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Isolation and Structural Characterization of New Anthocyanin-Derived Yellow Pigments in Aged Red Wines

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Two newly formed yellow pigments that revealed unique spectral features were detected and isolated from an aged Port red wine by TSK Toyopearl HW-40(s) gel chromatography and characterized by UV−visible spectrophotometry, ^{1}H NMR and ^{13}C NMR, and mass spectrometry (LC-ESI/MS). The UV−vis spectra of these pigments showed maximum absorption at 478 nm that is significantly hypsochromically shifted from those of original grape anthocyanins and other pyranoanthocyanins, exhibiting a more yellow hue in acidic solution. The structures of these pigments correspond to methyl-linked pyranomalvidin 3-glucoside and its respective coumaroyl glucoside derivative. They were shown to arise from the reaction between acetoacetic acid and genuine grape anthocyanins. Isolation and NMR identification using 1D and 2D NMR techniques are reported for the first time for this new family of anthocyanin-derived yellow pigments occurring in red wines.

KEYWORDS: Red wine; aging; yellow color; anthocyanins; acetoacetic acid; methyl pyranoanthocyanins; isolation; NMR

INTRODUCTION

Grape anthocyanins are the main polyphenolic compounds that contribute to the color in red wines. During winemaking and maturation, they are progressively displaced by newly formed pigments to give rise to more stable compounds with different physical−chemical features associated with important changes in the color and flavor properties of wines. Different chemical transformations have been shown or proposed to participate in such changes involving reactions between anthocyanins and/or flavanols with small molecules released from fermentative metabolism. Processes such as condensations between anthocyanins and flavanols directly (*1*−*4*) or mediated by aldehyde (*5*−*9*) or reactions of anthocyanins or flavanols with pyruvic acid (*10*−*13*), vinylphenol (*14*, *15*), cinnamic acid derivatives (*16*), or glyoxylic acid (*17*−*19*) have already been demonstrated to yield new pigment families. All of these events result in the formation of new pigments that could stabilize wine color and change it from the red-purple of young wines to the more orange-red hue of aged wines.

The color evolution of red wines during aging is a very complex process, and increasing numbers of wine pigments are being identified continuously due to the development of mass spectrometry coupled to HPLC and NMR techniques. Over recent years, several new pigment families occurring directly in wine have been detected (*8*, *20*, *21*) and structurally characterized as pyranoanthocyanins or vinylpyranoanthocyanins bearing different flavanol substituents (*22*−*25*), the latter showing a maximum absorption in the UV−vis spectrum greatly bathochromically shifted from that of anthocyanins and presenting a blue color. In the present work, two new pigments isolated from aged Port wines have been detected, exhibiting a unique yellow color that may contribute to the overall expression of the orange-red color of aged wines. Isolation and full characterization of this new pigment family from red wines were achieved by ESI/MS and UV−vis and NMR spectroscopy, together with a proposed mechanism of formation of these pigments elucidated here for the first time.

MATERIALS AND METHODS

Sample. The pigments were extracted from 3-year-old Port red wine [pH 3.6, 18% alcohol (v/v), total acidity = 6.5 g L^{-1}, total SO_2 = 20 mg L^{-1}] made from grapes of Touriga National (*Vitis vinifera*) grown in the Douro demarcated region (northern Portugal).

Wine Fractionation. A Port wine sample (5 L) was directly deposited onto a 10 cm diameter medium-porosity sintered glass funnel loaded with TSK Toyopearl gel HW-40(s) and connected to standard vacuum filtration glassware and then eluted with 20% aqueous methanol, yielding the original anthocyanins and some pyruvic acid adducts of the major anthocyanins. When practically no more colored compounds were eluted from the funnel, the other anthocyanin-derived

