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Structural diversity of anthocyanin-derived pigments in port wines

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Abstract

One year-old Port wine made from grapes of the Douro Valley was analysed for its anthocyanin-like pigment composition. The samples were purified by low pressure Toyopearl gel column chromatography, yielding two fractions. Fraction A was eluted with water/methanol 20% (v/v) and fraction B was eluted with methanol 100% (v/v). Structural elucidation of the pigments detected was attempted using LC/DAD-MS. Several anthocyanin-derived pigments were detected. Fraction A was mainly comprised of malvidin 3-glucoside and its pyruvic acid adducts. Additionally, other minor anthocyanin-pyruvic acid derivatives were also detected. Fraction B was shown to contain three groups of anthocyanin-derived pigments: (1) pigments in which anthocyanins are linked to a catechin unit via an ethyl linkage; (2) pigments in which anthocyanins are linked to a catechin unit or a procyanidin dimer via a vinyl linkage; (3) pigments in which anthocyanins are linked to a 4-vinylphenol group. The UV-visible spectral characteristics of most of these pigments show that they are contributing to the changing colour of Port wine from purple-red to a more orange-red hue.

Keywords: Red wine; Anthocyanin; Pyruvic acid; Acetaldehyde; LC-MS

1. Introduction

The colour evolution during red wine vinification and ageing has been attributed to the progressive changes in the phenolic compounds extracted from the grape berries. The original grape anthocyanins, which are responsible for the initial purple-red colour of young red wines, are displaced progressively and supposedly involving irreversible mechanisms by more stable pigments that stabilise wine colour, which changes to a more brick red hue. These pigments arise from the interactions between anthocyanins and other phenolic compounds, especially flavan-3-ols, such as catechins and proanthocyanidins (condensed tannins), and their formation may involve other non-phenolic compounds, such as acetaldehyde or pyruvic acid. Different reactions have been proposed, such as the direct condensation between anthocyanins and flavanols (Jurd & Somers, 1970; Liao, Cai, & Haslam, 1992; Remy, Fulcrand, Labarbe, Cheynier, & Moutounet, 2000; Santos-Buelga, Bravo-Haro, & Rivas, 1995; Somers, 1971) or the condensation between them through ethyl bridges (Bakker, Picinelli, & Bridle, 1993; Baranowsky & Nagel, 1983; Dallas, Ricardo da Silva, & Laureano, 1996; Escribano-Bailon, Dangles, & Brouillard, 1996; Francia-Aricha, Guerra, Rivas-Gonzalo, & Santos-Buelga, 1997; Rivas-Gonzalo, Bravo-Haro, & Santos-Buelga, 1995; Roggero, Coen, Archier, & Rochville-Divorne, 1987; Timberlake & Bridle, 1976). The isolation and identification of such pigments has proved difficult, especially because their levels are much lower than those of original anthocyanins. Nevertheless, some work has recently been carried out to detect and elucidate their structure in red wines, wine pomace or model solutions, using nuclear magnetic resonance (NMR) and mass spectrometry techniques (Bakker et al., 1997; Benabdeljalil, Cheynier, Fulcrand, Hakiki, Mosaddak, & Moutounet, 2000; Francia-Aricha, Guerra, Rivas-Gonzalo, & Santos-Buelga, 1997; Fulcrand, Benadeljalil, Rigaud, Cheynier, & Moutounet, 1998; Fulcrand, Cameira dos Santos, Sarni-Manchado, Cheynier, & Bonvin, 1996; Vivar-Quintana, Santos-Buelga, Francia-Aricha, & Rivas-Gonzalo, 1999). Amongst these more stable pigments,

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malvidin-pyruvic acid adducts have recently been reported in red wines and grape pomace (Bakker et al., 1997; Fulcrand et al., 1998). The structures of these malvidin-derived pigments involve a fourth ring, resulting from the cyclisation between carbon 4 and the hydroxyl group at carbon 5 of the original flavylium moiety. Additionally, similar pigments, derived from the reaction of malvidin 3-glucoside with ethanal (Bakker & Timberlake, 1997) and 4-vinylphenol (Cameira dos Santos, Brillouet, Cheynier, & Moutounet, 1996) have been detected in red wines. All these pigments have been found to be more resistant to bisulphite bleaching than the original anthocyanins, which is attributed to the substitution of the position 4 of the flavylium cation (Bakker & Timberlake, 1997).

