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Anthocyanin Composition of Cabernet Sauvignon and Tempranillo Grapes at Different Stages of Ripening

JOSÉ-MARÍA RYAN† AND EUGENIO REVILLA*

Departamento de Química Agrícola, Geología y Geoquímica, Universidad Autónoma de Madrid, 28049 Madrid, Spain

Changes in anthocyanins during ripening of Cabernet Sauvignon and Tempranillo grapes were studied over a three year period. The accumulation of anthocyanins showed variations during ripening, especially during the first three weeks after veraison, and the accumulation pattern of those molecules changed only slightly from one year to another. On the other hand, the percentages of the different anthocyanins studied were different for each cultivar, and some changes were observed in both cultivars depending on the weather conditions of the growing season. In warm years the percentages of primitive anthocyanins (delphinidin 3-*O*-glucoside and petunidin 3-*O*-glucoside) were slightly lower than in a relatively cool year. Nevertheless, the anthocyanin fingerprints of Cabernet Sauvignon and Tempranillo grapes seem to be rather stable during ripening, despite the sugar content of the grapes.

KEYWORDS: Anthocyanins; red grapes; ripening; anthocyanin fingerprint

INTRODUCTION

The color of red wines is a remarkable sensory attribute, related to the extraction of anthocyanins from grape skins during wine-making. During the maturation of red wines, anthocyanins may react with flavanols, leading to the formation of colored polymeric pigments responsible for the color stability of aged red wines (*1*−*4*). Furthermore, young red wines contain free anthocyanins at a relatively high concentration. The anthocyanin fingerprint of young red wines, determined by HPLC, has been proposed as a useful analytical tool to identify the cultivar used in wine-making, especially if it is mentioned in bottle labels (*5*−*8*). The use of this analytical tool to determine the grape cultivar used for wine-making needs a careful evaluation of the influence of different technological procedures on the anthocyanin fingerprints (*9*). Moreover, the usefulness of this tool in routine analysis will depend on a given cultivar's anthocyanin fingerprint not being affected by the degree of maturity of grapes and also on the rate of extraction of different anthocyanins during wine-making not being dependent on different technological procedures.

Anthocyanins are accumulated in berry skins during the ripening of grapes, and several agroecological factors, such as cultivar, climate, soil conditions, canopy management, crop level, and irrigation, have been related to anthocyanin accumulation in red grape skins (*10*, *11*). Several studies have been carried out to examine the accumulation of anthocyanins on grape berries during ripening over one or two seasons in a number of cultivars, using HPLC as the analytical tool (*11*−*16*). These studies have shown that the pattern of accumulation of anthocyanins differed with cultivar and growing season, even when a cultivar grown in the same vineyard was examined for two consecutive years. Nevertheless, the HPLC anthocyanin fingerprint of grape skins appears to be closely related to genetic characteristics, and these molecules have been postulated as chemical markers to differentiate grape cultivars (*8*, *17*−*19*). Unfortunately, there are not enough experimental data to ensure that, for a given cultivar grown in a specific location, the proportions of different anthocyanins in grape skins are or not rather constant from year to year.

The aim of our research has been to investigate the pattern of accumulation of anthocyanins in the skins of two red grape cultivars (Cabernet Sauvignon and Tempranillo) under warm conditions and to establish if the anthocyanin fingerprint of those cultivars, grown in the same location, changes from year to year. The study was carried out over three consecutive years in order to consider growing seasons with different weather conditions.

MATERIALS AND METHODS

Grape Samples and Sample Preparation. Samples of Cabernet Sauvignon and Tempranillo grapes, trained in Guyot cordon, were collected in a commercial vineyard located in Escalona del Alberche (Toledo, Spain), which lies at 40° 07′ N and 4° 25′ W, and 480 m above sea level, with a density of 2500 vines/ha. Sampling took place from veraison to harvest, in 1995, 1996, and 1997. In 1996, harvest took place 41 and 43 days after veraison for Cabernet Sauvignon and Tempranillo, respectively, to avoid the development of gray mold. That year, rainfall was quite intense in the beginning of September, as indicated by data collected at Estación Meteorológica de la Higueruela, located very close to the vineyard (40° 03′ N, 4° 25′ W, 450 m above sea level). Each sample consisted of 25 clusters picked randomly from 25 different plants located in 25 different rows, and clusters from different plants were picked in every sampling. Once in the laboratory, berries were separated from clusters, and three replicates of 100 grapes

* Author to whom correspondence should be addressed (fax +34-91-3973826; e-mail eugenio.revilla@uam.es).
† Present address: Bodegas CVNE, 26100 Haro, Spain.

