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A new vinylpyranoanthocyanin pigment occurring in aged red wine

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Abstract

A new pyranoanthocyanin–vinylphenol pigment was detected in an aged Port red wine. The UV–Vis spectrum of this pigment was found to have a λ_{max} of 538 nm that is bathochromically shifted from that of original anthocyanins, exhibiting a more purple hue in acidic solution. This newly formed pigment was synthesized in model solution through the reaction between malvidin 3-*O*-glucoside–pyruvic acid adduct and vinylphenol and its structure was assigned by NMR and mass spectrometry. This pigment is reported herein for the first time.

Keywords: Pyranoanthocyanin; Vinylphenol; Pyruvic acid; Pigment; Red wine; Aging; colour

1. Introduction

During wine aging, the initial red-purple colour of young red wines is progressively shifted towards more orange-like hues. These changes are generally attributed to the transformation of the original grape anthocyanins to new pigments through different reactions, including oxidation–reduction reactions and complexation with other compounds, such as carbohydrates, proteins, metals or flavanols. The newly formed red pigments were first thought to result mainly from reactions between anthocyanins and flavanols mediated, or not, by acetaldehyde (Bakker, Picinelli, & Bridle, 1993; Jurd, 1969; Liao, Cai, & Haslam, 1992; Remy, Fulcrand, Labarbe, Cheynier, & Moutounet, 2000; Rivas-Gonzalo, Bravo-Haro, & Santos-Buelga, 1995; Somers, 1971; Timberlake & Bridle, 1976). Nevertheless, reactions between anthocyanins and/or flavanols with other compounds, such as pyruvic acid (Bakker et al., 1997; Bakker & Timberlake, 1997; Fulcrand, Benabdeljalil, Rigaud, Cheynier, & Moutounet, 1998; Mateus, Silva, Vercauteren, & De Freitas, 2001; Romero & Bakker, 1999), vinylphenol (Cameira dos Santos, Brillouet, Cheynier, & Moutounet, 1996; Fulcrand, Cameira dos Santos, Sarni-Manchado, Cheynier, & Bonvin, 1996), vinylcatechol (Schwarz, Wabnitz, & Winterhalter, 2003), α-ketoglutaric acid (Benabdeljalil et al., 2000), acetone (Benabdeljalil et al., 2000; Hayasaka & Asenstorfer, 2002; Lu & Foo, 2001), 4-vinylguaiacol (Hayasaka & Asenstorfer, 2002) and glyoxylic acid (Es-Safi et al., 1999; Fulcrand, Cheynier, Oszmiansky, & Moutounet, 1997), have been demonstrated to yield new families of anthocyanin-derived pigments, namely pyranoanthocyanins with

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spectroscopic features that may contribute to a more orange-red colour of wine. Until very recently, most of the conclusions obtained regarding the identity of new pigments and their mechanisms of formation were derived from studies carried out in model solutions. Over recent years, the advances in mass spectrometry and NMR have allowed detection and structural characterisation of several new pigment families occurring directly in wine (Hayasaka & Asenstorfer, 2002; Mateus et al., 2003; Mateus, Pascual-Teresa, Rivas-Gonzalo, Santos-Buelga, & De Freitas, 2002; Mateus, Silva, Rivas-Gonzalo, Santos-Buelga, & De Freitas, 2002; Vivar-Quintana, Santos-Buelga, Francia-Aricha, & Rivas-Gonzalo, 1999). Among these, some structures have been found to correspond to bluish anthocyanin-derived pigments (Mateus, Silva, Rivas-Gonzalo, Santos-Buelga, & De Freitas, 2003). These compounds were shown to arise from the reaction between anthocyanin pyruvates and vinylflavanol adducts, derived either from the cleavage of ethyl-linked flavanol oligomers or from the dehydration of the flavanol–ethanol adduct formed after reaction of flavanols with acetaldehyde. Anthocyanin pyruvates, which result from the reaction between anthocyanins and pyruvic acid, are the major wine pigments after 1–2 years of aging, whilst anthocyanins decrease significantly, in amount, during the same period of time (Mateus & De Freitas, 2001). These pyruvic acid adducts become the main precursors of new pigments through reactions with other molecules, such as flavanols.

