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Structure Elucidation of Peonidin 3,7-*O*-*β*-Diglucoside Isolated from Garnacha Tintorera (*Vitis vinifera* L.) Grapes

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Red wines made from Garnacha Tintorera grapes (also known as Alicante Bouschet and one of the few so-called teinturier *Vitis vinifera* grape cultivars, because its berry flesh is also red-colored) are usually blended with other red wines as a natural way for enhancing the color intensity of red wines. The phenolic composition of Garnacha Tintorera grapes showed already described interesting features such as the occurrence of unusual anthocyanins (pelargonidin-based anthocyanins and a peonidin dihexoside) that had not been previously described in *V. vinifera* cultivars. The isolation and further structure elucidation (GC-MS analysis of sugar moieties, UV−vis, LC-MS^n, and ^1H and ^{13}C NMR spectroscopic data) have allowed the identification of the aforementioned peonidin dihexoside as peonidin 3,7-*β*-*O*-diglucoside. This peonidin derivative, in combination with the previously reported pelargonidin-type anthocyanins, can be suggested as a suitable chemical marker for this singular *V. vinifera* grape cultivar and the wines made totally or partially from this grape cultivar.

KEYWORDS: Anthocyanins; chemical marker; cultivar; Garnacha Tintorera; peonidin diglucoside; grape; *Vitis vinifera*; wine

INTRODUCTION

Garnacha Tintorera (*Vitis vinifera* L.) is one of the few *V. vinifera* grape cultivars having a red-colored berry flesh, so it is also known as a teinturier cultivar. Garnacha Tintorera has been described as a Spanish autochthonous grape cultivar. However, molecular analysis of representative grapevine accessions cultivated in Spain under the name Garnacha Tintorera demonstrated that they included three different teinturier genotypes, with the major genotype being Alicante Bouschet (*1*). The red wines made from Garnacha Tintorera are deeply red-colored, and they are highly appreciated as a natural way to enhance the color intensity of other red wines by blending them. The very intense red-purple color shown by young Garnacha Tintorera wines can reach values of color intensity as high as 25 units (*2*), thus indicating important anthocyanin copigmentation and self-association effects in such wines. Therefore, interest in the phenolic compounds involved in Garnacha Tintorera red wine color must be focused not only on their anthocyanins but also on their copigments (mainly flavonols and hydroxycinnamic acid derivatives).

In a previous work (*3*) we found that anthocyanins were unequally distributed between grape flesh and skins of Garnacha Tintorera grapes. 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^n evidence suggested the presence of small amounts of peonidin 3,5-diglucoside together with 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 their flavonol profile showed lower contribution of trisubstituted flavonoid structures (myricetin, laricitrin, and syringetin) when compared to skin. The skin of Garnacha Tintorera grapes contained hydroxycinnamic acids in higher amounts than in flesh, with caftaric acid constituting the main derivative in both berry parts and the proportion of coutaric acid being higher in skin than in flesh. 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 use of LC-MS^n allowed the detection of the aforementioned pelargonidin-based anthocyanins and peonidin dihexoside in red wine made from Garnacha Tintorera grapes, which were suggested as chemical markers for this grape cultivar and also their corresponding wines (*3*).

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The aim of this work is the isolation and complete structure elucidation of the peonidin dihexoside found in Garnacha Tintorera grapes and wines. Knowledge of the structure of this peonidin dihexoside can help to deepen our knowledge of the biosynthetic pathway of anthocyanins in this singular teinturier *V. vinifera* grape cultivar. Countercurrent chromatography and column chromatography were used for concentration of this minor peonidin dihexoside that was further isolated by semipreparative high-performance liquid chromatography and purified by solid phase extraction. The structure elucidation has been achieved by UV−vis, LC-MS^n, and ^{1}H and ^{13}C NMR spectroscopic data and also by hydrolysis of the glycoside and subsequent derivatization of released hexosides and their analysis by GC-MS.

MATERIALS AND METHODS

Chemicals and Grape Samples. All solvents were of HPLC quality and all chemicals of analytical grade (> 99%). Water was of Milli-Q quality. Commercial standards of peonidin 3,5-diglucoside, malvidin 3-glucoside, and malvidin 3,5-diglucoside were from Phytolab (Vestenbergsgreuth, Germany), whereas cyanidin 3-glucoside, cyanidin 3,5-diglucoside, cyanidin 3-(2″-glucosyl)-glucoside (cyanidin 3-sophoroside), and cyanidin 3-(6″-rhamnosyl)-glucoside (cyanidin 3-rutinoside) were from Extrasynthese (Genay, France). Amberlite XAD7HP (XAD-7), pyridine, and methoxyamine were purchased from Sigma (Tres Cantos, Madrid, Spain). *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was purchased from Aldrich (Tres Cantos, Madrid, Spain). D-Glucose, D-galactose, methanol, formic acid, and HCl were supplied by Panreac (Castellar de Vallès, Barcelona, Spain).

