Identification of Anthocyanin-Flavanol Pigments in Red Wines by NMR and Mass Spectrometry

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Three newly formed Port wine pigments were isolated by Toyopearl HW-40(s) gel chromatography and semipreparative HPLC. Furthermore, the pigments were identified by mass spectrometry (LC/MS) and NMR techniques (1D and 2D). These anthocyanin-derived pigments showed UV−visible spectra different from those of the original grape anthocyanins. These pigments correspond to malvidin 3-glucoside linked through a vinyl bond to either (+)-catechin, (−)-epicatechin, or procyanidin dimer B3 [(+)-catechin-(+)-catechin]. NMR data of these pigments are reported for the first time.

INTRODUCTION

Anthocyanins constitute a large family of polyphenols widespread in plants and are responsible for many of the fruit and floral colors observed in nature. They are the main polyphenolic compounds that contribute to the color in red wines. The color evolution of red wines is a complex process that is in part attributed to copigmentation phenomena (*1−3*) and to the progressive displacement of the original anthocyanins by newly formed pigments. These pigments usually arise from the interaction between anthocyanins and other phenolic compounds, especially flavan-3-ols such as catechins and procyanidins (condensed tannins). Different mechanisms have been suggested to explain the formation of these new pigments. Processes such as direct reaction between anthocyanins and flavanols (*4−8*), reaction between anthocyanins and flavanols through ethyl bridges (*9−15*), or, more recently, the reaction between anthocyanins and other small compounds such as glyoxylic acid (*16*), vinylphenol (*17, 18*) and pyruvic acid (*19−21*) have already been demonstrated in model solutions. All these events result in the formation of more stable pigments that stabilize wine color, changing it to a more brick-red hue. The major studies have been performed in wine-model solutions, because red wines have a highly complex chemistry that makes it difficult to isolate new pigments, which can be obtained only in very small quantities. ^{1}H and ^{13}C NMR analyses of anthocyanin-derived pigments isolated from red wine have only recently appeared in the literature, owing to the difficulty in obtaining analyzable spectra, and have been reported to be pyruvic acid adducts of original grape anthocyanins (*19, 21, 22*). The present work deals with the structural characterization of two new colored derivatives with the same mass (*m/z* 805) and another pigment with the same mass (*m/z* 1093) as a pigment previously detected by Francia-Aricha et al. in model solutions (*13*). The latter has been shown to result from the reaction of procyanidin dimer B2 with malvidin 3-glucoside in the presence of acetaldehyde.

MATERIALS AND METHODS

Source. The pigments were extracted from a monovarietal Touriga Nacional (*Vitis vinifera*) Port wine with two years of oak aging (pH 3.6, 18.5% alcohol (v/v), total acidity 6.5 g·L^{-1}, total SO$_2$ 20 mg·L^{-1}), made from grapes of the Douro Demarcated Region (Portugal).

Pigment Purification. Port wine samples were directly applied on a 250 × 16 mm i.d. Toyopearl HW-40(s) gel column (Tosoh, Japan). Flow rate was regulated at 0.8 mL/min using a peristaltic pump. A first elution was performed with 20% aqueous ethanol yielding anthocyanidin 3-glucosides and pyruvic acid adducts (*22*). When practically no more colored compounds were eluted from the column, the solvent was changed to 40% aqueous ethanol yielding three major pigments. These three pigments were then purified by semipreparative HPLC on a 250 × 4.6 mm i.d. C18 ODS column (Merck, Darmstadt) with an injection volume of 500 μL. The pigments were then collected, concentrated under vacuum, and applied on a 150 × 16 mm i.d. Toyopearl HW-40(s) gel column (Tosoh, Japan), which was eluted with aqueous 40% methanol for a final purification of the product.

HPLC Conditions. The fraction eluted with 40% methanol in water from the Toyopearl gel column was analyzed by HPLC using the above C18 ODS column (250 × 4.6 mm i.d.), and detection was carried out at 520 nm using a diode array detector (Merck-Hitachi L-7450A) (**Figure 1**). The solvents were A, H$_2$O/HCOOH (9:1), and B, CH$_3$CN/H$_2$O/HCOOH (3:6:1). The gradient consisted of 20−85% B for 70 min, 85−100% B for 5 min, and then isocratic for 10 min at a flow rate of 1 mL/min (*10*).

