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Red-Color Related Phenolic Composition of Garnacha Tintorera (*Vitis vinifera* L.) Grapes and Red Wines

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Garnacha Tintorera (also known as Alicante Bouschet) is one of the few *V. vinifera* grape cultivars with red-colored berry flesh. The study of the phenolic composition of both berry flesh and skin of Garnacha Tintorera grapes shows interesting findings. Anthocyanins were asymmetrically distributed within grape flesh and skins. Malvidin derivatives dominated in skin, followed by peonidin-type anthocyanins; in contrast, the flesh almost exclusively contained peonidin 3-glucoside. In addition, LC-UV−vis and LC-MS evidence suggest the presence of small amounts of peonidin 3,5-diglucoside and a second peonidin dihexoside derivative, and, very likely, the first report of the occurrence of pelargonidin 3-glucoside and its acetyl and *p*-coumaroyl derivatives in *V. vinifera* grapes. Flavonols also occurred in the flesh of Garnacha Tintorera grapes, but its flavonol profile showed lower contribution of trisubstituted flavonoid structures (myricetin, laricitrin, and syringetin) when compared to that of skin. The skin of Garnacha Tintorera grapes contained hydroxycinnamic acids in higher amounts than in flesh, caftaric acid being the main derivative found, and coutaric acid accounting for its highest proportion in the skin. The phenolic composition of the whole grape berries reflected the average of the differences described for the two aforementioned berry parts, and subsequently, the red wines made from these grapes had a phenolic composition closer to that shown by the whole berries. The formation of anthocyanin-derived pigments in Garnacha Tintorera red wines makes necessary the use of LC-MS for detecting the minor pelargonidin-based anthocyanins and peonidin dihexoside, which could be suggested as chemical markers for cultivar authentication of this grape cultivar and its wines.

KEYWORDS: Alicante Bouschet; anthocyanins; flavonols; Garnacha Tintorera; hydroxycinnamic acids; pelargonidin; peonidin dihexoside; grape; *Vitis vinifera*; wine

INTRODUCTION

Garnacha Tintorera is one of the few *V. vinifera* grape cultivars with a red-colored berry flesh, so it is also known as a teinturier cultivar. Most teinturier varieties now cultivated worldwide were developed in the nineteenth century by Louis and Henri Bouschet. They are hybrids derived from crosses which were designed to increase the color intensity of well-known, high quality red wine varieties cultivated at that time (*1*). Garnacha Tintorera has been described as a Spanish autochthonous grape cultivar and has long been considered the only tenturier cultivated in Spain and different from Alicante Bouschet, the best known teinturier grape variety developed by Henri Bouschet. However, the molecular analysis of representative grapevine accessions cultivated in Spain under the name Garnacha Tintorera demonstrated they included three different teinturier genotypes, the majority genotype being that of Alicante Bouschet (*2*).

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The very intense red-purple color shown by young Garnacha Tintorera wines can reach values of color intensity as high as 25 units (*3*), thus indicating copigmentation should be a phenomenon of great importance in such wines. Therefore, the study of the phenolic compounds involved in Garnacha Tintorera red wine color must consider not only their anthocyanins but also the so-called copigments. Several works have revealed the best copigments found in red wines are flavonols and also hydroxycinnamic acids (*4*). In addition, hydroxycinnamic acid participates in the formation of new anthocyanin-derived pigments in wine, known as hydroxyphenyl-pyranoanthocyanins (*5*,*6*). Since the Garnacha Tintorera berry has red-colored flesh, a similar anthocyanin composition in both flesh and skin could be expected, but an asymmetrical distribution of anthocyanins within these two berry parts has been found (*3*, *7*). Anthocyanin from *V. vinifera* grapes has been extensively studied and characterized as acylated and nonacylated 3-glucosides of five anthocyanidin structures, the disubstituted cyanidin and peonidin, and the trisubstituted delphinidin, petunidin, and malvidin (*8*). A unique monosubstituted anthocyanidin, called pelargonidin, can be found in other plants,

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and it has been recently reported to occur as 3-glucoside in non-vinifera grapes and juices of the cultivars Concord, Rubired, and Salvador (*9*, *10*). The berry skin of Garnacha Tintorera grapes mainly contains malvidin 3-glucoside (39−48%, on a molar basis) as expected for most of the *V. vinifera* cultivars, but it also contains unusually high amounts of peonidin 3-glucoside (19−31%, on a molar basis) when compared to that of most representative *Vitis vinifera* cultivars, such as Cabernet Sauvignon or Tempranillo (*11*); the main acylated anthocyanins in the skins are the *p*-coumarylated ones. In contrast, the berry flesh of Garnacha Tintorera grapes predominantly contains peonidin 3-glucoside (*3*, *7*), an usual pattern shown by other teinturier grape cultivars, such as Tinta de Santarem and Viera de Natividade from Portugal (*7*). In a recent paper, the flavonol profile of Garnacha Tintorera grape skin was determined (*12*), and to the best of our knowledge, flavonols have been reported to occur only in the grape berry skin (*13*, *14*), and there is no report about the presence of flavonols in the berry flesh of any *V. vinifera* grape cultivar. With regard to hydroxycinnamic acids, they can occur in both berry flesh and skin (*13*), but we have not found references dealing with differences in the composition of hydroxycinnamic acids within the flesh and the skin of Garnacha Tintorera grape berries.

The aim of this work has been to study the phenolic composition of the two red-colored parts of the Garnacha Tintorera grape berry, namely, the skin and also the flesh, the origin of the color of the red wines which can be elaborated using these grapes. Our attention has been focused not only on anthocyanins but also on other phenolic compounds that can help in extracting and stabilizing anthocyanins during winemaking by means of copigmentation; these compounds are flavonols and hydroxycinnamic acids. We report on two main findings: the detection of flavonols in the flesh of a *Vitis vinifera* grape cultivar and the detection of a new peonidin dihexoside (not peonidin 3,5-diglucoside) and pelargonidin-based anthocyanins in a *Vitis vinifera* grape cultivar and their corresponding red wines.

MATERIALS AND METHODS

Chemicals and Grape and Wine Samples. All solvents were of HPLC quality and all chemicals of analytical grade (>99%). Water was of Milli-Q quality. Commercial standards from Phytolab (Vestenbergsgreuth, Germany) were used: malvidin 3-glucoside, pelargonidin 3-glucoside, malvidin 3,5-diglucoside, peonidin 3,5-diglucoside, caffeic acid, and *p*-coumaric acid. Commercial standards from Extrasynthese (Genay, France) were used: cyanidin 3-glucoside, cyanidin 3,5-diglucoside, cyanidin 3-(2″-glucosyl)-glucoside (cyanidin 3-sophoroside), cyanidin 3-(6″-rhamnosyl)-glucoside (cyanidin 3-rutinoside), the 3-*O*-glucosides of quercetin, kaempferol, isorhamnetin and syringetin, and the 3-*O*-galactosides of quercetin and syringetin. Other noncommercial flavonol standards (myricetin 3-*O*-glucoside, quercetin 3-*O*-glucuronide, and kaempferol 3-*O*-glucuronide) were kindly supplied by Dr. Ullrich Engelhardt (Institute of Food Chemistry, Technical University of Braunschweig, Germany), or they were isolated from Petit Verdot grape skins (myricetin 3-galactoside and laricitrin 3-glucoside) in a previous work (*15*). A sample of *t*-caftaric acid was kindly supplied by Dr. Vrhovsek (IASMA Research Centre, San Michele all'Adige, Italy). All of the standards were used for identification and quantification by means of calibration curves, covering the expected concentration ranges (usually 0−100 mg/L, with the exception of malvidin 3-glucoside covering a range of 0−1000 mg/L). When a standard was not available, the quantification was made using the calibration curve of the most similar compound: malvidin 3-glucoside was used for all grape anthocyanins with the exception of pelargonidin-based anthocyanins (calibration curve obtained for pelargonidin 3-glucoside was used) and peonidin dihexoside (peonidin 3,5-diglucoside used for calibtration curve); *p*-coumaric acid was used for *t*- and *c*-coutaric acids; ferulic acid was used for *t*-fertaric acid; flavonol 3-glycosides was used with nonavailable standard as their corresponding 3-glucoside derivatives.