Healthy Garnacha Tintorera grapes were collected at optimum ripeness for harvesting (estimated alcoholic strength of around 13%, v/v). The grapes were grown in the experimental vineyard of the Instituto de la Vid y el Vino de Castilla-La Mancha (Tomelloso, Ciudad Real, Spain).

Obtaining of Raw Grape Extract. Several batches of 1 kg of healthy grape berries were extracted with 1.5 L of a solvent mixture of methanol, water, and formic acid (50:48.5:1.5 v/v/v) with the help of a domestic blender for 5 min. After a maceration time of 2 h, the slurry was centrifuged at 2500*g* at 5 °C for 10 min. Methanol was eliminated in a rotary evaporator (40 °C), and the resulting aqueous solution was freeze-dried until further use.

Cleaning and Fractionation of Raw Grape Extract by Column Chromatography (CC). A preparative glass chromatographic column (80 cm length and 6 cm i.d.) was filled with 1 kg of Amberlite XAD-7 suspended in methanol. After packaging, the methanol was eluted, and then the column was washed using 2 L of water. The freeze-dried raw grape extract corresponding to 5 kg of grape berries was dissolved in 2.5 L of aqueous HCl (0.05 N) and applied to the chromatographic column. The nonadsorbed compounds were eluted by washing the column with 2.5 L of aqueous HCl (0.05 N). The elution of the adsorbed compounds with methanol/acetic acid (19:1 v/v) yielded a grape phenolic extract. Methanol was evaporated in vacuo (40 °C), and the resulting aqueous solution was freeze-dried and stored (−18 °C) until fractionation by high-speed countercurrent chromatography (HSCCC).

A second batch of raw grape extract was applied to the column, washed as described above, and then eluted with 20% methanol in 0.05 N HCl, yielding several fractions enriched in peonidin dihexoside together with other very polar phenolic compounds. Finally, the column was rinsed using methanol/acetic acid (19:1 v/v) and conditioned for further uses by sequential washing with methanol and water. The fractions enriched in peonidin dihexoside were mixed, the methanol was rotary evaporated (40 °C), and the resulting aqueous solution was freeze-dried and stored (−18 °C) until fractionation by semipreparative HPLC.

Fractionation of Grape Phenolic Extract by HSCCC. Grape phenolic extract was fractionated by HSCCC following experimental conditions based on a previously described method for the isolation of anthocyanidin 3,5-diglucosides from red wine (*4*) using a high-speed model CCC-1000 (I) manufactured by Pharma-Tech Research Corp. (Baltimore, MD) and equipped with three preparative coils, connected in series (diameter of tubing, 2.6 mm; total volume, 850 mL). The separations were run at a revolution speed of 1000 rpm. A solvent system consisting of ethyl acetate/*n*-butanol/water (2:3:5) acidified with 0.1% trifluoroacetic acid was used. The elution mode was head to tail with the less dense layer being the stationary phase. The flow rate was set at 3 mL/min and delivered by a Biotronik HPLC pump BT 3020. Freeze-dried grape phenolic extract was dissolved in a 1:1 mixture of light and heavy phase and injected into the system by loop injection. The amount of sample injected was 1 g. Stationary phase retention was in the range of 53−75%. Fractions (10 mL) were collected with a Pharmacia LKB Super Frac fraction collector. Elution was monitored with a Knauer UV−vis detector at 520 nm, and chromatograms were recorded on a Knauer L 250 E plotter.

Isolation and Purification of Peonidin 3,7-*O*-*β*-Diglucoside. Isolation of peonidin 3,7-*O*-*β*-diglucoside from the enriched fraction obtained by column chromatography was achieved by repeated HPLC runs using the same equipment and the same chromatographic conditions as for analytical HPLC described below. With the help of an analytical fraction collector (Agilent G1364C) we made repeated injections (ca. 200 injections) of the column chromatography fraction enriched in the peonidin dihexoside.

The collected fraction containing peonidin 3,7-*O*-*β*-diglucoside was dried in a rotary evaporator (40 °C) and cleaned up from residual non-anthocyanin compounds using ECX SPE cartridges (40 μm, 500 mg, 6 mL; Scharlab, Sentmenat, Barcelona, Spain). The latter cartridges combine reversed-phase and cationic-exchange separation mechanisms and show a performance similar to the Oasis MCX cartridges used in the procedure described for obtaining free-anthocyanin flavonol fractions from grape extracts and red wines (*5*), which was adapted as follows: after elution of non-anthocyanin compounds with methanol, the adsorbed peonidin 3,7-*O*-*β*-diglucoside was eluted using 2% NH_4OH in 80% methanol as a deep blue solution; acidification with 2 N HCl restored the red color. After removal of methanol by rotary evaporation (40 °C), the eluate was applied to a C18 SPE cartridge (Sep-Pak Vac, 3 cm^3/500 mg 55−105 μm; Waters) previously conditioned with methanol and water. All of the polar compounds (mainly excess of ammonium chloride) were removed by rinsing with water, and the resulting purified peonidin 3,7-*O*-*β*-diglucoside was recovered by elution with 2% HCl in 80% methanol. Methanol was evaporated, and the sample was freeze-dried and stored at −18 °C until analysis.