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Table 1. Weight of 100 Berries, Sugar Content and Titratable Acidity of Must, and Total Anthocyanins (Determined by HPLC) in Grape Skins for Cabernet Sauvignon Grapes[a]

sample	days after veraison	wt of 100 berries (g)	sugar content of must (g/L)	titratable acidity of must (g/L)	total anthocyanins (mg/kg of grapes)
			1995		
CS-95-1	15	104.7	171.5	7.8	716
CS-95-2	36	104.2	214.8	3.0	847
CS-95-3	46	112.3	233.4	2.8	944
CS-95–4	51	113.6	240.3	2.9	738
CS-95-5	56	105.8	228.7	2.8	621
CS-95-6	62	107.1	247.7	2.4	767
			1996		
CS-96-1	3	63.6	131.6	25.2	264
CS-96-2	10	83.4	153.7	11.4	492
CS-96-3	18	87.1	176.0	7.4	739
CS-96-4	24	93.2	188.6	6.0	650
CS-96-5	31	103.2	211.4	5.2	747
CS-96-6	36	92.9	219.5	5.6	937
CS-96-7	41	101.7	226.4	5.3	868
			1997		
CS-97-1	8	77.5	153.7	9.6	495
CS-97-2	15	98.9	162.6	7.4	558
CS-97-3	22	109.7	171.5	7.1	629
CS-97-4	29	92.6	191.9	6.2	781
CS-97-5	36	105.3	202.2	4.0	748
CS-97-6	43	108.4	217.2	3.5	836
CS-97-7	50	108.4	245.0	3.3	839
CS-97-8	57	104.8	249.7	2.7	785

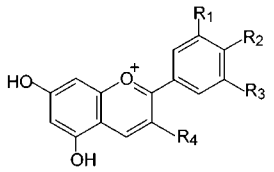
[a] Data are mean values of three replications.

Table 2. Weight of 100 Berries, Sugar Content and Titratable Acidity of Must, and Total Anthocyanins (Determined by HPLC) in Grape Skins for Tempranillo Grapes[a]

sample	days after veraison	wt of 100 berries (g)	sugar content of must (g/L)	titratable acidity of must (g/L)	total anthocyanins (mg/kg of grapes)
			1995		
T-95-1	19	166.6	158.1	5.9	684
T-95-2	40	170.1	226.4	2.5	916
T-95-3	52	183.8	219.5	2.3	865
T-95-4	55	165.3	221.7	2.3	804
T-95-5	60	176.8	224.1	2.3	713
T-95-6	66	166.6	219.5	2.2	890
			1996		
T-96-1	3	123.2	124.0	11.6	254
T-96-2	10	123.6	150.3	9.2	346
T-96-3	17	144.7	170.4	6.3	357
T-96-4	25	156.2	190.8	4.8	732
T-96-5	31	144.3	200.0	4.6	665
T-96-6	38	167.4	203.3	4.0	634
T-96-7	43	167.6	218.3	3.7	758
			1997		
T-97-1	12	194.8	149.2	5.6	292
T-97-2	19	192.0	171.5	3.7	487
T-97-3	26	217.7	187.4	3.6	628
T-97-4	33	206.8	194.2	2.9	625
T-97-5	40	243.5	201.1	2.6	509
T-97-6	47	216.0	210.3	2.7	719
T-97-7	54	234.3	224.1	2.4	555
T-97-8	61	241.2	228.7	2.3	512

[a] Data are mean values of three replications.

were randomly sampled and weighed. Then, the skins of each replicate were separated from the pulp and seeds, and the anthocyanins were extracted from grape skins following the extraction procedure proposed by Bourzeix et al. (*20*), which involves the use of several solvents (methanol, acetone, and water) without the addition of any acid, mineral, or organic. Sugar content and total acidity of grapes were measured in grape must after ~2 kg of berries had been crushed. Sugar content of grapes was determined by refractometry and titratable acidity by potentiometry (*21*). Skin extracts were stored at 4 °C prior to their analysis.



Compound	R_1	R_2	R_3	R_4
1. Delphinidin-3-*O*-glucoside	OH	OH	OH	*O*-glucose
2. Cyanidin-3-*O*-glucoside	OH	OH	H	*O*-glucose
3. Petunidin-3-*O*-glucoside	OCH_3	OH	OH	*O*-glucose
4. Peonidin-3-*O*-glucoside	OCH_3	OH	H	*O*-glucose
5. Malvidin-3-*O*-glucoside	OCH_3	OH	OCH_3	*O*-glucose
6. Malvidin-3-*O*-acetylglucoside	OCH_3	OH	OCH_3	*O*-acetylglucose
7. Malvidin-3-*O*-*p*-coumarylglucoside	OCH_3	OH	OCH_3	*O*-*p*-coumarylglucose

Figure 1. Chemical structures of seven anthocyanins analyzed in grapes.