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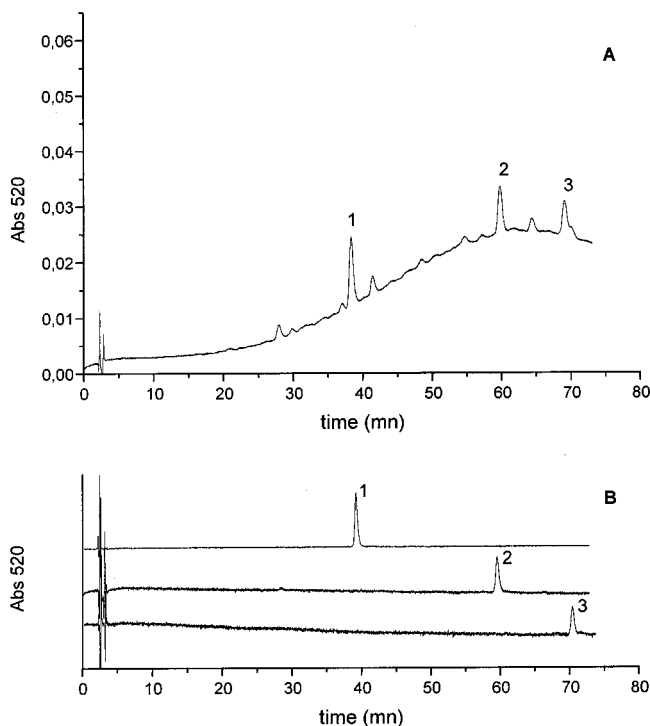*J. Agric. Food Chem.*, Vol. 50, No. 7, 2002   **2111**

Figure 1. HPLC chromatogram recorded at 520 nm of the fraction eluted from Toyopearl gel column with 40% methanol in water, showing three major peaks, 1, 2, and 3, before the HPLC semipreparative purification (A), and after the HPLC semipreparative and final Toyopearl gel column purification (B).

MS Analyses. Mass spectrometry was performed using a Finnigan LCQ equipped with an API source, using an electrospray ionization (ESI) probe. Pigments 1, 2, and 3 were injected directly into the MS spectrometer with a pump at a flow rate of 3 μL/min. The capillary temperature and voltage used were 180 °C and 3V, respectively, and spectra were obtained in positive ion mode. When the molecular ion of the pigment was detected, its MS^2 spectrum was obtained using a relative energy of collision of 20. In the case of pigments 2 and 3, MS^3 of the main fragment ion in the MS^2 was also obtained using a relative energy of collision of 30.

NMR Analysis. ^{1}H NMR (500.13 MHz) and ^{13}C NMR (125.77 MHz) spectra were measured in CD$_3$OD/TFA (98:2) on a Bruker-AMX500 spectrometer at 303 K and with TMS as internal standard. ^{1}H chemical shifts were assigned using 1D and 2D ^{1}H NMR (gCOSY), while ^{13}C resonances were assigned using 2D NMR (gHMBC and gHSQC) techniques (*23, 24*). The delay for the long-range C/H coupling constant was optimized to 7 Hz.

RESULTS AND DISCUSSION

Isolation. Elution of Port wine from a Toyopearl gel column with 20% aqueous ethanol yielded the original anthocyanidin 3-glucosides and some pyruvic acid adducts of the three major anthocyanidin 3-glucosides (malvidin 3-glucoside, malvidin 3-acetylglucoside, and malvidin 3-coumaroylglucoside), as previously reported (*22*). Furthermore, another elution was performed with 40% aqueous ethanol, yielding three major pigments (**Figure 1**). The retention times during RP-HPLC of these three compounds were found to be different from the ones of the pyruvic acid adducts previously identified. The UV–Vis spectra of these pigments recorded from the HPLC diode array detector are shown in **Figure 2**. The λ_{max} of the three pigments were hypsochromically shifted from the λ_{max} of original grape anthocyanins. The λ_{max} of peak 1 was situated around 512 nm, while the other two pigments had a λ_{max} at 503
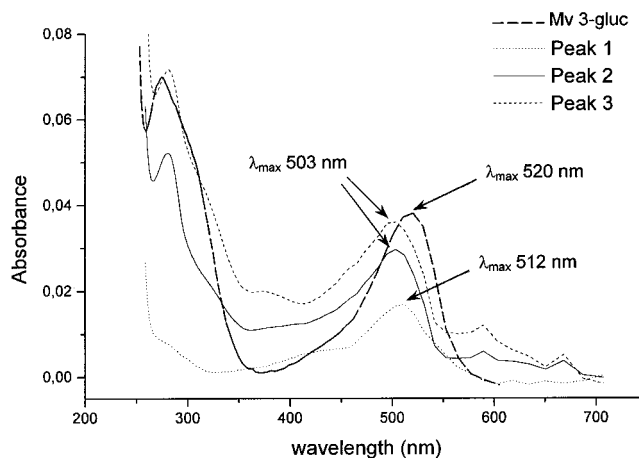