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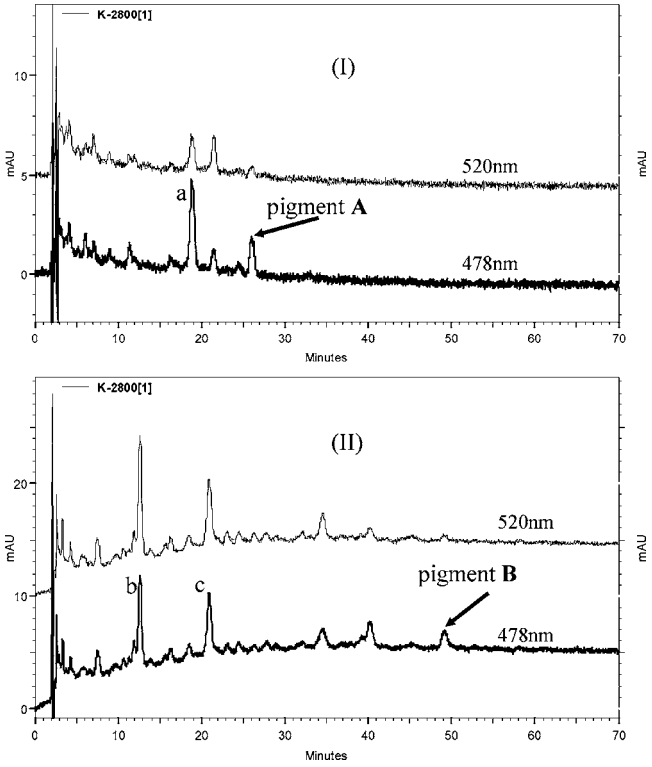


Figure 1. HPLC chromatograms recorded at 520 and 478 nm of fractions I and II isolated on polyamide with 30% methanol (v/v) from a Port wine extract obtained after fractionation on Toyopearl gel HW-40(s), showing peaks A (I) and B (II) that correspond to the two new yellow pigments isolated and other well-known compounds: (a) vitisin b; (b) malvidin 3-glucoside; (c) vitisin a.

pigment fraction was recovered by elution with 50% acidified methanol. After concentration under vacuum, the eluate was fractionated on a 250 × 16 mm i.d. Polyamide resin column (60−80 mesh, SINOPEC, Hunan, China), prepared and balanced with acidified water. The pigments were eluted with 30% aqueous methanol, and two fractions were collected thereafter and investigated by HPLC-DAD. Together with other colored pigments, two chromatographic peaks (A and B) showing maximum absorption at about 478 nm were detected in each fraction, respectively (**Figure 1**). The pH of the used eluents was set to 2.0 with HCl.

HPLC Conditions. The fractions eluted from the Polyamide resin column were analyzed by HPLC (model 96, Knauer, Berlin, Germany) on a 250 × 4.6 mm i.d. reverse-phase C18 column (Merck, Darmstadt, Germany), and detection was carried out using a diode array detector (Knauer, K-2800). An HPLC pump Knauer K-1001 was used together with a Kauer K-3800 autosampler. The solvents were A, H_2O/HCOOH (9:1), and B, CH_3CN/H_2O/HCOOH (8:1:1). The gradient consisted of 15−35% B over 70 min, 35−80% B over 5 min, and then isocratic for 10 min at a flow rate of 1.0 mL/min.

Semipreparative HPLC Conditions. Pigments A and B were purified by semipreparative HPLC using the above indicated reverse-phase C18 column with an injection volume of 500 μL and the same gradient program. Each pigment was collected, concentrated under vacuum, and applied on a 150 × 16 mm i.d. Toyopearl HW-40(s) gel column (Tosoh), which was eluted with distilled methanol for a final purification of the product. Finally, about 4 mg of pigment A was obtained, whereas much less quantity of pigment B (only 1 mg) was recovered.

LC-MS Conditions. A Hewlett-Packard 1100 series liquid chromatograph, equipped with an AQUA (Phenomenex, Torrance, CA) reverse-phase column (150 × 4.6 mm, 5 μm, C18) thermostated at 35 °C was used. Solvents were (A) aqueous 0.1% trifluoroacetic acid and (B) acetonitrile, using the gradient previously reported (*26*). Double-online detection was done in a photodiode spectrophotometer and by mass spectrometry. The mass detector was a Finnigan LCQ (Finnigan Corp., San Jose, CA), equipped with an API source, using an electrospray ionization (ESI) interface. The capillary voltage was 3 V, and the capillary temperature was 190 °C. Spectra were recorded in positive ion mode between *m*/*z* 120 and 1500. MS-MS spectra were registered using relative collision energies of 30 and 60V.