In the present work, a new pigment, whose structure corresponds to the pyruvic acid adduct of malvidin 3-*O*-glucoside linked to a vinylphenol group, was synthesized in model solution and structurally characterized by NMR and mass spectrometry. This pigment was also detected in a red wine sample by LC/DAD-MS. This outcome reinforces the fact that anthocyanin–pyruvic acid adducts are important precursors for the formation of new pigments in aged red wines.

2. Materials and methods

2.1. Red wine

The studied wine sample was a 2-year old Port red wine (pH 3.6, 18% alcohol (v/v), total acidity 6.5 g/l, total SO_2 20 mg/l), made from Touriga Nacional and Touriga Francesa varieties (*Vitis vinifera*) grown in the Douro Demarcated Region (Northern Portugal).

2.2. Wine fractionation

Port wine samples were applied directly onto a 250 × 16 mm i.d. TSK Toyopearl gel HW-40(S) column and eluted with 40% aqueous ethanol, yielding the original anthocyanidin 3-*O*-glucosides and some pyruvic acid adducts of the three major anthocyanidin 3-*O*-glucosides (malvidin 3-*O*-glucoside, malvidin 3-*O*-acetylglucoside and malvidin 3-*O*-coumaroylglucoside), together with other pyranoanthocyanin pigments, as previously reported (Mateus, Silva, et al., 2002). When practically no more coloured compounds were eluted from the column, the solvent was changed to 60% ethanol (v/v) and different fractions were collected thereafter. The pH of the used eluents was set to 2.0 with HCl.

2.3. HPLC conditions

The fractions eluted from the TSK Toyopearl gel column were analyzed by a Knauer K-1001 HPLC chromatograph on a 250 × 4.6 mm i.d. reversed-phase C18 column (Merck, Darmstadt), with detection at 520 nm, using a Knauer K-2800 diode array detector. The solvents were A: H_2O/HCOOH (9:1), and B: CH_3CN/H_2O/CH_3COOH (8:1.95:0.05). A linear gradient from 20% to 85% B in 70 min was used with a flow rate of 1.0 ml/min.

2.4. LC–MS conditions

A Hewlett–Packard 1100 series liquid chromatograph, equipped with an AQUA™ (Phenomenex, Torance, CA, USA) reversed-phase column (150 × 4.6 mm, 5 μm, C18) maintained at 35 °C was used. Solvents were A: aqueous 0.1% trifluoroacetic acid, and B: acetonitrile, using the gradient previously reported (Pissarra, Mateus, Rivas-Gonzalo, Santos-Buelga, & De Freitas, 2003). 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 60.

2.5. Malvidin 3-O-glucoside–pyruvic acid adduct synthesis

The formation of the malvidin 3-*O*-glucoside–pyruvic acid adduct was achieved through reaction of malvidin 3-*O*-glucoside with sodium pyruvate (Sigma–Aldrich®, Germany) in water (pH 2.6, 35 °C) at a molar ratio pyruvate/malvidin-3-glucoside of 600:1 over 5 days (Mateus, Oliveira, Pissarra, & De Freitas, 2003). The reaction was monitored by HPLC-DAD. Malvidin 3-*O*-glucoside–pyruvic acid adduct was 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 pigment was 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 20% ethanol for a final purification of the product, which was characterized by NMR and MS (Mateus et al., 2001).

2.6. Formation of vinylpyranomalvidin 3-*O*-glucoside–phenol

The synthesis of vinylpyranomalvidin 3-*O*-glucoside–phenol was performed at 35 °C in 20% aqueous ethanol (pH 2.6) in a screw-cap vial containing 40 mg of malvidin-3-glucoside–pyruvic acid adduct extract and 1.2 ml of vinylphenol (Sigma–Aldrich®, Germany) yielding a molar ratio of vinylphenol/anthocyanin–pyruvate of 250:1. After 3 days of reaction, the solution was analyzed by HPLC (Knauer K-1001), using a reversed-phase C18 column; detection was carried out with a diode array detector (Knauer K-2800). The solvents and the gradient were those referred to above. Pigment A was 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 pigment was collected, concentrated under vacuum and applied onto a 150 × 16 mm i.d. Toyopearl HW-40(s) gel column (Tosoh®, Japan), which was eluted with aqueous 60% ethanol for a final purification of the product, which was structurally characterized by NMR and mass spectroscopy.