Figure 2. UV–Vis spectra of malvidin 3-glucoside and peaks 1, 2, and 3 recorded from HPLC diode array detector, showing the λ_{max} values for malvidin 3-glucoside ($\lambda_{max} = 520$ nm), peak 1 ($\lambda_{max} = 512$ nm), and peaks 2 and 3 ($\lambda_{max} = 503$ nm).
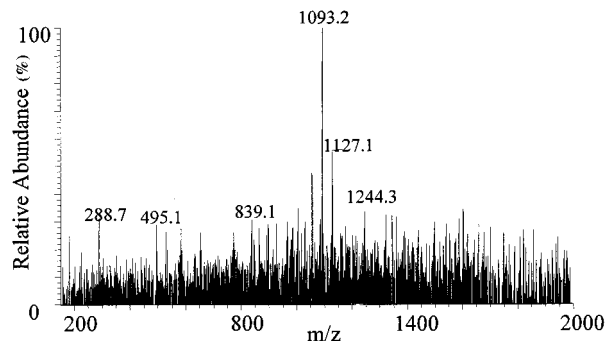

Figure 3. MS analysis of peak 1 performed with an ion spray source in the positive-ion mode.

nm, which is similar to the λ_{max} of the pyruvic acid adducts (*22*).

Mass Spectrometry. After a final purification through Toyopearl gel, the three pigments obtained were analyzed individually by MS using direct injection into the spectrometer. Pigment 1 revealed a [M]$^+$ ion at *m/z* 1093 (**Figure 3**) which fits exactly with the mass of the structure shown in **Figure 4**. MS^2 of the molecular ion yielded two fragments at *m/z* 931 (loss of a glucose residue) and at *m/z* 803, that results from the loss of a catechin monomer. A pigment with the same mass was previously detected by Francia-Aricha et al. in wine-model solutions and was obtained from the reaction of procyanidin dimer B2 with malvidin 3-glucoside in the presence of acetaldehyde (*13*). The mass spectrometric data obtained for pigments 2 and 3 were identical. A molecular ion [M]$^+$ was found at *m/z* 805 (**Figure 5**) which fits with the structure shown in **Figure 7**. This structure is similar to the one proposed for peak 1, except for the flavanol moiety that consists of a catechin monomer instead of a procyanidin dimer. MS^2 released a major fragment at *m/z* 643 corresponding to the loss of a glucose moiety. MS^3 analysis of this fragment yielded a major ion peak at *m/z* 491, which probably resulted from the retro Diels–Alder decomposition of the aglycon (**Figure 6**).

^{1}H NMR. The proton NMR chemical shifts of peaks 1, 2, and 3 in CD$_3$OD/TFA (98:2) are shown in **Table 1** and **Table 2**. All the aromatic protons were easily assigned for peak 2 and peak 3, whereas those of peak 1 were more difficult to identify because of the low quantities of product and the crowded aromatic region of the spectrum. The ^{1}H chemical shifts were attributed using one and two-dimensional analyses (gCOSY).
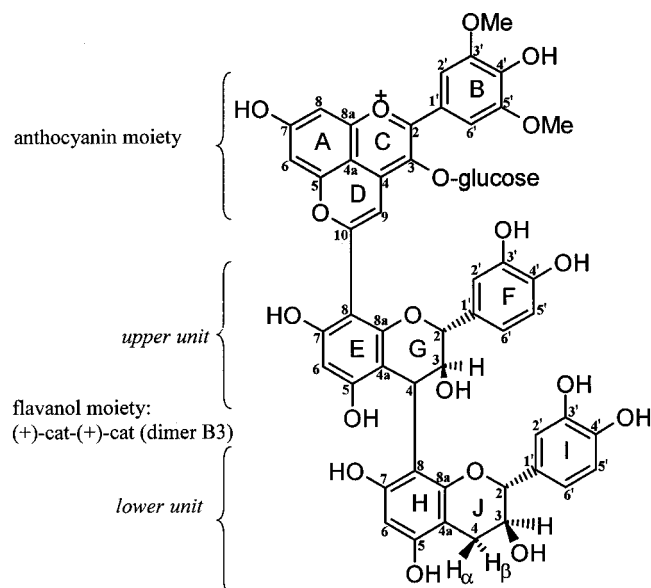
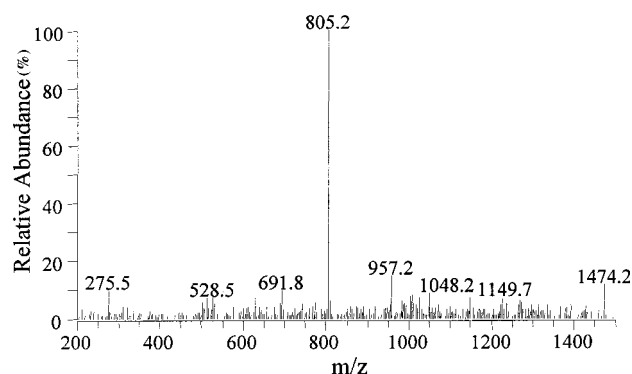**Figure 4.** Structure of peak 1 (flavylium structure, *m/z* 1093).