Healthy Garnacha Tintorera grapes were collected at optimum ripeness for harvesting (estimated alcoholic strength of around 13%, v/v). The grapes were grown in both the experimental vineyard of the Instituto de la Vid y el Vino de Castilla-La Mancha (Tomelloso, Ciudad Real, middle-southern Spain) and also in the vineyards of Tintoralba cellar (Higueruela, Albacete, middle-southern Spain), and they were labeled as GTG-1 and GTG-2, respectively. The sampling was randomly made by picking berries from the top, central, and bottom parts of the cluster, following a zigzag path between two marked rows of 10 vines. We tried to sample berries from both exposed and shaded clusters by picking berries of 4−5 clusters per vine. The size of the sample was around 300 berries, which were bulked and separated in 3 subsamples of approximately 100 berries. Wine samples were an experimental wine made at the Instituto de la Vid y el Vino de Castilla-La Mancha using grapes of the same vineyard and vintage as those analyzed from this institution (labeled as GTW-1) and two commercial wines supplied by the Tintoralba cellar, a young wine of the vintage 2008 (labeled as GTW-2) and a shortly aged wine of the vintage 2007 (labeled as GTW-3). All the samples were analyzed in duplicate.

Grape and Wine Sample Preparation. A weighed sample of 100 healthy grape berries was manually and carefully peeled taking care to immediately dip the resulting skins and the peeled berries in separated glasses, each containing 150 mL of a solvent mixture of methanol, water, and formic acid (50:48.5:1.5 v/v), thus avoiding oxidation. In addition, a second weighed sample of 100 whole berries was dipped in a third glass containing the same extraction solvent mixture (150 mL). Finally, the seeds of a third weighed sample of 100 grape berries were separated, washed with water, softly dry with paper, and dipped in a fourth glass containing 150 mL of the extraction solvent mixture. Furthermore, the content of the four glasses were separately crushed and homogenized (Heidolph DIAX 900) during 3 min and then centrifuged at 2500*g* at 5 °C for 10 min. A second extraction of the resulting pellets was made, and the combined supernatants for each sample (berry skins, peeled berries or berry flesh, whole berries, and berry seeds) were stored at −18 °C until use.

Anthocyanin-free flavonol fraction from grape skin, peeled berry, and whole berry extracts were obtained following the procedure previously described (*12*) using SPE cartridges (Oasis MCX cartridges, Waters Corp., Mildford, MA; cartridges of 6 mL capacity filed with 500 mg of adsorbent). The eluate containing flavonols was separately dried in a rotary evaporator (40 °C), resolved in 3 mL of 25% methanol, and stored at −18 °C until use. Wine samples were similarly processed for obtaining their respective anthocyanin-free fractions (*12*). Berry seed extract was used directly.

HPLC-DAD-ESI-MS^n Analysis of Anthocyanins. HPLC separation, identification, and quantification of grape berry and wine anthocyanins were performed on an Agilent 1100 Series system (Agilent, Germany), equipped with DAD (G1315B) and a LC/MSD Trap VL (G2445C VL) electrospray ionization mass spectrometry (ESI-MS^n) system, and coupled to an Agilent Chem Station (version B.01.03) data-processing station. The mass spectra data were processed with the Agilent LC/MS Trap software (version 5.3). The berry skin, peeled berry, whole berry, and berry seed extracts were diluted with 0.1 N HCl before injection (1:10, v/v). The diluted extracts and the wine samples were injected (50 μL) after filtration (0.20 μm, polyester membrane, Chromafil PET 20/25, Macherey-Nagel, Düren, Germany) on a reversed-phase column Zorbax Eclipse XDB-C18 (4.6 × 250 mm; 5 μm particle; Agilent, Germany), thermostatized at 40 °C. The chromatographic conditions were adapted from the OIV method for analysis of anthocyanins in red wines (*16*). The solvents were water/acetonitrile/formic acid (87:3:10, v/v/v, solvent A; 40:50:10, v/v/v, solvent B), and the flow rate was 0.63 mL/min. The linear gradient for solvent B was as follows: 0 min, 6%; 15 min, 30%; 30 min, 50%; 35 min, 60%; 38 min, 60%; 46 min, 6%. For identification, ESI-MS^n was used employing the following parameters: positive ionization mode; dry gas, N_2, 11 mL/min; drying temperature, 350 °C; nebulizer, 65 psi; capillary, −2500 V; capillary exit offset, 70 V; skimmer 1, 20 V; skimmer 2, 6 V; compound stability, 100%; scan range, 50−1200 *m*/*z*. For quantification, DAD-chromatograms were extracted at 520 nm for native grape anthocyanins, and their concentrations were expressed as equivalents of malvidin 3-glucoside, with the exception of pelargonidin 3-glucoside (a specific calibration curve was obtained for it) and peonidin diehexoside (quantified as peonidin 3,5-diglucoside).

Table 1. Genotype of Studied *V. vinifera* Accessions at 12 Microsatellite Loci[a]

loci	Garnacha Tinta (Grenache Noir, Alicante)		Garnacha Tintorera (Alicante Bouschet)		Petit Bouschet[b]	
VVS2	137	*145*	*133*	*145*	*133*	153
VVMD5	*222*	236	*222*	*234*	230	*234*
VVMD7	*236*	*240*	*236*	*240*	*236*	*240*
VVMD27	*190*	190	*177*	*190*	*177*	185
ZAG62	*187*	187	*187*	*187*	*187*	197
ZAG79	*256*	256	*242*	*256*	*242*	244
VVMD21	200	*202*	198	*202*		
VVMD28	*242*	242	*242*	258		
VVMD36	*261*	*265*	*261*	*265*		
ZAG64	*128*	146	*128*	136		
ZAG67	*133*	139	*133*	156		
ZAG83	*191*	*193*	*191*	*193*		

[a] Allele sizes are given in base pairs. Synonyms are indicated in parentheses. Parentage relationships are given in italics (one allele of each parent). [b] Data obtained from Spanish Vitis Microsatellite Database (*28*).