In view of the initial wine chemical complexity, it may be expected that many other anthocyanin-derived pigments, which contribute to the red wine colour are also formed. Thus, the objective of the current study was to tentatively identify newly formed pigments in red wines, using LC–MS analysis. With this aim, a method involving low pressure and high-performance liquid chromatography (HPLC) was employed, to purify pigment-containing fractions from Port wines. The use of electrospray mass spectrometry, coupled to HPLC further allowed the detection of a large number of pigments, as well as to elucidate their overall structure.

2. Materials and methods

2.1. Wine samples

The study was conducted with a 1 year-old monovarietal Touriga Nacional (*Vitis vinifera*) Port wine, vintage 1999, made from grapes of the Douro Demarcated Region (Portugal).

2.2. Fractionation

Port wine samples were directly applied onto a Toyopearl HW-40(s) gel column (250×16 mm i.d.). A first elution was performed with 20% methanol in water (fraction A). A second elution was performed with 100% methanol (fraction B). Flow rate was regulated at 0.8 ml/min, using a peristaltic pump. The two pigment fractions collected were concentrated under vacuum and lyophilised.

2.3. LC–MS analysis

A Hewlett-Packard 1100 Series liquid chromatograph, equipped with an AQUA™ (Phenomenex, Torrance, CA, USA) reversed-phase column (150×4.60 mm, 5 μm, C18), thermostatted at 35 °C, was used. Solvents were (A) aqueous 0.1% trifluoracetic acid and (B) 100% HPLC-grade acetonitrile, establishing the following gradient: isocratic 10% B over 5 min, from 10 to 15% B over 15 min, isocratic 15% B over 5 min, from 15 to 18% B over 5 min, and from 18 to 35% B over 20 min, at a flow rate of 0.5 ml min^{-1}. Double online detection was done in a photodiode spectrophotometer and by mass spectrometry. The mass detector was a Finnigan LCQ (Finnigan Corporation, San Jose, USA), equipped with an API source, using an electrospray ionisation (ESI) interface. Both the auxiliary and the sheath gas were a mixture of nitrogen and helium. The capillary voltage was 3 V and the capillary temperature 190 °C. Spectra were recorded in positive ion mode between m/z 120 and 1500. The mass spectrometer was programmed to do a series of three scans: a full mass, a zoom scan of the most intense ion in the first scan and a MS–MS of the most intense ion, using a collision energy of 30 V. The HPLC system was connected to the probe of the mass spectrometer via the UV cell outlet.

3. Results and discussion

3.1. Fraction A

Fraction A, eluted from Toyopearl with 20% methanol, consisted mainly of eight compounds (Fig. 1): malvidin 3-glucoside (mv3glc) and seven anthocyanin-derived pigments, whose structural identities (Fig. 2) were ascertained from the LC–MS data obtained (Table 1). Peak 1 (mv3glc) showed a molecular ion at m/z 493 with an ion fragment at m/z 331 (aglycone moiety). The molecular ion of pigment 3 (m/z 561) coincides with the mv3glc-pyruvic acid adduct described by Fulcrand et al. (1998) and that of vitisin A proposed by Bakker et al. (1997). Peaks 5 and 7 showed molecular ions at m/z 603 and 707, respectively, consistent with the acetyl and coumaroyl esters of the mv3glc-pyruvic acid adduct. All these three pyruvic acid derivatives (py) had a major fragment ion at m/z 399, which corresponded to their aglycone moiety. These pigments were already reported in red wines, grape pomace and model solutions (Bakker et al., 1997; Fulcrand et al., 1998; Romero & Bakker, 1999) and are known to be more abundant in fortified red wines, such as Port wine, than in red table wines (Bakker & Timberlake, 1986). In fact, mv3glc-py and its acylated forms together with mv3glc, are the major pigments detected in the Port wine studied after 1 year of bottle-ageing and are those with greater contributions to colour. Beside them, two other minor anthocyanin-py derivatives were detected in fraction A. Peak 4 showed an ion signal at m/z 589, with a major ion fragment at m/z 385, which may be assigned, respectively, to petunidin 3-acetylglucoside-py adduct and its aglycone moiety. The molecular ion at m/z 677