HPLC Analysis. HPLC analyses were performed using the equipment described previously (*9*). For samples collected in 1995 and 1996, a Linear UVIS 200 variable-wavelength visible−UV detector, set at 520 nm with a sensitivity of 0.01 AUFS, and a Spectra Physics SP-4290 integrator were used. Separation was carried out on a 150 mm × 3.9 mm i.d., 5 μm, Novapak C18 steel cartridge, using a 20 mm × 3.9 mm i.d. Sentry Nova-Pak C18 guard cartridge (Waters), both thermostated in a water bath at 32 °C. The mobile phase was a linear gradient of water/acetonitrile (40:60) with 0.6% perchloric acid (solvent B), in water/acetonitrile (95:5) with 0.6% perchloric acid (solvent A), at a flow rate of 1.5 mL/min. The following proportions of solvent B were used: 0−5 min, 10%; 5−25 min, 10−20%; 25−45 min, 20−30%; 45−54 min, 30−33%; 54−55 min, 33−100%; 55−58 min, 100%; 58−59 min, 100−10%. The different anthocyanins analyzed were identified on the basis of their retention times and UV−vis spectra by comparison with standards isolated from grapes, as previously described (*22*). The quantification of the different substances was carried out in the chromatograms recorded at 520 nm by an external standard procedure, using a series of solutions of malvidin 3-*O*-glucoside isolated from grapes. Results were expressed as malvidin 3-*O*-glucoside equivalents.

Statistical Analysis. Multivariate analyses to test differences among years and cultivars were run using the Statgraphics 2.0 Plus statistical package.

RESULTS AND DISCUSSION

Changes in Sugar Content and Tritatable Acidity of Musts and in Total Anthocyanins in Skins during Ripening of Tempranillo and Cabernet Sauvignon Grapes. Tables 1 and **2** show changes in berry weight, sugar content of grape must, titratable acidity of must, and total anthocyanins in grape skin extracts, measured by HPLC, in samples of Cabernet Sauvignon and Tempranillo grapes collected during ripening in 1995, 1996, and 1997. Final berry weights were quite similar in the three years studied for Cabernet Sauvignon, but Tempranillo showed a higher berry weight during ripening in 1997 than in the other two years. Rainfall in July was higher in 1997 than in the other two years, and this may explain the weight differences shown by Tempranillo. Nevertheless, both cultivars were grown in adjoining vineyards, and similar management practices were carried out in them, but berry weight in Cabernet Sauvignon was similar at the end of ripening during the three years studied. The accumulation of sugar in grape must was gradual during ripening except in 1995 for Tempranillo grapes, which achieved a maximum 40 days after veraison and then remained virtually stable. The loss of titratable acidity during ripening was less

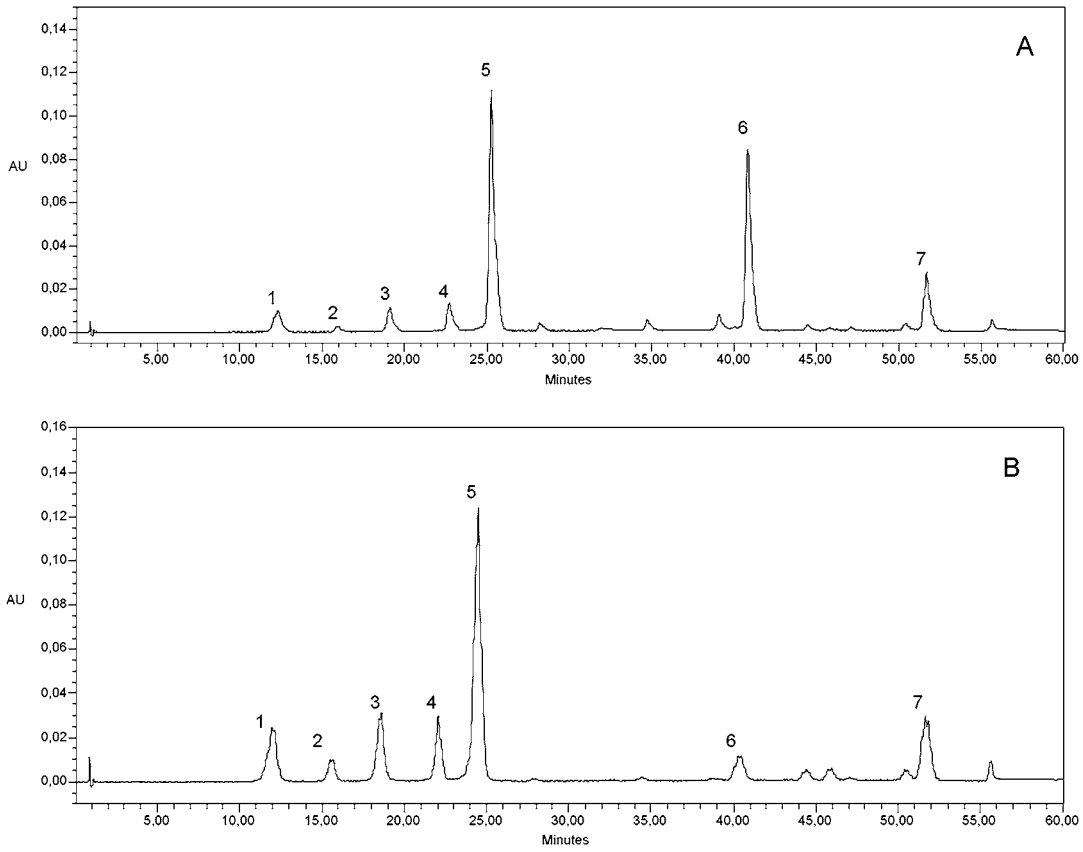


Figure 2. Chromatogram of skin extracts of Cabernet Sauvignon (A) and Tempranillo (B) grapes recorded at 520 nm. For key to substances, see **Figure 1**.

