

New Anthocyanin Pigments Formed after Condensation with Flavanols

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The formation of three major pigments has been detected in model solutions imitating wine containing malvidin 3-monoglucoside (Mv-3-glu) and procyanidin B2 in the presence of acetaldehyde. Two of them were reddish-blue compounds whose visible spectra are bathochromically shifted with regard to that of Mv-3-glu. They were assigned to two enantiomers containing Mv-3-glu and B2 moieties linked across their C-8 by an ethyl bridge. A molecular ion at m/z 1097, consistent with such a structure, has been established by LC/MS for both of them. The third majority pigment was an orange-red product possessing a spectrum with a maximum wavelength in the visible region at 511 nm and a molecular ion at m/z 1093. A possible structure containing two flavylum mesomeric forms, corresponding to malvidin and to pelargonidin, has been suggested for this compound. This pigment proved to be more stable than the first two, as well as more resistant to discoloration by increased pH values and SO₂. Similar pigments, whose spectra possess maximum wavelengths in the visible region between 490 and 511 nm, have also been found in equivalent solutions containing other flavan-3-ols [(+)-catechin, (–)-epicatechin, or procyanidin B3]. Evidence for the formation in red wine of pigments with similar spectral characteristics is also contributed.

Keywords: Malvidin 3-monoglucoside; flavan-3-ols; procyanidins; acetaldehyde; anthocyanin-derived pigments; LC/MS

INTRODUCTION

During conservation and aging a change is produced in the color of red wine which is attributed to the progressive formation of condensed pigments resulting from the interaction between the anthocyanins and other phenolic compounds, particularly flavanols (catechins, procyanidins). Different mechanisms have been suggested which could intervene in the formation of these new pigments. Processes such as copigmentation (Brouillard *et al.*, 1990; Dangles *et al.*, 1992; Figueiredo *et al.*, 1996; Mistry *et al.*, 1991), direct condensation between anthocyanins and flavanols (Jurd, 1967; Jurd and Somers, 1970; Liao *et al.*, 1992; Santos-Buelga *et al.*, 1995), and reactions between them mediated by acetaldehyde (Bakker *et al.*, 1993; Baranowski and Nagel, 1983; Dallas *et al.*, 1996a,b; Escribano-Bailon *et al.*, 1996; Rivas-Gonzalo *et al.*, 1995; Roggero *et al.*, 1987; Timberlake and Bridle, 1976) have been demonstrated in diverse studies carried out using model assays. However, in spite of the evidence obtained in simple models, attempts to isolate the pigments really formed in wine had failed and hypotheses offered up to now to explain the changes of color which are produced in wine have been merely speculative.

In a study performed by our group (Guerra *et al.*, 1995), in the HPLC chromatograms of red wines obtained in microvinifications peaks corresponding to new pigments possessing spectra whose maxima in the visible region were between 495 and 510 nm were detected. Recently two pigments showing similar spectra, with maxima wavelength of absorption at 507 nm, were extracted by Cameira-dos-Santos *et al.* (1996) from the material absorbed in polymeric membranes used for the cross-flow microfiltration of red wine. The structure

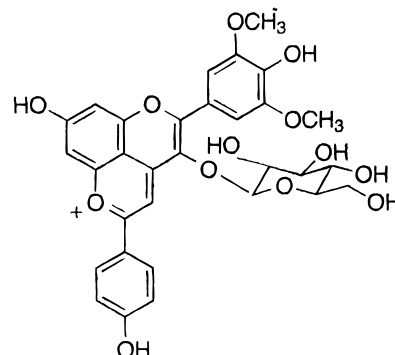


Figure 1. Structure of a malvidin-derived pigment identified by Fulcrand *et al.* (1996a).

of these pigments was associated to that presented in Figure 1 (Fulcrand *et al.*, 1996a) containing two possible mesomeric flavylum forms, one corresponding to malvidin and the other to pelargonidin. The cyclation between the C-4 and the hydroxyl group of the C-5 of the flavylum nucleus with the double bonding of the 4-vinylphenol was suggested as a mechanism for its formation.