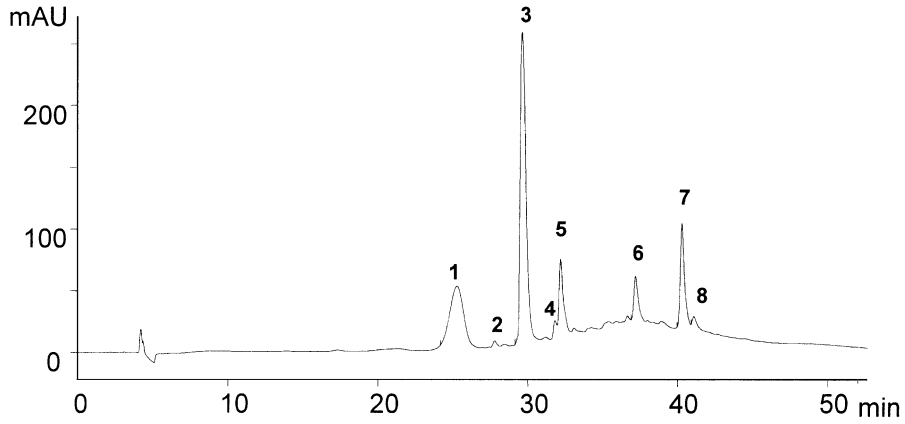
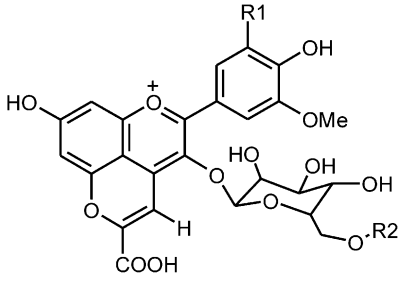


Fig. 1. HPLC chromatogram, recorded at 520 nm, of fraction A eluted from Toyopearl gel column with 20% methanol. See Table 1 for peak identification.

(peak 8) and its ion fragment at *m/z* 369 (aglycone moiety) would be consistent with a paeonidin 3-coumaroylglucoside-py adduct and its aglycone moiety. Finally, peak 2 showed a molecular ion at *m/z* 517, corresponding to a mv3glc with a cyclisation between C4 and the OH at C5 through a vinyl group. This pigment has also been referred to as vitisin B and it has been suggested that its formation involves ethanal (Bakker & Timberlake, 1997).



m/z 561 ⇒ R1 = OMe; R2 = H

m/z 603 ⇒ R1 = OMe; R2 = acetyl group

m/z 677 ⇒ R1 = H; R2 = coumaroyl group

m/z 707 ⇒ R1 = OMe; R2 = coumaroyl group

Fig. 2. Structures of the major anthocyanin-pyruvic acid adducts detected in fraction A.

3.2. Fraction B

A further elution from the Toyopearl gel column, with 100% methanol, yielded fraction B. The HPLC chromatogram of this fraction (Fig. 3) revealed a large number of peaks, most of them corresponding to malvidin-derived pigments. Many of them were shown to arise from the association of mv3glc and flavanol via ethyl or vinyl linkages (Table 2 and Fig. 4). The signals, detected at *m/z* 805, 847, 951, 1093, 1135 and 1239, are consistent with pigments in which anthocyanins are vinyl-linked to either catechin (cat) or epicatechin (epi) or a procyanidin dimer (PC), and similar to those described by Francia-Aricha et al. (1997) in studies carried out in model solutions. The molecular masses of 805 (peaks 5 and 10), 847 (peaks 7 and 12) and 951 (peak 15) are, respectively, attributed to mv3glc, mv 3-acetylglucoside and mv 3-coumaroylglucoside, linked through a vinyl bridge to a catechin monomer. These pigments yielded the same ion fragments at *m/z* 643 (aglycone moiety: mv-4-vinyl-cat/epi) and at *m/z* 491,

