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A NEW CLASS OF WINE PIGMENTS GENERATED BY REACTION BETWEEN PYRUVIC ACID AND GRAPE ANTHOCYANINS

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Key Word Index—Anthocyanins; pyruvic acid; pigments; wine; fermentation; electrospray; mass spectrometry.

Abstract—A new class of stable red pigments detected in grape pomace was analysed by electrospray ionisation mass spectrometry. They were shown to be pyruvic acid derivatives of genuine grape anthocyanins by synthesis experiments. The major product was identified by NMR (^{1}H, NOE, HSQC, HMBC) experiments as the malvidin-3-monoglucoside pyruvic acid adduct. Its formation results from cyclisation between C-4 and the hydroxyl group at C-5 of the original flavylium moiety with the double bond of the enolic form of pyruvic acid, followed by dehydration and rearomatisation steps. This type of reaction leads to increased colour stability. Various yeast metabolites other than pyruvic acid were shown to react with grape anthocyanins following this mechanism, suggesting that it may be an important route of conversion into stable pigments during the maturation and ageing of wine.

INTRODUCTION

Anthocyanins are significant components of red wine colour, which is a major attribute of wine quality. Though colour is relatively easy to assess, factors which govern it are more difficult to determine. These can include grape cultivars, winemaking methods, other phenolics content, wine pH and redox potential, presence of oxygen, acetaldehyde and sulfur dioxide [1]. An intrinsic factor affecting wine colour with time is the structural instability of anthocyanins. According to Somers [2], these molecules are gradually converted into polymeric pigments upon ageing. Their structural modifications result in a characteristic change of colour hue, passing from "full red" in young wines to "tawny" in older wines [3, 4]. The newly-formed pigments are more stable than genuine anthocyanins, thus increasing the colour stability of aged wines. Nevertheless, their structures, as well as their origins are not yet clearly established, although several hypotheses have been proposed. Essentially, these involve condensed tannins and anthocyanins either in non-covalent associations such as copigmentation and self-association phenomena [5–7] or in addition reactions mediated or not by acetaldehyde [6, 8–15].

Recent work in our laboratory [16] has revealed the presence, in some wines, of pigments other than monomeric anthocyanins in grapes. They exhibited quite different colour properties, reminiscent of those encountered in old wines. Their structural elucidation [17] allowed us to demonstrate, for the first time, a new type of reactivity in anthocyanin molecules. Thus, their formation was shown to result from a cycloaddition process, involving both C-4 and the hydroxyl at C-5 of the anthocyanin, with an ethylenic bond provided by another molecule. This reaction strongly increases the stability of the product obtained, particularly at higher pH values, sulfur dioxide and temperature [18]. After the discovery of these pigments, several other series exhibiting similar properties were detected in grape pomaces and wines [19, 20]. Herein, we report the full characterization of one of them. Structural elucidation indicated that a reaction had occurred between pyruvic acid and grape anthocyanins through a similar reaction pathway as we described earlier for anthocyanin-vinylphenol adducts [15]. A compound showing the same spectral characteristics has also been described by Bakker *et al.* [21, 22] but the NMR data led them to propose a different structure and no mechanism of formation was postulated.

RESULTS AND DISCUSSION

HPLC analysis of an extract of red grape pomace revealed unusual pigments showing an absorbance

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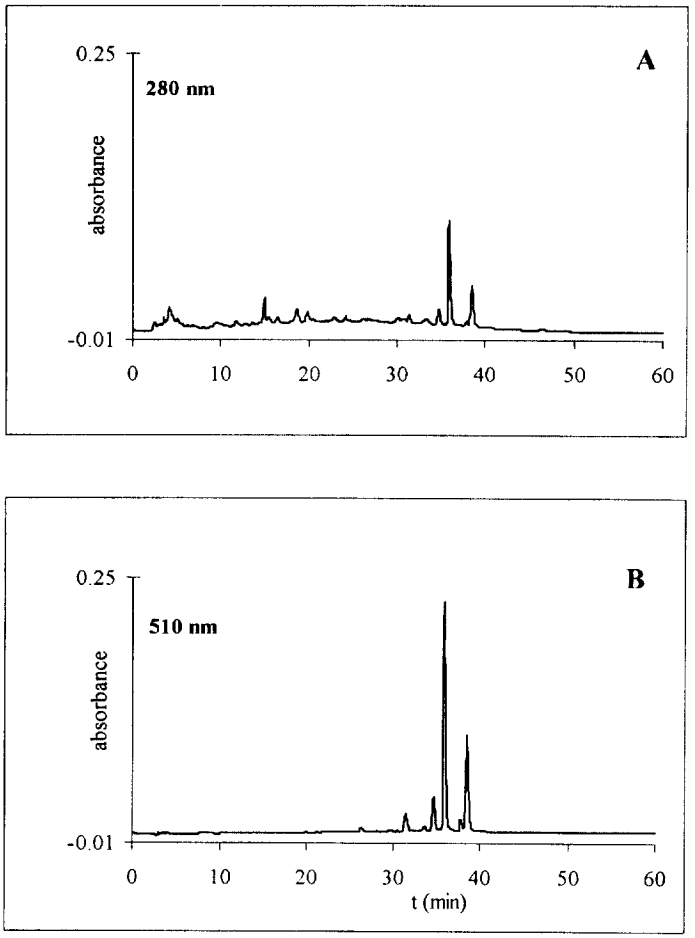