GC-MS Analysis of Sugars. We follow a previously described method (*6*). A drop of the aqueous solution of purified peonidin 3,7-*O*-*β*-diglucoside ready for freeze-drying was dried (rotary evaporator, 40 °C) and taken up in 500 μL of 1% HCOOH/MeOH, 50 μL of which was hydrolyzed in 1 mL of 1 M HCl (80 °C, 30 min). After hydrolysis, the solvent was removed under a stream of N_2 at 50 °C and the residue derivatized with 50 μL of methoxyamine solution (20 mg/mL in pyridine, 80 °C, 30 min), followed by 50 μL of MSTFA (80 °C, 30 min). Ten microliters of the sugar standard solutions (5 mg/mL in water) was evaporated to dryness under a stream of N_2 at 50 °C, and the residue was derivatized as above.

Gas chromatography−mass spectrometry was performed on a ThermoQuest TraceGC gas chromatograph coupled with a DSQII single-quadrupole mass detector (Thermofisher Scientific, Waltham, MA). The separation column was a 30 m × 0.25 mm i.d. × 0.25 μm film thickness Agilent J&W DB-XLB-DG (J&W Scientific, Folsom, CA). The temperature was programmed from 40 °C (held for 1 min) to 300 at 7 °C/min (held for 4 min). The derivatized sugar residues were identified by comparison of retention times and fragmentation patterns with authentic standards. Derivatized hexoses (glucose or galactose) m/z (rel intensity): 319 (36), 205 (26), 147 (30), 73 (100).

HPLC-DAD-ESI-MS^n Analysis of Anthocyanins. HPLC separation and identification of anthocyanins were performed on an Agilent 1100 series system (Agilent, Germany), equipped with DAD (G1315B) and 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 grape berry extracts were diluted with 0.1 N HCl before injection (1:10, v/v). The diluted extracts 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, Waldbronn, Germany), thermostated at 40 °C.

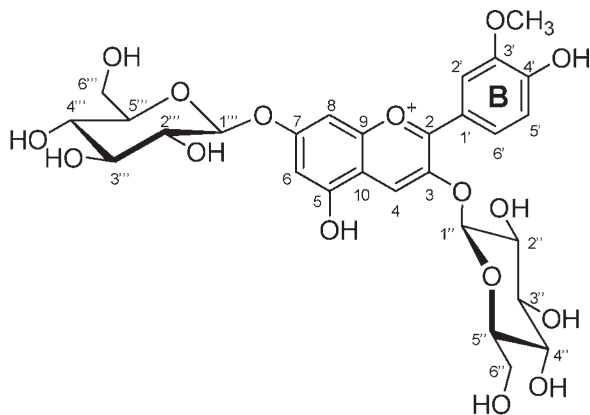

Figure 1. Structure of peonidin 3,7-*O*-β-diglucoside isolated from Garnacha Tintorera grapes.

The chromatographic conditions were adapted from the OIV method for analysis of anthocyanins in red wines (*7*). 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, *m*/*z* 50−1200. DAD chromatograms were extracted at 520 nm for characterization of online DAD-UV−vis spectra of anthocyanins.

^{1}H and ^{13}C Nuclear Magnetic Resonance. The assignment of the proton (^{1}H) and carbon (^{13}C) peaks was done by ^{1}H NMR, ^{1}H−^{1}H COSY, selective 1D-TOCSY, ^{1}H−^{13}C HSQC, and ^{1}H−^{13}C HMBC experiments in CD_3OD/CF_3COOD (95:5, v/v). The NMR experiments were carried out using a Varian Inova NMR spectrometer operating at 499.772 MHz for ^{1}H and at 125.678 MHz for ^{13}C. The spectrometer was equipped with a gradient amplifier and a four-nucleus 5 mm 1H{^{15}N−^{31}P}PFG high-field indirect detection probe. All 1D and 2D experiments (COSY, selective 1D-TOCSY (15−150 spin-lock time), HSQC, and HMBC) were performed at 298 K using standard pulse sequences from the Varian library.

RESULTS AND DISCUSSION

Isolation and Purification of Peonidin 3,7-*O*-β-Diglucoside from Garnacha Tintorera Grapes. Peonidin 3,7-*O*-β-diglucoside (**Figure 1**) is a very minor compound occurring in Garnacha Tintorera grapes (**Figure 2A**). Hence, for its successful isolation from grape extracts a concentration step is required. HSCCC has been successfully used for the enrichment, fractionation, and isolation of natural products including grape and wine anthocyanins and anthocyanin-related red wine pigments (*6*, *8*−*13*). This chromatographic preparative technique allows the processing of relatively high sample amounts but sometimes does not provide sufficiently pure compounds. Thus, a further purification step, usually by preparative HPLC, has to be performed. In our case, the complexity of the initial grape extract and the very low concentration of the compound of interest did not allow an acceptable separation (**Figure 3**). The desired peonidin dihexoside eluted too early in the three minor fractions F2, F3, and F4 that partially overlapped with the first fraction (F1), mainly containing polymeric pigments as shown in the HPLC chromatogram of fraction F3 (**Figure 2B**). Further preparative HPLC of these fractions did not yield sufficient amounts of the pure peonidin dihexoside to achieve its structure elucidation. However, the fractionation by HSCCC led to an enrichment of very minor and polar compounds present in the grape phenolic extract and thus facilitated their detection (**Figure 2B**). In addition to the 3,7-diglucoside and the 3,5-diglucoside of peonidin, already detected in phenolic