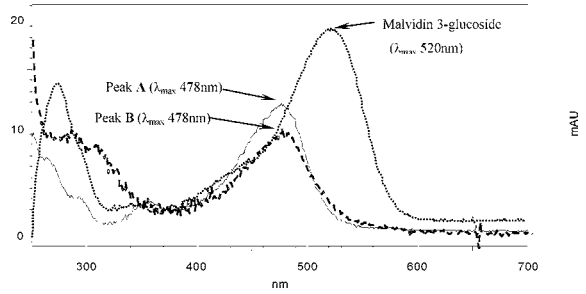


Figure 2. UV–visible spectra of malvidin 3-glucoside and peaks A/B as recorded with the HPLC diode array detector.

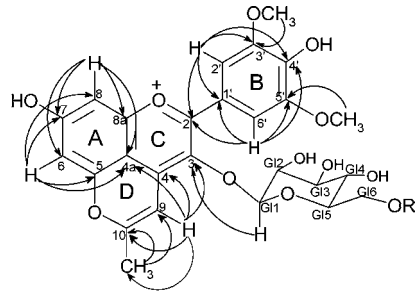
NMR Analysis. ^{1}H NMR (500.13 MHz) and ^{13}C NMR (125.77 MHz) spectra were measured in CD_3OD/TFA (98:2) on a Bruker-AMX500 spectrometer at 303 K and with TMS as internal standard. ^{1}H chemical shifts were assigned using 1D and 2D ^{1}H NMR (COSY and NOESY), whereas ^{13}C resonances were assigned using 2D NMR techniques (gHMBC and gHSQC) (*27*−*29*). The delay for the long-range C/H coupling constant was optimized to 7 Hz.

Formation of Pigments. Formation of the yellow pigments was monitored at 30 °C in 20% aqueous ethanol (pH 3.2) in a screw-cap vial containing 10 mg of malvidin 3-coumaroylglucoside previously isolated and characterized (*13*) and 17 mg of lithium acetoacetate (Sigma-Aldrich, Madrid, Spain), yielding a molar ratio of acetoacetate/anthocyanin of 10:1. The reaction was monitored by HPLC-DAD using the conditions described above. When the maximum intensity of the new pigment was obtained in 10 days, the pigment was then purified by semipreparative HPLC on a C18 ODS column and finally applied on a Toyopearl gel column for a last purification as descriptions referred to above. About 6 mg of the resulting pigment was obtained.

RESULTS AND DISCUSSION

Detection and Isolation. Two colored wine fractions collected from the elution of several Port wine samples from a polyamide resin column with 30% methanol (v/v) were thoroughly analyzed by HPLC-DAD. The HPLC chromatogram recorded generally at 520 nm for detection of the absorption of anthocyanins showed the presence of several colored pigments, with retention times and UV−vis spectra identical to those of the known anthocyanins and their derivatives (vitisins a and b) indicated in the legend of **Figure 1** as previously reported (*11*−*13*, *30*). Furthermore, the recording of the HPLC chromatogram at 478 nm revealed the presence of an additional peak in the end of each profile (**Figure 1**), although in a very tiny or disappearing intensity when detected at 520 nm. The UV−vis spectra of both peaks recorded from the HPLC diode array detector are shown in **Figure 2**. Both pigments have a distinct λ_{max} in the visible region at 478 nm, significantly hypsochromically shifted with respect to those of the original anthocyanins and other pyranoanthocyanins. The two pigments (named A and B) were isolated by semipreparative HPLC for their structural characterization. During the final purification of each pigment by Toyopearl gel column chromatography in acidic conditions (pH 2.0), a yellow band was observed, in agreement with the UV−vis spectrum of the pigments.

