# Occurrence of Anthocyanin-Derived Pigments in Red Wines

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Several anthocyanin-derived pigments that showed UV−visible spectra different from those of the original grape anthocyanins were detected by HPLC-DAD analysis in 1-year-old bottled Port wines from the Douro region. Among these, three malvidin 3-glucoside derived pigments were detected in large amounts, representing ∼60% of the total anthocyanidin monoglucosides content. These pigments were isolated, purified, and identified by LSI-MS and NMR ($^1$H, DQF-COSY, ROESY, HSQC, and HMBC) techniques. The major pigment is malvidin 3-glucoside pyruvic adduct, previously characterized, and the other two corresponded to its respective acetyl and coumaroyl glucoside derivatives. The latter is reported for the first time in red wines.

**Keywords:** *Red wine; aging; pigments; anthocyanins; pyruvic acid*

## INTRODUCTION

It has been stated that the most rapid change in wine color composition occurs during the first year of maturation when the wine is normally in bulk storage (*1*). This phase is considered to be quite distinct from the latter "aging phase", when the wine is in bottle and well protected from any further contact with air (*2*, *3*). The color changes during wine maturation are usually attributed to the formation of new pigments resulting from the interaction between anthocyanins and other phenolic compounds, especially flavan-3-ols such as catechins and procyanidins (condensed tannins). These newly formed pigments are thought to arise from a copigmentation phenomenon (*4*−*6*), direct reaction between anthocyanins and flavanols (*1*, *7*−*9*), or reaction between anthocyanins and flavanols through ethyl bridges (*10*−*16*). All of these events result in the formation of more stable pigments that stabilize wine color, changing it to a more brick red hue. Besides these newly formed pigments, anthocyanin-derived compounds have been reported in red wines over recent years (*17*−*19*). New malvidin 3-glucoside derived pigments, named vitisin A and vitisin B, together with their acylated glucoside derivatives, were isolated; vitisin A was determined to have a visible maximum absorption at 511 nm, whereas the $\lambda_{max}$ of vitisin B is ∼20 nm lower. Additionally, these pigments were found to be more resistant to bleaching by sulfur dioxide (*20*). These pigments were synthesized by other authors, but a slightly different structure was attributed (*21*, *22*). The evolution of vitisins in a model solution has shown that high temperature and acetaldehyde diminish their levels (*23*, *24*). All of these derived pigments were found to arise from the reaction of pyruvic acid with original anthocyanins initially extracted from grape skin (*21*−*23*, *25*), and acetaldehyde was found to be implicated in their formation (*16*, *24*).

The present work reports the occurrence of a malvidin−pyruvic acid adduct and its acetylated and *p*-coumaroyl derivatives in 1-year-old bottled Port wines. Identification of the latter was achieved using LSI-MS and NMR analyses.

## MATERIALS AND METHODS

**Wines.** The 1-year-old Port wines (Touriga Nacional and Touriga Francesa *Vitis vinifera* varieties) were provided by Barros, Almeida & C[a] - Vinhos S.A. Touriga Nacional Port wine was used as a source of the derived pigments.

Microvinifications were performed in triplicate for each wine studied. $SO_2$ was added to a final concentration of 60 mg/L. Twenty-five kilograms of grapes from the Douro Demarcated region were randomly sampled, destemmed, and crushed into stainless steel wine vats. When about half of the original sugar content had been converted to alcohol, the must fermentation was stopped by the addition of wine spirit (ratio of wine/wine spirit ≈ 5). Every wine was separated from pomace by filtration and pumped to other vats. The final alcohol content was set to 20% (v/v) for all the wines.

**Pigment Isolation.** Two liters of fortified wine (Touriga Nacional) was directly applied to a Toyopearl HW-40(s) gel column (200 × 16 mm i.d., 0.8 mL/min) (Tosohaas, Stuttgart, Germany). Elution with water/ethanol 20% (v/v) yielded a fraction containing the anthocyanidin monoglucosides and three derived pigments. Malvidin 3-glucoside, malvidin 3-(6-*p*-coumaroyl)glucoside, and the three malvidin-derived detected pigments were purified by semipreparative HPLC on a Beckman Ultrasphère (C18) ODS (250 × 4.6 mm i.d.) column (Merck, Darmstadt, Germany). The pigments were then collected, concentrated under vacuum, and lyophilized.

