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Analysis of Grape and Wine Anthocyanins by HPLC-MS

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The development and application of valuable analytical tools suitable for the varietal authentication of premium red wines are matters of interest in order to avoid fraud. In this study, an HPLC-MS procedure has been developed using trifluoroacetic acid as an acid modifier in the mobile phase. This method may be used as a routine method using UV−vis detection and allows the simultaneous analysis of the structural features of anthocyanins by MS under the same chromatographic conditions. Twenty different anthocyanins have been detected in 19 different samples of both grape extracts and wines. Cis and trans isomers of *p*-coumaryl derivatives have been identified for the first time. Important qualitative and quantitative differences among cultivars have been detected.

KEYWORDS: Grapes; wines; anthocyanins; HPLC-MS; electrospray ionization

INTRODUCTION

Premium varietal red wines play an important role in the world wine market. This has stimulated research on analytical tools to certify their authenticity and to protect consumers against fraud, especially if the grape cultivar is mentioned on bottle labels. For this purpose, different techniques have been proposed to determine the varietal, geographical, and technological origin of a wine (*1*). The differentiation of wines in terms of their variety can be performed by analyzing various physicochemical parameters, such as proteins (*2*), color and polyphenols (*3*−*9*), amino acids and aroma compounds (*10*, *11*), or DNA analyses (*12*, *13*). The latter seemed to be the most suitable method, but it has been found that the low concentration of residual DNA in musts and wines is a limiting factor (*14*, *15*).

Analytical tools based on color and phenolic analyses seem to fit the authentication purposes. Among them, the analysis of nine anthocyanins and their ratios has been proposed for validating the identity of the grapes used during wine-making. Several research groups have evinced important differences on the anthocyanin fingerprint of red grapes (*16*−*23*). These pigments are partially transferred into wines during red wine-making, so that every varietal wine has a typical and characteristic anthocyanin profile that differs from others (*3*, *4*, *7*−*9*, *22*, *24*). This pattern can remain quite constant with time after malolactic fermentation, regardless of whether the wine is stored in stainless steel tanks or aged in oak barrels (*25*). Usually, routine analyses of anthocyanins involve spectrophotometric and chromatographic techniques. These procedures have proven to be very useful, and HPLC coupled to photodiode array (PDA) detection has become the method of choice for monitoring anthocyanic profiles. However, sometimes PDA detection is not sufficient to discriminate between compounds with similar spectroscopic characteristics. Mass spectrometry (MS) has therefore been used as a supporting technique in anthocyanin characterization (*26*−*31*).

In the literature, a number of different procedures have been described to extract anthocyanins from grapes and to analyze them in grapes and wines by HPLC (*17*, *19*, *25*, *32*−*35*). In some cases, acid solvents that may cause the partial hydrolysis of acylated anthocyanins are used during extraction, leading to analytical results that would not be valid for chemotaxonomic purposes. On the other hand, HPLC procedures that use an organic acid as acid modifier may cause the formation of analytical artifacts, such as those described when formic acid is employed (*17*). In the recent literature, structural elucidation by MS of anthocyanins is performed either after sample fractionation or using different HPLC conditions depending on whether PDA or MS analyses are carried out (*26*, *30*).

In this investigation, a method that allows the structural determination of anthocyanins of grapes and wines by MS with positive electrospray ionization (ESI) coupled to HPLC has been developed. An important objective reached with this method is that HPLC-MS analyses can be performed under the same chromatographic conditions as with HPLC-PDA routine analysis.

MATERIALS AND METHODS

Reagents and Standards. Deionized water was purified with a Milli-Q water system (Millipore, Bedford, MA) before use. Acetonitrile of HPLC gradient grade was purchased from Merck (Darmstadt, Germany). Trifluoroacetic acid of analytical reagent grade was obtained from Fluka (Buch, Switzerland). All other chemicals (analytical reagent grade) were obtained from Panreac (Mollet del Valles, Spain). Standards of several anthocyanins were prepared from fresh red grape skins as previously described in the literature (*17*).

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Table 1. Grape Cultivars and Varietal Wines Studied in 2001 and Abbreviations Used in the Tables and Text

sample	type	abbreviation
Alicante Bouschet	skin extract	ABS-g
Bobal	skin extract	BOB-g
Cariñena	skin extract	CAR-g
Crujidera	skin extract	CRU-g
Garnacha Peluda	skin extract	GAP-g
Monastrell	skin extract	MON-g
Moristel	skin extract	MOR-g
Morrastel Bouschet	skin extract	MBS-g
Petit Bouschet	skin extract	PBS-g
Prieto Picudo	skin extract	PRP-g
Tempranillo	skin extract	TEM-g
Vitadillo	skin extract	VIT-g
Alicante Bouschet	wine	ABS-w
Bobal	wine	BOB-w
Cabernet Sauvignon	wine	CBS-w
Garnacha	wine	GAR-w
Merlot	wine	MER-w
Monastrell	wine	MON-w
Tempranillo	wine	TEM-w

Samples. *Grapes*. Grape samples of 12 cultivars commonly grown in Spain for making premium red wines (**Table 1**) were collected at harvest at the Germplasm Bank (BGV) of the IMIA (Madrid, Spain) in September 2001. Each sample consisted of 10 bunches picked randomly from eight different plants. Once in the laboratory, berries were separated from clusters, and a set of 100 grapes was randomly sampled and weighed. Then, the skins of each set of berries were separated from pulp and seeds. Anthocyanin extraction was performed on skins following the procedure of Bourzeix et al. (*36*), which uses several solvents (methanol, acetone, and water) with no acid addition. Pulps and seeds were discarded. No preparation was needed prior to HPLC analysis.