HPLC-DAD-ESI-MS^n Analysis of Nonanthocyanin Phenolic Compounds. HPLC separation, identification, and quantification of nonanthocyanin phenolic compounds were performed on the same chromatographic system as that formerly described for anthocyanins. The samples were injected (50 μL) after filtration (0.20 μm, polyester membrane, Chromafil PET 20/25, Machery-Nagel, Düren, Germany) on a reversed-phase column Zorbax Eclipse XDB-C18 (4.6 × 250 mm; 5 μm particle; Agilent, Germany), thermostatized at 40 °C. Hydroxycinnamic acid derivatives were analyzed by injection of berry skin, peeled berry, whole berry, and berry seed extracts and wines using the same chromatographic conditions as those described for anthocyanins but setting negative ionization mode conditions (capillary, +2500 V; compound stability, 40%), and DAD-chromatograms were extracted at 320 nm for identification and quantification as previously described (*6*). Flavonols were analyzed by injection of the anthocyanin-free flavonol fractions isolated from berry skin, peeled berry, whole berry, and berry seed extracts and wines. The chromatographic conditions were those previously reported and specifically developed for grape and wine flavonol analysis (*15*). The solvents were as follows: solvent A (acetonitrile/water/formic acid, 3:88.5:8.5, v/v/v), solvent B (acetonitrile/water/formic acid, 50:41.5:8.5, v/v/v), and solvent C (methanol/water/formic acid, 90:1.5:8.5, v/v/v). The flow rate was 0.63 mL/min. The linear solvents gradient was as follows: 0 min, 96% A, and 4% B; 7 min, 96% A, and 4% B; 38 min, 70% A, 17% B, and 13% C; 52 min, 50% A, 30% B, and 20% C; 52.5 min, 30% A, 40% B, and 30% C; 57 min, 50% B, and 50% C; 58 min, 50% B, and 50% C; 65 min, 96% A, and 4% B. For identification, ESI-MS^n was used in both positive and negative ionization modes (*15*), setting the following parameters: dry gas, N_2, 11 mL/min; drying temperature, 350 °C; nebulizer, 65 psi; capillary, −2500 V (positive ionization mode) and +2500 V (negative ionization mode); target mass, 600 *m*/*z*; compound stability, 40% (negative ionization mode) and 100% (positive ionization mode); trap drive level, 100%; scan range, 50−1200 *m*/*z*. For quantification of flavonols, DAD-chromatograms were extracted at 360 nm.

DNA Microsatellite Analysis for Grape Cultivar Authentication. The grape cultivar authentication was performed by means of microsatellite analysis. DNA was extracted from vine shoots from the same vines used for grape sampling by the CTAB method according to an existing method (*17*), adapted for small volumes. A set of 12 microsatellite loci were selected for amplification following the conditions previously reported (*18*). The 6 core loci, as per the recommendation of the EU project Genres081 (*19*), were VVS2, VVMD5, VVMD7, VVMD27, VrZAG62, and VrZAG79; samples were also analyzed at 6 additional loci, VrZAG64, VrZAG67, VrZAG83, VVMD21, VVMD28, and VVMD36.

RESULTS AND DISCUSSION

Cultivar Confirmation of Garnacha Tintorera Grapes. The studied grapevine accessions (GTG-1, GTG-2) were identified as Garnacha Tintorera synonymy Alicante Bouschet (Petit Bouschet × Grenache Noir). Garnacha Tinta (a *V. vinifera* cultivar also known as Grenache Noir or Alicante) was used as reference, in order to compare the obtained data with other existing microsatellite libraries (*20*, *21*). **Table 1** shows allele sizes in base pairs at 12 microsatellite loci chosen for this study.

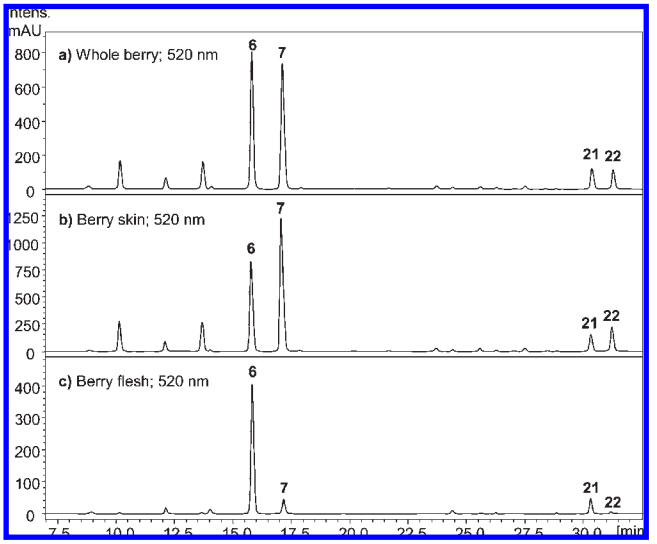

Figure 1. DAD-chromatograms (detection at 520 nm) of Garnacha Tintorera grape cultivar anthocyanins: (**a**) whole berry; (**b**) grape skin; (**c**) grape flesh. Peak numbering as in **Table 2**: 6, peonidin 3-glucoside; 7, malvidin 3-glucoside; 21, peonidin 3-(6″-*t*-coumaroyl)-glucoside; 22, malvidin 3-(6″-*t*-coumaroyl)-glucoside.

Identification of Anthocyanins of Garnacha Tintorera Grapes. First of all, we did not separate seeds and flesh when we homogenized the peeled berries, in order to avoid oxidation due to excessive sample handling. We confirmed the lack of anthocyanins, flavonols, and hydroxycinnamic acids in separated seeds of Garnacha Tintorera grape berries (data not shown). Therefore, we considered that peeled berry extract represented the composition of the berry flesh with regards to anthocyanins, flavonols, and hydroxycinnamic acid derivatives, and we are going to refer to the composition as berry flesh from now on.

The two red-colored parts of Garnacha Tintorera berries, namely, the skin and the flesh, contained the same anthocyanins, but their distribution was different depending upon, we consider, the berry skin or flesh. Nonacylated anthocyanins were the main class of grape anthocyanins, and coumaroylated derivatives were the most important within the minor acylated anthocyanins. Berry skin anthocyanins were dominated by malvidin 3-glucoside, but peonidin 3-glucoside was also a very important anthocyanin (**Figure 1b**). In contrast, peonidin 3-glucoside was the almost exclusive anthocyanin detected in berry flesh, and malvidin 3-glucoside was merely a minor anthocyanin (**Figure 1c**). Because of the asymmetrical distribution of anthocyanins within the berry skin and flesh and the respective amounts in which they are accounted for in each berry part, the average anthocyanin profile of the whole berry was finally dominated by both peonidin and malvidin 3-glucosides (peaks 6 and 7 in **Figure 1a**), accounting for both anthocyanins in very similar amounts (around 34% each, on a molar basis; **Table 2**).