Fig. 1. HPLC profiles of the orange fraction recorded at 280 nm (A) and 510 nm (B), showing the derived pigments.

maximum around 510 nm, along with a complex mixture of polyphenols. The pomace extract was deposited on a Toyopearl column for fractionation. When a water wash was performed to remove residual sugars, an unexpected orange fraction was first eluted, which was then analysed by HPLC (Fig. 1). The results indicated that it corresponded to the unknown pigments detected in the crude extract. Their spectral features, obtained by means of the diode array detector coupled with the chromatographic system, were similar to those of wine-derived pigments previously characterized [16]. Likewise, their visible maximum was shifted to shorter wavelength (i.e. 511 nm) and their visible to UV absorbance ratio was twice that of genuine anthocyanins (Fig. 2). On the other hand, they showed a pronounced band around 370 nm, characteristic of 4-substituted anthocyanins [23]. Structural investigation was continued with LC-mass spectrometric analysis of the orange fraction. Assays were performed using an ion-spray source and detection was achieved both in positive and negative ion modes. Unlike genuine anthocyanins, which are more easily detected in the positive ion mode as the flavylium cations $[M]^+$, the new pigments responded

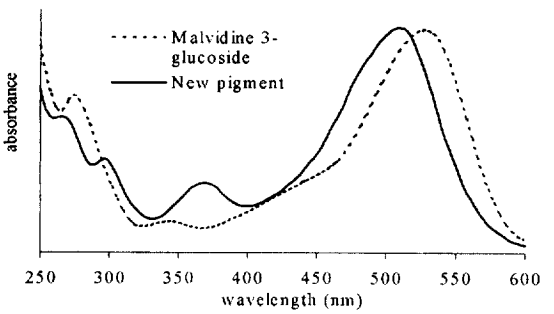

Fig. 2. Comparison between absorbance spectra (recorded on-line in the HPLC solvents) between the major genuine grape anthocyanin, malvidin-3-monoglucoside and the corresponding derived pigment.

better in the negative ion mode for which m/z values corresponded to $[M-2H]^-$. An ion peak series was thus detected from m/z 529.2 to 601.2 (Fig. 3). Given the elution order, the M_r related to each m/z value could be ascribed to that of one genuine anthocyanin plus an additional 68 mu (Table 1). An increase of orifice voltage showed characteristic fragmentations,