Table 1
Anthocyanin-derived pigments detected in fraction A eluted from Toyopearl gel column with 20% methanol

Peak	Rt (min)	*m/z* (M+)	Peak identity	*m/z*	Structure
1	25.26	493	Mv 3-gluc	331	Mv
2	27.77	517	Mv 3-gluc-4-vinyl	355	Mv-4-vinyl
3	30.45	561	Mv 3-gluc-py-derivative	399	Mv-py-derivative
4	31.67	589	Pt 3-(acetyl)gluc-py-derivative	385	Pt-py-derivative
5	32.64	603	Mv 3-(acetyl)gluc-py-derivative	399	Mv-py-derivative
6	37.55	693	Pt 3-(coumaroyl)gluc-py derivative	385	Pt-py-derivative
7	40.64	707	Mv 3-(coumaroyl)gluc-py derivative	399	Mv-py-derivative
8	41.48	677	Pn 3-(coumaroyl)gluc-py derivative	331	Pn-py-derivative

Mv, malvidin; Pt, petunidin; Pn, paeonidin; py, pyruvic acid; gluc, glucoside.

corresponding to a Retro Diels-Alder product from the catechin unit (Fig. 5). The signals at m/z 805 and m/z 847 were detected twice in the HPLC chromatogram at different retention times, suggesting one pigment to have a (+)-catechin unit and the other a (−)-epicatechin unit. Similarly, the molecular ions at m/z 1093, 1135 and 1239 would correspond respectively to mv3glc and its acetyl and coumaroyl derivatives linked to a PC dimer. These three oligomeric pigments were shown to have a similar major ion fragment at m/z 931 which corresponded to their aglycone moiety (mv-4-vinyl-PC). These vinyl-liked pigments are proposed to result from the reaction of the mv3glc cation with a flavanol moiety possessing a vinyl residue at C-8, following a mechanism similar to that indicated for Fulcrand et al. (1996) for the formation of 4-vinylphenol anthocyanin-derived pigments. The vinyl-flavanol adduct may derive either from the cleavage of ethyl-linked flavanol oligomers resulting from the acetaldehyde-induced condensation of flavanols (Es-Safi, Fulcrand, Cheynier, & Mounounet, 1999a) or from the direct dehydration of the flavanol–ethanol adduct formed after reaction with acetaldehyde (Fig. 6). These processes are expected to be favoured when pH is not very low; at very acidic pH values the