In this study new evidence is contributed that concerns the formation of pigments possessing spectra of this type, in model solutions containing Mv-3-glu and flavanols in the presence of acetaldehyde, and a possible structure for them is suggested.

MATERIALS AND METHODS

Isolation of Malvidin 3-Monoglucoside and Procyanidins B2 and B3. The procedure described by Rivas-Gonzalo *et al.* (1995) was used for the isolation of the Mv-3-glu from grape skin. Procyanidins B2 and B3 were isolated from grape seed, according to the protocol described by Escribano-Bailón *et al.* (1992), though the final purification was carried out by preparative HPLC using a Waters model

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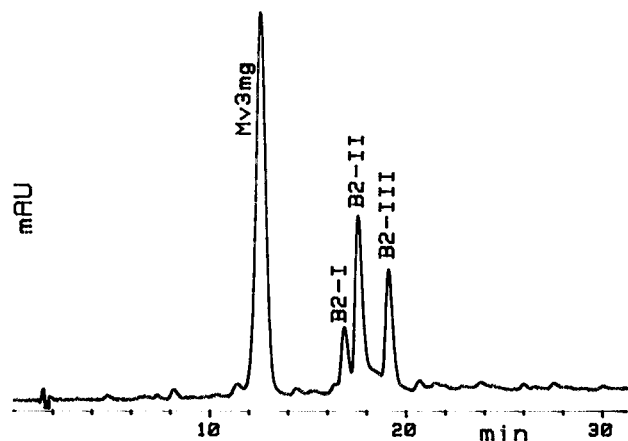


Figure 2. HPLC chromatogram recorded at 525 nm of a model solution containing malvidin 3-monoglucoside + procyanidin B2 + acetaldehyde at pH 3.2 after 30 days of reaction at 15 °C.

600 controller pump and a reversed-phase radial compression cartridge Nova-pak C18 6 μ m (25 \times 100 mm). The solvents were A, acetic acid at 2%, and B, methanol. The gradient was 5% B isocratic for 5 min and 5%–50% B for 25 min, at a flow rate of 18 mL/min. Detection was carried out at 280 nm. The compounds obtained were freeze-dried, and their purity was established by HPLC/DAS.

Preparation of Model Solutions. Equimolar solutions of Mv-3-glu (200 mg/L, 0.4 mM) plus procyanidin B2 (250 mg/L, 0.4 mM) in the presence of acetaldehyde (200 mg/L, 4.6 mM) were prepared in a medium that contained tartaric acid at 5% (w/v) and 10% ethanol and were adjusted to pH 3.2 or 4 with NaOH. Similar model solutions were also prepared using (+)-catechin (Cat), (–)-epicatechin (Ec), or procyanidin B3. The mixtures were put in glass vials, which were closed with a septum of Teflon and silicone and maintained at 15 °C. Samples were taken periodically by puncturing the septum over a period of 4 months. The first sample (day 0) was taken 1 h after the preparation of the solution.

Analysis. The samples were analyzed by HPLC/DAS, and their spectra were determined before and after bleaching with SO₂, in the range of wavelengths between 250 and 650 nm (Rivas-Gonzalo *et al.*, 1995).

LC/MS. An LC/MS analysis was carried out on the solution containing procyanidin B2, Mv-3-glu, and acetaldehyde at pH 3.2 at day 45 of the assay. The chromatographic conditions (pump, solvents, and gradient) were similar to those used for the HPLC analysis, but a flow rate of 1 mL/min was used to adjust to the exigencies of the mass detector's electrospray source. The detection was carried out using a Finnigan MAT LCQ ion trap detector provided with an electrospray ionization probe. The MS detector was programmed to obtain the FulScan between 220.0 and 1850.0 amu in positive mode. Detection was also carried out at 280 nm.

