 ± 6.9 α,A	57.9 ± 1.7 α,A	50.4 ± 4.1 α,A
	6.5%	52.0 ± 1.6 α,A	50.6 ± 1.7 β,A	50.6 ± 0.4 α,A
	13%	39.1 ± 4.4 β,A	33.5 ± 2.1 χ,A	33.9 ± 1.3 β,A
		two-factor ANOVA: ripening $p < 0.085$; ethanol $p < 0.0001$		
hue	0%	37.3 ± 0.2 α,A	39.9 ± 0.7 α,A	43.0 ± 1.7 α,B
	6.5%	39.6 ± 0.8 β,A	42.0 ± 1.0 β,B	41.5 ± 0.3 α,AB
	13%	42.6 ± 0.5 χ,A	46.5 ± 0.6 χ,B	45.6 ± 0.6 β,B
		two-factor ANOVA: ripening $p < 0.0001$; ethanol $p < 0.0001$		

[a] All data are expressed as the average values of three replicates ± standard deviation ($n = 3$). Statistical analysis: two-factor ANOVA and Scheffe's test (both, $p = 0.05$). Different letters indicate statistical differences. Greek letters (α, β, χ) are used to compare ethanol influence. Latin letters (A, B, C) are used to compare the ripening influence.

Table 4. Influence of Grape Ripening and Ethanol Content on Total Phenolic Compounds and Proanthocyanidins[a]

		stage of ripening		
	ethanol	1	2	3
IPT skins	0%	36.7 ± 1.2 α,A	47.3 ± 2.1 α,B	43.7 ± 1.5 α,B
	6.5%	42.3 ± 1.5 β,A	60.0 ± 1.0 β,B	62.3 ± 2.1 β,B
	13%	45.3 ± 1.5 χ,A	72.3 ± 1.5 χ,B	67.3 ± 1.5 χ,C
		two-factor ANOVA: ripening $p < 0.0001$; ethanol $p < 0.0001$		
IPT seeds	0%	6.7 ± 0.1 α,A	9.1 ± 0.6 α,B	8.4 ± 0.3 α,B
	6.5%	9.0 ± 0.4 β,A	12.6 ± 0.8 β,B	12.9 ± 0.9 β,B
	13%	12.9 ± 0.9 χ,A	17.6 ± 0.7 χ,B	15.7 ± 1.2 χ,B
		two-factor ANOVA: ripening $p < 0.0001$; ethanol $p < 0.0001$		
proanthocyanidins skins (g/L)	0%	1.36 ± 0.11 α,A	1.54 ± 0.09 α,A	1.26 ± 0.13 α,A
	6.5%	1.59 ± 0.04 β,A	2.06 ± 0.09 β,B	2.10 ± 0.07 β,B
	13%	1.92 ± 0.08 χ,A	2.70 ± 0.14 χ,B	2.55 ± 0.11 χ,B
		two-factor ANOVA: ripening $p < 0.0001$; ethanol $p < 0.0001$		
proanthocyanidins seeds (g/L)	0%	0.46 ± 0.01 α,A	0.59 ± 0.05 α,B	0.56 ± 0.03 α,B
	6.5%	0.63 ± 0.02 β,A	0.86 ± 0.08 β,B	0.94 ± 0.08 β,B
	13%	0.94 ± 0.09 χ,A	1.35 ± 0.08 χ,B	1.23 ± 0.10 χ,B
		two-factor ANOVA: ripening $p < 0.0001$; ethanol $p < 0.0001$		
proanthocyanidin–polysaccharide skins (g/L)	0%	0.10 ± 0.01 α,A	0.14 ± 0.01 α,B	0.22 ± 0.01 α,C
	6.5%	0.13 ± 0.02 α,A	0.22 ± 0.03 β,B	0.38 ± 0.05 β,C
	13%	0.17 ± 0.02 β,A	0.27 ± 0.02 β,B	0.39 ± 0.04 β,C
		two-factor ANOVA: ripening $p < 0.0001$; ethanol $p < 0.0001$		
astringency skins (g/L)	0%	0.257 ± 0.004 α,A	0.364 ± 0.026 α,B	0.267 ± 0.035 α,A
	6.5%	0.320 ± 0.034 β,A	0.468 ± 0.034 β,B	0.388 ± 0.036 β,A
	13%	0.386 ± 0.016 χ,A	0.576 ± 0.038 χ,B	0.524 ± 0.016 χ,C
		two-factor ANOVA: ripening $p < 0.0001$; ethanol $p < 0.0001$		
astringency seeds (g/L)	0%	0.074 ± 0.003 α,A	0.095 ± 0.006 α,B	0.076 ± 0.006 α,A
	6.5%	0.112 ± 0.006 β,A	0.145 ± 0.006 β,B	0.128 ± 0.007 β,C
	13%	0.152 ± 0.013 χ,A	0.186 ± 0.007 χ,B	0.165 ± 0.005 χ,A
		two-factor ANOVA: ripening $p < 0.0001$; ethanol $p < 0.0001$		