Wines. Seven varietal wines (**Table 1**) were made on a microvinification scale in the experimental cellar of the IMIA. Grapes were collected at maturity from vines grown at the BGV of the IMIA, vintage 2001. A typical red wine-making procedure was performed on them. A sulfited solution ($K_2S_2O_5$, 80 mg/L) was added to crushed grapes before fermentation, which was carried out spontaneously in stainless steel tanks ranging from 50 to 100 L. After fermentation, wines were kept at 18 °C in the same tanks until their transfer into bottles after the end of malolactic fermentation. Once bottled, wines were stored at 4 °C. No preparation was needed prior to HPLC analysis.

Equipment. The HPLC-MS analyses were carried out using an HP 1100 MSD system with a PDA UV−vis detector coupled to a mass spectrometer (quadrupole analyzer) equipped with an ESI interface (Agilent Technologies). Chromatographic separation was carried out using a 250 × 4.6 mm i.d., 5 μm Nova-Pak C_{18} steel column with a 20 × 3.9 mm i.d. Sentry Nova-Pak C18 guard cartridge (Waters, Milford, MA), both thermostated at 50 °C. All samples were analyzed in duplicate.

Analytical Conditions. *HPLC*. The mobile phase was a linear gradient of water/acetonitrile (50:50) (solvent B) in water/acetonitrile (95:5) (solvent A), both adjusted to pH 1.3 with trifluoroacetic acid, at a flow rate of 0.6 mL/min. The following gradient was used: 0 min, 15% B; 0−20 min, 15−30% B; 20−25 min, 30−35% B; 25−35 min, 35−40% B; 35−42 min, 40% B; 42−43 min, 40−100% B; 43−48 min, 100% B; and 48−49 min, 100−15% B.

PDA. Spectra were recorded every second between 250 and 600 nm, with a bandwidth of 1.2 nm, and chromatograms were acquired at 520 nm.

MS Analyses. MS parameters were as follows: capillary voltage, 4000 V; fragmenter ramped from 90 to 120 V; drying gas temperature, 325 °C; gas flow (N_2), 12 mL/min. The instrument was operated in positive ion mode scanning from *m*/*z* 50 to 2000 at a scan rate of 1.47 s/cycle.

RESULTS AND DISCUSSION

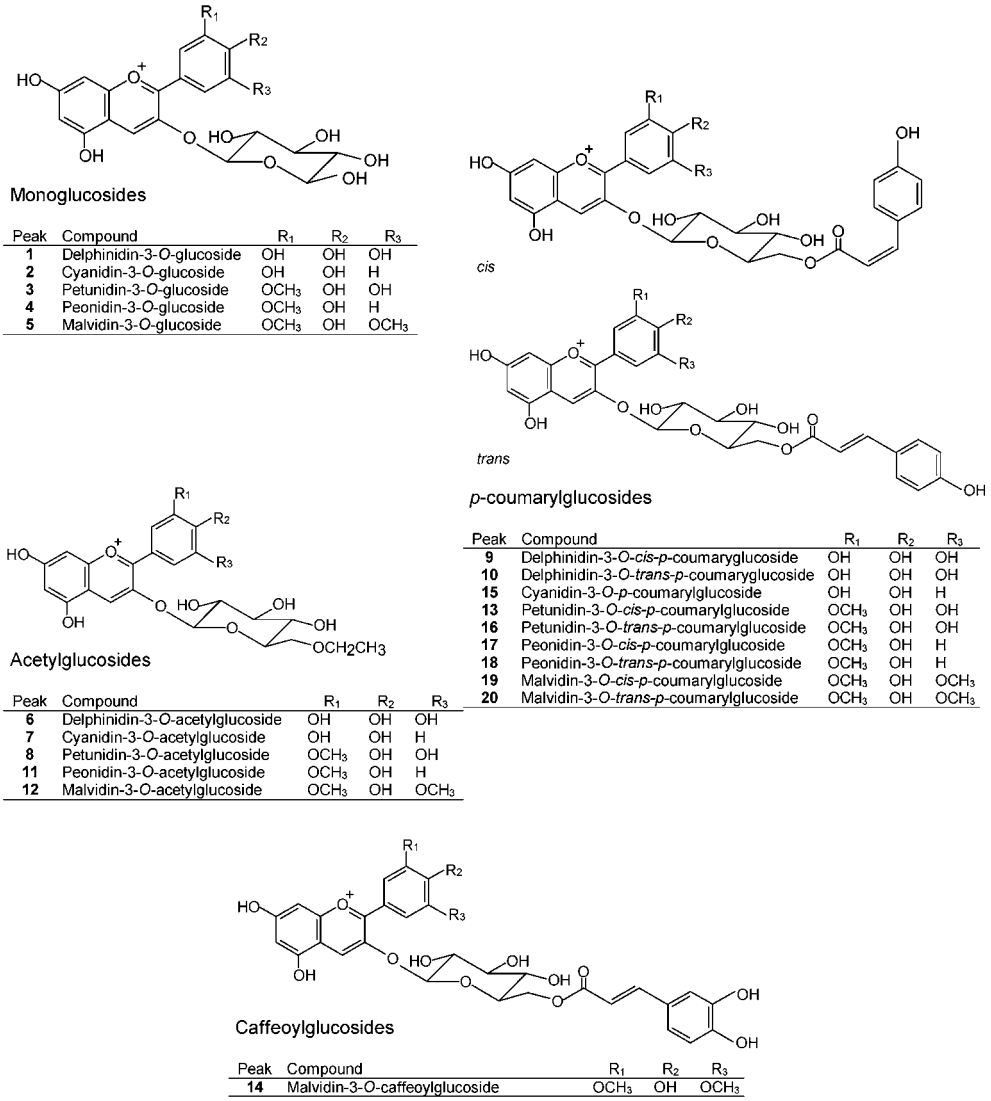
Reliability of the Method. An HPLC prodedure has been successfully applied to the analysis of anthocyanins in 19 samples of different nature (12 skin extracts and 7 wines of different cultivars). This method uses trifluoroacetic acid at very low proportion (~0.6%) as acid modifier in the mobile phase to limit the formation of ionic pairs that may decrease the detection sensibility by MS. To analyze anthocyanins by HPLC, pH values of the mobile phase must range from 1 to 2. When formic acid is used as acid modifier, high proportions of this acid (5−10%) are used to reach this pH, and it may decrease the sensibility of detection. Twenty different anthocyanins (**Figure 1**) have been successfully determined in 1 h. To evaluate the reliability of the method, the repeatabilities of the absolute retention time and relative retention index related to malvidin 3-*O*-glucoside (**5**) were calculated for several anthocyanins, considering all of the samples studied (**Table 1**); data are shown in **Table 2**. In some cases, certain anthocyanins were absent, as samples differ quantitatively and qualitatively. This was the case for peaks 17 and 18, present only in 10 and 13 samples, respectively. Anthocyanins present in 9 or fewer samples (which supposes 50% of the whole pool of samples) were not considered to evaluate the reliability of this method. This was the case for the six peaks 6−10 and 13.