Figure 5. MS analysis of peak 2 with an ion spray source in the positive-ion mode. MS analysis of peak 3 yielded the same data.

Figure 6. Structural hypothesis for the formation of the fragment ion (*m/z* 491) resulting from peaks 2 and 3.

Figure 7. Structure of peak 3 (flavylium structure) showing the long range ^1H–^{13}C correlations found in the HMBC spectrum.

Peak 1 − Malvidin 3-glucoside-vinyl-(+)-catechin-(+)-catechin. The spectrum of peak 1 showed the presence of H-2′,6′ and two methoxyl groups of the B ring, which were located at 7.51 and 3.97 ppm, respectively. The protons H-6 and H-8 of the A ring were assigned to the two broad singlets located at 6.89 and 7.06 ppm respectively and the proton H-9 of the D ring was located at 7.53 ppm. These proton chemical shifts of the flavylium moiety are consistent with those of malvidin-pyruvic acid adduct previously reported (*19*, *22*).

With respect to the flavanol moiety, the protons H-4α and H-4β of ring J were easily assigned to the double doublet at 3.01 ppm and to an unresolved peak at 2.35 ppm through the characteristic AMX spin system of the flavanol pyran ring observed in the COSY spectrum. The proton H-3J was assigned to the multiplet at 3.10 ppm from its weak (4αJ) or strong (4βJ) correlation with the protons H-4αJ and H-4βJ. H-3J also correlates with H-2J, which was thus assigned to the doublet at 5.08 ppm (J = 3.6 Hz). The analogous protons of the upper unit of the flavanol moiety were attributed using similar correlations. The proton H-3G was attributed to an unresolved peak at 4.30 ppm that correlates in the COSY spectrum with a doublet at 4.45 ppm (J = 7.9 Hz), which was attributed to H-2G, and with an unresolved peak around 3.41 ppm, which was attributed to H-4G. The ^1H NMR data obtained for the G and J rings led us to suppose that the flavanol moiety consisted of two (+)-catechin units linked through an interflavanoid bond. In the literature, the relative 2,3-stereochemistry is usually
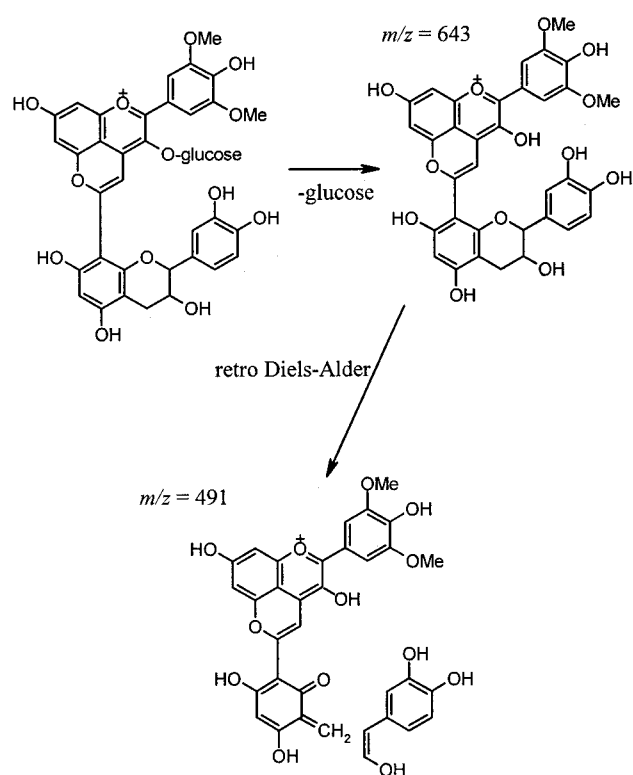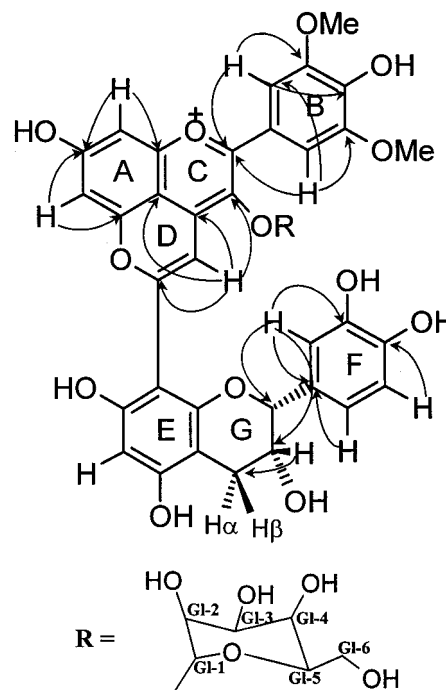
deduced from the coupling constant of the H-2 proton. Indeed, a large doublet (8−10 Hz) indicates a catechin moiety, whereas a broad singlet indicates an epicatechin moiety (*25*). Likewise, if the H-4 proton resonates as a large doublet, the relative 3,4-stereochemistry is concluded to be trans, whereas observation of a broad singlet does not allow the stereochemistry to be determined (*26*). The small observed $J_{2J,3J}$ coupling constant (3.6 Hz) may be the result of a distortion of the J ring, modifying the usual dihedral angles (*27*). The interflavanoid linkage (C4−

