Comparison of Several Procedures Used for the Extraction of Anthocyanins from Red Grapes

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Several procedures for the extraction of grape anthocyanins, based on different methods described in the literature, have been assayed. Results have shown that the use of solvents containing up to 1% of 12 N hydrochloric acid for the extraction of grape anthocyanins resulted in the partial hydrolysis of malvidin 3-*O*-acetylglucoside, leading to important changes in the relative content of anthocyanins in those extracts, despite their higher efficiency in extracting total anthocyans in some cases. Nevertheless, the use of different neutral solvents resulted in a similar relative content of anthocyanins in the extracts, and some of them could be as efficient in extracting total anthocyanins as other procedures that used an acid solvent for extraction. These data suggest that the use of solvents containing up to 1% of 12 N hydrochloric acid in the extraction of anthocyanins from grapes or from any other plant material containing acetylated anthocyanins should be avoided, especially if the work is related with any type of taxonomic studies.

Keywords: *Anthocyanins; acylated anthocyanins; grapes; extraction; HPLC*

INTRODUCTION

Anthocyanins are a group of naturally occurring phenolic compounds that are related with the color of several aerial and subterranean organs in many plants (Mazza and Miniatti, 1994). In grapevines (*Vitis vinifera* L.) anthocyanins are accumulated in leaves during senescence and are responsible for the coloration of grape skins in red and rosé cultivars, and in some grape cultivars, named teinturier or redjuice dyer cultivars, these pigments are also accumulated in the grape pulp (Boulton et al., 1995). The accumulation of anthocyanins in grapes begins after the phenological stage known as veraison and is affected by weather conditions, especially light intensity and temperature (Kliewer, 1977), which limit the cultivation of red grape cultivars in regions too cool or too warm during grape maturation. When grapes are harvested, the content of anthocyanins in grapes may be as high as 1000 mg/kg of grapes and even more (Boulton et al., 1995), especially when sugar content in grapes rises to 22%. In red grape cultivars used for wine-making, the amount of anthocyanins present in grapes when harvested may be important in conducting the fermentation and the postfermentation processes in an adequate way to obtain high-quality products. For this reason, several approaches have been taken to understand what changes occur in the content of anthocyanins from veraison to the harvest in several grape cultivars used for wine-making (Piergiovanni and Volonterio, 1983; Darné, 1988; González-San José et al., 1990; Cacho et al., 1992; Fernández-López et al., 1992), showing that the amount of some anthocyanins may decrease in the later stages of the maturation process.

Anthocyanins are unique molecules among plant phenolics because they can be present in plant tissues as different chemical species. At low pH, they are predominantly present in the form flavylium cation, giving a reddish color in aqueous solutions. At higher pH, the flavylium cation is converted into other species, some of them being uncolored and, under certain conditions, that conversion can be virtually irreversible (Cheminat and Brouillard, 1986). The flavylium cation form is red and stable at a highly acid medium, and this chemical feature of anthocyanins has probably led to a worldwide use of solvents containing mineral or organic acids for the extraction of anthocyanins from plant organs (Ribéreau-Gayon, 1972; Macheix et al., 1990). Nevertheless, Anderson et al. (1970), who identified acetic acid as an acylating agent of anthocyanin pigments in grapes, reported that facile hydrolysis of anthocyanin acetates occurs upon the exposure to trace quantities of mineral acid during the extraction processes, and some authors working on chemotaxonomic studies have reported that the extraction of some acylated anthocyanins under acid conditions may cause their partial or total hydrolysis (Van Sumere et al., 1985; Van Wyck and Winter, 1994). Moreover, Bakker and Timberlake (1985) have shown that artifacts can be obtained by using methanol containing 2% formic acid as the extraction solvent. Despite these reports, several authors have continued to use extraction solvents containing formic acid or hydrochloric acid for studying anthocyanins in grapes (Hebrero et al., 1988; González-San José et al., 1990; Cacho et al., 1992; Fernández-López et al., 1992; Gao and Cahoon, 1995), and some of these methods are also highly time-consuming. Moreover, the use of acid solvents for the extraction of anthocyanins may produce the generation of anthocyanidins from flavanols and proanthocyanidins (Hemingway, 1989), which are present in grape skins to some extent (Bourzeix et al., 1986), and this reaction may result in an overestimation of the total anthocyan content if a colorimetric method is used for this purpose. All of these facts could limit the potential use of those