It should be noted that the matrix effects of different kinds of samples have been considered, as both grape extracts and wines were analyzed. This HPLC-PDA method has been thought to be a routine analytical tool to check anthocyanin profiles of grapes and wines and to confirm the nature of these compounds

Table 2. Repeatabilities of Retention Time (t_R) and Retention Index (i_R), Relative to Malvidin 3-*O*-Glucoside, for 14 Anthocyanins Detected in Skin Extracts and Wines of Several Cultivars Studied[a]

peak	anthocyanin	*n*	$t_R \pm$ SD (min)	CV	$i_R \pm$ SD (min)	CV
1	delphinidin 3-*O*-glucoside	19	8.90 ± 0.19	2.13	0.49 ± 0.00	0.35
2	cyanidin 3-*O*-glucoside	18	11.60 ± 0.28	2.42	0.64 ± 0.01	1.14
3	petunidin 3-*O*-glucoside	19	13.01 ± 0.28	2.12	0.72 ± 0.00	0.31
4	peonidin 3-*O*-glucoside	19	16.61 ± 0.31	1.89	0.92 ± 0.00	0.15
5	malvidin 3-*O*-glucoside	19	18.11 ± 0.33	1.83	1.00	
11	peonidin 3-*O*-acetylglucoside	17	30.15 ± 0.32	1.07	1.67 ± 0.01	0.58
12	malvidin 3-*O*-acetylglucoside	19	31.45 ± 0.34	1.07	1.74 ± 0.01	0.76
14 + 15	malvidin 3-*O*-caffeoylglucoside + cyanidin 3-*O*-*p*-coumarylglucoside	15	33.54 ± 0.42	1.26	1.85 ± 0.01	0.80
16	petunidin 3-*O*-*trans*-*p*-coumarylglucoside	14	34.77 ± 0.44	1.25	1.92 ± 0.01	0.75
17	peonidin 3-*O*-*cis*-*p*-coumarylglucoside	10	36.30 ± 0.50	1.37	2.01 ± 0.02	0.95
18	malvidin 3-*O*-*cis*-*p*-coumarylglucoside	13	37.06 ± 1.27	3.44	2.04 ± 0.05	2.40
19	peonidin 3-*O*-*trans*-*p*-coumarylglucoside	19	39.92 ± 0.47	1.18	2.21 ± 0.02	0.73
20	malvidin 3-*O*-*trans*-*p*-coumarylglucoside	19	40.90 ± 0.53	1.29	2.26 ± 0.02	0.94

[a] *n*, number of samples; SD, standard deviation; CV, coefficient of variation.