RESULTS AND DISCUSSION

In the solutions containing B2, Mv-3-glu, and acetaldehyde, the progressive formation of three new pigments (marked B2-I, B2-II, and B2-III in Figure 2) was observed. Pigments B2-I and B2-II were also reported by Dallas *et al.* (1996a), but no evidence was presented for the presence of a pigment similar to B2-III. Pigments B2-I and B2-II are bluish-red, and their spectra have the maximum wavelength in the visible region bathochromically displaced in relation to that of the Mv-3-glu (Figure 3). Identical molecular ions were determined at m/z 1097 by LC/MS analysis for both B2-I and B2-II. This mass corresponds exactly to a structure in which Mv-3-glu and procyanidin B2 are linked by an ethyl bridge. According to the evidence recently contributed (Escribano-Bailón *et al.*, 1996; Rivas-Gonzalo

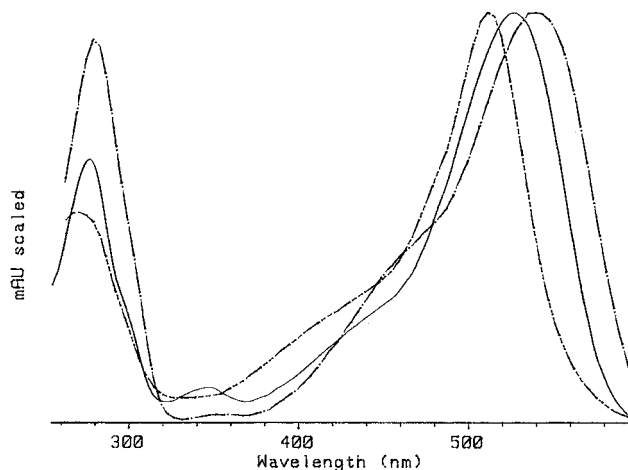


Figure 3. Spectra of malvidin-3-monoglucoside (—) and pigments B2-II (---) and B2-III (····).

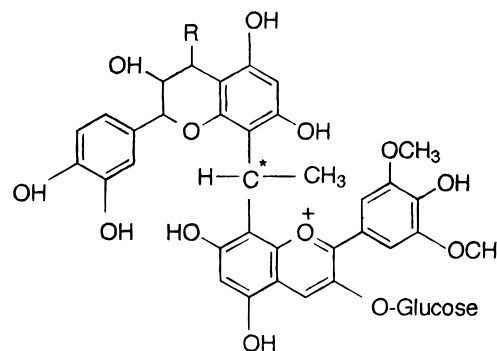


Figure 4. Proposed structure for pigments B2-I and B2-II; R = epicatechin unit.

et al., 1995), anthocyanin and flavanol would be linked across their C-8 and such pigments correspond to two enantiomers, taking into account the presence of an asymmetric carbon in the ethyl bridge (Figure 4). The formation of two pigments similar to I and II was also observed by ourselves (Rivas-Gonzalo *et al.*, 1995) and by other authors (Bakker *et al.*, 1993; Dallas *et al.*, 1996a,b; Escribano-Bailón *et al.*, 1996; Roggero *et al.*, 1987) in solutions containing other flavanols different from B2, such as Cat, Ec, B3, or B2-3'-O-gallate. A molecular ion at m/z 809 was determined by Bakker *et al.* (1993) for the two pigments formed in solutions containing Cat, Mv-3-glu, and acetaldehyde, also consistent with an anthocyanin–ethyl–catechin structure. The spectra of type I pigments show a more pronounced shoulder at around 440 nm than type II pigments, and the former elute first in our HPLC conditions. For all the flavanols assayed, higher amounts of one of the bluish-red pigments were always formed. However, the disproportion between pigments I and II was lower when they were derived from monomeric catechins, a circumstance also noted by Dallas *et al.* (1996b). In the case of the dimers the existence of sterical hindrances might prevent the formation of one of the pigments.