**HPLC Conditions.** Aliquot wine samples were chromatographed by HPLC using a Beckman Ultrasphère (C18) ODS (250 × 4.6 mm i.d.) column, and detection was carried out at 520 nm using a diode array detector. The solvents were (A) $H_2O$/HCOOH (9:1) and (B) $CH_3CN/H_2O$/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 (*11*). The anthocyanidin 3-monoglucoside and respective acy-

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10.1021/jf001505b CCC: $20.00 
Published on Web 09/05/2001

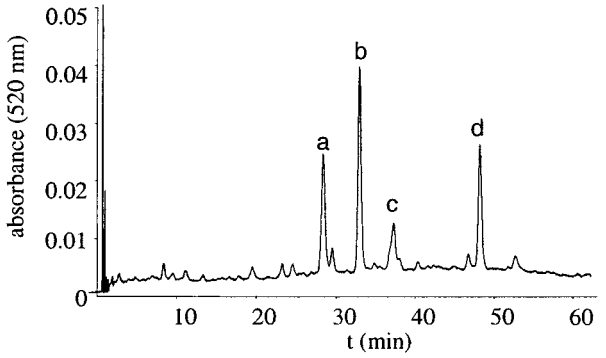



**Figure 1.** HPLC profile recorded at 520 nm of 1-year-old Port wine after elution from Toyopearl gel column with water/ethanol 20% (v/v), showing (a) malvidin 3-glucoside, (b) malvidin 3-glucoside pyruvic adduct, (c) malvidin 3-acetylglucoside pyruvic adduct, and (d) malvidin 3-coumaroylglucoside pyruvic adduct.

lated glucoside derivatives were identified on the basis of their UV−visible spectra and retention times. The acylated esters were isolated by semipreparative HPLC, and their structures were elucidated by acid hydrolysis. The released anthocyanidins and phenolic acids were identified by HPLC by comparison with authentic standards. The calibration curves were obtained by injecting different concentrations of malvidin 3-glucoside (Extrasynthèse, Lyon, France) for anthocyanidin monoglucosides and malvidin−pyruvic adducts. The pigment calibration curves were obtained by injecting standards with different concentrations of malvidin 3-glucoside pyruvic adduct. The range of the linear calibration curves ($r^2 > 0.98$) was from 0.01 (limit of detection) to 1.0 mg/L for the lower concentration compounds and from 1.0 to 100.0 mg/L for the higher concentration compounds. Unknown concentrations were determined from regression equations. Repeatability of this method from extraction to HPLC analysis for four samples of the same batch of wines gave a coefficient of variation of <7%.

**LSI Mass Spectrometry.** A small quantity of pigment was dissolved in the minimum volume of $CH_3OH$/TFA (98:2) and then mixed in a matrix of glycerol. The LSI-MS spectra were recorded using a VG Autospec EQ mass spectrometer, equipped with a $Cs^+$ gun (beam energy = 35 keV) (Fisons Instruments). Calibration was performed using cesium iodide.

**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 with TMS as internal standard. $^1$H chemical shifts were assigned using DQF-COSY and ROESY experiments (*26*), whereas $^{13}$C resonances were assigned using 2D NMR (HMBC and HSQC) techniques (*27*, *28*). 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 water and ethanol 20% (v/v) yielded large amounts of malvidin 3-glucoside and mainly three derived pigments with retention times different from those of the known grape anthocyanins (Figure 1). Their UV−vis, mass spectrometric, and NMR characteristics indicated that these pigments were a malvidin 3-glucoside pyruvic adduct, malvidin 3-acetylglucoside pyruvic adduct, and malvidin 3-coumaroylglucoside pyruvic adduct. The latter is reported here for the first time in red wines. The UV−vis spectra of these pigments recorded from the HPLC diode array detector are shown in Figure 2. These spectra were different from those of the original anthocyanins and showed a $\lambda_{max}$ of ∼503 nm, whereas anthocyanins have their maximal absorption at 520 nm, as previously reported (*18*, *19*, *21*).