A detailed assignment of the anthocyanin peaks detected in Garnacha Tintorera whole berry extract is shown in **Figure 2** and **Table 2**. The complete series of nonacylated (peaks 2, 3, 4, 6, and 7), acetylated (peaks 8, 9, 10, 13, and 14), and *trans*-coumaroylated (peaks 12, 15, 17, 21, and 22) derivatives of the 3-glucosides of the five anthocyanidins, delphinidin, cyanidin, petunidin, peonidin, and malvidin, as well as malvidin 3-(6″-caffeoyl)-glucoside (peak 16), which are common to all of the *V. vinifera* grape cultivars, were easily identified (UV−vis and MS data). With the

Table 2. Chromatographic Elution Order and Anthocyanin Profiles (Mean Value and Standard Deviation, $n = 4$, of Molar Percentage of Each Individual Anthocyanin over the Total Summation) Shown by Whole Grape and Berry Flesh and Skin for Garnacha Tintorera Grapes

peak	anthocyanin	whole berry	berry flesh	berry skin
1	peonidin dihexoside	0.60 ± 0.02	1.72 ± 0.36	0.25 ± 0.01
2	delphinidin 3-glucoside	5.12 ± 1.64	0.60 ± 0.34	6.12 ± 1.66
3	cyanidin 3-glucoside	2.35 ± 0.32	3.07 ± 0.49	2.01 ± 0.34
4	petunidin 3-glucoside	5.42 ± 1.33	0.88 ± 0.45	6.52 ± 1.37
5	pelargonidin 3-glucoside	0.45 ± 0.13	1.47 ± 0.60	0.16 ± 0.01
6	peonidin 3-glucoside	33.57 ± 0.93	67.19 ± 2.97	23.62 ± 0.86
7	malvidin 3-glucoside	34.46 ± 1.73	11.11 ± 4.17	41.61 ± 2.21
8	delphinidin 3-(6″-acetyl)-glucoside	0.16 ± 0.02	ND	0.20 ± 0.01
9	cyanidin 3-(6″acetyl)-glucoside	0.11 ± 0.03	0.17 ± 0.03	0.10 ± 0.02
10	petunidin 3-(6″-acetyl)-glucoside	0.19 ± 0.03	ND	0.25 ± 0.02
11	pelargonidin 3-(6″-acetyl)-glucoside[a]	ND	ND	ND
12	delphinidin 3-(6″-*t*-coumaroyl)-glucoside	0.82 ± 0.14	0.12 ± 0.01	1.04 ± 0.22
13	peonidin 3-(6″-acetyl)-glucoside	0.73 ± 0.30	1.56 ± 0.49	0.55 ± 0.17
14	malvidin 3-(6″-acetyl)-glucoside	1.11 ± 0.56	0.32 ± 0.01	1.44 ± 0.54
15	cyanidin 3-(6″-*t*-coumaroyl)-glucoside	0.49 ± 0.04	0.48 ± 0.07	0.47 ± 0.03
16	malvidin 3-(6″-*t*-caffeoyl)-glucoside	0.07 ± 0.03	ND	0.09 ± 0.04
17	petunidin 3-(6″-*t*-coumaroyl)-glucoside[b]	0.89 ± 0.09	0.12 ± 0.04	1.10 ± 0.11
18	peonidin 3-(6″-*c*-coumaroyl)-glucoside[b]	ND	ND	ND
19	malvidin 3-(6″-*c*-coumaroyl)-glucoside	0.18 ± 0.06	ND	0.23 ± 0.07
20	pelargonidin 3-(6″-*t*-coumaroyl)-glucoside	0.07 ± 0.00	0.18 ± 0.03	0.03 ± 0.00
21	peonidin 3-(6″-*t*-coumaroyl)-glucoside	6.14 ± 0.01	9.33 ± 0.31	5.26 ± 0.08
22	malvidin 3-(6″-*t*-coumaroyl)-glucoside	7.07 ± 1.65	1.67 ± 0.37	8.93 ± 1.63

[a] Only detectable by LC-MS, it was not quantified by DAD-HPLC. [b] Coeluting peaks, peak 18 being in trace amounts.

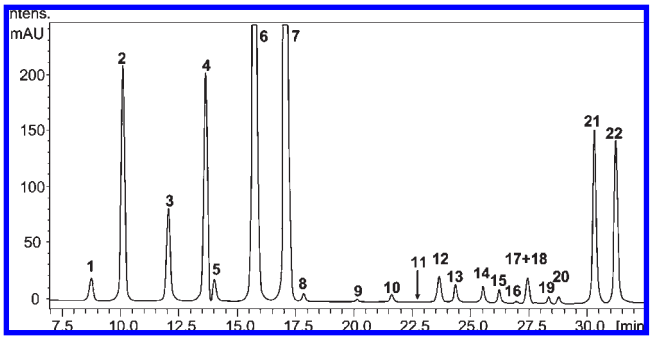

Figure 2. Expanded DAD-chromatogram (detection at 520 nm) of Garnacha Tintorera whole grape extracts showing all of the detected anthocyanins. Peak numbering as in **Table 2**.

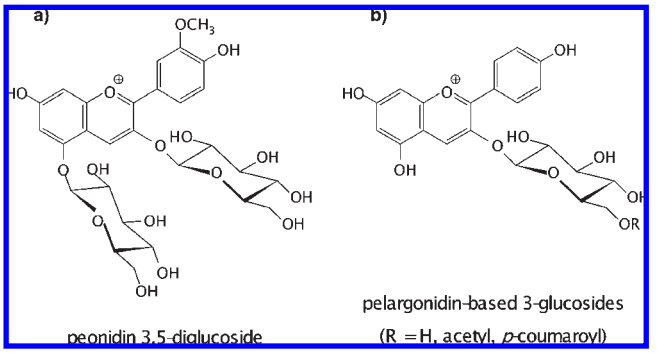

Figure 3. Structure of some anthocyanins newly found in Garnacha Tintorera grapes and wine: (**a**) peonidin 3,5-diglucoside and (**b**) pelargonidin-based 3-glucosides.

help of the mass spectrometry detector, it was possible to clearly detect the presence of the *cis* isomers of the 3-(6″-coumaroyl)-glucosides of peonidin and malvidin (peaks 18 and 19), which are not frequently reported when only DAD detector is used. The *cis*-isomers assigned to peaks 18 and 19 were confirmed by increasing their proportions after UV-light irradiation as previously reported (*22*). In addition, experimental evidence of the presence of an unexpected peonidin dihexoside (peak 1) and pelargonidin-based anthocyanins (peaks 5, 11, and 20) were found.

Peak 1 eluted before peonidin 3-glucoside in the reversed-phase column used, thus indicating higher polarity, and its online-DAD spectrum suggested the presence of a peonidin derivative (anthocyanin-like UV−vis spectrum with two absorbance maxima at 280 and 514 nm; at around 440 nm, a shoulder seems to appear, but the shape was not as clear as that for peonidin 3-glucoside). ESI-MS and ESI-MS/MS mass spectral data obtained for peak 1 in electrospray positive ionization conditions showed a molecular ion ($[M]^+$) at m/z 625 and two fragmentation signals at m/z 463 ($[M-162]^+$) and 301 ($[M-162-162]^+$), which were compatible with a peonidin-based structure linked to two hexose units. The first option was to assign peak 1 as peonidin 3,5-diglucoside (**Figure 3a**); although anthocyanidin 3,5-diglucosides have been considered as markers for nonvinifera grape varieties and their hybrids (*9*, *23*−*25*), they have been also found as very minor compounds in *V. vinifera* grape varieties and their wines (*26*−*28*). However, a true standard of peonidin 3,5-diglucoside did not match with peak 1 (**Figure 4a** and **b**), although their respective MS/MS spectra were almost identical (**Figure 4c** and **d**); moreover, the extracted ion chromatograms (m/z selection at 625 and 301) showed that peonidin 3,5-diglucoside was also detectable as a trace compound in addition to peak 1 (data not shown). The second possible assignment of peak 1 could be peonidin 3,7-diglucoside; this compound has been found in Tempranillo wine (*27*), but it elutes after peonidin 3,5-diglucoside, and it gives an ESI-MS/MS spectrum characterized by a very low-abundance fragment ion at m/z 463 (Y_0^+ ion) due to the high lability of the glycosidic linkage at C-7 position of peonidin that is more easily cleaved than that in position C-3 (*29*). The third suggestion for peak 1 is a peonidin 3-hexosylhexoside since the existence of anthocyanidin 3-(2″-glucosyl)-glucosides (anthocyanidin 3-sophorosides) and anthocyanidin 3-(6″-glucosyl)-glucosides (anthocyanidin 3-gentiobiosides) has been reported (*30*). Because of the lack of appropriate standards, we tested the fragmentation patterns under our ESI-MS/MS conditions for some commercially