2.7. 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 (*g*COSY), while ^{13}C resonances were assigned using 2D NMR techniques (*g*HMBC and *g*HSQC) (Bax & Subramanian, 1986; Bax & Summers, 1986). The delay for the

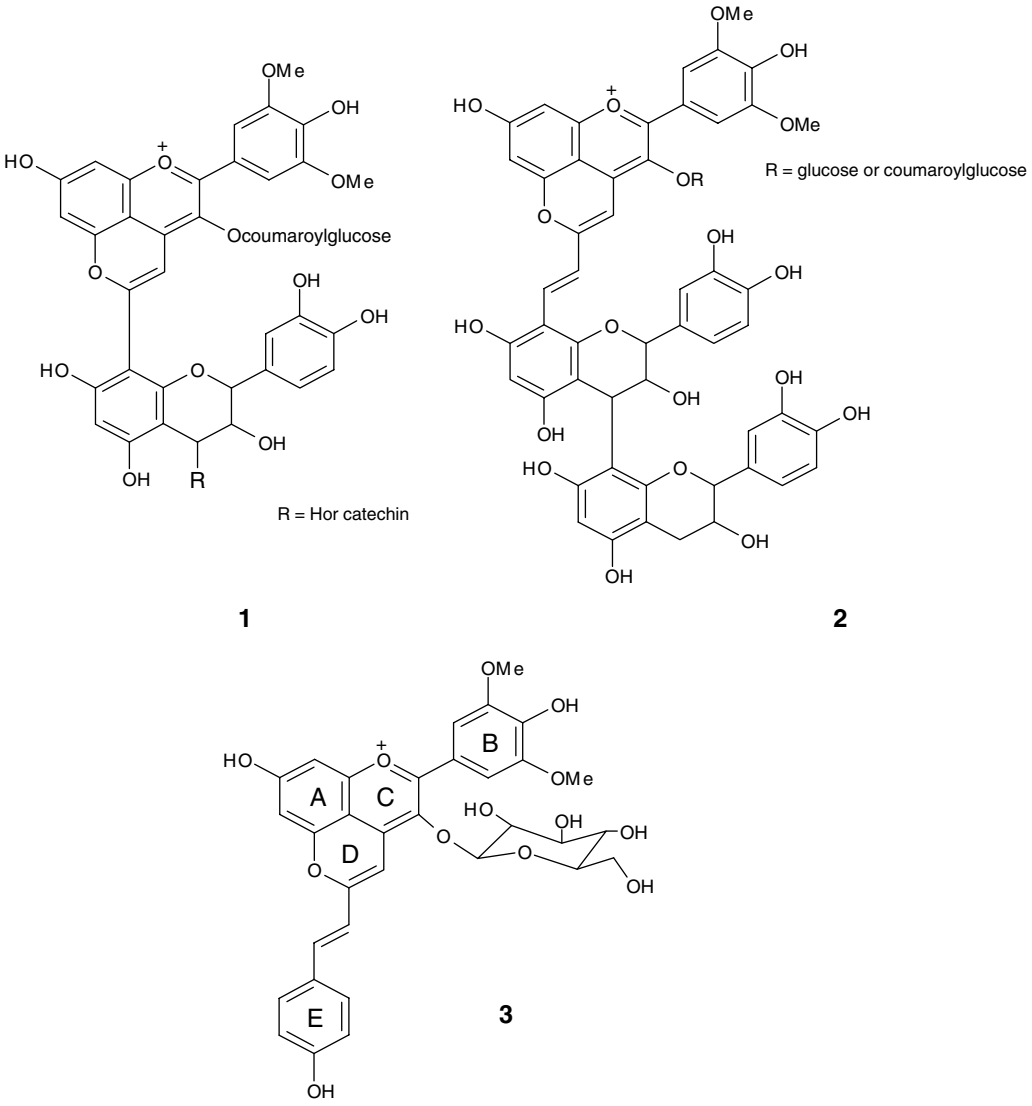

Fig. 1. Structure of: (1) acylated pyranoanthocyanin–flavanol pigments (Mateus et al., 2003) and (2) blue vinylpyranoanthocyanin–flavanol pigments (Mateus, Silva, et al., 2003) and (3) vinylpyranomalvidin 3-*O*-glucoside–phenol.

long-range C/H coupling constant was optimized to 7 Hz.

3. Results and discussion

3.1. Detection and identification of vinylpyranomalvidin 3-O-glucoside–phenol

The collected wine fractions were thoroughly analyzed by HPLC/DAD-MS. The use of mass spectrometry, coupled to high performance liquid chromatography, allowed the tentative identification of several acylated pyranoanthocyanins previously isolated from Port wine and characterized by MS and NMR (Mateus et al., 2003; Mateus, Silva, et al., 2002) (Fig. 1). Moreover, two recently reported blue pigments (Fig. 1), corresponding to vinylpyranoanthocyanins linked to a procyanidin dimer, were also detected by LC–MS (Mateus, Silva, et al., 2003). In addition, a chromatographic peak was observed, although in a much lower significance, comparatively, to the other pyranoanthocyanins previously reported. The mass spectrum of this new pigment