intense in 1996 than in the other two years considered; this may be explained by the fact that during August and September 1996 the weather was not so warm as in 1995 and 1997. Nevertheless, the accumulation of anthocyanins followed a different pattern from that shown by sugars in must. In 1995, the content of total anthocyanins in Tempranillo grapes achieved a maximum 40 days after veraison, when the sugar content of must reached 225 g/L. Afterward, the sugar content of must remained virtually unchanged, but total anthocyanins showed a decreasing trend during 20 days, and then total anthocyanins increased again. A similar behavior was observed that year in Cabernet Sauvignon grapes, as total anthocyanins achieved a maximum 46 days after veraison, when the sugar content of must reached 230 g/L. The total content of anthocyanins then decreased, but a second maximum was achieved at harvest. In 1996 and 1997, the accumulation of anthocyanins was quite intense for both cultivars in the first stages of ripening, and a maximum was achieved when the sugar content of must was 175−200 g/L. Afterward, the content of anthocyanins decreased, and a second maximum was achieved when the sugar content of must reached 210−220 g/L in Tempranillo and 220−245 g/L in Cabernet Sauvignon. Data obtained in 1996 and 1997 are quite different from those reported by several authors (*11*, *13*, *14*, *23*), who observed that the anthocyanin content of grape skins reached a maximum at harvest. Nevertheless, they are consistent with the findings of Somers (*24*) in Shiraz grapes, which reached a maximum for total anthocyanins content 20−30 days after veraison when the sugar content of must was 21−24 °Brix, and with results obtained in other studies on Shiraz (*12*), Cabernet Sauvignon and Merlot (*16*), and Tempranillo (*25*) grapes. A similar decline in anthocyanin content of grapes during the period of over-ripening was reported by Ribéreau-Gayon (*26*), but the concept of over-ripening must be used with care because its significance differs among various viticultural areas.

Changes in the Content of Different Anthocyanins during Ripening. Seven different anthocyanins (3-*O*-monoglucosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin, malvidin 3-*O*-acetylglucoside, and malvidin 3-*O*-*p*-coumaryl-glucoside) were determined in skin extracts except in those corresponding to the 1995 vintage, because levels of cyanidin 3-*O*-glucoside in those samples were too low. Chemical structures of those anthocyanins are given in **Figure 1**. Typical chromatograms of skin extracts of Cabernet Sauvignon and Tempranillo grapes, recorded at 520 nm, are displayed in **Figure 2**.

5 was the major anthocyanin in Cabernet Sauvignon grapes, which also contained a remarkable amount of two acylated derivatives of that anthocyanin (**6** and **7**). The other four anthocyanins (**1**−**4**) were present in low amounts, <50 mg/kg of grapes (**Figure 3**). In the case of Tempranillo, **5** was also the major anthocyanin, and there was a remarkable amount of **7**; **1**, **3**, **4**, and **6** were present at lower amounts, not exceeding 85 mg/kg, and the levels of **2** were very low (**Figure 4**).

In both cultivars, **1** was accumulated in the earliest stages of ripening in 1996 and also in 1997, but at a lower extent. Thereafter, its concentration remained stable and even decreased. In 1995, the concentration of this pigment sharply decreased 46−50 days after veraison. These results are consistent with others reported previously (*12*, *27*), which considered **1** to be a primitive pigment, taking into account the biosynthetic pathways that lead to the formation of different anthocyanins in grapes. **3** showed a pattern of accumulation similar to that presented by **1**. This may be explained by taking into account that some authors have proposed that **3** is obtained from **1** and then