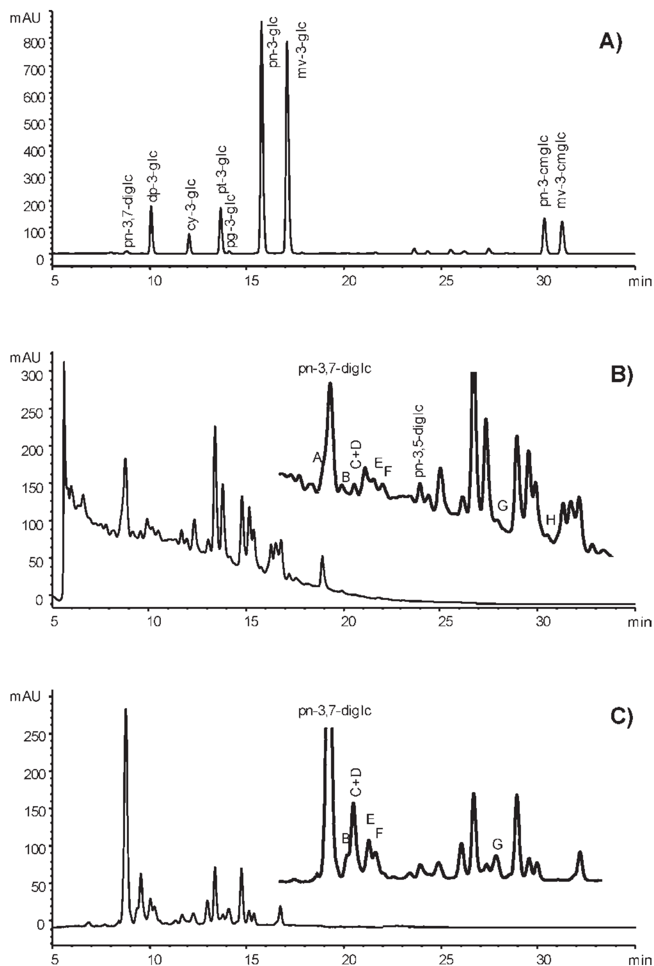

Figure 2. HPLC-DAD-chromatograms (detection at 520 nm) of (**A**) Garnacha Tintorera grape extract, (**B**) fraction F3 separated by HSCCC from Garnacha Tintorera grape extract, and (**C**) fraction enriched in peonidin 3,7-*O*-β-diglucoside obtained by CC on Amberlite XAD-7 from the Garnacha Tintorera grape extract. Capital letters in expanded chromatograms **B** and **C** indicate minor anthocyanidin dihexosides and hexosylhexosides detected in these chromatographic fractions (see **Table 2**). Abbreviations: dp, delphinidin; cy, cyanidin; pt, petunidin; pn, peonidin; pg, pelargonidin; mv, malvidin; glc, glucoside; cmglc, 6″-cumaroylglucoside.

extracts of Garnacha Tintorera grape berries, other anthocyanin derivatives linked to two hexose units (peaks A−H in **Figure 2B**) could now be detected (**Table 1**), which were not previously found by direct analysis of nonfractionated grape extracts (*3*). On the basis of the LC-MS^n data the newly detected anthocyanins seem to possess different substitution patterns, that is, anthocyanidin dihexosides, having two independent hexose units linked to different positions of the flavylium cation (peaks A−F). This was concluded from their MS^2 spectra exhibiting signals for two successive losses of hexoses ($[M - 162]^+$ and $[M - 324]^+$). Further newly detected compounds were anthocyanidin hexosylhexosides, having two linked hexose units, and the resulting disaccharide is attached to only one position of the flavylium cation (peaks G and H). It is assumed that the latter could be 3-(2″-glucosyl)-glucosides (or 3-sophorosides) because practically only one signal ($[M - 324]^+$) was observed in the MS^2 spectra. The expected signal attributable to the loss of the hexosyl moiety linked to the hexoside ($[M - 162]^+$) exhibited a very low intensity (around 2%). The aforementioned assignations are further discussed and referenced in the section dealing with the structure elucidation of peonidin 3,7-*O*-β-diglucoside. None of