Mass Spectrometry. Pigments A and B isolated were analyzed individually by LC-ESI/MS in positive ion mode. Analysis of pigment A produced a $[M]^+$ ion at *m*/*z* 531, and its MS^2 spectrum showed a major fragment ion $[M - 162]^+$ at *m*/*z* 369, corresponding to the loss of a glucosyl moiety. Pigment



Pigment A: R = H,

Pigment B: R =

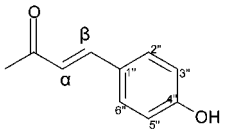


Figure 3. Structures of the newly formed pigments A and B and the long-range ^{1}H–^{13}C correlations found in the HMBC spectrum.

Table 1. ^{1}H and ^{13}C Assignments and HMBC and HSQC Correlations of Pigment A Isolated from a 3-Year-Old Port Wine, Determined in CD_3OD/TFA (98:2)[a]

position	δ (^{1}H); J (Hz)	δ (^{13}C)	position	δ (^{1}H); J (Hz)	δ (^{13}C)
		Pyranoanthocyanidin Moiety			
2		164.3	9	7.21; s	103.1
3		134.6	10		174.2
4		109.8	CH_3	2.66; s	21.7
4a		109.5	1′		120.3
5		155.3	2′,6′	7.62; s	108.9.3
6	7.08; s	101.2	3′,5′		149.3
7		168.7	4′		143.6
8	7.23; s	101.1	OMe	3.96; s	57.2
8a		154.3			
		Sugar Moiety			
Gl-1	4.66; d, 7.7	104.9	Gl-5	3.11; t, 7.7	79.0
Gl-2	3.59; t, 8.5	75.6	Gl-6a	3.68; d, 11.4	62.7
Gl-3	3.34; *	78.0	Gl-6b	3.38; m	62.7
Gl-4	3.24; *	71.3			

[a] Key: *, unresolved; s, singlet; d, doublet; t, triplet; m, multiplet.

B revealed a $[M]^+$ ion at m/z 677 and the same fragment ion at m/z 369 in the MS^2 spectrum, which could correspond to the loss of a coumaroylglucosyl residue $[M - 308]^+$. In addition, the fragmentation pattern in the respective MS^3 spectrum of both pigments released elimination masses of 16, 32, 44, 61, and 89 amu, characteristic of malvidin-derived pigments (*31*). The fact that pigments A and B present the same fragment ion masses in the MS^2 and MS^3 spectra and the same maximum absorption in the visible region suggests that they are probably the same family of anthocyanin-derived pigments with different glucoside moieties, the latter being likely to be the coumaroyl derivative of the former. Indeed, these molecular ion masses fit exactly with the structures shown in **Figure 3**.

Structural Characterization by NMR. The unambiguous identification of these yellow wine pigments via their isolation is highly desirable for structural characterization and confirmation of their presence in wine. Pigment A was isolated in sufficient amounts to be characterized by NMR. Its structure was identified by ^{1}H and ^{13}C NMR spectroscopy in CD_3OD/TFA (98:2) as shown in **Table 1**. Pigment B was identified by comparison with the retention time and MS of the standards obtained by synthesis and characterized by NMR as described below. Practically all of the proton and carbon resonances of pigment A were fully attributed. The proton chemical shifts were attributed using 1D and 2D NMR techniques (*g*COSY), whereas the carbons were achieved by two-dimensional analyses (*g*HSQC and *g*HMBC).