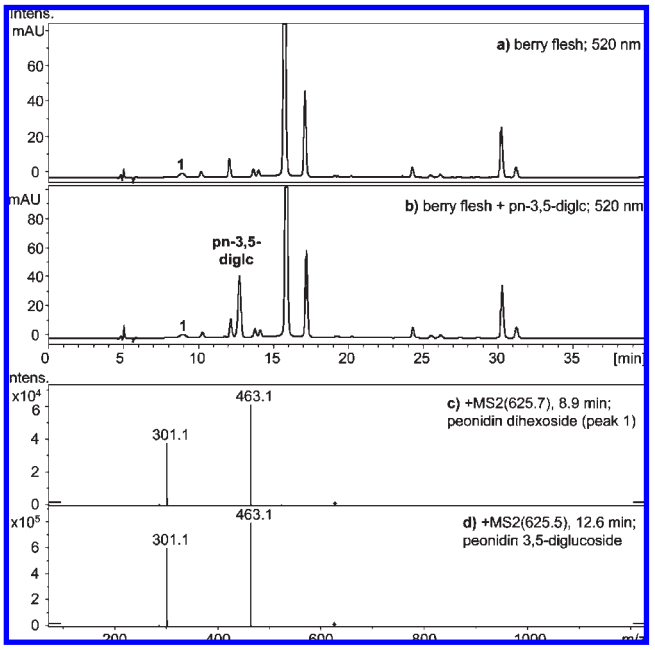

Figure 4. DAD-chromatograms (detection at 520 nm) of (**a**) a sample of Garnacha Tintorera berry flesh extract; (**b**) the same extract spiked with a standard of peonidin 3,5-diglucoside (pn-3,5-diglc). From these chromatograms were obtained the ESI-MS/MS spectra of (**c**) suggested peonidin dihexoside (peak 1) and (**d**) added peonidin 3,5-diglucoside.

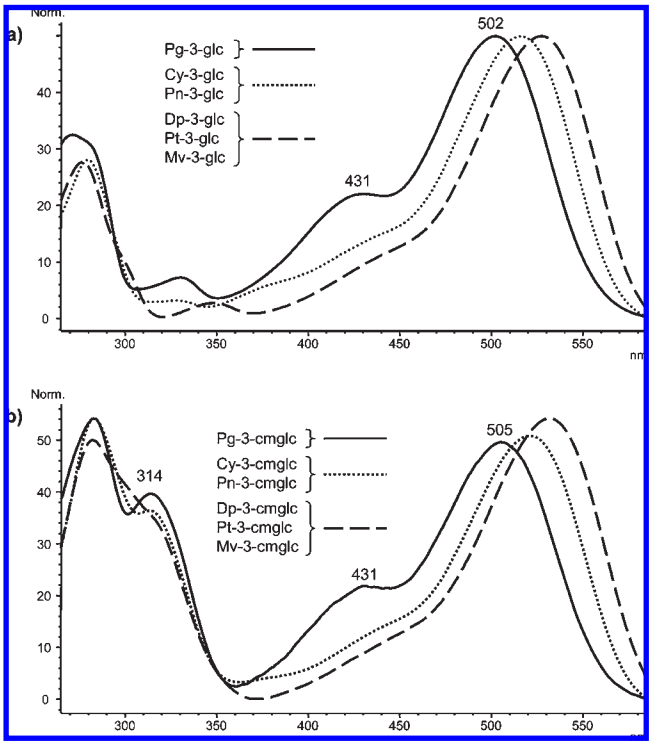

Figure 5. Comparison between the online DAD-UV−vis spectra of different mono-, di-, and trisubstituted anthocyanins found in Garnacha Tintorera grapes: (**a**) 3-glucosides (3-glc) of pelargonidin (pg; monosubstituted), cyanidin, and peonidin (cy, pn; disubstituted), and delphinidin, petunidin, and malvidin (dp, pt, mv; trisubstituted); (**b**) 3-(6″-*t*-coumaroyl)-glucosides (3-cmglc) of pelargonidin (pg; monosubstituted), cyanidin, and peonidin (cy, pn; disubstituted), and delphinidin, petunidin, and malvidin (dp, pt, mv; trisubstituted).

available closely-related compounds, that is, cyanidin diglycosides (the 3,5-diglucoside, the 3-sophoroside, and the 3-rutinoside). Cyanidin 3,5-diglucoside fragmented in the same way we found for peonidin 3,5-diglucoside, giving rise to two intense Y_0^+ ions (*m*/*z* 449 and 287) corresponding to the independent losses of the two glucose units, thus confirming the validity of the standards for the purpose of comparison of different glycoside fragmentation patterns. In contrast, the 3-(2″-glucosyl)-glucose unit of cyanidin 3-sophoroside was lost in one step, and only the signal for the Y_0^+ ion (the cyanidin aglycone, *m*/*z* 287), was present in the MS/MS spectrum; moreover, cyanidin 3-sophoroside eluted after cyanidin 3,5-diglucoside. However, the MS/MS spectrum of cyanidin 3-rutinoside (cyanidin 3-(6″-rhamnosyl)-glucoside) showed a low-abundance fragment ion at *m*/*z* 449 (Y_1^+ ion), attributable to the loss of the rhamnosyl moiety of the disaccharide, in addition to the highest-abundance Y_0^+ ion (*m*/*z* 287). The presence of Y_1^+ ions with low relative abundance in the MS/MS spectra of anthocyanidin 3-rutinosides has been previously reported (*31*, *32*), whereas for other anthocyanidin 3-diglycosides (such as 3-sophorosides and 3-sambubiosides), only the presence of the molecular and the aglycone fragment ions have been reported under electrospray ionization mass spectrometry conditions (*31*). No commercial standards of anthocyanidin 3-gentiobiosides were found, and to the best of our knowledge, no references on the fragmentation pattern of anthocyanidin 3-gentiobiosides have been reported. Considering the fragmentation pattern shown by cyanidin 3-rutinoside, it could be suggested that anthocyanidin 3-gentiobiosides (having a 6″-glycosidic linkage like in 3-rutinosides) could originate a Y_1^+ ion signal, but it would be likely of low-abundance as shown by 3-rutinosides. On the basis of the aforementioned results and literature data, we can suggest that peak 1 is likely a peonidin dihexoside, the two hexoses probably being separately linked to the peonidin aglycone, but we cannot determine the linkage positions nor the nature of the involved hexoses. It is remarkable that peonidin 3,5-diglucoside and the suspected peonidin dihexoside (peak 1) were the only anthocyanidin dihexosides detected in our samples of Garnacha Tintorera grapes.