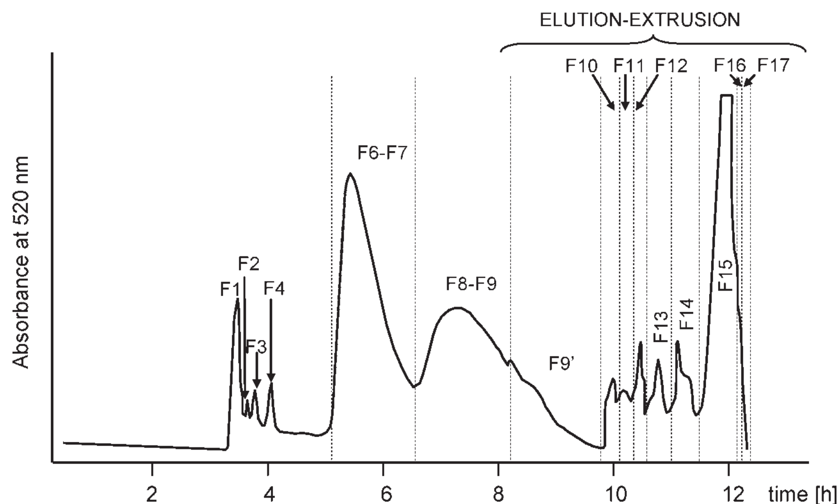

Figure 3. HSCCC chromatogram (detection at 520 nm) of Garnacha Tintorera grape extract. Fractions F2 to F4 contained peonidin 3,7-*O*-β-diglucoside.

Table 1. HPLC Chromatographic (Retention Time, t_R) and ESI-MS^n Data (*m*/*z* Values) of the Minor Anthocyanins with Two Hexoses Detected in Some Fractions (F2, F3, and F4) Collected by HSCCC of Garnacha Tintorera Grape Phenolic Extract

peak	t_R (min)	assignation	MS *m*/*z*	MS^2 (% rel intensity) *m*/*z*
A	8.6	cyanidin dihexoside	611	449 (53), 287 (100)
pn-3,7-diglc	8.9	peonidin 3,7-diglucoside	625	463 (63), 301 (100)
B	9.2	delphinidin dihexoside	627	465 (45), 303 (100)
C	9.6	malvidin dihexoside 1[a]	655	493 (71), 331 (100)
D	9.7	peonidin dihexoside	625	463 (86), 301 (100)
E	10.0	malvidin dihexoside 2[a]	655	493 (95), 331 (100)
F	10.6	petunidin dihexoside	641	479 (100), 317 (65)
pn-3,5-diglc	11.9	peonidin 3,5-diglucoside[b]	625	463 (59), 301 (100)
G	14.5	peonidin 3-hexosylhexoside	625	463 (2), 301 (100)
H	15.7	malvidin 3-hexosylhexoside	655	493 (2), 331 (100)

[a] No matching with a true standard of malvidin 3,5-diglucoside. [b] Matching with a true standard of peonidin 3,5-diglucoside.

the two newly detected malvidin dihexosides or the cyanidin dihexoside matched with true standards of malvidin 3,5-glucoside or cyanidin 3,5-diglucoside, respectively. The first eluting of the two malvidin dihexosides is suggested to be malvidin 3,7-diglucoside, because the elution order of peonidin derivatives was 3,7-diglucoside, dihexoside, and 3,5-diglucoside, but the latter kind of compound was not found in the case of malvidin.

As an alternative approach for the enrichment of the so far unknown peonidin dihexoside, a fractionation by column chromatography (CC) on Amberlite XAD-7, combined with a subsequent semipreparative HPLC and solid phase extraction on consecutive ECX and C18 cartridges, was performed. In this case, the high polarity of the compound of interest allowed its elution from the XAD-7 column by adding methanol (up to 20%) to the initial washing solvent (0.05 N HCl). The collected fractions contained peonidin 3,7-*O*-β-diglucoside as the main compound (**Figure 2C**) together with some of the above-mentioned minor anthocyanidin dihexosides and hexoxylhexosides (peaks B−G in **Figure 2C**). These fractions were combined and submitted to further separation by semipreparative HPLC on analytical equipment, thus obtaining the compound of interest with very high chromatographic purity (97.3%) at 520 nm. However, the isolated fraction also contained other non-anthocyanin compounds, which were detected at 280 nm, making the interpretation of NMR spectra difficult (data not shown). These impurities were removed by successive solid phase extraction on ECX and C18 cartridges, and the resulting purified fraction showed a similar chromatographic purity at 520 nm (97.8%), but the chromatographic purity at 280 nm increased from 55.3 to 90.5% after the cleaning step.