Peak	Compound	R_1	R_2	R_3
1	Delphinidin-3-*O*-glucoside	OH	OH	OH
2	Cyanidin-3-*O*-glucoside	OH	OH	H
3	Petunidin-3-*O*-glucoside	OCH_3	OH	OH
4	Peonidin-3-*O*-glucoside	OCH_3	OH	H
5	Malvidin-3-*O*-glucoside	OCH_3	OH	OCH_3

Peak	Compound	R_1	R_2	R_3
9	Delphinidin-3-*O*-*cis*-*p*-coumaryglucoside	OH	OH	OH
10	Delphinidin-3-*O*-*trans*-*p*-coumaryglucoside	OH	OH	OH
15	Cyanidin-3-*O*-*p*-coumarylglucoside	OH	OH	H
13	Petunidin-3-*O*-*cis*-*p*-coumarylglucoside	OCH_3	OH	OH
16	Petunidin-3-*O*-*trans*-*p*-coumarylglucoside	OCH_3	OH	OH
17	Peonidin-3-*O*-*cis*-*p*-coumarylglucoside	OCH_3	OH	H
18	Peonidin-3-*O*-*trans*-*p*-coumarylglucoside	OCH_3	OH	H
19	Malvidin-3-*O*-*cis*-*p*-coumarylglucoside	OCH_3	OH	OCH_3
20	Malvidin-3-*O*-*trans*-*p*-coumarylglucoside	OCH_3	OH	OCH_3

Peak	Compound	R_1	R_2	R_3
6	Delphinidin-3-*O*-acetylglucoside	OH	OH	OH
7	Cyanidin-3-*O*-acetylglucoside	OH	OH	H
8	Petunidin-3-*O*-acetylglucoside	OCH_3	OH	OH
11	Peonidin-3-*O*-acetylglucoside	OCH_3	OH	H
12	Malvidin-3-*O*-acetylglucoside	OCH_3	OH	OCH_3

Peak	Compound	R_1	R_2	R_3
14	Malvidin-3-*O*-caffeoylglucoside	OCH_3	OH	OCH_3

Figure 1. Structures of the 20 anthocyanins detected in skin extracts and wines.

with a coupled mass spectrometry system. It was therefore important to test the chromatographic conditions with different cultivars and different kinds of samples. Also, the different separation properties of columns, the reproducibility of the gradient program, and the effect of room temperature (only the column was thermostated) were taken into account. **Table 2** shows that standard deviations were very low and quite similar to others mentioned in the literature (*22*, *25*). The procedure allows a good separation of all of the anthocyanins considered and may be adequate for the estimation of their relative contents in different grape extracts and wines.

HPLC-MS Identification of Anthocyanins in Grape Extracts and Wines. Figure 2 shows a chromatogram of a Bobal grape extract recorded at 520 nm. It can be seen that there are 17 peaks corresponding to 18 different anthocyanins. This is one of the most complex chromatograms obtained in this work. These compounds have been identified by their HPLC retention times, elution order, spectroscopic characteristics, and fragmentation pattern (**Table 3**). Three main groups can be clearly distinguished: the monoglucosides of five anthocyanidins (peaks 1−5); the acetylated anthocyanins (peaks 6−8, 11, and 12); and the cinnamoyl derivatives (peaks 9, 10, and 13−20).

Monoglucosides. The presence of 3-*O*-glucoside derivatives of delphinidin (**1**), cyanidin (**2**), petunidin (**3**), peonidin (**4**), and malvidin (**5**) in grape skin extracts and wines of *Vitis vinifera* L. has been confirmed. All of them show a similar fragmentation pattern. The mass spectra present two signals, the molecular ion M^+ and the fragment resulting from the loss of a glucose molecule, $M^+ - 162$ Da, corresponding to the aglycon. These five compounds are always present in grape extracts, generally in high concentrations, and in wines, with the exception of **2**, which can be absent or in low concentration (**Table 4**). Despite this, the five monoglucosides have been always considered for chemotaxonomic purposes (*7*−*9*, *18*, *19*, *21*, *22*).

Acetylglucosides. Five acetylglucoside derivatives (**6**−**12**) of delphinidin, cyanidin, petunidin, peonidin, and malvidin, respectively, have been detected. The mass spectra of these molecules show two signals, relating to the molecular ion M^+ and the fragment $M^+ - 204$ Da. The 204 Da value corresponds to the acetylglucoside moiety (gluAc), so the fragment $M^+ - 204$ Da corresponds to the related aglycon. **11** and **12** are commonly found in grapes and wines and belong to the set of nine anthocyanins considered when testing grape and wine identities. The other three acetylglucosides were present only in certain samples. **8** was detectable in nine samples and was present in trace amounts in the rest (**Table 4**). **6** and **7** were present only in certain wines (Cabernet Sauvignon, Merlot, and Monastrell) but not in the grape sample pool of this work. The presence of these minor anthocyanins has been described in *V.*