showed an ion mass $[M]^+$ at *m/z* 635 and a major fragment ion $[M - 162]^+$ at *m/z* 473 which could correspond to the loss of a glucose moiety (data not shown). This mass is consistent with the structure where a vinylpyranomalvidin 3-*O*-glucoside unit is linked to a phenol group, as shown in Fig. 1. With the exception of the glucose moiety, no other fragment ions were observed in the MS^2 spectrum under the conditions used. This indicates that such compounds present a higher stability, as previously reported for vinylpyranoanthocyanin–flavanol pigments (Mateus, Silva, et al., 2003). The whole structure of this pigment suggests that it could result from the addition of *p*-vinylphenol to a vinylpyranomalvidin 3-*O*-glucoside moiety.

3.2. Formation of vinylpyranomalvidin 3-O-glucoside–phenol

Vinylpyranomalvidin 3-*O*-glucoside–phenol was tentatively synthesized in model solution using the malvidin 3-*O*-glucoside–pyruvic acid adduct extract previously obtained from the reaction between malvidin 3-*O*-glucoside and sodium pyruvate. After 3 days of reaction between the malvidin 3-*O*-glucoside–pyruvic acid adduct and *p*-vinylphenol, HPLC/DAD-MS analysis showed the presence of a very significant peak with the same retention time and UV–Vis spectrum as the vinylpyranoanthocyanin–phenol pigment detected in Port wine. The mass spectrum of this pigment revealed a