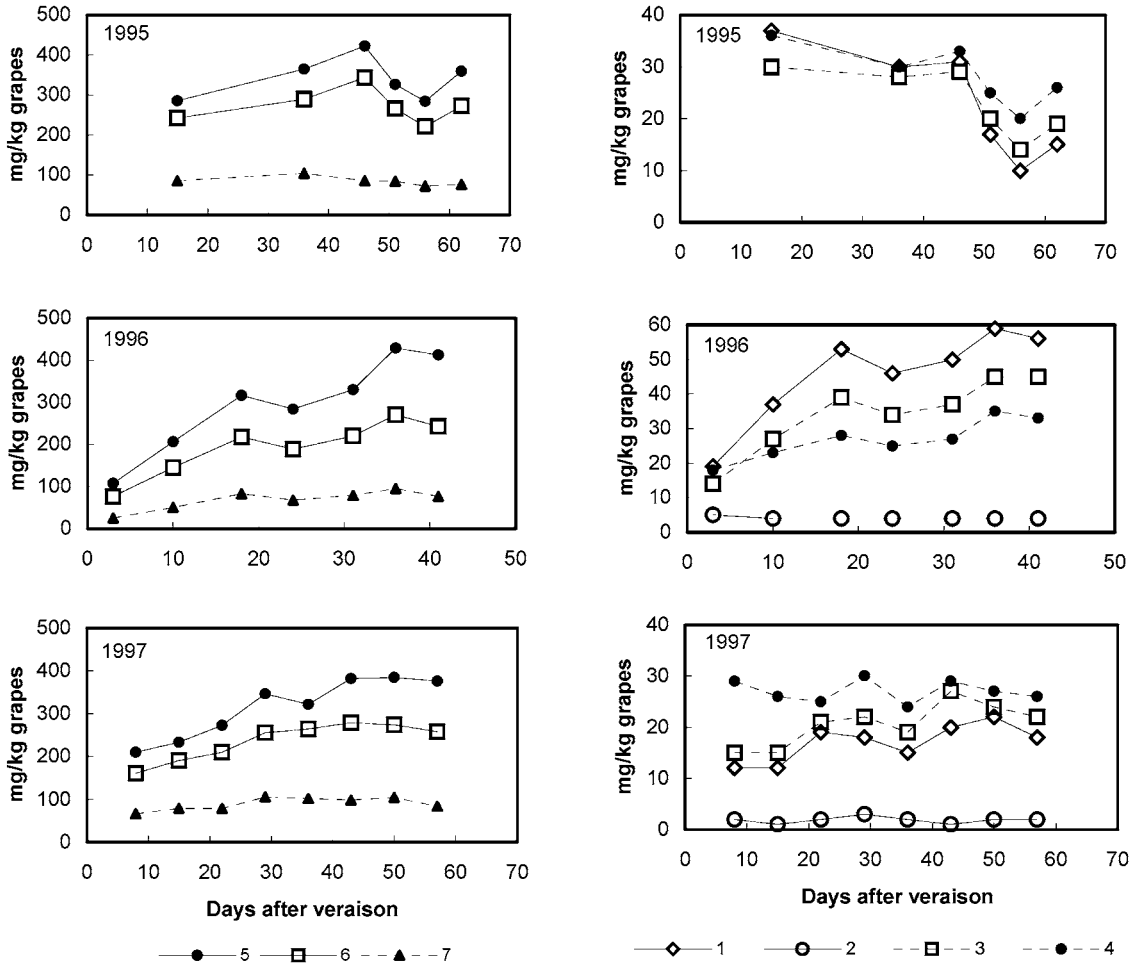


Figure 3. Changes undergone by the content of seven anthocyanins in skins of Cabernet Sauvignon grapes (mg/kg of grapes) during ripening in 1995, 1996, and 1997. Data are mean values of three replications. For key to anthocyanins, see **Figure 1**.

transformed into **5** by two similar reactions induced by a methyltransferase (*12*). Nevertheless, other authors have suggested that **5** is obtained directly from **1** by the action of methyltransferases (*27*).

The behavior of **5** was quite similar to that observed for total anthocyanins. Thus, despite its sharp accumulation after veraison in 1996 and 1997, its content decreased after a first maximum, observed when the sugar content of must reached ~190 g/L in Cabernet Sauvignon (1996) and Tempranillo (both years) and ~170 g/L in Cabernet Sauvignon (1997). Its concentration then increased again after 35−40 days of ripening. In each cultivar, changes in the content of the acylated derivatives of **5** (**6** and **7**) were quite similar to those observed for **5**. The behavior of **4** was quite similar to that observed for **5**, especially in Tempranillo. This observation agrees with those of Roggero et al. (*12*) and Boss et al. (*27*), who considered that **4** and **5** are stable anthocyanins, representing the ultimate forms in the sequence of transformations that take place during anthocyanin biosynthesis. Our results are difficult to compare with other reported changes of the anthocyanin pattern in Tempranillo grapes during ripening (*11*, *13*) because the extraction of anthocyanins was carried out with solvents containing a remarkable amount of hydrochloric acid, which leads to the hydrolysis of acetylated anthocyanins (*28*). Nevertheless, they agree with those reported on Cabernet Sauvignon grapes (*16*).

Anthocyanin Fingerprint of Cabernet Sauvignon and Tempranillo Grapes during Ripening. The anthocyanin fingerprint of red grapes and wines has been postulated as a useful tool to differentiate grape cultivars and the varietal origin of wines. For this reason, the study of changes undergone by the anthocyanin fingerprint of Cabernet Sauvignon and Tempranillo grapes during ripening may give valuable information on the reliability of that tool.