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Table 1. ^{1}H Assignments of Peak 1 Isolated from Two-Year-Old Port Wine, Determined in CD_3OD/TFA (98:2)^a

position	$\delta\,^1$H; *J*(Hz)
anthocyanin moiety	
6A	6.89; *bs*
8A	7.06; *bs*
2′B, 6′B	7.51; *s*
OMe	3.97; *s*
9	7.53; *s*
flavanol moiety	
2G	4.45; *d*, 7.9
2J	5.08; *d*, 3.6
3G	4.30; *
3J	3.10; *m*
4G	3.41; *
4αJ	3.01; *dd*, 8.10/16.15
4βJ	2.35; *
6E	6.6–6.9;*
6H	6.6–6.9;*
2′F	6.81;*
2′I	6.6–6.9;*
5′F	6.76;*
5′I	6.6–6.9;*
6′F	6.73;*
6′I	6.6–6.9;*
sugar moiety	
Gl-1	4.85; *d*,7.6
Gl-2	3.60;*
Gl-3	3.14;*
Gl-4	na
Gl-5	na
Gl-6a	na
Gl-6b	na

^{a} Key: *, unresolved; bs, broad singlet; s, singlet; m, multiplet; d, doublet; dd, double doublets; na, not attributed.

C8 or C4–C6) cannot be fully ascertained from the data yielded from the NMR experiments. Nevertheless, a C4–C8 inter-flavanoid linkage is expected for peak 1, as the C4–C8 procyanidin dimers (B1 to B4) are more abundant in grapes and resulting Port wines than their respective C4–C6 counterparts (B5 to B8) (*28, 29*).

Finally, the protons of the E, F, H, and I rings appeared to be difficult to assign, because all the crucial signals fell into a crowded area comprising the protons H-6E, H-6H, H-2′F, H-2′I, H-5′I, H-5′F, H-6′I, and H-6′F situated between 6.6 and 6.9 ppm. The protons H-2′F, H-5′F, and H-6′F were attributed to unresolved peak signals at 6.81, 6.76, and 6.73 ppm, respectively, although these protons are not conclusively assigned to the upper or lower catechin unit.

For the glucosyl moiety, the anomeric proton chemical shift was found in the region of 4.85 ppm as a doublet with a large coupling constant (7.6 Hz). This coupling constant is characteristic of a β configuration of the sugar moiety. The proton Gl-2 at 3.60 ppm was attributed from its correlation with the anomeric proton (Gl-1), and the proton Gl-3 was assigned at 3.14 ppm from its correlation with Gl-2. The other glucosyl proton signals were situated in the complex region of 3.0–3.7 ppm, mostly masked by the methanol peak.

Peak 2 – Malvidin 3-glucoside-vinyl-(+)-catechin. The protons H-2′,6′ and the two methoxyl groups of the B ring were situated at 7.60 and 3.95 ppm, respectively. The protons H-6 and H-8 of the A ring were assigned to the two doublets with a low coupling constant (*J* = 1.8 Hz) at 6.92 and 7.12 ppm, respectively. The proton H-9 of the D ring was assigned to the singlet peak at 7.62 ppm.