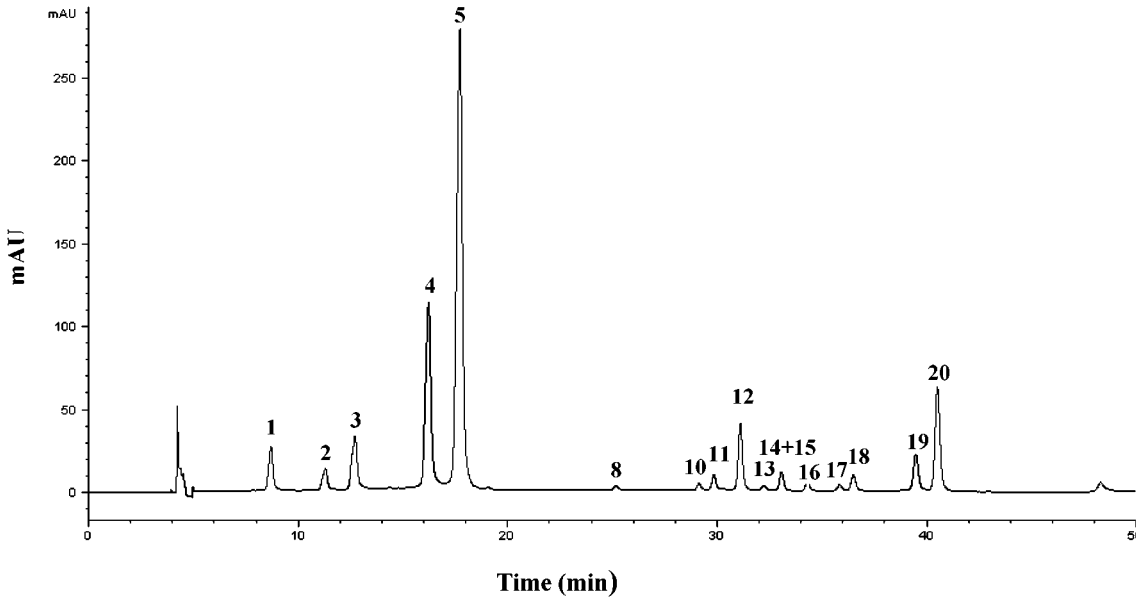


Figure 2. Chromatogram of a skin extract of Bobal grapes recorded at 520 nm. Peaks are labeled according to **Table 3**.

Table 3. Characteristics of the Anthocyanins Found in Skin Extracts and Wines of Different Cultivars Studied, Related to Their Retention Time (t_R), Spectroscopic Characteristics (λ_{max}), and Fragmentation Pattern (M^+, $M^+ - X$)[a]

peak	t_R (min)	anthocyanin	λ_{max}	M^+	$M^+ - X$
1	8.91	delphinidin 3-*O*-glucoside	522	465	303 (M^+ – glu)
2	11.60	cyanidin 3-*O*-glucoside	514	449	287 (M^+ – glu)
3	13.03	petunidin 3-*O*-glucoside	522	479	317 (M^+ – glu)
4	16.62	peonidin 3-*O*-glucoside	515	463	301 (M^+ – glu)
5	18.12	malvidin 3-*O*-glucoside	524	493	331 (M^+ – glu)
6	19.39	delphinidin 3-*O*-acetylglucoside	522	507	303 (M^+ – gluAc)
7	23.73	cyanidin 3-*O*-acetylglucoside	514	491	287 (M^+ – gluAc)
8	25.52	petunidin 3-*O*-acetylglucoside	522	521	317 (M^+ – gluAc)
9	26.07	delphinidin 3-*O*-*cis*-*p*-coumarylglucoside	312,[b] 527	611	303 (M^+ – gluCou)
10	29.67	delphinidin 3-*O*-*trans*-*p*-coumarylglucoside	312,[b] 527	611	303 (M^+ – gluCou)
11	30.13	peonidin 3-*O*-acetylglucoside	522	505	301 (M^+ – gluAc)
12	31.47	malvidin 3-*O*-acetylglucoside	527	535	331 (M^+ – gluAc)
13	32.33	petunidin 3-*O*-*cis*-*p*-coumarylglucoside	313,[b] 532	625	317 (M^+ – gluCou)
14	33.55	malvidin 3-*O*-caffeoylglucoside	308,[b] 522	655	331 (M^+ – gluCf)
15	33.55	cyanidin 3-*O*-*p*-coumarylglucoside	308,[b] 522	595	287 (M^+ – gluCou)
16	34.78	petunidin 3-*O*-*trans*-*p*-coumarylglucoside	313,[b] 532	625	317 (M^+ – gluCou)
17	36.26	peonidin 3-*O*-*cis*-*p*-coumarylglucoside	311,[b] 522	609	301 (M^+ – gluCou)
18	37.06	malvidin 3-*O*-*cis*-*p*-coumarylglucoside	317,[b] 532	609	331 (M^+ – gluCou)
19	39.94	peonidin 3-*O*-*trans*-*p*-coumarylglucoside	311,[b] 522	639	301 (M^+ – gluCou)
20	40.93	malvidin 3-*O*-*trans*-*p*-coumarylglucoside	317,[b] 532	639	331 (M^+ – gluCou)

[a] M^+, molecular ion; glu, glucoside; gluAc, acetylglucoside derivative; gluCou, *p*-coumarylglucoside derivative; gluCf, caffeoylglucoside derivative. [b] Characteristic shoulder of cinnamoyl derivatives.

vinifera cv. Colorino (*26*), hybrid grapes (*30*), and Cabernet Sauvignon wines (*37*), although in very low concentrations.