Structure Elucidation of Peonidin 3,7-*O*-β-Diglucoside. The first evidence of the structure of peonidin 3,7-*O*-β-diglucoside was given by its ESI-MS^n spectra. From a molecular ion (M^+, MS conditions) at *m*/*z* 625, two product ions were generated in the ion trap (MS^2 conditions) by independent losses of two hexose units, giving rise to similar intense signals corresponding to $[M - 162]^+$ (*m*/*z* 463, 63% of relative intensity) and $[M - 324]^+$ (*m*/*z* 301, 100% of relative intensity). On the basis of the data published by Alcalde-Eon et al. (*14*) the identification as peonidin 3,7-diglucoside was initially considered rather unlikely because some anthocyanidin 3,7-diglucosides in red wine were found to elute after their respective 3,5-diglucoside derivatives. Also, the ESI-MS^2 spectra of suspected anthocyanidin 3,7-diglucosides (*14*) showed an intense signal corresponding to the product ion $[M - 324]^+$, whereas only a low intense signal (< 5% of relative intensity) was observed for the product ion $[M - 162]^+$. The aforementioned results were explained by the lability of the *O*-glucosidic bond at C-7 as reported by Vallejo et al. (*15*) for flavonol 3,7-diglucosides analyzed under negative ionization mode MS^2 conditions. However, what the work of Vallejo et al. (*15*) really indicated was that the signal attributed to the product ion corresponding to the loss of only the glucose moiety linked to C-7 ($[M - 162]^+$) must be very intense in the respective ESI-MS^2 spectra. Therefore, the MS^2 fragmentation pattern shown by the compound of our interest with the product ion $[M - 162]^+$ signal of 63% relative intensity could be in agreement with a 3,7-diglucoside structure, although these results were obtained using positive ionization mode conditions. Moreover, anthocyanidin 3,7-diglucosides and 3,5-diglucosides have been found in other natural sources, and their structures have been unequivocally elucidated by ^{1}H NMR data (*16*−*18*). These latter studies reported that 3,7-diglucosides of anthocyanidins (delphinidin and petunidin) eluted in HPLC reversed-phase conditions before their respective 3,5-diglucosides. A similar order of elution is therefore also likely for peonidin 3,7-diglucoside with regard to peonidin 3,5-diglucoside. Both kinds of anthocyanidin diglucosides showed the signals of the two expected product ions ($[M - 162]^+$ and $[M - 324]^+$) in their respective FAB mass spectra. On the basis of all the aforementioned data we could tentatively assign the new compound as peonidin 3,7-diglucoside.

Additional evidence for the structure of peonidin 3,7-*O*-β-diglucoside came from the DAD-UV−vis spectral data. The *O*-glucosidic substitution pattern of anthocyanidin 3-glucoside and

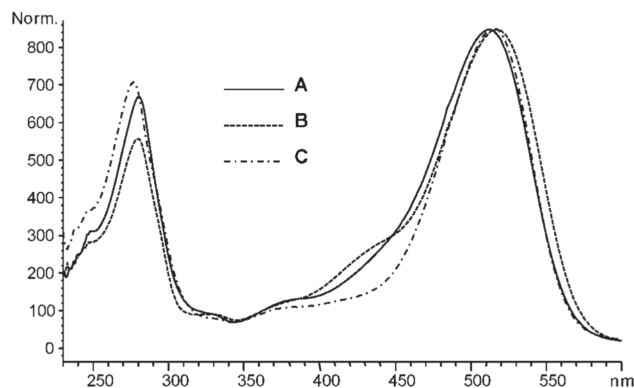

Figure 4. Online DAD-UV−vis spectra of (**A**) isolated peonidin 3,7-*O*-*β*-diglucoside, (**B**) peonidin 3-*O*-*β*-glucoside, from grape phenolic extract DAD chromatogram shown in **Figure 2A**, and (**C**) standard of peonidin 3,5-*O*-*β*-diglucoside.

anthocyanidin 3,5-diglucoside introduce well-known differences in their UV−vis spectra, mainly the disappearance of the characteristic shoulder at 440 nm and the enhancement of the absorbance around 280 nm in the case of the 3,5-diglucosides. For anthocyanidin 3,7-diglucosides Alcalde-Eon et al. (*14*) proposed the presence of the shoulder at 440 nm as proof to distinguish them from anthocyanidin 3,5-diglucosides. As the UV−vis spectra of the suggested malvidin 3,7-diglucoside completely matched that of malvidin 3-glucoside, it is more likely that these compounds were anthocyanidin 3-glycosylglycosides. In our case, the UV−vis spectra did not match those of either peonidin 3-glucoside or peonidin 3,5-diglucoside (**Figure 4**): The band at 280 nm was more intense than that of 3-glucoside but a little bit less intense than that of 3,5-diglucoside; a shoulder around 440 nm was not clearly defined as for 3-glucoside; and the vis band was not as symmetrical as for 3,5-diglucoside. The reported UV−vis spectrum registered in methanol with 0.1% HCl of delphinidin 3,7-diglucoside shows two maxima at 278 and 539 nm, together with a shoulder at 440 nm, with a ratio between the shoulder and vis maximum absorbances (E_{440}/E_{539}) of 21% (*19*); in a recent work, the measured ratio E_{440}/E_{525} for cyanidin 3,7-diglucoside was 25% in the same solvent (*20*); in our case, the HPLC-DAD online spectrum of the suggested peonidin 3,7-diglucoside showed maxima at 280 and 512 nm and a nonclear shoulder around 440 nm, with a ratio E_{440}/E_{512} of 30%.