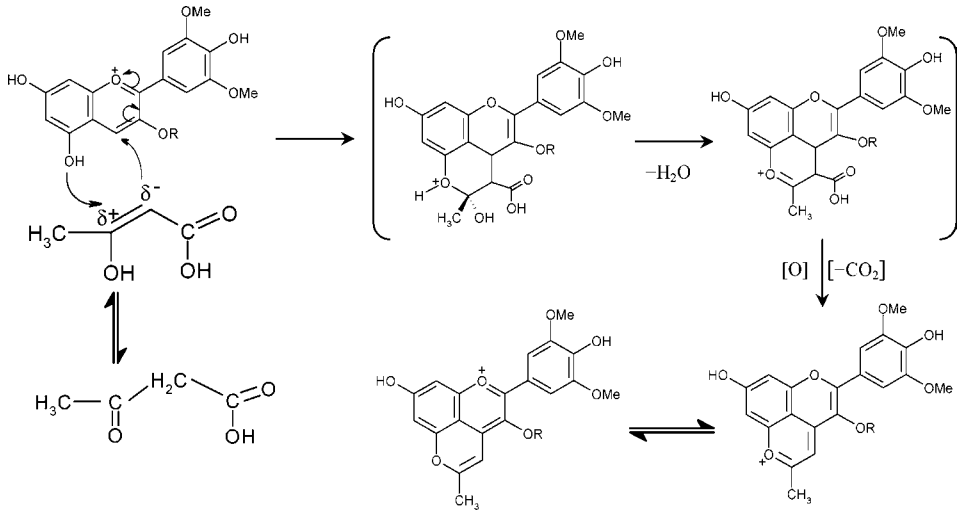
The ^{1}H spectrum showed the presence of H-2′, H-6′ and the two methoxyl groups of ring B, which were located at δ 7.62 and 3.96, respectively. Protons H-6 and H-8 of the A ring were assigned to the two singlets at δ 7.08 and 7.23, respectively, and proton H-9 of ring D was located at δ 7.21. These proton chemical shifts of the flavylium moiety are consistent with those of malvidin-derived pyranoanthocyanins previously reported (*13*, *22*). With respect to the methyl group, a sharp singlet peak integrating for three protons and showing a strong HMBC correlation with the carbon C-9D and C-10D was observed at δ 2.66 and thus attributed unambiguously to the three protons of the methyl group linked with the pyran ring D.

For the glucosyl moiety, the anomeric proton Gl-1 chemical shift was found at δ 4.66 as a doublet with a large coupling constant (7.7 Hz), suggesting a β configuration of the sugar moiety. Proton Gl-2 was attributed to a triplet located at δ 3.59 ($J = 8.5$ Hz) from its correlation with the anomeric proton (Gl-1), and proton Gl-3 was assigned to an unresolved peak around 3.17 ppm from its correlation with Gl-2 in the COSY spectrum. The two protons of Gl-6 that revealed a clear correlation in the COSY spectrum were attributed to a broad doublet at δ 3.68 ($J = 11.4$ Hz) and to a multiplet at δ 3.38. Proton Gl-5 was assigned to a triplet at δ 3.11 ($J = 7.7$ Hz) from its correlation with Gl-6, whereas the last proton, Gl-4, was assigned to an unresolved peak at δ 3.24.

The assignment of the carbon resonance obtained using two-dimensional techniques is presented in **Table 1**. Important correlations found in the HMBC spectrum are shown in **Figure 3**. The correlation observed in the HMBC spectrum between the methoxyl proton resonances and that of the carbons at δ 149.3 allowed their assignment to C-3′ and C-5′. Carbon C-10 was assigned at δ 174.2 through its strong HMBC correlations with H-9 and protons of the CH_3 group. Despite having similar chemical shifts, carbon C-4a assigned at δ 109.5 was differentiated from C-4 assigned at δ 109.8 from its $^3J_{C,H}$ coupling with H-6A and H-8A (**Figure 3**), as both carbons showed long-range ^{1}H$-^{13}$C correlations (HMBC) with proton H-9. Carbon C-9 was assigned at δ 103.1 through its direct ^{1}H$-^{13}$C correlation (HSQC) with H-9 and long-distance ^{1}H$-^{13}$C correlation (HMBC) with protons of CH_3 group. The quaternary carbons C-5, C-7, and C-8a were assigned from their long-distance correlation with protons H-8A and H-6A observed in the HMBC spectrum at δ 155.5, 168.7, and 154.3, respectively. These ^{13}C NMR data are in good agreement with the ones obtained for malvidin-derived pyranoanthocyanins previously reported (*13*, *22*). Finally, the carbon chemical shift of the methyl group was assigned at δ 21.7 through direct HSQC correlation with its protons and $^3J_{C,H}$ coupling with H-9 in the HMBC spectrum.

All of the carbons of the glucosyl moiety were easily assigned through direct ^{1}H$-^{13}$C correlations in the HSQC spectrum and were situated between δ 63 and 79, except for that at the anomeric position, which was assigned to the signal at δ 104.9.