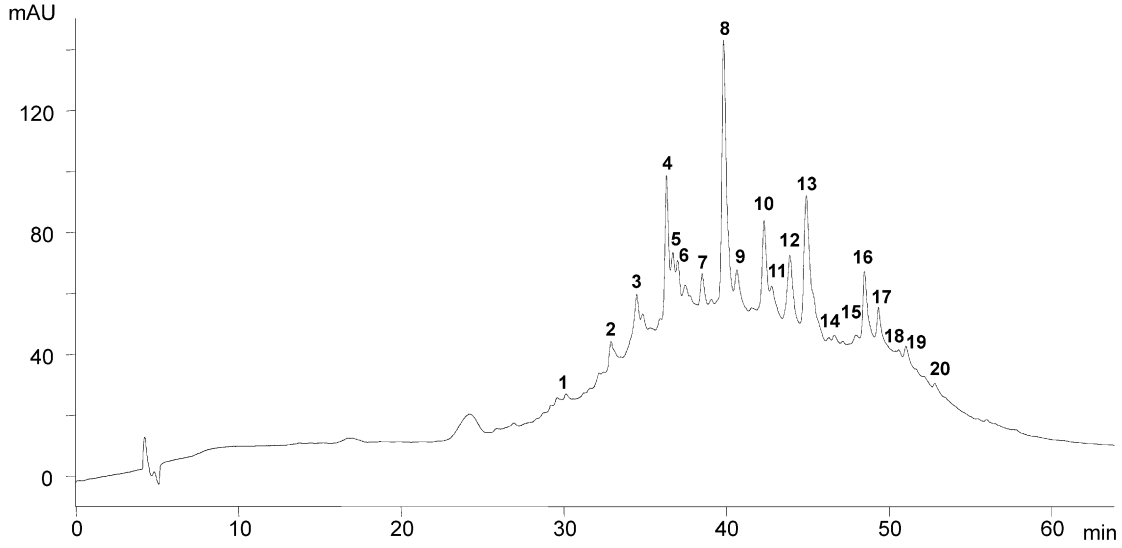


Fig. 3. HPLC chromatogram, recorded at 520 nm, of fraction B eluded from Toyopearl gel column with 100% methanol. See Table 2 for peak identification.

Table 2
Anthocyanin-derived pigments detected in fraction B eluted from Toyopearl gel column with 100% methanol

Peak	Rt (min)	m/z (M+)	Peak identity	m/z	Structure
1	31.06	809	Mv 3-gluc-4-ethyl-cat	517	Mv 3-gluc-4-vinyl
2	33.90	1093	Mv 3-gluc-4-vinyl-PC dimer	931	Mv-4-vinyl-PC dimer
3	35.05	1135	Mv 3-(acetyl)gluc-4-vinyl-PC dimer	931	Mv-4-vinyl-PC dimer
4	36.21	677	Pn 3-(coumaroyl)gluc-py derivative	331	Mv
5	37.05	805	Mv 3-gluc-4-vinyl-cat	643; 491	Mv-4-vinyl-cat; fragment X (Fig. 5)
6	38.75	533	Dp 3-gluc-py derivative	371	Dp-py derivative
7	39.32	847	Mv 3-(acetyl)gluc-4-vinyl-cat	643; 491	Mv-4-vinyl-cat; fragment X (Fig. 5)
8	40.21	707	Mv 3-(coumaroyl)gluc-py derivative	399	Mv-py derivative
9	40.81	1239	Mv 3-(coumaroyl)gluc-4-vinyl-PC dimer	931	Mv-4-vinyl-PC dimer
10	41.59	805	Mv 3-gluc-4-vinyl-cat	369	Mv-4-vinyl-cat; fragment X (Fig. 5)
11	42.57	955	Mv 3-(coumaroyl)gluc-4-ethyl-cat	665; 357	Mv 3-(coumaroyl)gluc-4-ethyl; Mv-4-ethyl
12	43.01	847	Mv 3-(acetyl)gluc-4-vinyl-cat	643; 491	Mv-4-vinyl-cat; fragment X (Fig. 5)
13	44.21	639	Mv 3-(coumaroyl)gluc	331	Mv
14	46.10	575	Dp 3-acetylgluc-py derivative	371	Dp-py derivative
15	46.87	951	Mv 3-(coumaroyl)gluc-4-vinyl-cat	643; 491	Mv-4-vinyl-cat; fragment X (Fig. 5)
16	48.17	609	Mv 3-gluc-4-vinylphenol	447	Mv-4-vinylphenol
17	51.49	771	Mv 3-(caffeoyl)gluc-4-vinylphenol	679	Mv 3-(caffeoyl)gluc-4-vinyl
18	52.53	725	Pn 3-(coumaroyl)gluc-4-vinylphenol	355	Mv-4-vinyl
19	52.59	755	Mv 3-(coumaroyl)gluc-4-vinylphenol	355	Mv-4-vinyl
20	52.68	651	Mv 3-(acetyl)gluc-4-vinylphenol	355	Mv-4-vinyl

Mv, malvidin; Dp, delphinidin; Pt, petunidin; Pn, paeonidin; py, pyruvic acid; gluc, glucoside; cat, (+)-catechin or (−)-epicatechin; PC, procyanidin.

formation of an ethyl-flavanol cation and, consequently, of ethyl-linked pigments, would be favoured (Rivas-Gonzalo et al., 1995).