For the flavanol moiety, all the protons were also easily assigned. The two protons H-4αG and H-4βG were attributed

Table 2. ^{1}H and ^{13}C Assignments of Peak 2 and Peak 3 Isolated from Two-Year-Old Port Wine, Determined in CD_3OD/TFA (98:2)^a

	peak 2	peak 3	
position	$\delta\,^1$H; *J*(Hz)	$\delta\,^1$H; *J*(Hz)	$\delta\,^{13}$C
anthocyanin moiety			
2C	-	-	162.0
3C	-	-	135.0
4C	-	-	107.9
4aA	-	-	108.1
5A	-	-	146.5
6A	6.92; *d*, 1.8	7.03; *d*, 1.8	100.6
7A	-	-	168.8
8A	7.12; *d*, 1.8	7.18; *d*, 1.8	100.9
8aA	-	-	152.7
9	7.62; *s*	7.68; *s*	106.8
10	-	-	162.8
1′B	-	-	121.5
2′B, 6′B	7.60; *s*	7.62; *s*	108.6
3′B, 5′B	-	-	148.5
4′B	-	-	142,0
OMe	3.95; *s*	4.00; *s*	58.1
flavanol moiety			
2G	4.85; *d*, 7.6	5.00; *s*	84.1
3G	4.11; *m*	4.29; *m*	68.0
4αG	2.85; *dd*, 5.6/16.1	2.96; *dd*, 5.1, 17.5	29.0
4βG	2.66; *dd*, 6.9/16.1	2.83; *dd*, 2.3, 17.5	29.0
4aE	-	-	na
5E	-	-	na
6E	6.20; *s*	6.21; *s*	108.7
7E	-	-	na
8E	-	-	na
8aE	-	-	na
1′F	-	-	122.4
2′F	6.88; *bs*	7.00; *bs*	115.5
3′F	-	-	146.5
4′F	-	-	146.3
5′F	6.76; *bd*, 8.1	6.80; *bd*, 8.1	116.1
6′F	6.80; *bd*, 8.1	6.88; *bd*, 8.1	119.5
sugar moiety			
Gl-1	4.75; *d*, 7.7	4.75; *d*, 7.7	104.1
Gl-2	na	3.62; *	72.6
Gl-3	na	3.21; *t*, 9.4	72.2
Gl-4	na	3.13; *	67.2
Gl-5	na	3.06; *	72.6
Gl-6a	na	3.68; *dd*, 10.6/1.5	63.2
Gl-6b	na	3.39; *	63.2

^{a} Key: *, unresolved; bd, broad doublet; s, singlet; m, multiplet; d, doublet; dd, double doublets; t, triplet; na, not attributed.