The definitive evidence that unequivocally confirmed the structure of peonidin 3,7-*O*-*β*-diglucoside was obtained by ^{1}H and ^{13}C NMR spectroscopy (**Table 2** and **Figure 1**), including 1D and 2D experiments (selective 1D-TOCSY, ^{1}H−^{1}H COSY, ^{1}H−^{13}C HSQC, and ^{1}H−^{13}C HMBC). Prior to NMR analyses, we confirmed the nature of the hexose substituents by hydrolysis and derivatization of the released hexoses and subsequent analysis by GC-MS. The results showed that the only kind of hexose linked to our compound was glucose (**Figure 5**) and that other previously considered structures, like those in which glucose was partially or totally replaced by galactose (*3*), were not possible. By means of selective 1D-TOCSY experiments it was possible to obtain the ^{1}H chemical shifts and coupling constants (J_{HH}) of the two hexoses units. The measured value for the coupling constant (J_{HH}) between the couples of sugar protons H-1″ and H-2″ (8 Hz) and H-1‴ and H-2‴ (7 Hz) indicated a *β*-configuration for the *O*-glycosidic linkage. The assignation of both sugar moieties as glucose was based on the high values found for the coupling constants (J_{HH}) involving every consecutive couple of the sugar protons from H-1″ to H-5″ and from H-1‴ to H-5‴, ranging within 7−10 Hz. Moreover, the ^{1}H NMR data of the glucose units matched very well previously reported data of the

Table 2. ^{1}H and ^{13}C NMR Chemical Shifts (δ), Coupling Constants (J, Hz), and HMBC and HSQC Correlations of Peonidin 3,7-*O*-*β*-Diglucoside Recorded in CD_3OD/CF_3COOD (95:5, v/v)

position[a]	δ ^{1}H (J_{HH})	δ ^{13}C	HMBC	HSQC
benzopyran ring				
2		164.7	H-4	
3		146.1	H-4, H-1″	
4	9.08	134.8		H-4
5		157.5	H-4, H-6	
6	6.88 (2)	103.2		H-6
7		166.5	H-8, H1‴	
8	7.38 (2)	94.8		H-8
9		na[b]		
10		114.1	H-4	
B-ring				
1′		119.8	H-4, H-2′, H-5′	
2′	8.30 (2)	114.5		H-2′
3′		148.9	H-2′, H-5′, OCH_3	
4′		156.7	H-5′, H-6′	
5′	7.08 (9)	116.6		H-5′
6′	8.40 (9, 2)	129.0		H-6′
OCH_3	4.04	55.8		OCH_3
glucose at C-3				
1″	5.40 (8)	103.3		H-1″
2″	3.67 (9)	73.7		H-2″
3″	3.56 (9)	73.6[c]		H-3″
4″	3.42 (10)	70.2		H-4″
5″	3.60 m	76.7[c]		H-5″
6″a	3.70 m	61.3		H-6″a, H-6″b
6″b	3.94 (12)			
glucose at C-7				
1‴	5.21 (7)	101.9		H-1‴
2‴	3.53[d] m	73.6[c]		H-2‴
3‴	3.54[d] m	76.7[c]		H-3‴
4‴	3.41 (10)	70.2		H-4‴
5‴	3.63 (9)	77.8		H-5‴
6‴a	3.71 (12, 6)	61.4		H-6‴a, H-6‴b
6‴b	3.97 (12)			

[a] Position numbering as in **Figure 1**. [b] na, not assigned. [c] Signal assignations may be interchanged. [d] Signal assignations may be interchanged.

3,7-diglucosides of delphinidin and petunidin (*18*, *19*). According to the latter works, the assignation of the anomeric proton H-1″ from the glucose linked to C-3 corresponded to the higher value of chemical shift (5.40 ppm), whereas the assignation of H-1‴ from the glucose linked to C-7, corresponded to the lowest value (5.21 ppm). The chemical shift of H-2″ (3.67 ppm) was similarly affected in comparison to that of H-2‴ (3.53 ppm). The B-ring substitution pattern of peonidin was confirmed by both ^{1}H and ^{13}C NMR data, which were in agreement with previously reported data of different peonidin-type anthocyanins (*20*−*22*). With regard to the benzopyran ring, a comparative analysis of the reported data for the 3-glucosides and 3,5-diglucosides of anthocyanidins (*21*−*24*) showed that C-5 is shielded 2−3 ppm in the case of 3,5-diglucoside (around 156.5 vs 159.2 ppm for 3-glucosides), whatever the kind of substitution pattern in B-ring was. In our case, the chemical shift assigned to C-5 (157.5 ppm) matched well to nonglucosylated anthocyanins at C-5. On the basis of the latter results, we could expect a similar shielding caused by the *O*-glucosidation at C-7. In fact, the chemical shift assigned to C-7 of peonidin 3,7-*O*-*β*-diglucoside was 166.5 ppm, which is in good agreement with previously reported data for C-7 (166.9 ppm) of delphinidin 3,7-diglucoside (*20*). Data reported for nonglucosylated anthocyanins at C-7 (*21*−*23*) were around 170.0 ppm (shielding of 3.5 ppm). Finally, the ^{1}H−^{13}C HMBC experiment allowed us to observe key correlations (**Table 2**) that definitively confirmed the assignation suggested for C-3, C-5,