Peaks 5, 11, and 20 had UV−vis and mass spectrometry spectral data which were in accordance with pelargonidin-based anthocyanins. First, the UV−vis spectrum of peak 5 showed a characteristic visible absorption maximum at 502 nm, together with a visible absorbance shoulder at 431 nm, which were in a characteristic absorbance ratio (431 to 502 nm) value of 0.45 (**Figure 5a**). It is well known that the substitution pattern of the B-ring of anthocyanins affect the value of their characteristic visible maximum: the value of the maximum decreases in the order tri → di → monosubstituted B-ring, as was found when comparing the UV−vis spectra of peak 5 (suspected monosubstituted anthocyanidin) with those of peaks 3 and 6 (disubstituted anthocyanidins) and peaks 2, 4, and 7 (trisubstituted anthocyanidins). All of the aforementioned results were in agreement with those previously reported for pelargonidin 3-glucoside (*33*). Peak 20 showed a UV−vis spectrum very similar to that of peak 5 in the visible region (**Figure 5b**), but an additional UV absorbance maximum at 314 nm was now present; these results suggested to us that peak 20 could be assigned as pelargonidin 3-(6″-coumaroyl)-glucoside. Further confirmation of the suggested pelargonidin-based structure of peaks 5 and 20 was given by the study of mass spectra data and by comparison to a true standard of pelargonidin 3-glucoside. The extracted ion chromatogram at the *m*/*z* value of 271, which corresponds to the pelargonidin aglycone, showed three signals corresponding to peaks 5, 11, and 20 in the DAD-chromatogram. The fragmentation pattern found in the MS/MS experiments of these three peaks suggested that they could be assigned as pelargonidin 3-glucoside,

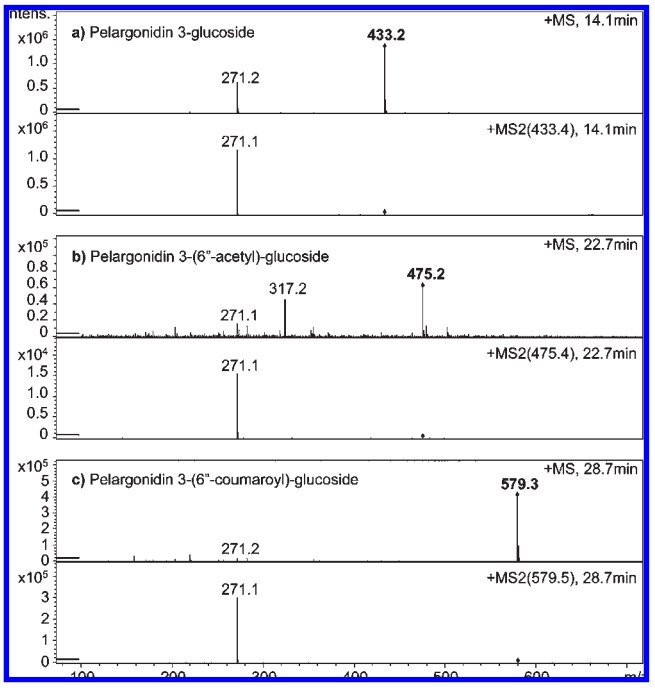

Figure 6. ESI-MS and MS/MS spectra corresponding to chromatographic peaks: (**a**) peak 5, pelargonidin 3-glucoside; (**b**) peak 11, pelargonidin 3-(6″-acetyl)-glucoside; (**c**) peak 20, pelargonidin 3-(6″-*t*-coumaroyl)-glucoside.

pelargonidin 3-(6″-acetyl)-glucoside, and pelargonidin 3-(6″-coumaroyl)-glucoside, respectively (**Figure 3b**) because of the corresponding observed losses of 162, 204, and 308 uma to give rise to the common fragment ion at *m*/*z* 271 (**Figure 6**). A commercial standard of pelargonidin 3-glucoside eluted at the same retention time as peak 5, giving the same UV−vis and mass spectra, and spiking experiments increased the area of peak 5 in the expected amounts with recovery percentages of ≈100% (data not shown). The acetyl derivative of pelargonidin 3-glucoside (peak 11) could not be assigned in the DAD-chromatogram because it was impossible to obtain its UV−vis spectra free of interferences and base noise (the peak was very small).

With regard to anthocyanin biosynthesis pathway in *V. vinifera* grape (**Figure 7**), the following six enzymes are needed to act sequentially (*34*): chalcone synthase (CHS), chalcone isomerase (CHI), flavanon-3-hydroxylase (F3H), dihydroflavonol-4-reductase (DFR), leucoanthocyanidin deoxygenase (LDOX), and UDP-glucose/flavonoid 3-*O*-glucosyltransferase (UFGT). Additional B-ring hydroxylation and methoxylation steps allow the completion of the series of six known anthocyanidins. It has been suggested that the absence of pelargonidin in the family Vitaceae indicates that the first hydroxylation in B-ring of the anthocyanidin molecule occurs in the initial flavanone (*35*, *36*). It is known that the preference of the enzyme DFR toward the three common dihydroflavonols varies markedly between species (*37*), with some enzymes showing little or no activity against dihydrokaempferol, the precursor of pelargonidin, and maybe the DFR of Vitaceae lies in this category. However, pelargonidin 3-glucoside has been reported to occur in nonvinifera grape cultivars (*9*, *10*), and to the best of our knowledge, we are now reporting for the first time on the occurrence of pelargonidin-based anthocyanins in a *V. vinifera* grape cultivar. A likely explanation of the detection of pelargonidin-based anthocyanin in Garnacha Tintorera grape can be suggested on the basis of recent findings on the factors influencing grape anthocyanin biosynthesis. A study of mutated Sangiovese grape biotypes (red-purplish, pink, pinkish-green, and green grape berries) has shown that only the expression of the gene encoding the enzyme UFGT (*ufgt*) is affected: its expression is normal in the red-purplish berries, lower in the biotypes pink and pinkish-green, and wholly lacking in the green one (*38*). Thus, anthocyanin biosynthesis in *V. vinifera* grapes seems to be largely controlled by transcription factors, particular proteins which operate according to a highly conserved mechanism among plant species, as was known for such species as *Zea mays* and *Arabidopsis thaliana* (*39*). In grapes, two specific transcription factors of the Myb family (*VvmybA1* and *VvmybA2*) were found to be involved in color regulation (*40*). Recently, it has been suggested that the *VvmybA1* gene very likely controls anthocyanin biosynthesis in grape berry skin through the transcription of the *Ufgt* gene (it produces the enzyme UFGT, that is critical for anthocyanin biosynthesis) and all other anthocyanin biosynthetic genes either directly or indirectly (*41*). Moreover, the *VvmybA1* gene has been found to be expressed in *V. vinifera* berry skin specifically in nonteinturier grape cultivars, whereas the origin of teinturier grape cultivars may be caused by a mutation related to organ-specific *VvmybA1* expression (*42*). The aforementioned genetic facts suggest that the mutation of *VvmybA1* causing the synthesis of anthocyanins in the berry flesh of Garnacha Tintorera grape could have affected the transcription of other implicated genes, such as those controlling the production of pelargonidin-based anthocyanins, specially the DFR enzyme that does not express in nonteinturier grapes toward dihydrokaempferol, and also those involved in 3,5-diglucosilation of peonidin which are usually expressed in nonvinifera grapes. Further studies dealing with the activity of *V. vinifera* DFR enzyme toward dihydrokaempferol (the way to pelargonidin) and the transcription factors affecting DFR gene expression are needed to clarify these unresolved biosynthetic doubts. The use of a recently prepared *V. vinifera* anti-DFR polyclonal antibody (*43*) could help in such a studies.