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Table 1. Some Characteristics of Extraction Procedures Carried out with Skins of Fresh Grapes

procedure	solvent(s)	vol (mL)	length of extraction (h)	extraction temp (°C)	no. of grapes
11	methanol	60	16	−25	100
	80% methanol	60	4	25	
12	methanol	60	16	−25	100
	80% methanol	60	4	room	
	50% methanol	60	4	room	
	deionized water	60	16	−25	
	75% acetone	60	1	room	
13	methanol	60	4	25	100
	methanol	60	12	25	
	methanol	60	4	25	
14	methanol/12 N HCl (99:1)	60	4	25	100
	methanol/12 N HCl (99:1)	60	12	25	
	methanol/12 N HCl (99:1)	60	4	25	
15	methanol/12 N HCl (99:1)	10 (9 times)	1 (9 times)	25	50
16	methanol	10 (9 times)	1 (9 times)	25	50
17	methanol/12 N HCl (98:2)	250 (once)	24 (once)	4	100
	methanol/12 N HCl (98:2)	100 (4 times)	24 (4 times)	4	

Table 2. Some Characteristics of Extraction Procedures Carried out with Entire Fresh Grapes

procedure	solvent(s)	vol (mL)	length of extraction (h)	extraction temp (°C)	no. of grapes
21	methanol	60	16	−25	100
	80% methanol	60	4	25	
22	methanol	60	16	−25	100
	80% methanol	60	4	room	
	50% methanol	60	4	room	
	deionized water	60	16	−25	
	75% acetone	60	1	room	
23	methanol	60	4	25	100
	methanol	60	12	25	
	methanol	60	4	25	
24	methanol/12 N HCl (99:1)	60	4	25	100
	methanol/12 N HCl (99:1)	60	12	25	
	methanol/12 N HCl (99:1)	60	4	25	
27	methanol/12 N HCl (98:2)	250 (once)	24 (once)	4	100
	methanol/12 N HCl (98:2)	100 (4 times)	24 (4 times)	4	

procedures for routine analysis of anthocyans in grapevine production.

The aim of our research has been to develop a rapid method, avoiding the use of acid extractions solvents, for the extraction of anthocyanins from grapes that could be used as a routine analysis for enhancing grapevine production. To achieve this purpose, several extraction procedures with different type of solvents (acid or neutral) have been compared using the same set of grapes, and their efficiency in the extraction of anthocyanins has been estimated by colorimetric and chromatographic methods.

EXPERIMENTAL PROCEDURES

Grape Samples. One hundred clusters (~10 kg) of Cabernet Sauvignon grapes were collected in October 1996 from 100 different vines, trained in Guyot double cordon, in a vineyard located in Escalona (province of Toledo, Spain), which has been surveyed by our group for several years. The clusters were collected in plastic bags and were transported to the laboratory at 4 °C. Once there, the grapes were separated from the clusters. Thirty sets of 100 grapes and six sets of 50 grapes were randomly selected and weighed.

Extraction Procedures. Inmediately after grape sets were randomly selected and weighed, all of them were processed by carrying out several extraction procedures consisting of two or more sequential steps, the characteristics of which are displayed in Tables 1 and 2. Each extraction procedure has been identified by a two-digit number; the first digit refers to the type of material to be extracted (skins of fresh grapes or entire fresh grapes, designated 1 and 2, respectively), and the second digit refers to the characteristics of the extraction process. Thus, the procedures 11 and 16 involved the extraction of fresh grape skins; and procedures 13 and 23 were carried out under the same conditions, but using skins of fresh grapes and entire fresh grapes, respectively. Some of the procedures used are based on methods for the extraction of phenolic compounds described in the literature. Procedures 12 and 22 are based on the method developed by Bourzeix et al. (1986) for extracting phenolic compounds from grape skins and grape seeds; procedures 17 and 27 are based on the method described by Hebrero et al. (1988) for the anthocyanin extraction from grape skins, and procedure 15 is based on the method proposed by Cacho et al. (1992) for extracting anthocyanins from grape skins. As can be noted, each procedure involves two or more successive extractions, and all of them include, as a first step, the inmersion of the sample in pure methanol (which contains an adequate proportion of 12 N hydrochloric acid if necessary), followed by grinding in a Kinematica PCU-2 blender for 1 min. When each extraction step was finished, the liquid extract was separated by centrifugation at 3500 rpm and 15 °C for 20 min in a Sorvall RC-5B refrigerated centrifuge, and the residue was submitted to extraction again. For each sample, all of the liquid extracts were combined, and their volume was raised to between 200 and 650 mL with methanol. The extracts were then stored at 4 °C prior to their analysis. Each extraction procedure was carried out in triplicate.