In both cultivars, the relative amount of **5** increased during ripening, and samples collected at harvest contained the highest relative amount of this pigment except in the case of Tempranillo in 1995. In contrast, the relative amounts of **1** and **3** usually showed a decreasing trend at the end of ripening, especially in Tempranillo, and the relative amount of **4** was quite stable in the late stages of ripening, especially in Cabernet Sauvignon. These results agree with observations made with three different clones of Shiraz (*12*). In both cultivars, the relative content of **6** was quite stable during ripening, but **7** usually increased in the first stages of ripening, especially in Tempranillo in 1995 and 1996, and decreased at the end of ripening. This difference was not observed in Shiraz by Roggero et al. (*12*), and it should be related to the biosynthetic reactions involved in the formation of those acylated derivatives. The different behavior presented by the percentages of **6** and **7** during ripening leads to fluctuations in the ratio of **6** to **7** in grapes, as shown in **Figure 5**. The ratio of acetylated to *p*-coumarylated anthocyanins has been proposed as a distinctive analytical parameter to differentiate wines made with different grape cultivars (*5*); thus, fluctuations mentioned above may explain to some extent the differences observed in that ratio in different wines made with the same grape cultivar (*29*, *30*). However, different technological

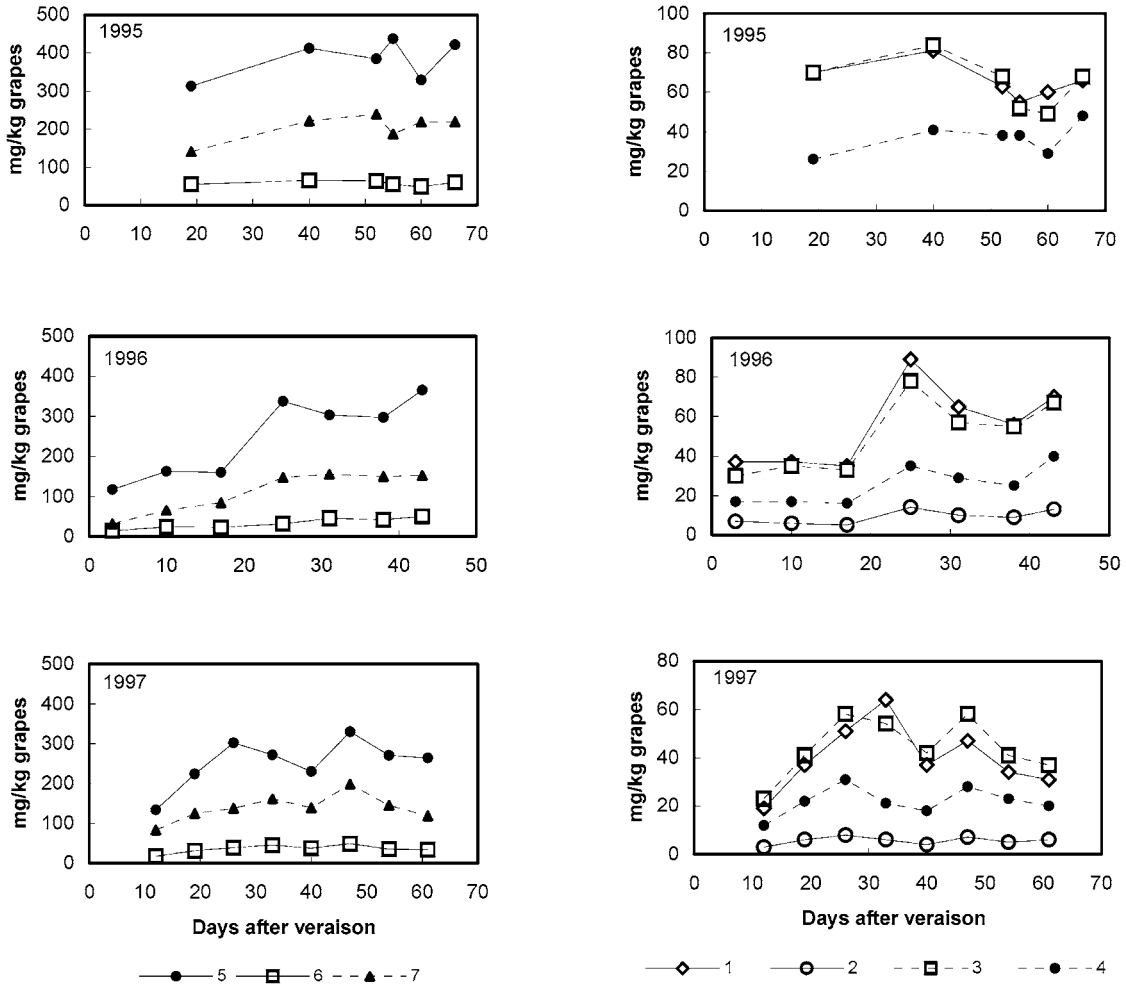


Figure 4. Changes undergone by the content of seven anthocyanins in skins of Tempranillo grapes (mg/kg of grapes) during ripening in 1995, 1996, and 1997. Data are mean values of three replications. For key to anthocyanins, see **Figure 1**.

procedures may affect that ratio in wines because the extraction kinetics of **6** and **7** during wine-making under the same conditions are different, as has been shown for 16 different grape cultivars (*8*).