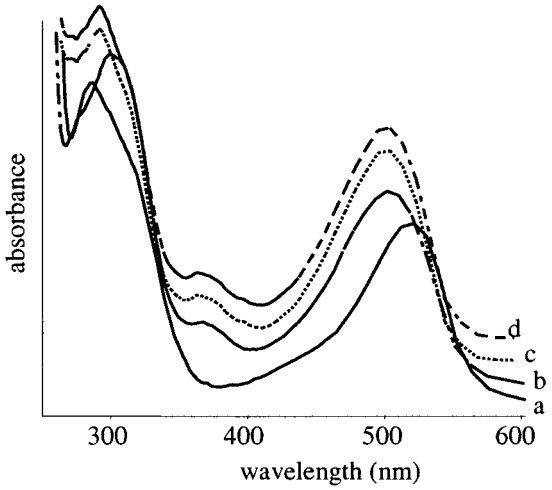



**Figure 2.** UV−visible spectra of (a) malvidin 3-glucoside, (b) malvidin 3-glucoside pyruvic adduct, (c) malvidin 3-acetylglucoside pyruvic adduct, and (d) malvidin 3-coumaroylglucoside pyruvic adduct recorded from HPLC diode array detector.

Another absorption maximum was also observed at 370 nm, which is characteristic of 4-substitued anthocyanins (*29*). The resistance of these compounds to sulfur dioxide bleaching results from this particular feature (*20*).

**Structural Analysis.** *Mass Spectrometry*. The LSI-MS analysis of pigment b revealed an $[M]^+$ ion at *m/z* 561, which corresponds to the mass of malvidin 3-glucoside pyruvic adduct adduct. An ion fragment at *m/z* 399 corresponding to the loss of a glucose residue (malvidin 3-glucoside pyruvic adduct aglycon) was also observed. Pigments c and d showed $[M]^+$ ions at *m/z* 603 and 707, respectively, which correspond to the sum of the malvidin 3-glucoside pyruvic adduct (399 amu) and the mass of the acetylglucoside group (204 amu) for pigment c and the coumaroylglucoside group (308 amu) for pigment d. The mass spectra of these pigments also revealed the presence of a signal at *m/z* 561, which corresponds to the molecular ion of the malvidin 3-glucoside pyruvic adduct.

*$^1$H NMR*. Proton NMR chemical shifts of the malvidin 3-glucoside pyruvic adduct, its acetyl and coumaroyl esters, and malvidin 3-(6-*p*-coumaroyl) monoglucoside in $CD_3OD$/TFA (98:2) are shown in Table 1, and the corresponding proposed structures are shown in Figure 4. All of the aromatic protons were easily assigned, whereas glucosyl protons were more difficult to identify due to the low quantities of product and the presence of the methanol peak in the sugar moiety region of the spectra. The $^1$H chemical shifts were attributed using one- and two-dimensional analyses (DQF-COSY). nOe information was also obtained from another two-dimensional experiment, ROESY, to elucidate the full structure of the malvidin 3-coumaroylglucoside pyruvic adduct.

The spectra of malvidin 3-glucoside pyruvic adduct and its acylated forms showed the presence of H-2′,6′ and two methoxyl groups of the B ring, which were located around 7.8 and 4.0 ppm, respectively. The protons H-6 and H-8 of the A ring were assigned to the two doublets located around 7.20 and 7.33 ppm, respectively, and the proton H-9 of the D ring was located at 8.01 ppm. The protons H-6 and H-8 were differentiated through ROESY experiments, which revealed nOe correlations between H-2′ and H-8 and between H-2′ and the methoxyl protons of C-3′. With respect to the

**Table 1. $^1$H and $^{13}$C Assignments of the Malvidin 3-Glucoside Pyruvic Adduct (mv-py), Malvidin 3-Acetylglucoside Pyruvic Adduct (mv-ac-py), Malvidin 3-Coumaroylglucoside Pyruvic Adduct (mv-coum-py), and Malvidin 3-(6-*p*-Coumaroyl) Monoglucoside (mv-coum) Isolated from 1-Year-Old Port Wines, Analyzed in $CD_3OD$/TFA (98:2)**