Colorimetric Analysis. Total phenolics were measured in the extracts according to the Folin−Ciocalteu method, following the procedure proposed by Singleton and Rossi (1965), and were expressed as gallic acid equivalents. Total anthocyans, expressed as malvidin 3-*O*-glucoside equivalents, were determined using the procedure proposed by Niketic-Aleksic and Hrazdina (1972). All of these measurements were carried out in a Turner 690 UV−vis spectrophotometer.

HPLC Analysis. Several anthocyanins present in the extracts were separated and determined by HPLC. Routine analyses were performed in a chromatograph that consisted of Waters M-510 and M-501 solvent delivery systems, a Waters 680 gradient controller, a Rheodyne 7725 injection valve furnished with a 20 μL loop, a Linear UVIS 200 variable wavelength UV−vis detector set at 520 nm with a sensitivity of 0.01 AUFS, and a Spectra-Physics SP-4290 integrator. In some cases, detection was carried out with a Waters 996 photodiode array detector, and data were acquired and processed in a Waters Millenium workstation, vs 2.15.01, the spectra being registered each second between 250 and 600 nm, with a bandwidth of 1.2 nm. The chromatographic separation was performed on a Waters Nova-Pak C18 cartridge, 150 mm × 3.9 mm, using a Waters Sentry Nova-Pak C18 guard column, 20 mm × 39 mm, both thermostated in a water bath at 32 °C. The mobile phase was a linear gradient of water/acetonitrile (60:40) adjusted at pH 1.2 with perchloric acid (solvent B) in deionized distilled water adjusted at pH 1.2 with perchloric acid (solvent A), at a flow rate of 1.5 mL/min, as it is shown in

Table 3. Linear Gradient Used for the Separation of Anthocyanins by HPLC

time (min)	solvent A (%)	solvent B (%)	time (min)	solvent A (%)	solvent B (%)
0	90	10	54	67	33
5	90	10	55	0	100
25	80	20	58	0	100
45	70	30	59	90	10

Table 4. Extraction of Total Anthocyans and Total Phenolics from Skins of Fresh Grapes[a]

extraction procedure	total anthocyans (mg/100 g of grapes)	total phenolics (mg/100 g of grapes)
11	79.7 ± 3.7a	182 ± 10b
12	82.8 ± 2.1ab	248 ± 2c
13	88.3 ± 3.1ab	173 ± 8b
14	94.0 ± 7.4b	243 ± 14c
15	91.3 ± 5.6ab	194 ± 15b
16	86.6 ± 4.4ab	111 ± 3a
17	113.9 ± 1.8c	267 ± 10c

[a] Data are mean values for three replications. Values in the same column followed by a common letter are not significantly different ($p < 0.01$).

Table 3. Under these conditions, which are quite close to those described by Bakker and Timberlake (1985), acylated anthocyanins are reasonably stable. The different anthocyanins that have been analyzed were identified on the basis of their retention times and UV−vis spectra by comparison with standards isolated from grapes, as indicated elsewhere. The quantitation of the different substances was carried out in the chromatograms registered at 520 nm by an external standard procedure, using a series of solutions of malvidin 3-*O*-glucoside isolated from grapes. Results are expressed as malvidin 3-*O*-glucoside equivalents.

Statistical Analysis. Statistical analyses of multiple treatment effects were conducted by using one-way analysis of variance with comparison of means made by Duncan's multiple-range test at the 1% level of significance (Duncan, 1957).