As has been demonstrated, the formation of pigments mediated by acetaldehyde is favored at more acidic pH (García-Viguera *et al.*, 1994), which has been attributed to the greater facility of the acetaldehyde to form a cation, necessary for the condensation to be produced (Rivas-Gonzalo *et al.*, 1995). In all of the solutions studied here a more rapid accumulation of pigments I and II, but also a faster subsequent decrease, is produced at more acidic pH. This can be observed in Figure 5, where the changes of these pigments in solutions

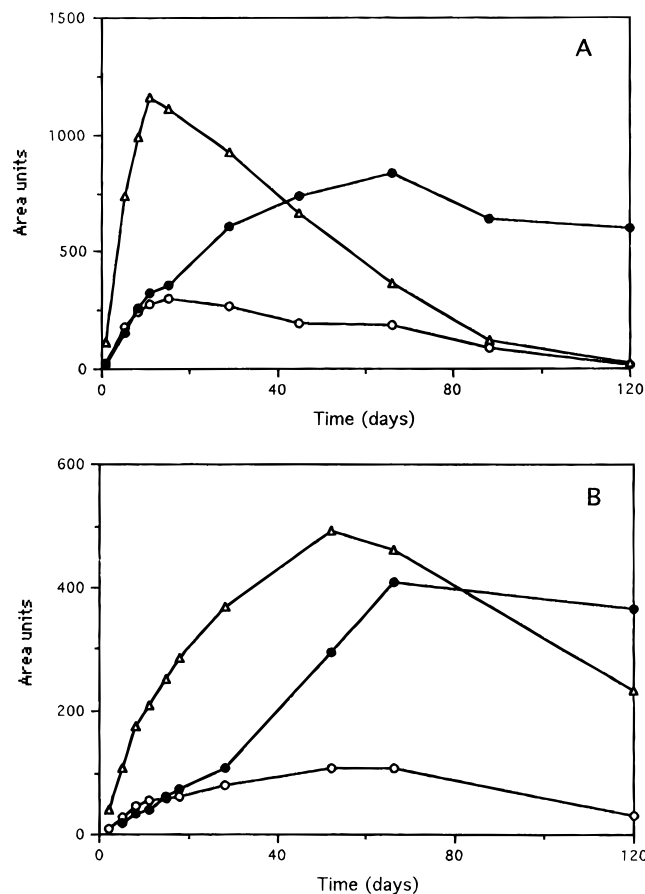


Figure 5. Changes in chromatographic areas of peaks B2-I (○), B2-II (△), and B2-III (●) recorded at 525 nm in solutions of malvidin 3-monoglucoside + procyanidin B2 + acetaldehyde at pH 3.2 (A) and pH 4 (B) kept at 15 °C.

containing B2, Mv-3-glu, and acetaldehyde at pH 3.2 and 4 are compared. The decrease is attributed to the formation of more condensed structures, which lead to their precipitation, as is shown by the appearance of a violet precipitate in the vials. The increase in size of the pigments could take place by the covalent incorporation of new flavanol units linked by ethyl bridges (Rivas-Gonzalo *et al.*, 1995), as is supported by the fact that more acidic pH's favor a faster progression of the process. This assumption has recently been confirmed in LC/MS studies on acetaldehyde-induced polymerization of flavan-3-ols and Mv-3-glu carried out by Es-Safi *et al.* (1996). It has also been suggested that non-covalent dimerization of pigments could take place, which may be followed by a covalent coupling between them; oligomers possessing two flavylum and two flavanol residues would be produced, which might be the early products in the polymerization process leading to the precipitation (Escribano-Bailón *et al.*, 1996). Neither process (incorporation of new units/non-covalent contact) is necessarily exclusive, and both could contribute to the precipitation.