| | mv-py | | mv-ac-py | mv-coum-py | | mv-coum |
|---|---|---|---|---|---|---|
| position | $\delta$ $^1$H; *J* (Hz) | $\delta$ $^{13}$C | $\delta$ $^1$H; *J* (Hz) | $\delta$ $^1$H; *J* (Hz) | $\delta$ $^{13}$C | $\delta$ $^1$H; *J* (Hz) |
| 2 | | 166.8 | | | 166.2 | |
| 3 | | 134.8 | | | 136.2 | |
| 4 | | 111.9 | | | 111.1 | 8.97; *s* |
| 4a | | 112.1 | | | 111.4 | |
| 5 | | 153.4 | | | 154.9 | |
| 6 | 7.19; *d*, 1.9 | 102.1 | 7.20; *d*, 1.9 | 6.94; *d*, 1.9 | 101.2 | 6.56; *d*, 1.9 |
| 7 | | 170.8 | | | 170.0 | |
| 8 | 7.33; *d*, 1.9 | 102.8 | 7.34; *d*, 1.9 | 7.07; *d*, 1.9 | 102.0 | 6.88; *d*, 1.9 |
| 8a | | 153.4 | | | 152.4 | |
| 9 | 8.01; *s* | 108.3 | 8.03; *s* | 7.91; *s* | 107.8 | |
| 10 | | 154.8 | | | 152.2 | |
| COOH | | 160.4 | | | 159.1 | |
| 1′ | | 120.7 | | | 119.9 | |
| 2′, 6′ | 7.78; *s* | 111.0 | 7.80; *s* | 7.78; *s* | 110.2 | 7.95; *s* |
| 3′, 5′ | | 148.4 | | | 150.8 | |
| OMe | 4.00; *s* | 56.8 | 4.00; *s* | 3.97; *s* | 58.0 | 3.99; *s* |
| 4′ | | 146.0 | | | 146.1 | |
| G1 | 4.72; *d*, 7.8 | 105.9 | 4.71; *d*, 7.8 | 4.69;*d*, 7.7 | 106.0 | 5.35;*d*, 7.6 |
| G2 | | 76.6 | | 3.64; *dd*, 8.0/7.7 | 77.0 | 3.66; *dd*, 8.6/7.7 |
| G3 | 3.37; *t*, 8.9 | 75.2 | | 3.39; *t*, 8.6 | 75.9 | 3.57; *t*, 8.7 |
| G4 | 3.23; *[a] | 72.7 | | 3.32; * | 72.2 | 3.43; * |
| G5 | 3.15; * | 78.4 | | 3.29; * | 79.0 | |
| G6a | 3.3−3.5; * | 62.0 | | 4.46; *dd*, 11.8/7.8 | 64.2 | 4.48; * |
| G6b | 3.3−3.5; * | 62.0 | | 4.07; *dd*, 11.8/1.9 | 64.2 | 4.21; * |
| acetate | | | 1.78; *s* | | | |
| R1$CO_2$R2 | | | | | 168.5 | |
| CH=C$H_\alpha$$CO_2$R | | | | 5.82; *d*, 16.1 | 114.9 | 6.18; *d*, 15.9 |
| C$H_\beta$=CH$CO_2$R | | | | 7.25; *d*, 16.1 | 147.2 | 7.41; *d*, 15.9 |
| 1″ | | | | | 127.4 | |
| 2″, 6″ | | | | 7.29; *d*, 8.7 | 132.2 | 7.28; *d*, 8.7 |
| 3″, 5″ | | | | 6.82; *d*, 8.7 | 117.2 | 6.78; *d*, 8.7 |
| 4″ | | | | | 162.1 | |

[a] An asterisk (*) signifies unresolved status.

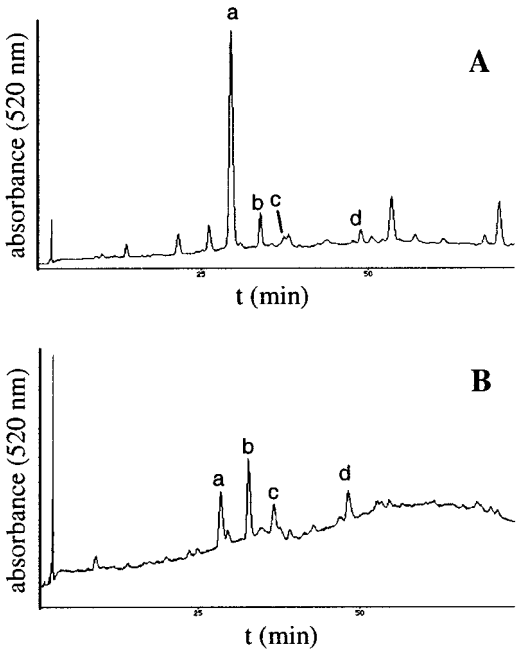
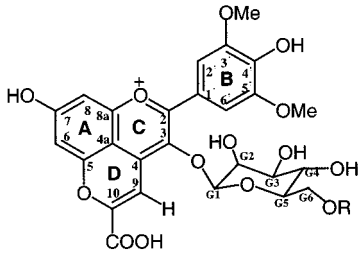
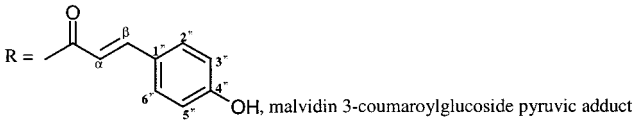



**Figure 3.** HPLC profile recorded at 520 nm of young (A) and 1-year-old (B) Port wine (Touriga Nacional): (a) malvidin 3-glucoside; (b) malvidin 3-glucoside pyruvic adduct; (c) malvidin 3-acetylglucoside pyruvic adduct; (d) malvidin 3-coumaroylglucoside pyruvic adduct.