RESULTS AND DISCUSSION

Total Phenolics and Total Anthocyans. Table 4 displays the data corresponding to the levels of total phenolics and total anthocyanins in the different extraction procedures carried out with skins of fresh grapes. As can be noted, the extraction of total phenolics clearly differs in some cases, and two factors seem to play an important role: the total time employed in the extraction and the use of solvents containing hydrochloric acid. Thus, procedure 16, which involves the use of methanol for 9 h, led to the lowest level of extraction of phenolics among the seven procedures used ($p < 0.01$). On the other hand, procedure 17, which used methanol containing 1% of 12 N HCl for a very long time (120 h), led to the highest extraction of phenolics among the seven procedures assayed, but procedures 12 and 14 were as efficient in extracting phenolics as procedure 17 ($p < 0.01$), despite the use of neutral solvents for extraction for the long time (procedure 12) or the relatively short time (20 h) skins were in contact with an acid extraction solvent (procedure 14). The dramatic effect of acidity on the extraction of phenolics is clearly shown by the results corresponding to procedures 15 and 16, which differ only by the presence of hydrochloric acid in the extraction solvent used in procedure 15, and it is a likely consequence of the association of phenolic compounds with cell wall polymers, although the presence of an acid in solvent should increase the rate of extraction of those substances.

Table 5. Extraction of Total Anthocyans and Total Phenolics from Entire Fresh Grapes[a]

extraction procedure	total anthocyans (mg/100 g of grapes)	total phenolics (mg/100 g of grapes)
21	83.5 ± 2.1b	311 ± 33a
22	70.8 ± 1.9a	430 ± 36b
23	86.1 ± 5.5b	357 ± 7a
24	81.1 ± 2.6ab	510 ± 26c
27	110.9 ± 6.4c	561 ± 7c

[a] Data are mean values for three replications. Values in the same column followed by a common letter are not significantly different ($p < 0.01$).

The effects of different factors in the extraction of anthocyans in experiments carried out with skins of fresh grapes were less evident than they were for total phenolics, but the acidity of solvents used for the extraction and the length of the extraction may play a relevant role in the extraction of those substances. Thus, procedure 17, which used methanol containing 2% of 12 N HCl as solvent, led to a significantly higher extraction of anthocyans than any other procedure ($p < 0.01$), and procedure 14, which used methanol containing 1% of 12 N HCl as solvent for 20 h, was more efficient ($p < 0.01$) than procedure 11, which used neutral solvents for 20 h. It is very probable that the higher efficiency of procedure 17 was a consequence of the much longer extraction time associated with it compared to others, but it could be hypothesized that the higher efficiency of acid solvents for extracting anthocyans could partially be explained because flavanols and proanthocyanidins, which are present in grape skins to some extent (Bourzeix et al., 1986), generate anthocyanidins in acid media (Hemingway, 1989). Nevertheless, this hypothesis needs further work to be confirmed.

Table 5 shows the data corresponding to the levels of total phenolics and total anthocyanins in the different experiments carried out with entire fresh grapes. Results obtained for total phenolics were higher than those obtained for assays carried out with skins of fresh grapes (Table 3), as grape seeds contain a very large amount of phenolics (Bourzeix et al., 1986), but the effects of two factors (acidity of solvents and total extraction time) on the extraction of total phenolics from entire fresh grapes were quite similar to those observed in the extraction assays carried out with skins of fresh grapes. It can be seen that the levels of total phenolics were significantly higher ($p < 0.01$) in the extracts corresponding to experiments carried out with acid solvents (procedures 24 and 27) than in the extracts obtained with neutral solvents (procedures 21−23), and this effect should be explained as mentioned above. However, the effects of different factors on the extraction of anthocyans were less evident than they were for total phenolics: procedure 27, which involved the use of an acid solvent for 120 h, apparently led to the highest extraction ($p < 0.01$) among the five procedures used, but the efficiency of procedures 21, 23, and 24, which lasted 20 h, was similar for extracting anthocyans ($p < 0.01$), despite the use of neutral solvents (procedures 21 and 23) or of a solvent containing hydrochloric acid (procedure 24). Finally, procedure 22 led to the extraction of a very low amount of anthocyans. As has been mentioned above, the apparently higher efficiency of procedure 27 for extracting anthocyans may be a consequence of the much longer extraction time associated with it compared to the others, but the possible