For a better understanding of differences shown by the anthocyanin fingerprints of Cabernet Sauvignon and Tempranillo grapes during ripening, different types of multivariate analysis were carried out. Principal component analysis (PCA) was performed to establish the relationships among six variables (percentages of **1** and **3**−**7**) and samples (21 of Cabernet Sauvignon and 21 of Tempranillo grapes). Percentages of **2** were not considered because its content in 1995 was too low to be successfully determined. Two principal components, with eigenvalues >1, explained 82.8% of the variability in the original data. Component 1 is mainly associated with primitive anthocyanins (**1** and **3**) and acylated derivatives of **5** (**6** and **7**), whereas component 2 is mainly associated with stable nonacylated anthocyanins (**4** and **5**). Thus, PCA suggests that differences observed in the anthocyanin pattern of Cabernet Sauvignon and Tempranillo grapes are primarily due to the different proportions of **1**, **3**, **6**, and **7** presented for each cultivar. Samples of each cultivar are clearly grouped in the plot of component 1 versus component 2 (**Figure 6**): Cabernet Sauvignon samples on the left side of the plot, close to the percentage of **6**, and Tempranillo samples on the right side. This classification has been achieved using the six variables mentioned above.

The relative contents of different anthocyanins and PCA point out that each cultivar studied has a characteristic anthocyanin fingerprint. Moreover, samples of each cultivar have been successfully classified by cluster analysis, using as variables the relative contents of six anthocyanins (**1** and **3**−**7**), and may be grouped into two clusters clearly differentiated, each cluster containing the samples belonging to a cultivar. This is shown in the dendrogram presented in **Figure 7**, obtained using Ward's method with squared euclidean distance. Thus, it can be concluded that Cabernet Sauvignon and Tempranillo grapes present different anthocyanin fingerprints at any stage of grape development during ripening.

Discriminant analysis, using a stepwise forward selection algorithm, was performed to develop a set of discriminating functions, derived from values of six quantitative variables (percentages of **1** and **3**−**7**), capable of predicting cultivar [Cabernet Sauvignon (CS) or Tempranillo (T)] and weather conditions of the year [warm (W) or relatively cool (C)]. We considered 1996 to be a relatively cool year, because means of maximum, minimum, and average temperatures from February to May were lower that year than in 1995 and 1997, leading to the delay of budbreak and, as a consequence, of veraison. Three variables (percentages of **1**, **3**, and **6**) were significant predictors of the four groups considered (CS-W, CS-C, T-W, and T-C), and two discriminant functions, associated with those variables, with P values of <0.05 were statistically significant at the 95% confidence level. These discriminant functions represented 100%

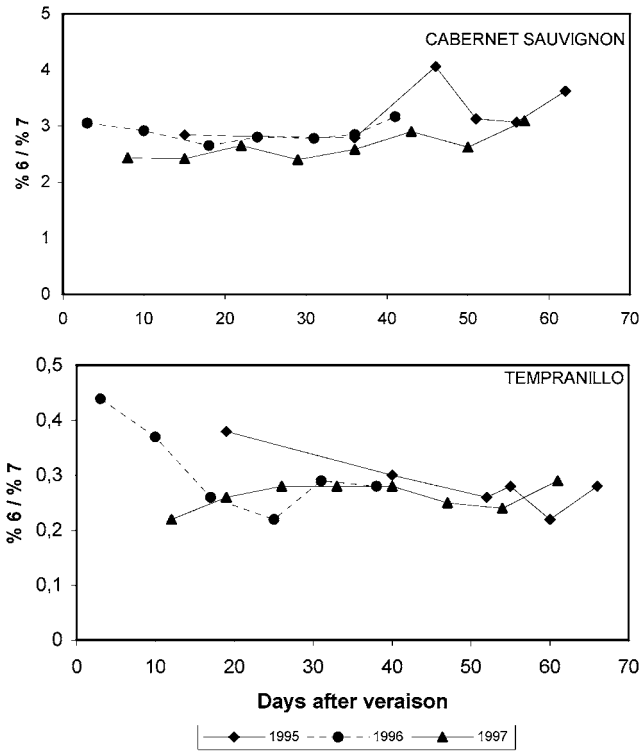


Figure 5. Changes undergone by the ratio of percentage of malvidin 3-*O*-acetylglucoside (**6**) to percentage of malvidin 3-*O*-*p*-coumarylglucoside (**7**) in Cabernet Sauvignon and Tempranillo grapes during ripening in 1995, 1996, and 1997.