R = H, malvidin 3-glucoside pyruvic adduct



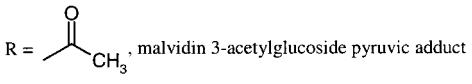




**Figure 4.** Structures of the newly formed pigments (flavylium cation).

glucosyl moiety, the anomeric proton chemical shift was always located in the region of 4.70 ppm as a doublet with a large coupling constant (7.7 Hz), suggesting a $\beta$ configuration. The other glucosyl proton signals were found in the region of 3.15−3.80 ppm for the malvidin 3-glucoside pyruvic adduct, 3.30−4.20 ppm for its acetyl ester, and 3.30−4.50 ppm for its coumaroyl ester. It is important to notice that the downfield shift (∼1 ppm) of the two glucosyl H-6 (G6) resonances of the malvidin 3-coumaroylglucoside pyruvic adduct relative to those of the malvidin 3-glucoside pyruvic adduct suggests the *p*-coumaroyl group is attached to this position (*30*). The protons of this coumaroyl ring were also fully assigned. Two doublets integrating one proton each and located

**Table 2. $^{1}H,^{13}C$ Correlations Found in the HMBC Experiments for the Malvidin 3-Glucoside Pyruvic Adduct (mv-py) and Malvidin 3-Coumaroylglucoside Pyruvic Adduct (mv-coum-py) Isolated from 1-Year-Old Port Wines, Analyzed in $CD_3OD$/TFA (98:2)**

| | $^{13}C$ correlations | |
|---|---|---|
| proton | mv-py | mv-coum-py |
| H-6 | 4a, 5, 7, 8 | 4a, 5, 7, 8, 8a |
| H-8 | 4a, 5, 6, 7, 8a | 4a, 5, 6, 7, 8a |
| H-9 | 4, 10, 4a,COOH | 4, 10, 4a |
| H-2′, 6′ | 2, 1′, 2′, 3′, 4′, 5′, 6′ | 2, 1′, 2′, 3′, 4′, 5′, 6′ |
| 3′,5′-$CH_3O$ | 3′, 5′ | 3′, 5′ |
| H−G1 | 3 | G3 |
| CH═C$H_\alpha$$CO_2$R | | −$CO_2$R, 1″ |
| C$H_\beta$═CHC$O_2$R | | −$CO_2$R, $C_\alpha$, 2″, 6″ |
| H-2″,6″ | | −$CO_2$R, $C_\beta$, 2″, 4″, 6″ |
| H-3″,5″ | | 1″, 3″, 4″, 5″ |

at 7.25 and 5.82 ppm were attributed to the olefinic $H_\alpha$ and $H_\beta$ of the coumaroyl moiety, respectively. Their large coupling constant (16.1 Hz), obtained using DQF-COSY experiments, indicated a *trans* stereochemistry. Two other doublets with a coupling constant of 8.7 Hz and integrating for two protons each were also observed at 7.29 and 6.82 ppm and were attributed to the four aromatic protons of the coumaroyl ring. These results are in agreement with the NMR data of malvidin 3-(6-*p*-coumaroyl) monoglucoside isolated from the same wine (Table 1). The acetate group of the malvidin 3-acetylglucoside pyruvic adduct was confirmed by the presence of a singlet located at 1.78 ppm and corresponded to three protons of the acetyl group.