Formation of Pigments. The formation mechanism of these anthocyanin-derived yellow pigments in red wines remains to be clearly explained. The structure of pigment A corresponds to methyl-substituted pyranomalvidin 3-glucoside, in reference to the carboxyl pyranomalvidin (malvidin-pyruvic acid adducts) (*10*). Cyanidin- and delphinidin-based methyl pyranoanthocyanins were first detected in the acetone extract of black currant seed as a result of solvent participation through the reaction of glucoside of cyanidin and delphinidin with acetone (*32*, *33*). The detection in wine of methyl pyranoanthocyanin is only



Pigment A: R = glucose

Pigment B: R = coumaroylglucose

Figure 4. Suggested mechanism for the formation of pigments A and B.

postulated on the basis of the mass data obtained from direct mass spectrometry without any HPLC profile (*31*), and their occurrence is therefore also claimed to be acetone adducts of malvidin, following the same mechanism previously proposed by Fulcrand et al. for the formation of anthocyanin−pyruvic acid adducts (*10*), as the carboxyl group of pyruvic acid being replaced, in the former, by a methyl group.

Hereupon a mechanism of formation of grape-derived methyl pyranoanthocyanins in red wines is proposed in **Figure 4** involving reaction between anthocyanins and acetoacetic acid instead of acetone, which is formed as a toxic side product in the common vinification in spite of its possible reactivity with anthocyanins. It is known that acetoacetate is a normal end product of fatty acid oxidation, which is usually oxidized further to CO_2 and water, which serve as fuels for energy production (*34*). Acetoacetic acid is very easily converted in metabolic activities from two molecules of acetyl-CoA, one of the most important intermediates, especially in the anaerobic starvation conditions of fermentation by microbial catabolism of lipids or fatty acids for the generation of energy (*34*, *35*). It is also expected to arise during the second wine fermentation and postfermentation aging on yeast lees under reducing conditions from the microbial conversion of acetyl-CoA. In the proposed mechanism, the initial bond formation between the C-4 position of anthocyanin and the C-2 position of acetoacetate is consistent with the strongly electrophilic nature of the flavylium cation unit and the nucleophilicity of the α-carbon of acetoacetic acid (**Figure 4**). The resulting intermediate can be trapped intramolecularly by the hemiacetal formation with the phenolic hydroxyl group of anthocyanin to form the new pyran ring, followed by a dehydration step. Finally, decarboxylation and oxidation are involved in the mechanism, leading to the newly formed structure with the extended conjugation of the π electrons allowing for the aromatization of the new pyran ring and the delocalization of the charge. This structural feature is likely to confer a higher stability to the resulting pigment.

To obtain further evidence to confirm the reactivity of acetoacetic acid involved in this mechanism, another experiment was hence performed with malvidin 3-coumaroylglucoside and acetoacetate in a wine-like model solution of pH 3.2. The HPLC-DAD analysis of the solution after 7 days of reaction revealed the presence of malvidin 3-coumaroylglucoside (a), together with a new significant peak (b) with the same retention time

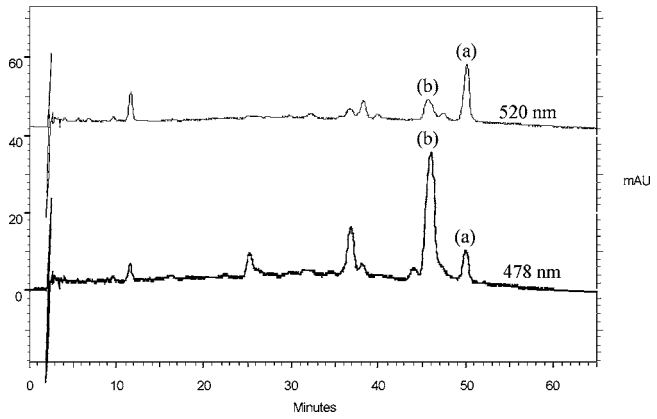


Figure 5. HPLC chromatograms recorded at 520 and 478 nm of the wine-like model solution containing acetoacetate and malvidin 3-coumaroylglucoside after 7 days of reaction: (a) malvidin 3-coumaroylglucoside; (b) methyl pyranomalvidin 3-coumaroylglucoside.