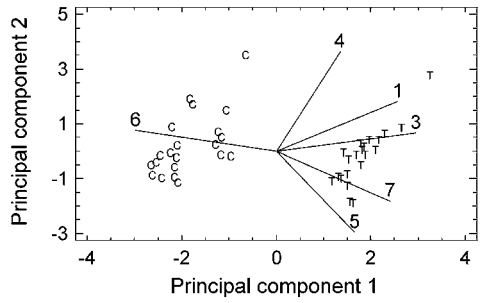


Figure 6. Plot of the first two principal components in PCA of Cabernet Sauvignon (C) and Tempranillo (T) grapes. Variables are percentages of six different anthocyanins. For key to anthocyanins, see **Figure 1**.

of total variability, and 97.6% of samples were correctly classified. **Figure 8** shows the different groups of samples in the biplot of discriminant functions 1 and 2. As can be noted, only a Tempranillo sample that actually belonged in group T-W was classified as T-C. All other samples were correctly classified. These results suggest that the anthocyanin fingerprint of grapes is related to cultivar and weather conditions of the growing season. Furthermore, the percentages of **1** and **3**, which are considered to be primitive pigments, can be significant predictors of weather conditions of the year. In warm years (1995 and 1997), percentages of **1** and **3** are lower than in a relatively cool year (1996). These results agree with observations on the anthocyanin fingerprints of Cabernet Sauvignon and Tempranillo grapes grown in temperate areas (*15*, *31*), which contain a higher percentage of **1** and **3** than grapes examined in this paper, grown in a warm region.

The accumulation of anthocyanins during the ripening of Cabernet Sauvignon and Tempranillo grapes showed a similar pattern. In both cultivars, the concentrations of **1** and **3**, which

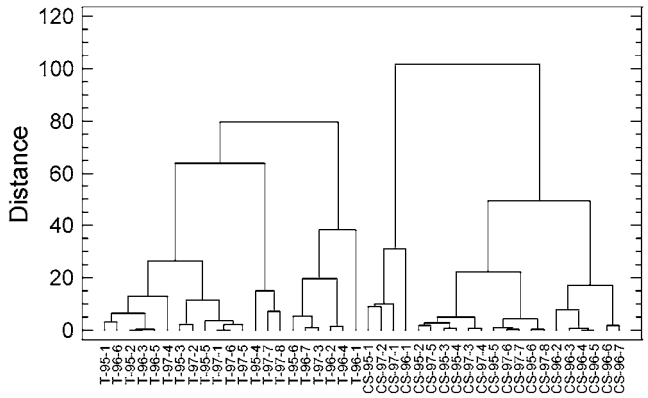


Figure 7. Dendrogram of Cabernet Sauvignon and Tempranillo grapes. For key to samples, see **Tables 1** and **2**.

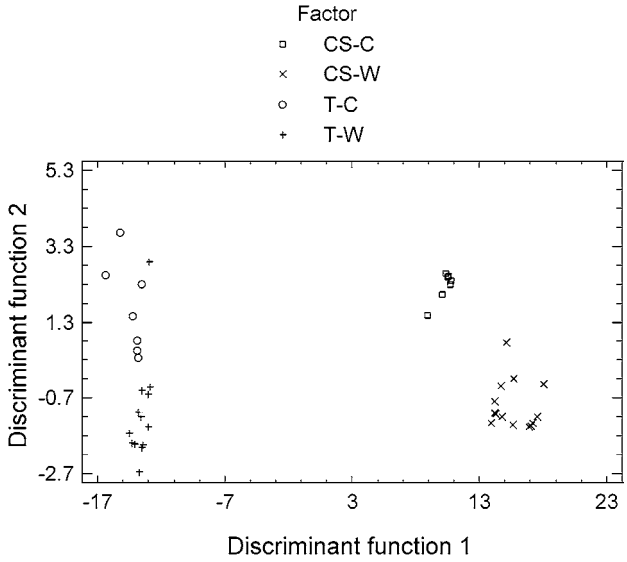


Figure 8. Plot of discriminant functions 1 and 2 in discriminant analysis of Cabernet Sauvignon (CS) and Tempranillo (T) grapes, grouped in warm years (W) and a relatively cool year (C).

are considered to be primitive anthocyanins, sharply increased after veraison. Thereafter, their concentrations remained stable and even decreased. On the other hand, the other four pigments (**4**−**7**) sharply increased after veraison, but their contents decreased after a first maximum and then increased during the late stages of ripening. As a consequence, the relative amounts of **1** and **3** usually decreased at the end of ripening, but percentages of other anthocyanins, especially **5**, usually increased during ripening. Nevertheless, each cultivar presented a distinctive anthocyanin fingerprint at any stage of grape development, as is indicated by cluster analysis. The anthocyanin fingerprint changed slightly during ripening, and PCA analysis suggested that those differences were primarily due to the different proportions presented by primitive anthocyanins (**1** and **3**) and by the acylated derivatives of **5** (**6** and **7**). Furthermore, discriminant analysis has shown that the percentages of two anthocyanins (**1** and **3**) may be significant predictors of weather conditions of the year (warm or relatively cool). All of these data suggest that the anthocyanin fingerprint of a grape cultivar grown in a given location changed slightly from year to year, probably as a consequence of the modulation of anthocyanin biosynthesis by weather conditions during ripening. Further research should be done to estimate the effect of those differences in the anthocyanin fingerprint of grapes on the color characteristics of wines.

Supporting Information Available: Dates of veraison and harvest in different years, climatic data, and results of principal component analysis (PCA). This material is available free of charge via the Internet at http://pubs.acs.org.

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