As indicated at first, in the solutions containing B2, Mv-3-glu, and acetaldehyde the formation of a third majority pigment (B2-III) was also observed. It is an orange-red product, whose spectrum possesses a maximum wavelength in the visible region at 511 nm (Figure 3). The formation of one pigment with similar spectral characteristics, but with λ_{\max} at 505 nm, has already been reported by our group in solutions of Cat, Mv-3-glu, and acetaldehyde (Rivas-Gonzalo *et al.*, 1995) and confirmed again in the new assays performed. In

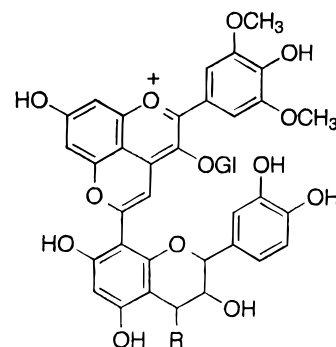


Figure 6. Proposed structure for pigment B2-III; R = epicatechin unit.

addition, similar pigments have also been detected in solutions containing (–)-epicatechin (λ_{\max} at 511 nm) or B3 (λ_{\max} at 490 nm). As far as we know, none of these products has been previously reported in similar model solutions (Bakker *et al.*, 1993; Dallas *et al.*, 1996a,b).

In the LC/MS analysis, a molecular ion at m/z 1093 was established for the pigment B2-III. This mass fits exactly with the structure shown in Figure 6, speculated from that established by Fulcrand *et al.* (1996a) (Figure 1) for a derived pigment showing a similar spectrum. As these authors showed, such a structure can yield two possible flavylum mesomeric forms, one corresponding to the malvidin type and the other to the pelargonidin type. The lower absorption maximum of this latter would explain the hypsochromic shift in the visible spectrum of pigment B2-III with regard to that of Mv-3-glu.

No definitive explanation is available for the possible mechanism of formation of such a structure in the model solutions used. As indicated above, different pigments were detected in the solutions containing different flavanols, showing that these latter were involved in their formation; if not, the same product should have been observed in all of them. The possibility of a cation from the depolymerization of the procyanidin being involved can be discarded, since similar pigments were also formed from monomeric catechins. Moreover, in solutions containing B2 and Mv-3-glu in the absence of acetaldehyde no such pigment was detected, but others showed xanthylum-like spectra (Santos-Buelga *et al.*, 1996). It seems clear that B2, Mv-3-glu, and acetaldehyde are involved in B2-III formation, since it was not observed in solutions containing any other combination among these compounds. Fulcrand *et al.* (1996a) showed that the pigment of Figure 1 results from the addition of 4-vinylphenol to the flavylum nucleus. Similarly, it could be speculated that pigment B2-III is formed by reaction of the Mv-3-glu cation with a B2 moiety possessing a vinyl residue at C-8. A fragment corresponding to vinylcatechin was found by Saucier *et al.* (1996) after fragmentation of catechin oligomers linked by ethyl bridges. Similar fragments could be expected to be formed from equivalent B2-ethyl oligomers or B2-ethyl-Mv-3-glu pigments (e.g., B2-I and B2-II). Likewise, an arrangement may occur in the adduct resulting from the substitution of the acetaldehyde at C-8 of the procyanidin B2 (the first step in the process of formation of pigments B2-I and II), which allows its addition to the flavylum nucleus. Higher levels of pigment B2-III are reached at more acidic pH (Figure 5A), and lower pH values favor both the anthocyanin flavylum form and the formation of the B2-acetaldehyde adducts.