and spectroscopic properties as pigment B in wine, with λ_{max} at 478 nm (**Figure 5**). The LC-MS analysis of this newly formed pigment showed a $[M]^+$ ion at *m*/*z* 677, which fits exactly with the postulated structure of pigment B in **Figure 3**. Additionally, the MS^2 data revealed one major fragment ion, $[M - 308]^+$ at *m*/*z* 369, corresponding to the loss of a coumaroylglucosyl residue, as observed for pigment B. These results strongly suggest that pigment B could be formed in wine by this facile and direct reaction between acetoacetic acid and malvidin 3-coumaroylglucoside. Thereafter, the methyl pyranoanthocyanin structure of the newly formed pigment B was further identified by NMR spectroscopy (**Table 2**). Overall, the NMR data of the pyranoanthocyanin moiety are in agreement with that of analogous pigment A, thus confirming the methyl pyranomalvidin core structure of the synthesized pigment B. With respect to the coumaroyl moiety, all of the proton resonances are clearly assigned, and the large coupling constant (15.8 Hz) of the protons of the vinyl group (H_α, H_β) suggests a trans stereochemistry. Thus, pigment B was unambiguously determined to be the coumaroyl glucoside derivative of methyl pyranomalvidin 3-glucoside (pigment A), together forming a new family of anthocyanin-derived yellow pigments in red wines.

Table 2. ^{1}H and ^{13}C Assignments of the Synthesized Pigments B, Determined in CD_3OD/TFA (98:2)[a]

position	δ (^{1}H); J (Hz)	δ (^{13}C)	position	δ (^{1}H); J (Hz)	δ (^{13}C)
Pyranoanthocyanidin Moiety					
2		162.4	9	6.89; s	103.2
3		133.3	10		173.3
4		107.5	CH_3	2.49; s	21.1
4a		107.7	1′		119.1
5		154.6	2′,6′	7.37; s	108.1
6	6.68; bs	100.9	3′,5′		148.8
7		167.5	4′		143.2
8	6.81; bs	100.4	OMe	3.76; s	56.5
8a		152.9			
Sugar Moiety					
Gl-1	4.43; d, 7.9	104.1	Gl-5	3.19;*	75.2
Gl-2	3.46; t, 7.4	74.6	Gl-6a	4.33; dd, 8.0/11.3	62.9
Gl-3	3.17; *	76.8	Gl-6b	3.87; d, 11.6	62.9
Gl-4	3.15; *	71.2			
Coumaroyl Moiety					
R1CO_2R2		167.2	2″,6″	7.10; d, 7.8	130.3
CH═$CH_\alpha CO_2$R	5.63; d, 15.8	113.3	3″,5″	6.66; d, 7.8	116.2
CH_β═CH CO_2R	7.08; d, 15.8	145.7	4″		160.8
1″		126.1			

[a] Key: *, unresolved; bs, broad singlet; s, singlet; d, doublet; dd, double doublets; t, triplet.

The isolation and structural characterization of these newly formed methyl pyranoanthocyanins from red wine represent the first direct evidence of anthocyanin-derived pigments, which exhibit a more yellowish hue in the acidic medium of red wines, although compounds with the same mass were detected in Shiraz wine and postulated from data obtained by direct nanoelectrospray mass spectrometry, but did not show any HPLC profile and spectral properties even after a multistep purification (*31*). Moreover, very recently, one compound appearing to correspond to pigment A was also detected by LC-MS (*36*). The structural confirmation of the presence of these yellow pigments in red wine provides further information regarding the diverse transformations of pigments involved in the complex color evolution from young red-purple wines to older orange-like wines. The unique spectroscopic features of these pigments formed during winemaking and maturation may contribute to the changing color of aging red wines. Nevertheless, further studies are still required to elucidate the factors that influence the formation of this family of yellow pigments and to assess their relative contribution to the overall expression of the color of aged red wines.

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