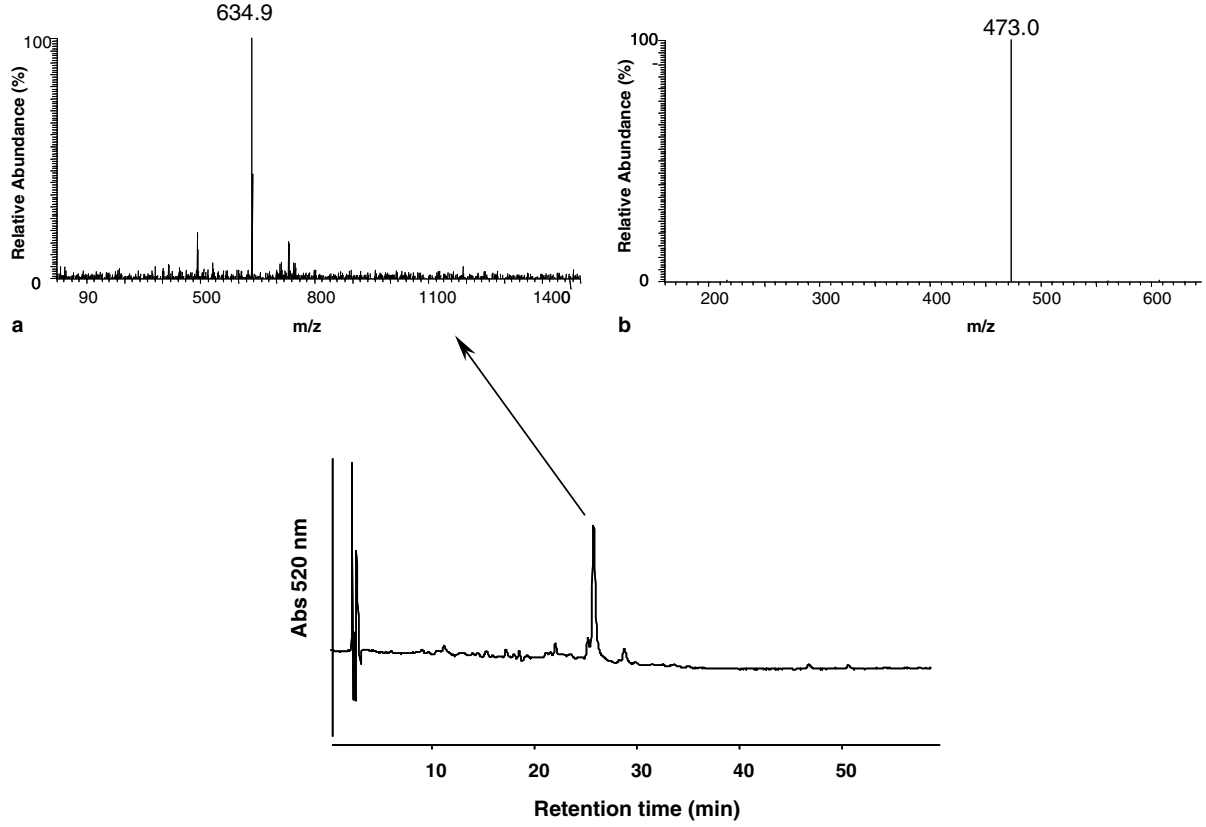

Fig. 2. HPLC chromatogram of vinylpyranomalvidin 3-*O*-glucoside–phenol after Toyopearl gel purification, and respective MS analysis performed with an ion spray source in the positive-ion mode: a, full mass; b, MS^2 of the major fragment obtained in full mass (*m/z* 635).

$[M]^+$ molecular ion at *m/z* 635 and a major fragment ion $[M - 162]^+$ at *m/z* 473, which is identical to the data referred to above (Fig. 2.). This outcome strongly suggests that such pigments were obtained in wine by reaction between vinylphenol and anthocyanin–pyruvic acid adducts.

The structure of pigment A was further confirmed by ^1H and ^{13}C NMR data, as shown in Table 1. The assignment of the protons was achieved by 1D and 2D experiments (*g*COSY), whereas the carbons were assigned through 2D experiments (*g*HSQC and *g*HMBC). Overall, the ^1H and ^{13}C NMR data of the pyranoanthocyanin moiety are in agreement with that of analogous pyranoanthocyanins previously described (Mateus et al., 2003; Mateus, Silva, et al., 2002, 2003). The protons and carbons of the vinylphenol group were all easily assigned. Two doublets, integrating one proton each and located at 7.97 and 7.16 ppm, were, respectively, attributed to protons H_α and H_β. Their high coupling constant (15.8 Hz) obtained using *g*COSY experiment suggests a *trans* stereochemistry. Two other doublets with a coupling constant of 8.6 Hz and integrating two protons each were also observed at 7.68 and 6.91 ppm and were attributed to the four aromatic protons of the phenol ring. The carbons of the vinylphenol group were assigned by direct ^1H,^{13}C correlation for carbons H_α, H_β, H-2″,6″, H-3″,5″, and by long distance ^1H, ^{13}C correlation for carbons H-1″and H-4″.