Cinnamoyl Derivatives. Ten different anthocyanins having UV−vis spectra corresponding to cinnamoyl derivatives (*p*-coumarylglucosides and caffeoylglucosides) were detected. Their spectra are characteristic and significantly different from others, as they present a shoulder between 310 and 320 nm. These compounds have been identified as *p*-coumarylglucosides of delphinidin (**9** and **10**), cyanidin (**15**), petunidin (**13** and **16**), peonidin (**17** and **19**), and malvidin (**18** and **20**) and caffeoylglucoside of malvidin (**14**). The mass spectra of the *p*-coumaryl derivatives showed two signals, corresponding to the molecular ion M^+ and the fragment $M^+ - 308$ Da. The 308 Da value belongs to the *p*-coumarylglucoside moiety (gluCou), so the fragment $M^+ - 308$ Da corresponds to the related aglycon. On the other hand, the caffeoylglucoside of malvidin also showed two signals, relating to the molecular ion M^+ and the fragment $M^+ - 324$ Da. The 324 Da value belongs to the caffeoylglucoside moiety (gluCf), so the fragment $M^+ - 324$ Da corresponds to the related aglycon.

It can be seen that two compounds, each corresponding to delphinidin, petunidin, peonidin, and malvidin *p*-coumaryl derivatives, have been identified. In every pair, both compounds (**9** and **10**, **13** and **16**, **17** and **19**, and **18** and **20)** match the same identity, as they showed equivalent UV−vis spectra and fragmentation patterns (**Figure 3A**,**B**). Thus, it must be inferred that those molecules are cis and trans isomers. To our knowledge, these isomers have not been described in the literature related to anthocyanins in grapes and wines, but, in a recent paper, cis and trans isomers of *p*-coumaric and *p*-coutaric acids have been detected in wines by RP-HPLC analyses (*38*). In that study, cis isomers always eluted first and were present in lower proportions than the trans isomers. We have assumed this pattern of elution and assigned the cis and trans labels to the first and second compounds of each couple, respectively. Following this criterion, cis isomers were always in lower

Table 4. Relative Amounts (Percent) for Anthocyanins Found in Skin Extracts and Wines

peak	anthocyanin	ABS-g	BOB-g	CAR-g	CRU-g	GAP-g	MON-g	MOR-g	MBS-g	PBS-g	PRP-g	TEM-g	VIT-g	ABS-w	BOB-w	CBS-w	GAR-w	MER-w	MON-w	TEM-w
1	delphinidin 3-*O*-glucoside	1.0	3.8	11.1	1.6	1.7	6.2	2.8	0.9	0.8	4.5	11.2	2.9	3.2	5.8	4.0	0.9	6.2	5.8	9.7
2	cyanidin 3-*O*-glucoside	1.4	2.1	4.1	3.9	4.0	9.8	2.6	1.2	1.7	3.4	5.6	1.9	0.7	0.9	0.4	nd[a]	0.8	3.0	0.4
3	petunidin 3-*O*-glucoside	2.1	5.0	10.7	4.0	4.8	8.1	5.2	0.7	1.4	6.7	9.9	4.7	4.7	8.0	4.8	2.0	7.9	11.4	12.7
4	peonidin 3-*O*-glucoside	40.0	18.1	11.4	35.9	33.6	13.4	29.2	55.4	59.7	10.4	11.7	7.3	21.0	12.6	2.2	15.7	8.2	12.2	6.5
5	malvidin 3-*O*-glucoside	30.1	46.5	40.5	39.5	42.8	41.5	40.0	26.4	24.4	35.8	38.7	41.7	55.6	59.2	50.7	72.0	46.6	55.3	55.3
6	delphinidin 3-*O*-acetylglucoside	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.0	nd	1.8	nd	nd
7	cyanidin 3-*O*-acetylglucoside	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.5	nd	0.9	0.6	nd
8	petunidin 3-*O*-acetylglucoside	nd	0.3	0.4	nd	nd	0.7	nd	nd	nd	0.6	0.6	nd	nd	0.6	1.2	nd	1.9	0.6	nd
9	delphinidin 3-*O*-*cis*-*p*-coumarylglucoside	nd	nd	0.3	nd	nd	nd	nd	nd	nd	nd	0.3	nd	nd	nd	nd	nd	nd	nd	nd
10	delphinidin 3-*O*-*trans*-*p*-coumarylglucoside	nd	0.7	1.9	nd	nd	1.1	nd	nd	nd	1.8	1.9	1.2	nd	nd	nd	nd	nd	nd	1.4
11	peonidin 3-*O*-acetylglucoside	1.2	1.1	0.3	0.6	0.6	nd	1.2	1.0	0.8	1.3	0.5	0.6	1.7	1.3	1.5	nd	3.2	0.9	0.4
12	malvidin 3-*O*-acetylglucoside	2.6	5.2	2.5	1.8	1.4	3.7	3.2	2.0	1.3	8.8	3.0	5.9	3.9	4.9	25.4	2.6	13.3	3.0	3.9
13	petunidin 3-*O*-*cis*-*p*-coumarylglucoside	nd	0.5	nd	0.4	nd	nd	0.4	0.2	0.5	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
14 + 15	malvidin 3-*O*-caffeoylglucoside + cyanidin 3-*O*-*p*-coumarylglucoside	1.0	1.7	1.3	0.9	0.9	3.4	1.2	0.9	0.6	3.0	1.4	2.5	nd	nd	0.3	nd	0.5	1.2	nd
16	petunidin 3-*O*-*trans*-*p*-coumarylglucoside	0.8	0.7	1.9	0.4	0.4	2.1	0.6	nd	nd	3.0	1.9	1.8	nd	nd	0.3	nd	0.6	0.7	0.9
17	peonidin 3-*O*-*cis*-*p*-coumarylglucoside	0.7	0.6	0.3	0.9	0.6	nd	0.7	0.4	0.8	nd	0.2	1.1	nd	nd	nd	nd	nd	nd	nd
18	malvidin 3-*O*-*cis*-*p*-coumarylglucoside	0.6	1.4	1.3	1.3	0.9	1.1	1.3	0.6	0.8	0.5	1.2	4.3	nd	nd	0.4	nd	nd	nd	nd
19	peonidin 3-*O*-*trans*-*p*-coumarylglucoside	8.4	3.1	2.1	3.6	3.3	1.9	4.3	4.1	3.7	4.0	1.9	4.1	3.6	1.8	0.7	1.7	2.0	1.4	1.4
20	malvidin 3-*O*-*trans*-*p*-coumarylglucoside	10.2	9.3	9.7	5.3	5.0	7.3	7.4	6.3	3.5	16.2	9.9	20.1	5.4	5.0	6.5	5.0	6.2	3.9	7.3