Anthocyanin Profiles of Red-Colored Berry Parts of Garnacha Tintorera Grapes. The whole berry had a content of anthocyanins that was different depending upon the analyzed sample, accounting for 2441 and 3593 mg/kg of grapes (as malvidin 3-glucoside) for GTG-1 and GTG-2 grape samples, respectively. However, the quantitative distribution of anthocyanins between berry flesh and skin was quite similar for the two analyzed samples, accounting for 18.9−21.4% in the berry flesh and 78.6−81.1% in the berry skin. The anthocyanin profiles of berry skin and flesh of the Garnacha Tintorera grape differed mainly in the relative importance of peonidin-based and malvidin-based anthocyanins (**Table 2**). The main anthocyanin, independent of the analyzed grape sample, was malvidin 3-glucoside in berry skin (41.61 ± 2.21%, on a molar basis) and peonidin 3-glucoside in berry flesh (67.19 ± 2.97%, on a molar basis), thus confirming previous reported data (*3*, *7*, *11*, *44*). Some minor anthocyanins were not detected in berry flesh, such as delphinidin and petunidin 3-(6″-acetyl)-glucosides (peaks 8 and 10), malvidin 3-(6″-caffeoyl)-glucoside (peak 16), and the *cis* isomers of peonidin and malvidin 3-(6″-coumaroyl)-glucosides (peaks 18 and 19). As the distribution of anthocyanins from each berry part was almost the same in the two grape samples, the whole berry showed almost the same anthocyanin profile for two grape samples, thus maintaining a characteristic anthocyanin profile for the whole berry. With regard to pelargonidin-based anthocyanins, their real contents were as follows (concentrations obtained with a specific calibration curve): pelargonidin 3-glucoside accounted for 10.15−13.70 and 4.63−6.77 mg/kg of grapes in the berry flesh and berry skin, respectively; pelargonidin 3-(6″-coumaroyl)-glucoside accounted for 1.95−2.59 and 1.29−1.59 mg/kg of grapes in the berry flesh and berry skin, respectively; and the 6″-acetyl derivative was not quantified because of its low signal in the DAD-chromatogram.

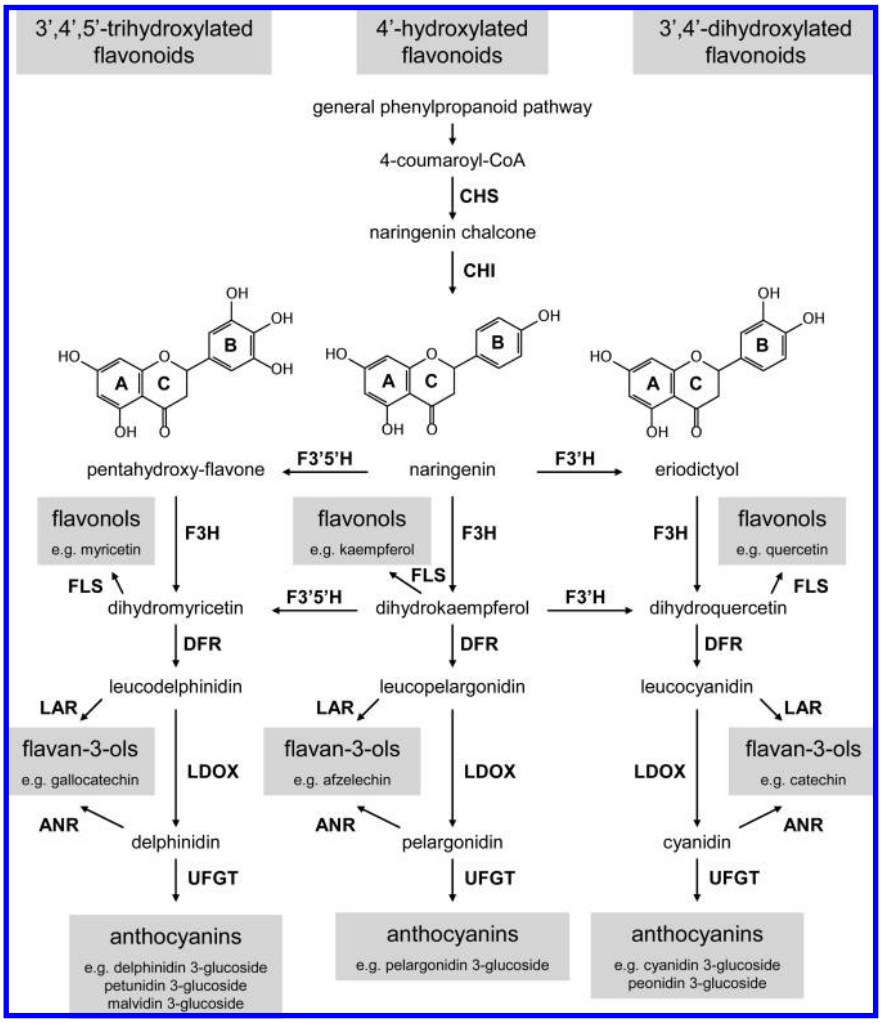

Figure 7. General flavonoid biosynthetic pathways. The B-ring methoxylation steps are not shown. Enzyme abbreviations: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; LDOX, leucoanthocyanidin deoxygenase; UFGT, UDP-glucose: flavonoid 3-*O*-glucosyltransferase; F3′H, flavanone 3′-hydroxylase; F3′5′H, flavanone 3′,5′-dihydroxylase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase. Adapted with permission from ref *40*. Copyright 2002 Springer.

Table 3. Hydroxycinnamoyl-tartaric Acid Profiles (Mean Value and Standard Deviation, $n = 4$, of Molar Percentage of Each Individual Hydroxycinnamoyl-tartaric Acid over the Total Summation) Shown by Whole Grape and Berry Flesh and Skin for Garnacha Tintorera Grapes

hydroxycinnamoyl-tartaric acid	whole berry	berry flesh	berry skin
t-caftaric acid	61.17 ± 2.13	82.69 ± 4.02	49.83 ± 1.68
t-coutaric acid	28.32 ± 2.69	13.28 ± 2.96	37.07 ± 0.70
c-coutaric acid	3.80 ± 0.36	1.90 ± 0.31	4.63 ± 0.64
t-fertaric acid	6.70 ± 0.91	2.13 ± 1.37	8.47 ± 1.61

Nonanthocyanin Red-Color-Related Phenolics of Garnacha Tintorera Grapes. On the basis of previous UV−vis and MS spectral data (*6*) and by comparison to a standard of *t*-caftaric acid, we were able to detect the hydroxycinnamoyl-tartaric acids derived from caffeic, *p*-coumaric, and ferulic acids (caftaric, coutaric, and fertaric acids, respectively) in both berry flesh and skin. The *trans* isomer was the only one detectable for caftaric and fertaric acids, and small amounts of the *cis* isomer were detected for coutaric acid, in addition to its main *trans* isomer. As was found for the anthocyanin profile, the hydroxycinnamic acid derivative profile also differed depending upon the berry flesh or skin considered (**Table 3**). *trans*-Caftaric acid was the most important hydroxycinnamic acid derivative found, although their proportion was higher in the berry flesh when compared to that in berry skin. The distribution of hydroxycinnamic acid derivatives accounted for a higher proportion in the skin (68.30−71.80%) than in the flesh (28.20−31.70%), independent of the total content of these compounds in the whole grape berries (GTG-1 contained 689 μmol/kg of grape, whereas GTG-2 contained 799 μmol/kg of grape).