For the pigments with ethyl linkages, the only molecular ions detected were m/z 809 (peak 1) corresponding to a mv3glc-4-ethyl-cat with an ion fragment at m/z 517, and m/z 955 (peak 11), which is assigned as mv 3-coumaroylglucoside-ethyl-cat, having two major ion fragments at m/z 665 and m/z 357 (Fig 6). These kind of pigments have been repeatedly demonstrated in model

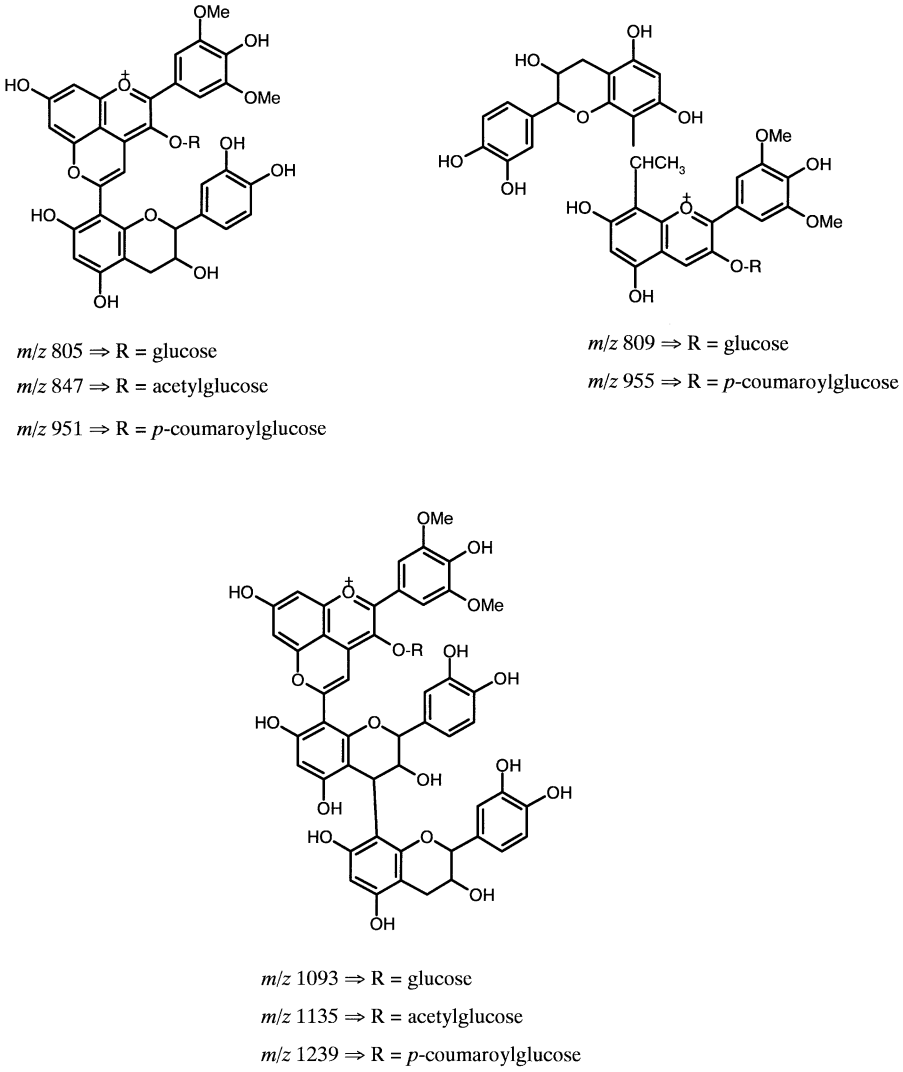


Fig. 4. Structures of anthocyanin-derived pigments detected in fraction B arising from condensation of anthocyanidin monoglucosides to either a catechin/epicatechin unit or procyanidin dimer.