to the double doublets at 2.85 ppm for H-4αG (*J* = 5.1, 16.1 Hz) and 2.66 ppm for H-4βG (*J* = 6.9, 16.1 Hz) through the characteristic AMX spin system of the pyran ring G observed in the COSY spectrum. The proton H-3G was assigned to the multiplet at 4.11 ppm from its weak (4αG) or strong (4βG) correlation with the protons H-4αG and H-4βG. H-3G also correlates with H-2G, which was thus assigned to the doublet at 4.85 ppm (*J* = 7.6 Hz). Similarly to malvidin 3-glucoside-vinyl-(+)-catechin-(+)-catechin, the monomeric unit of the flavanol moiety was found to be (+)-catechin because the proton H-2G resonates as a large doublet. This multiplicity results from the coupling with H-3G, suggesting a trans configuration relative to H-3G. The only proton of the E ring, H-6E, was attributed to the singlet at 6.20 ppm. Finally, the spectrum of peak 2 showed the presence of the protons of the F ring with H-2′F assigned to a broad singlet at 6.88 ppm, and the protons H-5′F and H-6′F attributed to broad doublets (*J* = 8.1 Hz) at 6.76 and 6.80 ppm, respectively. With respect to the glucosyl moiety, the anomeric proton chemical shift was detected at 4.75 ppm as a doublet with a large coupling constant (7.7 Hz) suggesting
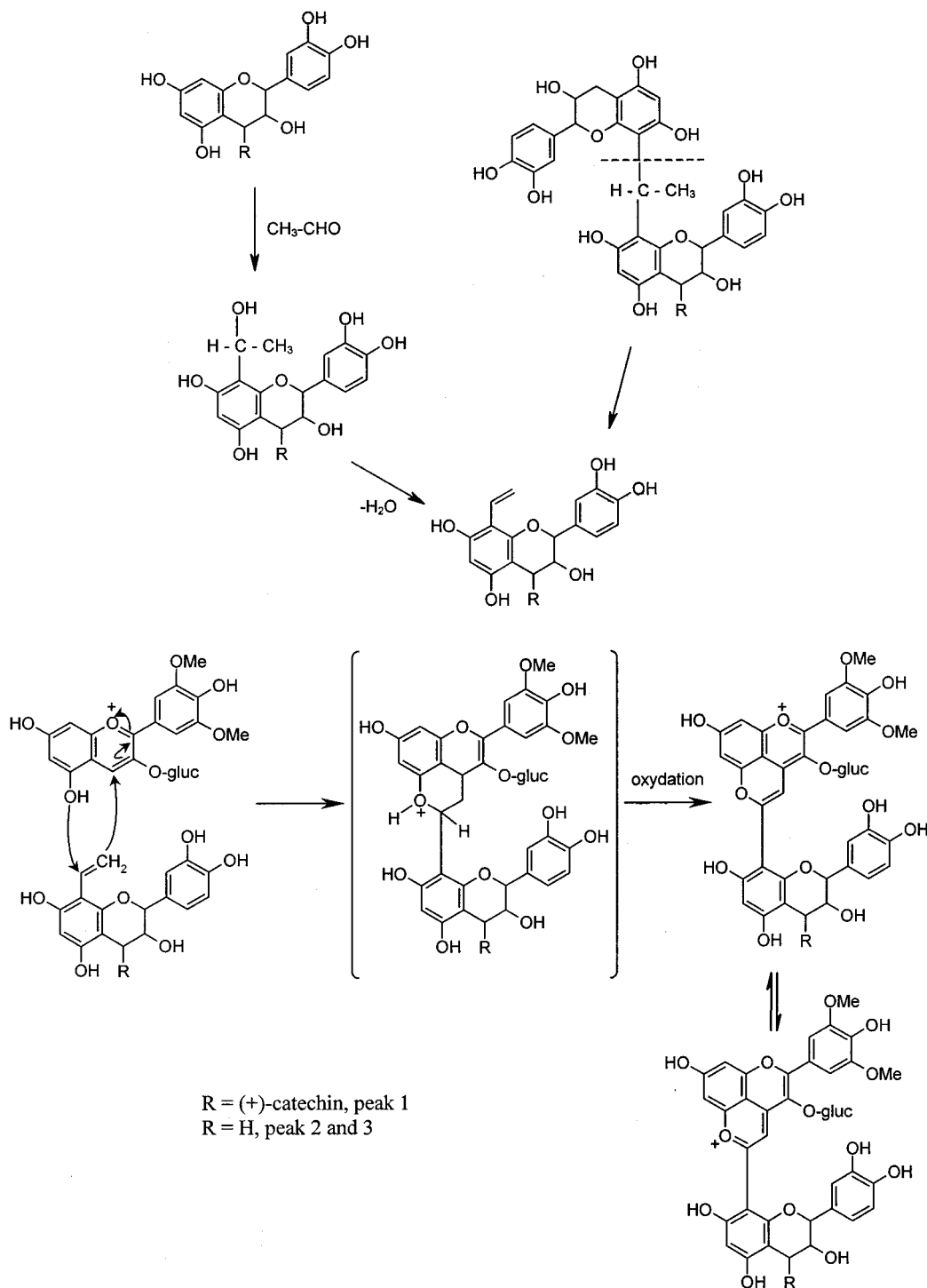
R = (+)-catechin, peak 1
R = H, peak 2 and 3

Figure 8. Mechanisms proposed for the formation of peaks 1, 2, and 3.

a β configuration of the sugar moiety. All the other glucosyl proton signals were situated in the region of 3.0–3.7 ppm, mostly masked by the methanol peak.

Peak 3 − Malvidin 3-glucoside-vinyl-(−)-epicatechin. Peak 3 was the pigment obtained in higher amounts, which allowed an easier attribution of all the protons (**Table 2**). The spectrum of peak 3 is extremely similar to the one of peak 2 already described above. The main difference is in the flavanol moiety. Thus, the proton H-2G was assigned to a singlet at 5.00 ppm, whereas the proton H-2G of peak 2 resonates as a doublet at 4.85 ppm. This feature suggests that the proton H-3G (assigned to the multiplet at 4.29 ppm) is weakly coupled with H-2G, suggesting a cis configuration of H-2G and H-3G. A strong

correlation between H-3G and H-4αG (2.96 ppm) and a weak one between H-3G and H-4βG (2.83 ppm) was observed in the COSY spectrum. The monomeric catechin unit of the flavanol moiety is thus attributed to (−)-epicatechin.