Table 1
^1H and ^{13}C chemical shifts of vinylpyranomalvidin-3-*O*-glucoside–phenol isolated from a 2-year old Port red wine, determined in CD_3 OD/TFA (98:2)

Position	δ^1H (ppm); *J* (Hz)	$\delta^{13}C$
Pyranomalvidin moiety		
2C		162.1
3C		n.a.
4C		n.a.
4aA		108.7
5A		154.9
6A	7.15; *bs*	108.4
7A		168.2
8A	7.33; *bs*	109.0
8aA		154.0
9D	8.04; *s*	107.0
10D		169.0
1′B		120.8
2′B, 6′B	7.67; *s*	110.0
3′B, 5′B		148.4
4′B		142.5
OMe	3.99; *s*	57.2
Vinylphenol group		
H_α	7.97; *d*, 15.8	145.5
H_β	7.16; *d*, 15.8	117.7
1″		127.1
2″, 6″	7.68; *d*, 8.6	133.5
3″, 5″	6.91; *d*, 8.6	118.2
4″		144.9
Glucose moiety		
Gl-1	4.75; *d*, 7.8	105.8
Gl-2	3.65; *dd*, 9.2/7.8	75.7
Gl-3	3.41; *	77.1
Gl-4	3.38; *	71.8
Gl-5	3.19; *	78.8
Gl-6a	3.72; *dd*, 11.6/1.9	62.9
Gl-6b	3.45; *	62.9

*, unresolved; *bs*, broad singlet; *s*, singlet; *d*, doublet; *dd*, double doublets; n.a., not assigned.

3.3. Mechanism of formation

The formation of vinylpyranomalvidin 3-*O*-glucoside–phenol is supposed to be similar to that previously reported for the blue vinylpyranoanthocyanin–flavanol pigments (Mateus, Silva, et al., 2003). Therefore, the pyruvic acid adduct of malvidin 3-*O*-glucoside is thought to react with *p*-vinylphenol, yielding this new pigment (Fig. 3). In wine, *p*-vinylphenol is thought to arise from decarboxylation of *p*-coumaric acid (Chatonnet, Dubourdieu, Boidron, & Lavigne, 1993) and the pyruvic acid adduct of malvidin 3-*O*-glucoside is formed through reaction of malvidin 3-*O*-glucoside with pyruvic acid (Fulcrand et al., 1998; Romero & Bakker, 1999). In the proposed mechanism (Fig. 4), anthocyanin–pyruvic acid derivatives are thought to react through their C-10 position with the vinyl group of *p*-vinylphenol. The last step of the formation involves the loss of a formic acid group and oxidation, yielding the new anthocyanin-derived pigment. The extended conjugation of the π electrons in this newly formed structure is likely to confer a higher stability on the molecule.

3.4. UV–Vis spectrophotometry

Although the chromophore moiety of vinylpyranomalvidin 3-*O*-glucoside–phenol is quite similar to that of previously reported blue pigments (Mateus, Silva, et al., 2003), the UV–Vis characteristics were found to be different. Indeed, the UV–Vis spectrum of this newly formed pigment revealed a λ_{max} located at 538 nm (Fig. 4), which is lower than those of the previously characterized blue pigments (575 nm). The main structural difference between these pigments, that could interfere in the chromophore features, lies in the substitutive groups of ring E, which consists of a phenol ring in the case of vinylpyranomalvidin 3-*O*-glucoside–phenol and a phloroglucinol ring of the flavanol moiety in the blue pigment structures. The increase of hydroxylation in ring E leads to a shift towards a more bluish colour as a result of the extended conjugation of the π electrons throughout the molecule. Therefore, a poly-hydroxylated substitutive group (such as a phloroglucinol ring) in the vinylpyranoanthocyanin structure is supposed to induce a higher bathochromic shift of the λ_{max}.