[a] All data are expressed as the average values of three replicates ± standard deviation ($n = 3$). Statistical analysis: two-factor ANOVA and Scheffe's test (both, $p = 0.05$). Different letters indicate statistical differences. Greek letters (α, β, χ) are used to compare the ethanol influence. Latin letters (A, B, C) are used to compare the ripening influence.

theoretically reproduce what really happens during the maceration/fermentation process.

Moreover, in our experimental conditions, all macerations only lasted 7 days. As shown in **Figures 2** and **3**, the kinetics of extracting total phenolic compounds from skins and seeds seems to be different. Whereas, the extraction of total phenolic compounds from skins seems to stabilize after 4 or 5 days of maceration, the extraction from seeds seems to continue progressively. Therefore, extending maceration beyond the end of alcoholic fermentation considerably increases the concentration of proanthocyanidins from the seeds. In longer macerations, the proportion of proanthocyanidins from the seeds would probably be greater.

In general terms, the concentrations of total phenolic compounds and proanthocyanidins in skins and seeds increased significantly between the first and second ripening steps. However, no significant changes are found at the point of greatest ripeness.

Although some authors have found that the proanthocyanidin concentration decreases in skins and seeds throughout ripening

(*19*, *20*, *22*, *45*), our results seem to indicate that they are easily extracted. The experience of winemakers indicates that riper grapes generally tend to yield wines with a greater tannic concentration, and our results seem to agree with experience.

On the other hand, the influence of the ethanol concentration is very clear. When the ethanol concentration is high, the extraction of skin and seed proanthocyanidins is significantly higher. This agrees with the results obtained by González-Manzano et al. (*16*).

The ethanol index makes it possible to quantify the proanthocyanidin−polysaccharide complexes. It has been reported that these complexes can diminish the capacity of proanthocyanidins to bind proteins, and therefore, the astringency of the wine decreases (*46*). This index can only be determined in skin extracts or wines, not in seed extracts. If it is used in seed extracts, the values are always close to zero. These proanthocyanidin−polysaccharide complexes increase significantly during ripening.

On the other hand, ripeness has a nonnegligible effect on astringency. The astringency of the skin and seed extracts increases significantly during the first stage of ripening, coinciding with the increase in the proanthocyanidin concentration. However, the astringency of the skin and seed extracts decreases significantly during the last phases of ripening even though the proanthocyanidin concentration is stable. It has been postulated that the combination of polysaccharides with proanthocyanidins may diminish their capacity to bind proteins (*46*). Therefore, the observed increase in the concentration of proanthocyanidin−polysaccharide complexes may be related to the decrease in the astringency of skin extracts.

The presence of ethanol also significantly increases the astringency of the skin and seed extracts probably because of its effect on proanthocyanidin extraction.

It can be concluded that ripeness has a considerable effect on the future extraction of color and phenolic compounds during winemaking. In general terms, the extraction of anthocyanins (from skins) and proanthocyanidins (from skins and seeds) is significantly higher when the grapes are riper. This increase is particularly significant between the first and second steps of ripening. The astringency of the skin and seed extracts is also influenced by ripeness. Although this negative sensory attribute increases between the first and second steps in ripening, it decreases significantly when the grapes begin to be ripen.

On the other hand, the presence of ethanol in the extraction medium facilitates anthocyanin and especially proanthocyanidin extraction. Therefore, the length of maceration can determine the proanthocyanidin concentration and the astringency of red wines. A short maceration will lead to wines with a low proanthocyanidin concentration and low astringency because seeds and skins have been in contact with a medium rich in ethanol for a short time. On the other hand, a long maceration will lead to wines with a high proanthocyanidin concentration and high astringency because skins and especially seeds have been in contact with a medium rich in ethanol for a long time. The effect on the astringency of the wine will be greater if the grapes are less ripe. Moreover, the potential ethanol content of the grapes can also determine the proanthocyanidin concentration and the wine astringency during winemaking.

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