The anomeric proton resonance was detected at 4.75 ppm as a doublet with a large coupling constant (7.7 Hz) suggesting a β configuration. The proton Gl-2 was attributed from its correlation with Gl-1 at 3.62 ppm. The proton Gl-3 was assigned to a triplet located at 3.21 ppm (J = 9.4 Hz), whereas Gl-4 and Gl-5 were assigned to unresolved peaks around 3.13 and 3.06 ppm, respectively. The two protons of Gl-6 were attributed to an unresolved peak at 3.39 ppm and to a double doublet at 3.68 ppm (J = 10.6, 1.5 Hz).

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¹³C NMR. The assignment of most of the carbon resonances was only possible for peak 3, which was obtained in sufficient amounts, and were obtained using two-dimensional (HSQC and HMBC) techniques. Correlations found in the HMBC spectrum of peak 3 are shown in **Figure 7**, and the most probable structure was deduced although five carbons could not be fully assigned. The correlation observed between the methoxyl proton resonances and that of the carbons at 148.5 ppm allowed their assignment to C-3′ and C-5′. C-10 was assigned at 162.8 ppm through its HMBC correlation with H-9. Despite the fact of having similar chemical shifts, carbon C-4 was differentiated from C4a because of the $^3J_{C,H}$ coupling of the latter with H-6. The carbon C-9 was assigned at 106.8 ppm through HSQC correlation with H-9. These ¹³C NMR data are in good agreement with the ones obtained for malvidin-pyruvic acid adduct previously reported (*19, 22*).

For the flavanol moiety, the carbons C-2′, C-5′, and C-6′ of the F ring were assigned from their direct ¹H−¹³C correlation (HSQC) with H-2′F, H-5′F, and H-6′F, at 115.5, 116.1, and 119.5 ppm, respectively. The carbons C-3′F and C-4′F were assigned at 146.5 and 146.3 ppm from their long distance ¹H−¹³C correlations (HMBC) with the protons H-2′F and H-5′F, respectively. Finally, the carbon C-1′ was attributed from its long range ¹H−¹³C correlation with H-2′F and H-6′F. The carbons C-2, C-3, and C-4 of the G ring were easily assigned at 84.1, 68.0, and 29.0 ppm from direct ¹H−¹³C correlations in the HSQC spectrum. The shielded resonance of C-4 (29.0 ppm) is typical of a doubly benzylic position. The only carbon of the E ring that could be assigned was C-6 at 108.7 ppm, because no long range ¹H−¹³C correlation could be detected.

All the carbons of the glucosyl moiety were easily attributed through direct ¹H−¹³C correlations in the HSQC spectrum and were situated between 63 and 73 ppm, except that of the anomeric proton (Gl-1) which was assigned to the signal at 104.1 ppm.

Formation of Pigments. The formation mechanism of these pigments remains to be clearly explained. The structure of these newly formed pigments arises from a malvidin 3-glucoside molecule and a flavan-3-ol (monomer for peak 2 and peak 3, or dimer for peak 1) through a vinyl linkage. Therefore, these pigments are proposed to result from the reaction of the malvidin 3-glucoside flavylium cation with a flavanol moiety possessing a vinyl residue at C-8, as proposed in **Figure 8**, following a mechanism previously proposed by Fulcrand et al. for the formation of 4-vinylphenol anthocyanin-derived pigments (*18*). The vinyl-flavanol adduct may derive either from the cleavage of ethyl-linked flavanol oligomers resulting from the acetaldehyde-induced condensation of flavanols (*14*) or from the dehydration of the flavanol-ethanol adduct formed after reaction with acetaldehyde (**Figure 8**). Such processes are expected to be favored when the pH is not very acidic, because in that case the formation of an ethyl-flavanol cation, and consequently of ethyl-linked pigments, would be favored (*11*). The last step of the formation mechanism is a cycloaddition whereby the vinyl-catechin residue binds to the flavylium moiety. This oxidative addition leads to the newly formed pigment with the extended conjugation allowing for the aromatization of ring D. This feature is likely to confer a higher stability to the resulting molecule.

Many other pigments detected in red wines are thought to contribute to the changing color from young red-purple wines to older brick-red wines (*30*). The structural confirmation of the presence of anthocyanin-flavanol pigments in red wine provides fundamental information for the study of newly formed pigments which contribute to the changing color of red wine during maturation and aging.

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