*$^{13}C$ NMR.* The assignments of the carbon chemical shifts of the malvidin 3-coumaroylglucoside pyruvic adduct and the malvidin 3-glucoside pyruvic adduct are presented in Table 1 and were obtained using two-dimensional (HSQC and HMBC) techniques. Correlations observed through HMBC of the malvidin 3-glucoside pyruvic adduct and its coumaroyl ester are shown in Table 2. The correlation observed between the methoxyl proton resonances and the carbons located at 148.4 ppm for the malvidin 3-glucoside pyruvic adduct, and at 150.8 ppm for its coumaroyl ester, allowed them to be assigned to C-3′ and C-5′. Carbon C-9 was assigned at 108.3 ppm for the malvidin 3-coumaroylglucoside pyruvic adduct and at 107.8 for the malvidin 3-glucoside pyruvic adduct through HSQC correlation with H-9. The weak signal detected at 160.4 ppm from an HMBC correlation with H-9 for the malvidin 3-coumaroylglucoside pyruvic adduct and at 159.1 ppm for its coumaroyl ester was attributed to the carboxylic carbon. C-10 was assigned at 154.8 and 152.2 ppm for the malvidin 3-glucoside pyruvic adduct and its coumaroyl ester, respectively, through its correlation with H-9. Despite the similar chemical shifts, carbon C4 was differentiated from C4a because of the $^{3}J$ (C,H) coupling of the latter with H-6.

**Pigment Concentrations in Port Wines.** HPLC analysis of young and 1-year-old Port wine is shown in Figure 3, and their anthocyanin contents are indicated in Table 3. It can be seen that most of the original anthocyanidin monoglucosides have disappeared, and the three derived pigments reported in this work were the major pigments in the analyzed wine samples. Malvidin 3-glucoside pyruvic adduct adduct is by far the most significant pigment after 1 year of bottle aging with a concentration 3−4-fold higher than those of all anthocyanidin 3-glucosides. These data showed the importance of such pigments in color contribution of Port wines during aging.

**Table 3. Concentrations[a] of Total Anthocyanidin Monoglucosides (AMG), the Malvidin 3-Glucoside Pyruvic Adduct (mv-py), Malvidin 3-Acetylglucoside Pyruvic Adduct (mv-ac-py), and Malvidin 3-Coumaroylglucoside Pyruvic Adduct (mv-coum-py) in Young (Y) and 1-Year-Old (O) Port Wines of Touriga Nacional (TN) and Touriga Francesa (TF) Varieties**

| | TN 1 | | TN 2 | | TF 1 | | TF 2 | |
|---|---|---|---|---|---|---|---|---|
| | Y | O | Y | O | Y | O | Y | O |
| AMG | 187.4 | 10.3 | 412.9 | 14.1 | 84.4 | 5.1 | 117.1 | 9.0 |
| mv-py | 46.9 | 51.1 | 49.6 | 51.2 | 18.8 | 25.4 | 19.3 | 27.9 |
| mv-ac-py | 9.3 | 10.9 | 11.4 | 10.1 | 4.0 | 4.9 | 4.1 | 5.9 |
| mv-coum-py | 12.9 | 16.5 | 7.8 | 8.6 | 5.7 | 6.1 | 4.3 | 5.3 |

[a] Levels of AMG expressed in mg of malvidin 3-glucoside/L; levels of pyruvic adducts expressed in mg of mv-py/L. Data are means of triplicate analyses.

Over recent years, the occurrence of new pigments has been reported in aging Port wines (*31*, *32*). The appearance of these pigments is crucial for the changing of wine color from bright red to a more brick red hue. The contribution of these anthocyanin-derived pigments is already significant after only 1 year of aging. Concerning the structural elucidation of malvidin−pyruvic acid adducts, our results support the structure proposed by Fulcrand et al. (*21*) (Figure 4) as indicated by the HSQC correlation between C-9 and H-9 shown in the HSQC spectrum of the malvidin 3-coumaroylglucoside pyruvic adduct.

From a mechanistic point of view, it has already been reported that these pigments arise from reaction between malvidin 3-glucoside (or its acylated forms) with pyruvic acid (which is excreted by yeast) (*21*, *25*). Other small molecules were also reported to react with anthocyanins similarly to pyruvic acid, giving rise to new anthocyanin-derived pigments (*22*). The structural identification of these pigments can be helpful to elucidate the complex evolution of wine color during aging. Furthermore, it is also extremely important to establish that anthocyanins are converted into stable pigments with structural features that improve their food color properties.

ACKNOWLEDGMENT

We thank Tristan Richard and Hilário Tavares for NMR analysis, CESAMO for LSIMS mass spectrometry analysis, and Nicolas Foucou for technical assistance.

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Received for review December 20, 2000. Revised manuscript received June 13, 2001. Accepted June 19, 2001. This research was supported by grants from FCT (Fundação para a Ciência e Tecnologia) of Portugal (PRAXIS/BD/15692/98 and PRAXIS/PCEX/C/QUI/105/96).

JF001505B