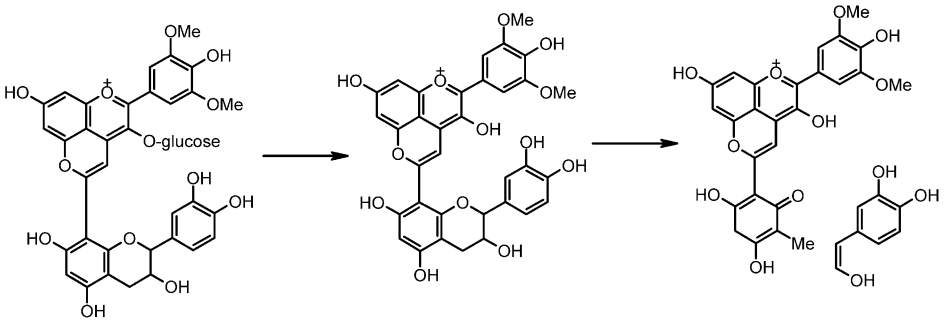


Fig. 5. Structural hypothesis for the formation of the fragment ion X (m/z 491) resulting from malvidin 3-glucoside-4-vinylcatechin.

solutions and their structures and mechanisms of formation are well known (Bakker et al., 1993; Escribano-Bailón et al., 1996; Es-Safi et al., 1999b; Rivas-Gonzalo et al., 1995; Timberlake & Bridle, 1976).

Another group of pigments detected in the last part of the HPLC chromatogram of fraction B is consistent with a group of anthocyanins linked to a 4-vinylphenol group (Fig. 7). The molecular ion of peak 16 (m/z at 609), and its major ion fragment (m/z 447), correspond to the pigment derived from the cycloaddition of *p*-vinylphenol to C4 and the OH at C5 of mv3glc and its aglycone, respectively, previously reported in red wines (Cameira dos Santos, Brillouet, Cheynier, & Mountounet, 1996; Fulcrand et al., 1996). The *p*-vinylphenol moiety can be formed in wines from the degradation of *p*-coumaric acid (Chatonnet, Dobourdieu, Boidron, & Lavigne, 1993). Similar pigments have also been shown to result from the mv 3-caffeoylglc (peak 17, m/z 771), mv 3-coumaroylglc (peak 19, m/z 755) and mv 3-acetylglc (peak 20, m/z 651) and paeonidin 3-coumaroylglc (peak 18, m/z 725).

In addition to all these anthocyanin-derived pigments, fraction B was also shown to contain a few minor anthocyanin-py derivatives, such as delphinidin 3-glucoside-py (peak 6, m/z 533), delphinidin 3-acetylglucoside-py (peak 14, m/z 575) and, especially, mv 3-coumaroylglucoside-py (peak 8, m/z 707) that was already detected in fraction A. This might be anticipated, since anthocyanidin coumaroylglucosides are not totally eluted from Toyopearl gel column with 20% methanol (v/v), as confirmed by the presence, in fraction B of mv 3-coumaroylglucoside (peak 13, m/z 639).

The structural diversity of anthocyanin-derived pigments found in 1-year old Port wine suggests different reactions, whereby original grape anthocyanins may associate with other colourless compounds. Most of these newly formed pigments have different UV-visible spectral characteristics, showing a hypsochromic shift in the visible spectrum with regard to that of the original anthocyanins. The λ_{max} of the py-derivatives is situated near 503 nm whereas the λ_{max} of the anthocyanin-flavanol vinyl-linked pigments is near 511 nm and that of the vinylphenol derivatives is about 507 nm. The only exception are the three ethyl-linked pigments detected, which have a more bluish-red hue showing λ_{max} above 530 nm. Thus, the formation of all these anthocyanin-derived pigments plays a crucial role in the changing colour of Port wine from purple-red (in young wines) to a more orange-red hue. Furthermore, the structural features of these pigments give them more stability than the original anthocyanins. The presence of a 4-substituent on the anthocyanin molecule give them resistance to colour bleaching by sulphur dioxide (Timberlake & Bridle, 1968) and to pH variations (Sarni-Manchado et al., 1996).