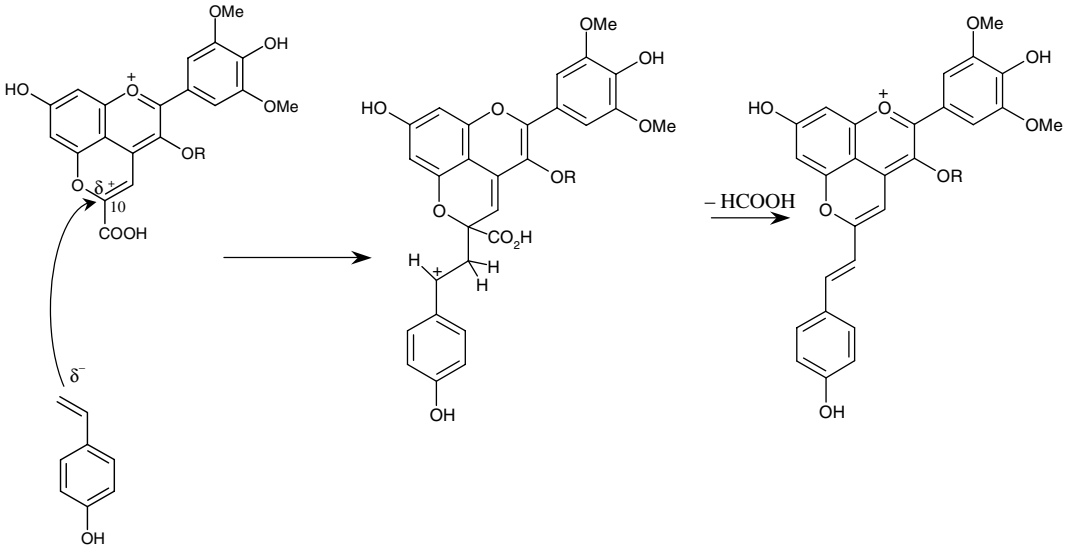

Fig. 3. Mechanism proposed for the formation of pigment A.

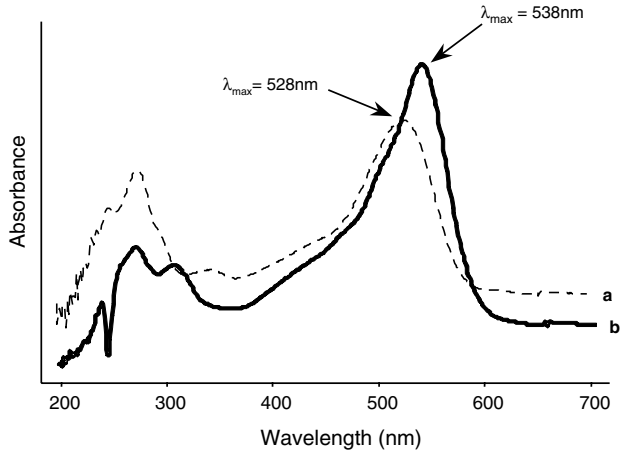

Fig. 4. UV–Vis spectra of malvidin 3-*O*-glucoside (a) and vinylpyranomalvidin 3-*O*-glucoside–phenol, recorded from the HPLC diode array detector.

4. Conclusion

The presence of this newly formed pigment in Port wine reinforces the idea that the vinylpyranoanthocyanin pigments family may be comprised of several compounds with great structural diversity. The first pigments of this family previously reported were shown to arise from the reaction between anthocyanin–pyruvic acid adducts and vinylflavanol adducts, whereas the present pigment was found to arise from the reaction between anthocyanin–pyruvic acid adducts and *p*-vinylphenol. Although anthocyanins constitute the major precursors for the formation of new pigments in young red wines, it seems that their pyruvic acid derivatives play a crucial role in the subsequent stages of colour evolution, overtaking anthocyanins as the main precursors for the appearance of anthocyanin-derived pigments in ageing red wine. The number and amount of pyranoanthocyanin pigments in red wine is still a matter of research for wine chemists and further studies are still required in order to assess the real contribution of each of them to the colour of ageing red wines.

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