[a] Not detected.

Figure 3. (A) Mass spectrum of malvidin 3-*O*-*cis*-*p*-coumarylglucoside, recorded at 36.5 min. (B) Mass spectrum of malvidin 3-*O*-*trans*-*p*-coumarylglucoside recorded at 40.5 min. (C) Mass spectrum of malvidin 3-*O*-caffeoylglucoside and cyanidin 3-*O*-coumarylglucoside recorded at 33.0 min. The three mass spectra were extracted from skin extracts of Moristel grapes.

proportions than trans ones in all of the samples analyzed in this paper. In our research, some of these compounds were detected in both grape extracts and wines, although their concentrations were always greater in the former. This fact can be easily explained as the anthocyanin extraction during alcoholic fermentation is not complete and the *p*-coumaryl derivatives extraction rate is very low, as previously reported (*22*). Wines were directly injected in the chromatograph with no preparation prior to analysis, so the presence of these compounds cannot be due to isomerization processes during sample preparation or analysis.

Another observation is the coelution of compounds **14** and **15** at ~33.5 min. The UV−vis spectrum did not reveal the presence of two compounds but the MS analysis showed two molecular ions M^+ at *m*/*z* 595 and 655 and two fragments $M^+ - X$ at *m*/*z* 287, corresponding to cyanindin, and *m*/*z* 331, corresponding to malvidin (**Figure 3C**). From this fragmentation pattern, there is only one possibility for their identification. An M^+ of 595 Da matches with $M^+ - X$ of 287 Da, and the