As is shown in Figure 7, an increase in the color density exists in the solution at pH 4, in spite of the

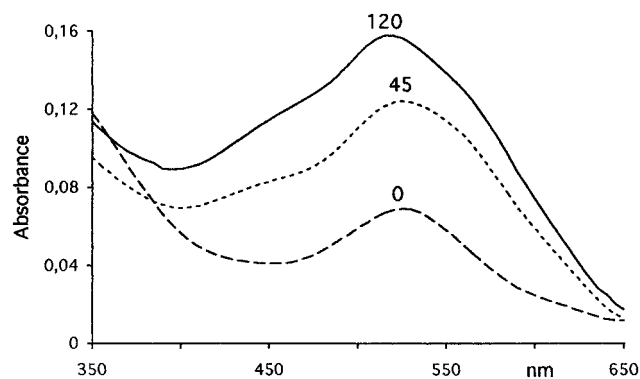


Figure 7. Changes in the spectrum of a solution of malvidin 3-monoglucoside + procyanidin B2 + acetaldehyde at pH 4 and 15 °C.

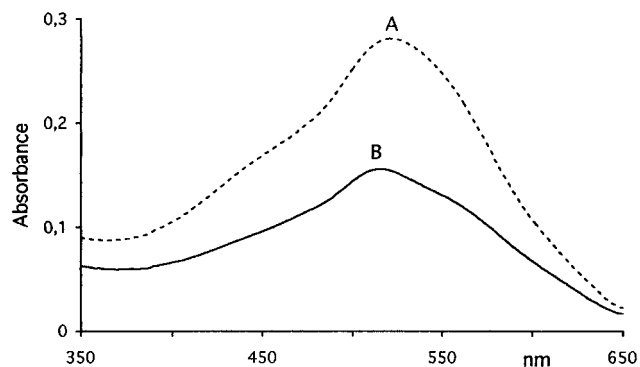


Figure 8. Spectrum of the solution of malvidin 3-monoglucoside + procyanidin B2 + acetaldehyde at pH 3.2 at day 30 before (A) and after (B) bleaching with SO₂.

loss of Mv-3-glu and the decrease observed in the levels of pigments B2-I and B2-II from day 45 of the assay (see Figure 5B). Therefore, the increase of B2-III must explain the higher color density found at day 120, as well as the displacement of the maximum of absorption toward lower wavelengths. It must be assumed that this product is more resistant to the discoloration that increased pH values exert on anthocyanin pigments. This suggests that it must be more protected from the hydration, leading to the formation of colorless pseudo-bases. Likewise, pigment B2-III shows also greater resistance to the bleaching by SO₂. In Figure 8 is shown the spectrum of a solution at pH 3.2 containing different pigments before and after the addition of bisulfite. The hypsochromic shift produced indicates that pigment B2-III is less sensitive to the SO₂ effect than Mv-3-glu, B2-I, and B2-II. Its relative resistance to the bleaching could confirm the substitution of the C-4 of Mv-3-glu, which protects the molecule from hydration and reaction with SO₂. The resistance to discoloration of anthocyanin-derived pigments with spectral characteristics similar to B2-III was also noted by Sarni-Manchado *et al.* (1996).

Traces of pigment B2-III are already detectable on the first day of the assay, and with the passage of time it becomes the majority pigment detected and the principal one responsible for the color in the solutions at pH 3.2 (Figure 5A). Its greater stability with regard to pigments B2-I and B2-II might be due to the fact that it shows no tendency to polycondense. The slight decrease which is produced in its levels in the final stage of the assays could be explained by the depletion of Mv-3-glu, which stops its accumulation and allows a certain degradation. The formation of type III pigments is not so favored when other flavanols different from B2 are