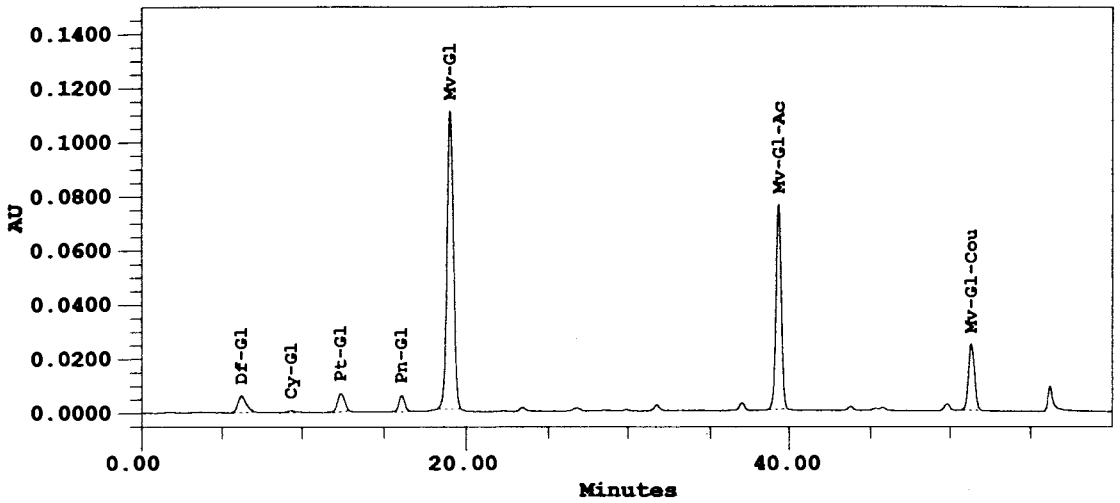

Figure 1. Chromatogram registered at 520 nm corresponding to an extract obtained from skins of fresh grapes, following procedure 11. For key to substances, see text.

Table 6. Extraction of Several Anthocyanins from Skins of Fresh Grapes[a]

	extraction procedure						
	11	12	13	14	15	16	17
Df-Gl	4.2 ± 0.5a	4.4 ± 0.4a	4.9 ± 0.5ab	5.6 ± 0.5b	5.8 ± 0.1b	4.3 ± 0.5a	5.6 ± 0.5b
Cy-Gl	0.3 ± 0.1a	0.3 ± 0.1ab	0.4 ± 0.1abc	0.5 ± 0.0c	0.5 ± 0.1bc	0.4 ± 0.1abc	0.5 ± 0.1c
Pt-Gl	3.2 ± 0.3a	3.4 ± 0.2ab	3.6 ± 0.3abc	4.4 ± 0.4bcd	4.6 ± 0.2cd	3.4 ± 0.4ab	5.0 ± 0.4d
Pn-Gl	2.2 ± 0.2a	2.3 ± 0.1a	2.4 ± 0.3a	3.2 ± 0.1b	3.4 ± 0.2bc	2.3 ± 0.3a	3.8 ± 0.2c
Mv-Gl	37.9 ± 2.7a	43.3 ± 2.0a	42.0 ± 1.2a	59.2 ± 4.7b	65.8 ± 2.0b	41.2 ± 3.8a	77.8 ± 2.2c
Mv-Gl-Ac	23.1 ± 2.2d	25.5 ± 0.3e	25.7 ± 1.1e	10.2 ± 0.6c	3.9 ± 0.1b	25.3 ± 2.1de	0.5 ± 0.1a
Mv-Gl-Cou	8.1 ± 0.7ab	9.1 ± 0.2bc	8.6 ± 0.4ab	10.1 ± 0.8cd	10.6 ± 0.1d	7.4 ± 0.8a	10.4 ± 0.3d

[a] Results are expressed as mg of Mv-Gl equivalents/100 g of grapes. Data are mean values for three replications. Values in the same row followed by a common letter are not significantly different ($p < 0.01$). For key to substances, see text.