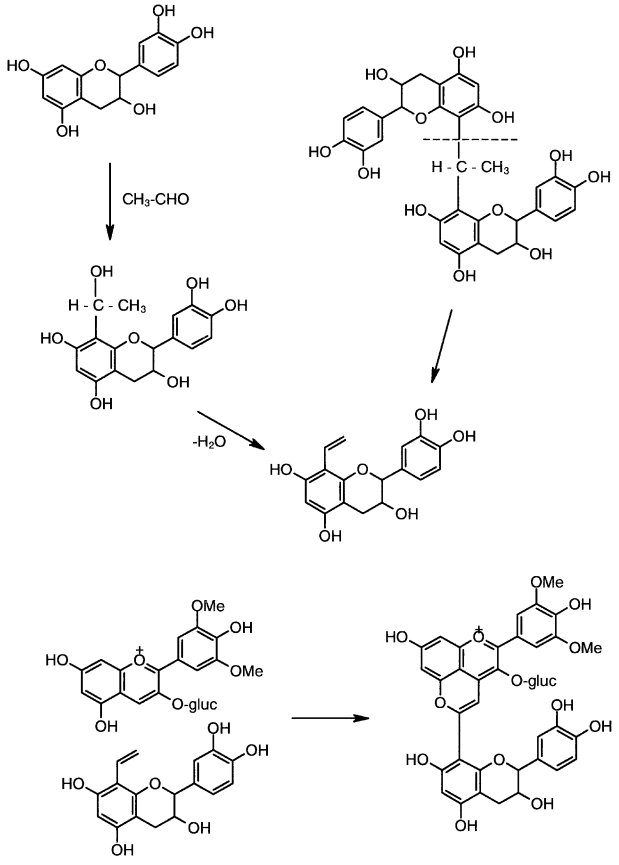
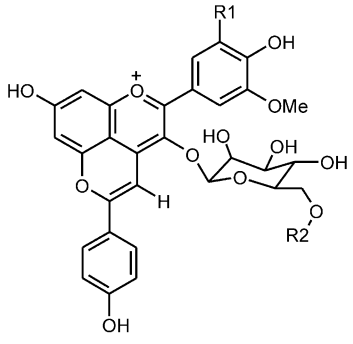


Fig. 6. Mechanisms proposed for the formation of vinyl-linked pigments.



m/z 609 ⇒ R1 = OMe; R2 = H

m/z 651 ⇒ R1 = OMe; R2 = acetyl group

m/z 755 ⇒ R1 = OMe; R2 = coumaroyl group

m/z 771 ⇒ R1 = OMe; R2 = caffeoyl group

m/z 725 ⇒ R1 = H; R2 = coumaroyl group

Fig. 7. Structures of vinylphenol derivatives detected in fraction B (Table 2).

4. Conclusion

The results reported herein show that several pigments with structural diversity can be easily detected in Port wines. The fractionation method employed in this work allowed to obtain a fraction rich in lower molecular weight pigments and another, containing more complex anthocyanin-derived pigments. Elution from a Toyopearl gel column, with 20% methanol (v/v), yielded mv3glc, along with anthocyanin-py derivatives, especially mv3glc-py and its acylated forms. These py-derivatives arise from the reaction of pyruvic acid with the original grape anthocyanins. A final elution of 100% methanol yielded a fraction containing three groups of anthocyanin-derived pigments: (1) pigments whereby anthocyanins are linked to a cat/epi unit via ethyl linkage; (2) pigments whereby anthocyanins are linked to either a cat/epi unit or a PC dimer via vinyl linkage; (3) pigments whereby anthocyanins are linked to a 4-vinylphenol group. Among all these detected pigments, only mv3glc-py and its acylated forms are present in significant amounts in red wines, whereas others are present in low concentration. For this reason, the former ones are the only ones that can usually be quantified in aged red wines, whilst the importance of the other pigments remains unknown and their relative contribution to wine colour can not be clearly assessed. The structural elucidation of anthocyanin-derived pigments will probably help understanding of the complex evolution of wine colour during ageing. Furthermore, it also becomes extremely important to prove as genuine anthocyanins are converted into stable pigments with structural features influencing wine colour.

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