With regard to flavonol 3-glycoside, the first remarkable finding was their detection in grape berry flesh (**Figure 8**) following a previous identification procedure (*15*). To the best of our knowledge, this is the first time flavonols are reported to occur in *V. vinifera* grape berry flesh. They were expected to occur only in the grape berry skin, and their distribution between berry flesh and skin were also asymmetrical as found for the other analyzed red-color-related phenolic compounds (**Table 4**). Flavonols accounted mainly in the berry skin (90.10−92.90%; 7.10−9.90% in the berry flesh), independent of the different content shown by the two analyzed grape samples (GTG-1, 732 μmol/kg of grape; GTG-2, 635 μmol/kg of grape). Because of the scarce contribution of berry flesh flavonols, the whole berry flavonol profile did not differ so much from that of berry skin, and it was in agreement with previously reported data (*12*). Myricetin 3-glucoside and, very closely, quercetin 3-glucoside were the most important flavonols in berry skin. In contrast, the importance of myricetin 3-glucoside decreased in the berry flesh, where the main flavonol

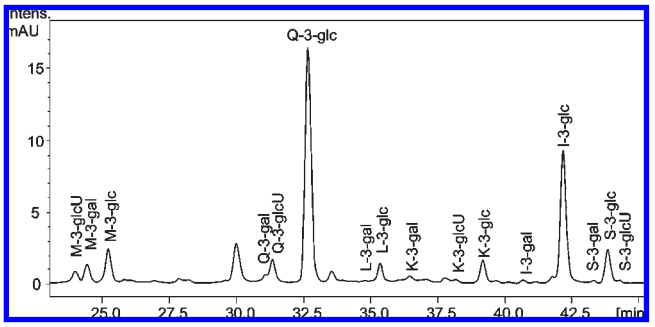

Figure 8. DAD-chromatogram (detection at 360 nm) showing the flavonol 3-glycosides detected in the flesh of Garnacha Tintorera grape berries. Peak assignment: M, myricetin; Q, quercetin; L, laricitrin; K, kaempferol; I, isorhamnetin; S, syringetin; glc, glucoside; glcU, glucuronide; gal, galactoside.

Table 4. Flavonol Profiles (Mean Value and Standard Deviation, $n = 4$, of Molar Percentage of Each Individual Flavonol-3-glycoside over the Total Summation) Shown by Whole Grape and Berry Flesh and Skin for Garnacha Tintorera Grapes

flavonol 3-glycoside	whole berry	berry flesh	berry skin
myricetin 3-glucuronide	1.97 ± 0.76	2.55 ± 0.55	1.38 ± 0.01
myricetin 3-galactoside	2.50 ± 0.29	4.40 ± 0.81	2.21 ± 0.05
myricetin 3-glucoside	29.18 ± 5.21	10.22 ± 2.96	31.21 ± 6.20
quercetin 3-galactoside	1.88 ± 0.64	0.37 ± 0.52	2.06 ± 0.56
quercetin 3-glucuronide	8.62 ± 0.11	2.94 ± 1.12	8.67 ± 0.59
quercetin 3-glucoside	25.12 ± 0.09	37.63 ± 5.87	23.68 ± 0.32
laricitrin 3-galactoside	0.24 ± 0.06	NQ[a]	0.28 ± 0.04
laricitrin 3-glucoside	7.60 ± 0.78	4.52 ± 0.73	8.20 ± 0.38
kaempferol 3-galactoside	1.42 ± 0.96	1.37 ± 0.08	1.75 ± 0.58
kaempferol 3-glucuronide	0.68 ± 0.25	0.77 ± 0.05	0.64 ± 0.31
kaempferol 3-glucoside	5.03 ± 1.82	3.00 ± 1.44	5.33 ± 1.65
isorhamnetin 3-galactoside	0.24 ± 0.15	0.68 ± 0.25	0.23 ± 0.15
isorhamnetin 3-glucoside	8.37 ± 0.03	24.00 ± 2.78	6.82 ± 0.75
syringetin 3-galactoside	0.08 ± 0.11	0.26 ± 0.37	0.07 ± 0.09
syringetin 3-glucoside	6.84 ± 1.21	7.29 ± 1.20	7.18 ± 0.87
syringetin 3-glucuronide	0.24 ± 0.03	NQ	0.29 ± 0.02

[a] NQ, not quantified due to their low peak area value.

was the B-ring disubstituted quercetin 3-glucoside and its methoxylation product, isorhamnetin 3-glucoside; in addition, quercetin 3-glucuronide was the third most important flavonol in berry skin and its importance also decreased in berry flesh. The aforementioned results suggested a different expression of the enzymes involved in flavonoid biosynthesis depending upon the berry part considered: in berry flesh, the trend is to produce B-ring disubstituted flavonoids (quercetin and isorhamnetin 3-glucosides in the case of flavonols; peonidin 3-glucoside in the case of anthocyanins); in addition, the glucuronation step of quercetin seems to be handicapped in berry flesh.

Phenolic Compounds as Markers for Cultivar Authenticity in Garnacha Tintorera Wines. The unexpected anthocyanins we have found in Garnacha Tintorera grape, namely, peonidin dihexoside (peak 1) and pelargonidin-based 3-glucosides (peaks 5, 11, and 20), could be suggested as chemical markers for Garnacha Tintorera wines. Indeed, the anthocyanin profile of Garnacha Tintorera wine samples showed the same anthocyanins as those found in the whole berry extracts, although the formation of new anthocyanin-derived pigments in wines handicapped the detection of some of the suggested markers by means of only DAD-chromatograms. For instance, peonidin dihexoside (peak 1) overlapped the well-known direct condensation product between catechin and malvidin 3-glucoside, but they could be distinguishable by their different MS spectral data. The presence of the three pelargonidin-based anthocyanins in wine samples was demonstrated by means of the extracted ion chromatograms (EIC) obtained at the m/z values of the molecular ions of the respective pelargonidin 3-glucoside (m/z 433), pelargonidin 3-(6″-acetyl)-glucoside (m/z 475), and pelargonidin 3-(6″-coumaroyl)-glucoside (m/z 579), together with the EIC recorded at m/z 271 that corresponds to the common pelargonidin aglycone. Quantification of the suggested markers in the analyzed wine samples yielded the following concentrations: peonidin dihexoside, 1.77−4.09 mg/L (as peonidin 3,5-diglucoside; quantification made by a calibration curve from EIC data at m/z 625); pelargonidin 3-glucoside, 0.63−2.01 mg/L; and pelargonidin 3-(6″-coumaroyl)-glucoside, 0.12−0.45 mg/L.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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