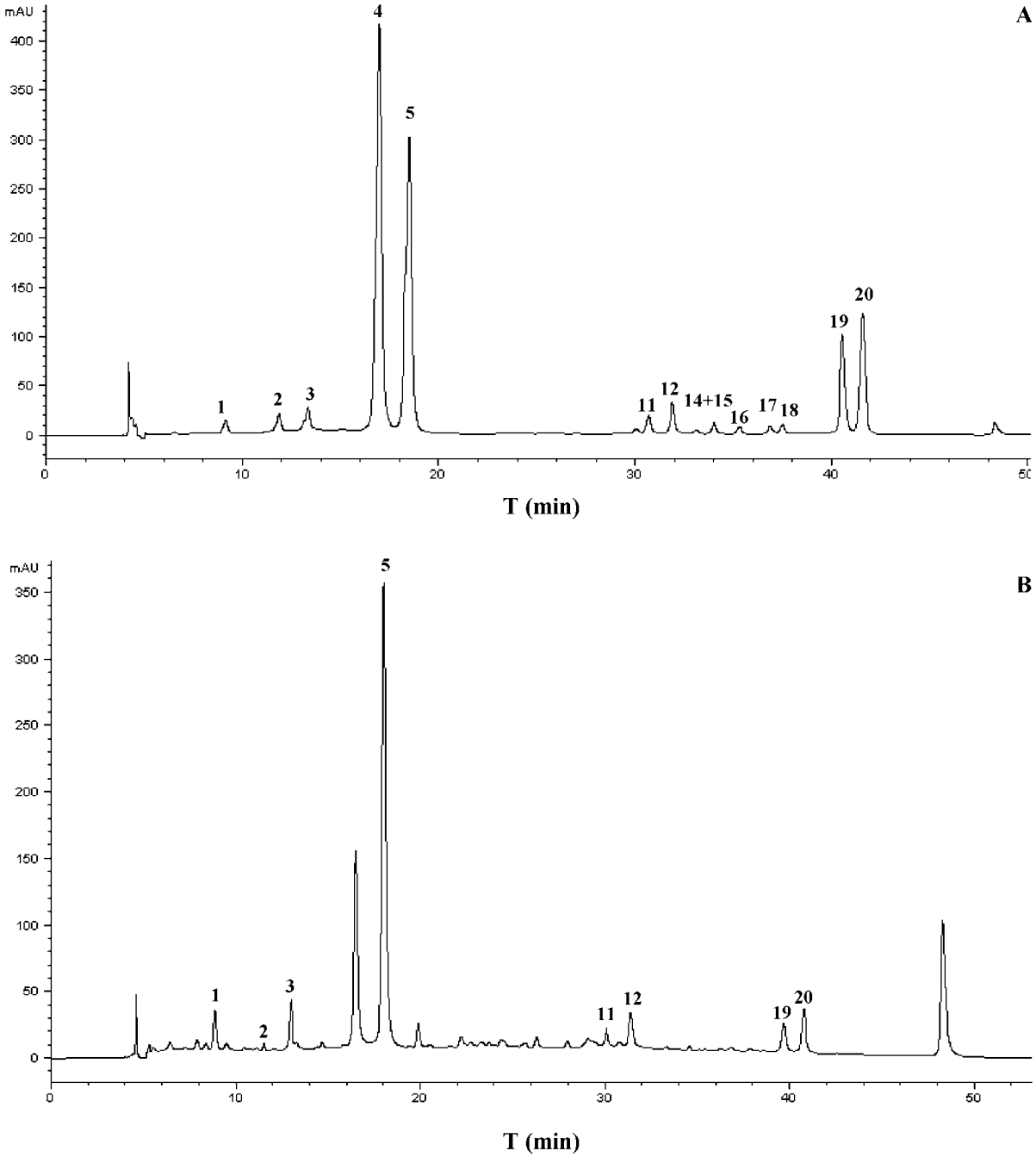


Figure 4. Comparison between chromatograms of skin extract (A) and wine (B) of Alicante Bouschet.

difference between both molecules corresponds to 308 Da, corresponding to a *p*-coumarylglucoside moiety. This compound is therefore identified as cyanidin 3-*O*-*p*-coumaryglucoside (**15**). Similarly, an M^+ of 655 Da matches with $M^+ - X$ of 331 Da, and the difference between both ions corresponds to 324 Da, a caffeoylglucoside moiety. The compound is therefore identified as malvidin 3-*O*-caffeolylglucoside (**14**). This confirms the great importance of HPLC-MS analysis of anthocyanins when taxonomic purposes are considered.

HPLC Anthocyanin Profile of Grape Skins and Wines. Table 4 shows the percentages of the 20 anthocyanins identified by HPLC-MS analyses in the whole samples. Remarkable qualitative and quantitative differences can be noticed among grapes of different cultivars, among wines of different cultivars, and between grapes and wines of the same cultivar. Grape samples were collected on an Ampelographic collection. Only a small number of plants of each cultivar (eight) were available for this study, and only one varietal wine could be done. Thus, no statistical analysis can be performed on our results.

Anthocyanin Fingerprint of Grape Extracts. Eighteen different anthocyanins were found in the skin extracts of the 12 cultivars studied. Only two acetylglucosides (**6** and **7**) were lacking in them. Grapes of each cultivar presented a distinctive fingerprint, as reported previously (*18*, *19*, *21*−*23*).

In most cases, **5** was the major anthocyanin, and its relative content ranged from 24.4% (Petit Bouschet) to 46.5% (Bobal). Nevertheless, **4** was the major anthocyanin in three cultivars, Alicante Bouschet, Morrastel Bouschet, and Petit Bouschet, revealing the teinturier nature of these three cultivars (*16*). As is well-known, the teinturier cultivars contain this pigment not only in skin cells but also in pulp, having significant amounts of this compund. Other cultivars (Crujidera, Garnacha Peluda,

and Moristel) contained a remarkable amount of **4**, up to 35.9%, but always lower than the amount of **5**. Only one cultivar (Prieto Picudo) had a high proportion of **12**, ~9%, and the rest of the samples contained amounts of this compound ranging from 1 to 5%. The content of **20** also presented a remarkable variability among the cultivars studied, with proportions ranging from 3.5 to 20.1%, being highest in Vitadillo and lowest in Petit Bouschet. The relative contents of **11** were very low in all cases (always <1%), but this was not the case for **19**, for which values varied between 1.9% (Tempranillo and Monastrell) and 8.4% (Alicante Bouschet). Teinturier cultivars have greater proportions of this pigment than nonteinturier grapes. The other anthocyanins identified (**8**−**10** and **13**−**18**) represented, as a whole, <10% of the total anthocyanin content. Therefore, their importance is not quantitative but qualitative, as there are cultivars with more complex anthocyanin fingerprints than others.

Anthocyanin Fingerprint of Wines. **Figure 4** shows the comparison between skin extract and wine chromatograms of the same cultivar (Alicante Bouschet). It can be seen that the anthocyanin patterns are clearly different. In wines, the most remarkable features related to the five monoglucosides are the predominance of **5** and the low quantities of **2**. The former is always the major anthocyanin, and the latter is always in trace amounts, or even lacking. The most important acylated pigments are two acetylglucosides (**11** and **12**) and two *trans*-*p*-coumarylglucosides (**19** and **20**), as described in the literature (*8*, *9*). The other minor anthocyanins are normally absent, with the exceptions of **6** and **7**, found only in Cabernet Sauvignon, Merlot, and Monastrell wines, but absent in all skin extracts analyzed. As a rule, wines contain higher contents of **5** than grapes, whereas all of the other anthocyanins are in lower proportion or absent (*22*).

Some authors consider that anthocyanin fingerprints of varietal wines can be used as an analytical tool to certify their authenticity (*8*, *9*). Certification purposes need extensive databases of varietal wines from different geographical and enological origins. The HPLC-MS method described here allows the successful identification of anthocyanins and may be of choice for these goals. Nevertheless, further research must be done, analyzing grapes and wines from different cultivars, geographical origins, and years.

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