Table 7. Extraction of Several Anthocyanins from Entire Fresh Grapes[a]

	extraction procedure				
	21	22	23	24	27
Df-Gl	4.2 ± 0.3a	3.7 ± 0.4a	3.8 ± 0.4a	5.1 ± 0.3b	5.5 ± 0.4b
Cy-Gl	0.3 ± 0.0ab	0.3 ± 0.1a	0.3 ± 0.1ab	0.4 ± 0.1ab	0.4 ± 0.1b
Pt-Gl	3.2 ± 0.2a	2.9 ± 0.1a	3.0 ± 0.3a	3.7 ± 0.2a	4.8 ± 0.4b
Pn-Gl	2.1 ± 0.1a	2.1 ± 0.1a	2.2 ± 0.1a	2.5 ± 0.1b	3.9 ± 0.2c
Mv-Gl	39.6 ± 1.7a	34.9 ± 1.1a	39.5 ± 1.7a	45.3 ± 1.4b	73.5 ± 3.2c
Mv-Gl-Ac	23.8 ± 1.9cd	21.8 ± 0.5c	24.9 ± 0.6d	13.1 ± 1.2b	0.3 ± 0.2a
Mv-Gl-Cou	8.6 ± 0.5b	7.4 ± 0.5a	8.8 ± 0.3b	9.2 ± 0.4b	10.9 ± 0.3c

[a] Results are expressed as mg of Mv-Gl equivalents/100 g of grapes. Data are mean values for three replications. Values in the same row followed by a common letter are not significantly different ($p < 0.01$). For key to substances, see text.

formation of anthocyanidins from flavanols and proanthocyanidins extracted from grape skins and seeds in an acid media should be considered to a certain extent (Hemingway, 1989).

Extraction of Individual Anthocyanins. Figure 1 shows a chromatogram corresponding to an extract from skins of fresh grapes obtained by procedure 11. Seven different peaks were quantitated and were assigned to delphinidin 3-*O*-glucoside (Df-Gl), cyanidin 3-*O*-glucoside (Cy-Gl), petunidin 3-*O*-glucoside (Pt-Gl), peonidin 3-*O*-glucoside (Pn-Gl), malvidin 3-*O*-glucoside (Mv-Gl), malvidin 3-*O*-acetylglucoside (Mv-Gl-Ac), and malvidin 3-*O*-*p*-coumarylglucoside (Mv-Gl-Cou) on the basis of their chromatographic and spectral characteristics, as has been previously reported (Wulf and Nagel, 1978; Bakker and Timberlake, 1985; Hebrero et al., 1988; Baldi et al., 1995).

Tables 6 and 7 display the data corresponding to the amount of seven anthocyanins determined by HPLC in the extracts obtained using the different procedures assayed for skins of fresh grapes and for entire fresh grapes, respectively. It can be noticed that the use of solvents which contained hydrochloric acid for extracting anthocyanins apparently led to a higher extraction of nonacylated anthocyanins, and especially malvidin 3-*O*-glucoside, which is the major anthocyanin in Cabernet Sauvignon grapes (Wulf and Nagel, 1978; Darné, 1988). The use of acid solvents (procedures 14, 15, and 17 in the case of experiments with skins of fresh grapes, and procedures 24 and 27 for experiments with entire fresh grapes) led to extracts that contained more malvidin 3-*O*-glucoside than those extracts obtained by procedures that used neutral solvents ($p < 0.01$). The effect was especially intense when methanol containing 2% of 12 N HCl was used as solvent. The other four nonacylated anthocyanins (3-*O*-glucosides of delphinidin, cyanidin, petunidin, and peonidin) behaved similarly, and procedures 17 and 27 led to the highest amount of these molecules in the extracts.