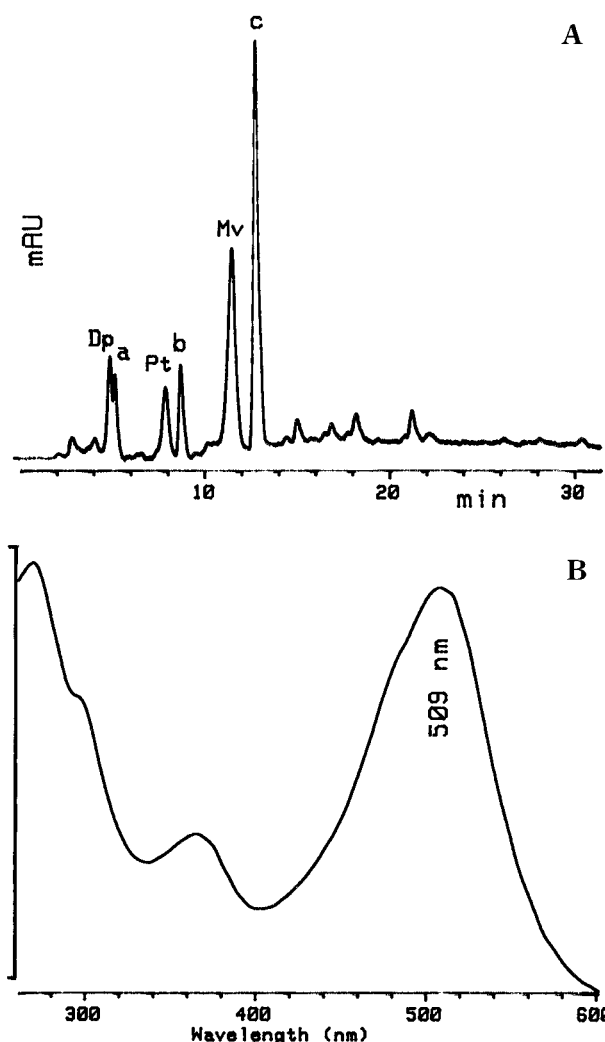


Figure 9. (A) HPLC chromatogram recorded at 525 nm of a mature red wine. Dp, Pt, and Mv correspond to the native anthocyanin 3-monoglucosides of the wine, and *a*, *b*, and *c* are new anthocyanin-derived pigments. HPLC conditions are the same as in Figure 2. (B) Spectrum of pigment *c*.

involved. In the solutions containing Cat, Ec, or B3, these pigments are detected later and very small amounts are accumulated.

The formation of pigments possessing similar spectra to that of type III compounds was detected by our group (Guerra *et al.*, 1995) in red wines obtained by microvinification of *Vitis vinifera* grapes (Tempranillo variety). In Figure 9A is shown the chromatogram corresponding to one of those wines, indicating the new derived pigments. The spectrum of pigment *c* is presented in the Figure 9B. These pigments elute in more polar phases of the HPLC gradient than do those found by Cameira-dos-Santos *et al.* (1996) or those formed in our model solutions. Moreover, each of the new pigments elutes close to a native anthocyanin, leading to the consideration that this could be its precursor (delphinidin 3-monoglucoside, petunidin 3-monoglucoside, and Mv-3-glu, in the respective cases of pigments *a*, *b*, and *c*). In the formation of these pigments, acetaldehyde also seems to be involved, as was observed in model solutions, since it is more favored in non-sulfited wines (Guerra *et al.*, 1995).

The detection of a new series of compounds presenting similar spectral characteristics has been reported by Fulcrand *et al.* (1996b) in the 18th International Conference on Polyphenols. It must be emphasized that,

according to our knowledge, this type of anthocyanin-derived pigments constitutes, up to the present, the only type whose presence has been detected in wine. This circumstance, together with their relative stability, makes these pigments particularly interesting, especially when it is borne in mind that they have an orange-red tone similar to that which red wine acquires during aging. Thus new and promising expectations are arising in the study of the changes of color that are produced in red wine during maturation and aging.

ACKNOWLEDGMENT

Thanks are due to Mr. J. García for technical assistance and to Mr. G. Jenkins for the translation.

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Received for review December 16, 1996. Accepted March 10, 1997.® This work was supported by Junta de Castilla y León (SA527/93) and CICYT (AL194-0704). E.F.-A. and M.T.G. are indebted to Universidad de Salamanca and Junta de Castilla y León, respectively, for pre-doctoral fellowships.

JF9609587

® Abstract published in *Advance ACS Abstracts*, May 15, 1997.