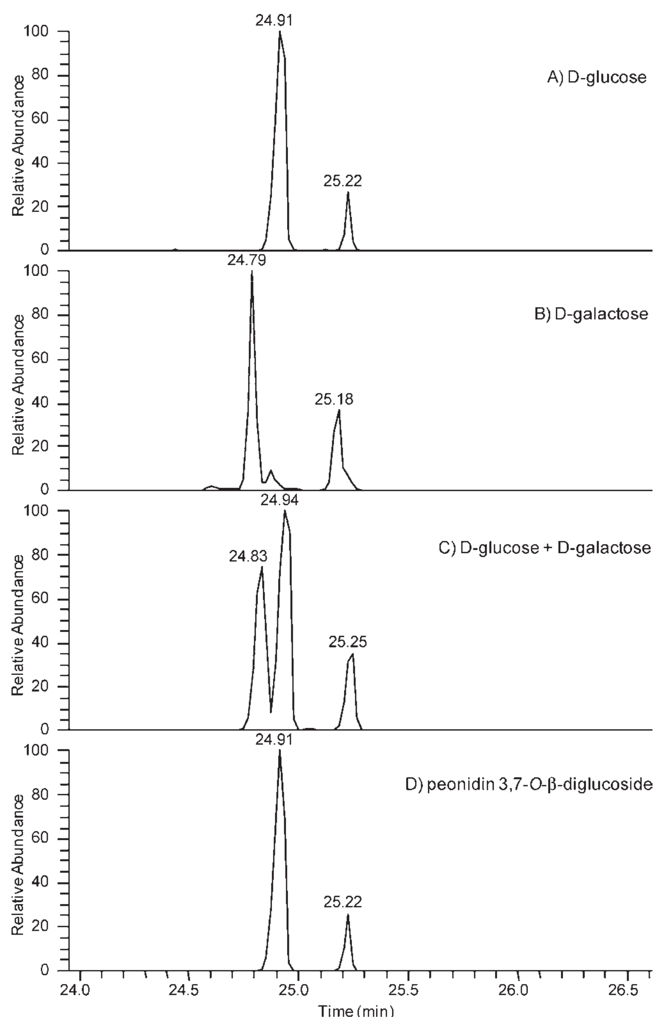

Figure 5. GC-MS chromatograms of derivatized sugar analysis released from (**A**) standard of D-glucose, (**B**) standard of D-galactose, (**C**) mixture of standards of D-glucose and D-galactose, and (**D**) purified peonidin 3,7-*O*-*β*-diglucoside after acidic hydrolysis.

and C-7 and, consequently, for the *O*-glucosylation positions: C-3 correlated with H-4 and H-1″; C-5 correlated with H-4 and H-6; and C-7 correlated with H-8 and H-1‴.

In this work we have unambiguously demonstrated the occurrence of peonidin 3,7-*O*-*β*-diglucoside in Garnacha Tintorera grapes and wines. Anthocyanidin 3,5-diglucosides have been reported as minor compounds in *V. vinifera* grapes, and these kinds of anthocyanins are usually major compounds in non-*vinifera* grapes. However, as far as we know, anthocyanidin 3,7-diglucosides were never found in *V. vinifera* grapes and wines, and the only known report suggesting their occurrence in Tempranillo wines (*14*) was not based on definitive evidence. Known anthocyanidin 3,7-diglucosides comprise some derivatives of cyanidin, delphinidin and petunidin found in flowers (*18*−*20*), being peonidin 3,7-*O*-*β*-diglucoside a pigment that has not been properly characterized before in any natural plant source. In this paper, we are now reporting for the first time on chromatographic and spectroscopic data of peonidin 3,7-*O*-*β*-diglucoside. The occurrence of peonidin 3,7-*O*-*β*-diglucoside together with previously reported pelargonidin-based anthocyanins (*3*) in Garnacha Tintorera grapes and wines offers a unique chance for authentication purposes. These anthocyanins can be suggested as chemical markers for this grape cultivar and its wines. Bearing in mind that Garnacha Tintorera wines are frequently blended with other red wines for enhancing its red color, the detection of such anthocyanins could indicate the blending with Garnacha Tintorera wine. The latter suggestion obviously needs confirmation that the aforementioned anthocyanins do not occur in other *V. vinifera* grape cultivars. In addition, due to the expected low concentration of the suggested markers, special techniques of extraction/concentration or very sensitive MS-based analysis may be needed to make them useful markers for authentication purposes.

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