The two acylated anthocyanins that have been analyzed (malvidin 3-*O*-acetylglucose and malvidin 3-*O*-*p*-coumarylglucose) behaved in different ways. Appar-

Table 8. Area Percentages of Seven Anthocyanins Determined by HPLC in Extracts Obtained from Skins of Fresh Grapes[a]

	extraction procedure						
	11	12	13	14	15	16	17
Df-Gl	5.3 ± 0.3a	5.0 ± 0.3a	5.6 ± 0.3abc	6.0 ± 0.1bc	6.1 ± 0.0c	5.1 ± 0.3a	5.4 ± 0.4ab
Cy-Gl	0.3 ± 0.1a	0.3 ± 0.1a	0.5 ± 0.1ab	0.5 ± 0.0b	0.5 ± 0.1ab	0.4 ± 0.0ab	0.5 ± 0.1ab
Pt-Gl	4.1 ± 0.1a	3.9 ± 0.1a	4.1 ± 0.3a	4.7 ± 0.1b	4.8 ± 0.0b	4.1 ± 0.1a	4.9 ± 0.2b
Pn-Gl	2.8 ± 0.1a	2.6 ± 0.1a	2.7 ± 0.3a	3.5 ± 0.2b	3.6 ± 0.1b	2.8 ± 0.1a	3.7 ± 0.1b
Mv-Gl	48.1 ± 0.6a	49.0 ± 0.9a	48.0 ± 0.1a	63.6 ± 0.3b	69.7 ± 0.4c	48.9 ± 0.0a	75.1 ± 0.7d
Mv-Gl-Ac	29.2 ± 0.5d	28.9 ± 1.1d	29.4 ± 0.7d	10.9 ± 0.2c	4.1 ± 0.1b	30.0 ± 0.0d	0.5 ± 0.1a
Mv-Gl-Cou	10.3 ± 0.2bc	10.3 ± 0.2bc	9.8 ± 0.1b	10.8 ± 0.0cd	11.2 ± 0.4d	8.7 ± 0.2a	10.1 ± 1.2b

[a] Data are mean values for three replications. Values in the same row followed by a common letter are not significantly different ($p < 0.01$). For key to substances, see text.

Table 9. Area Percentages of Seven Anthocyanins Determined by HPLC in Extracts Obtained from Entire Fresh Grapes[a]

	extraction procedure				
	21	22	23	24	27
Df-Gl	5.1 ± 0.1ab	5.0 ± 0.6ab	4.6 ± 0.3a	6.4 ± 0.2c	5.6 ± 0.2b
Cy-Gl	0.4 ± 0.0a	0.4 ± 0.1a	0.4 ± 0.1a	0.5 ± 0.1a	0.4 ± 0.0a
Pt-Gl	4.0 ± 0.2a	4.0 ± 0.1a	3.7 ± 0.3a	4.7 ± 0.2b	4.9 ± 0.2b
Pn-Gl	2.6 ± 0.2a	2.8 ± 0.1ab	2.7 ± 0.1a	3.1 ± 0.1b	3.9 ± 0.1c
Mv-Gl	48.4 ± 0.8a	47.9 ± 0.8a	47.9 ± 0.3a	57.2 ± 1.1b	74.0 ± 0.3c
Mv-Gl-Ac	29.0 ± 0.8c	29.9 ± 0.2c	30.2 ± 0.4c	16.5 ± 0.9b	0.3 ± 0.2a
Mv-Gl-Cou	10.5 ± 0.5ab	10.1 ± 0.4a	10.6 ± 0.5ab	11.6 ± 0.2b	11.0 ± 0.2ab

[a] Data are mean values for three replications. Values in the same row followed by a common letter are not significantly different ($p < 0.01$). For key to substances, see text.

ently, the extraction of malvidin 3-*O*-*p*-coumarylglucose followed the same patterns as the extraction of nonacylated anthocyanins. Certainly, the amount of malvidin 3-*O*-*p*-coumarylglucose in extracts obtained from skins of fresh grapes (Table 5) was higher in procedures that involved the use of hydrochloric acid (procedures 14, 15, and 17) than in the others, but differences were not so important as those observed for malvidin 3-*O*-glucoside, and extractions carried out by procedures 12 and 14 were not different ($p < 0.01$). A similar effect was observed for extracts obtained from entire fresh grapes: procedure 27, which involved the use of an acid solvent, led to a more intense extraction of malvidin 3-*O*-*p*-coumarylglucose than procedures 21−23, which used neutral solvents, but the extraction carried out by procedure 24, which involved the use of an acid solvent, was not different ($p < 0.01$) from the extractions carried out by procedures 21 and 22, which used neutral solvents. Consequently, it would be possible to hypothesize that the factors that affect the extraction of malvidin 3-*O*-*p*-coumarylglucose from grapes may be qualitatively different from those that influence the extraction of malvidin 3-*O*-glucoside, except if the high amount of this latter molecule in extracts obtained with acid solvents was the consequence of the hydrolysis of any other acylated derivative. On the other hand, procedures that involved the use of acidic solvents (procedures 14, 15, 17, 24, and 27) led to the extraction of a lower amount of malvidin 3-*O*-acetylglucose ($p < 0.01$) than procedures that used neutral solvents. The effect of using methanol containing 2% of 12 N HCl as solvent was especially dramatic, and the extracts obtained with this solvent contained a very low amount of malvidin 3-*O*-acetylglucose. The effect of other factors on the extraction of nonacylated anthocyanins and their acetyl derivatives, such as total extraction time, extraction temperature, or the number of sucessive extractions, seem to be less important than the use of acidic solvents for their extraction.

The results obtained suggest that the presence of hydrochloric acid in the extraction solvent originates the partial hydrolysis of acetylated anthocyanins, as it was reported previously by Anderson et al. (1970), Van Sumere et al. (1985), and Van Wyk and Winter (1994). Data on the relative content of the seven anthocyanins analyzed by HPLC in the extracts obtained in the different experiments, expressed as area percentages and summarized in Tables 8 and 9, would make this question clear. The relative contents of malvidin 3-*O*-glucoside were quite similar for all of the extracts obtained with neutral solvents (procedures 11−13, 16, and 21−23), ranging from 47.9% (procedure 22) to 49.0% (procedure 12), and, in the same way, the relative contents of malvidin 3-*O*-acetylglucose were quite similar for all of the extractions carried out with neutral solvents, ranging from 28.9% (procedure 12) to 30.2% (procedure 23). The use of acid solvents increased the relative content of malvidin 3-*O*-glucoside in the extracts ($p < 0.01$), which may be as high as 75.1% (procedure 17), and consequently reduced the relative content of malvidin 3-*O*-acetylglucose ($p < 0.01$), which may be as low as 0.3% (procedure 27).

Furthermore, the relative contents of the other four nonacylated anthocyanins (the 3-*O*-glucosides of delphinidin, cyanidin, petunidin, and peonidin) were higher in the extracts obtained by procedures that involved the use of acid solvents than in those obtained when neutral solvents were used, probably as a consequence of the hydrolysis of their acetylated derivatives, because the apparent extraction of those nonacylated anthocyanins was more intense when acid solvents were used (Tables 6 and 7). Unfortunately, it was not possible to evaluate the amount of the acetyl derivatives of those anthocyanins present in the extracts.

These data point out that the use of solvents containing up to 1% of 12 N hydrochloric acid produces the partial hydrolysis of some acetylated anthocyanins during extraction. As a consequence, the use of experi-

mental protocols for extracting anthocyanins using solvents that contained up to 1% of 12 N hydrochloric acid from grapes or from any other plant material containing acetylated anthocyanins should be avoided, especially if the work is related with any type of taxonomic studies. Any extraction protocol that uses other mineral and organic acids, and even solvents containing hydrochloric acid at lower concentrations than those used in our experiments, should be tested against an extraction procedure that involves the use of neutral solvents, to avoid partial hydrolysis of acetylated anthocyanins or even the formation of laboratory artifacts, as those mentioned by Bakker and Timberlake (1985) when formic acid is used for the extraction of anthocyanins. The relatively high amount of malvidin 3-*O*-acetylglucoside present in Cabernet Sauvignon grapes makes them good material for testing extraction solvents intended to be used for anthocyanin analysis.

Some of the procedures used, and especially procedure 23, could give information of interest about the content of anthocyanins in grapes shortly after sampling (~24 h) and even complementary information about the global phenolic status of grapes. These data could be useful for the better conducting of red wine fermentation, as anthocyanins and other phenolic compounds play a very relevant role in the characteristics of red wines.

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