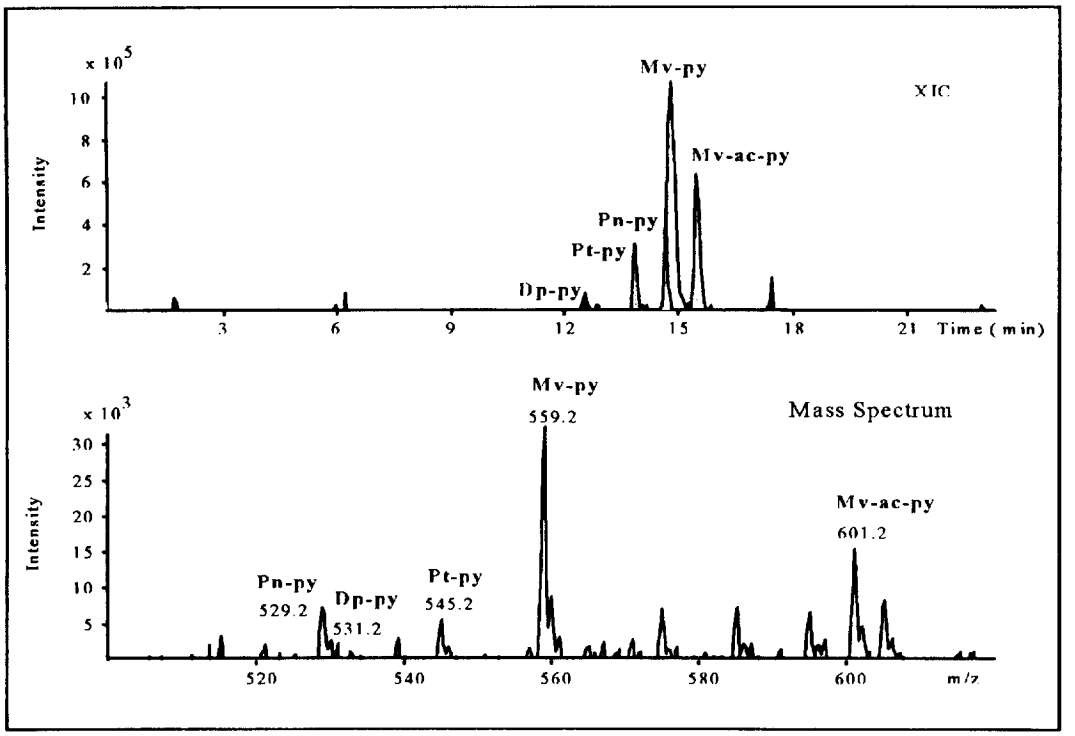

Fig. 3. LC/MS analysis of orange fraction, performed with an ion-spray source, in the negative-ion mode with the orifice voltage set at −60V. At the top, traces of ion currents extracted (XIC) from the total ion current chromatogram (TIC), each one corresponding to *m/z* value of a derived pigment. Below the mass spectrum obtained from XIC. Dp-py: delphinidin-3-monoglucoside derived-pigment; Pt-py: petunidin-3-monoglucoside derived- pigment; Pn-py: peonidin-3-monoglucoside derived- pigment; Mv-py: malvidin-3-monoglucoside derived- pigment; Mv-ac-py: malvidin-3-(6-acetyl)-monoglucoside derived-pigment.

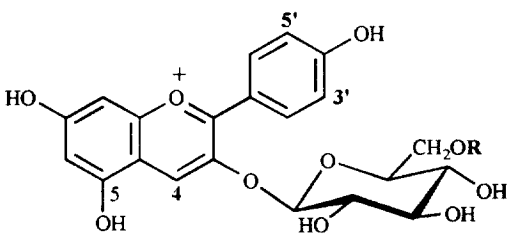

Table 1. M_r of grape anthocyanins and their derivatives in the orange fraction

Grape anthocyanins	Hydroxylation pattern			M_r	M_r of derivatives	Retention time (min)
	3′	5′	R			
Delphinidin-3-monoglucoside	OH	OH	H	Dp: 465.2	Dp-py: 533.2	12.53
Petunidin-3-monoglucoside	OCH_3	OH	H	Pt: 479.2	Pt-py: 547.2	13.88
Peonidin-3-monoglucoside	OCH_3	H	H	Pn: 463.2	Pn-py: 531.2	14.69
Malvidin-3-monoglucoside	OCH_3	OCH_3	H	Mv: 493.2	Mv-py: 561.2	14.83
Malvidin-3-(6-acetyl)-monoglucoside	OCH_3	OCH_3	CO-CH_3	Mv-ac: 535.2	Mv-ac-py: 603.2	15.51

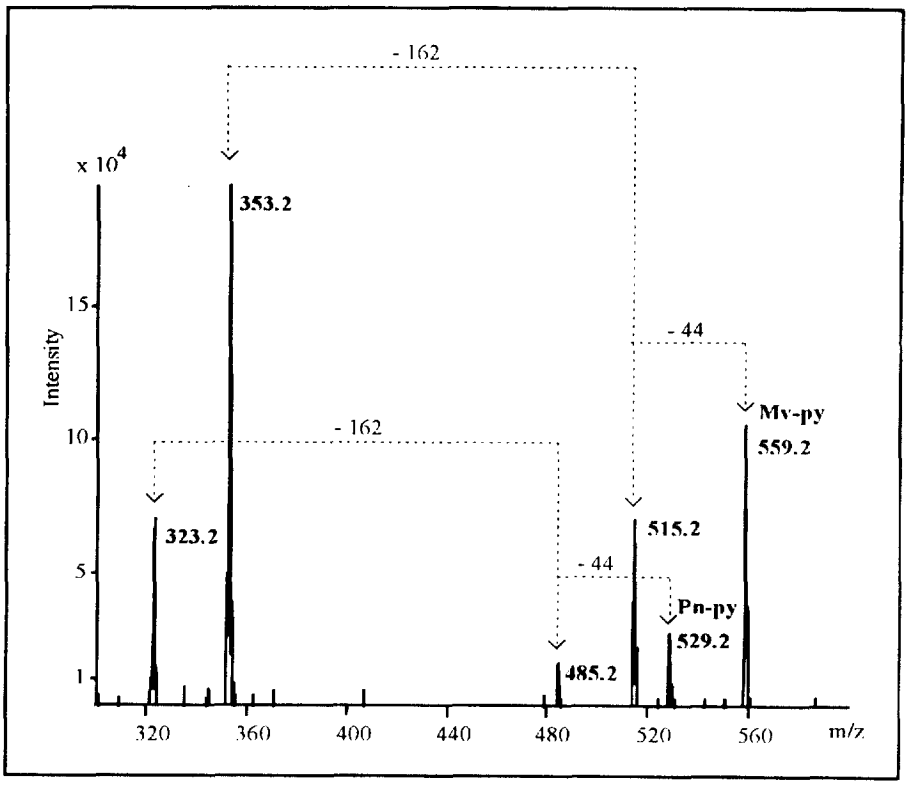

Fig. 4. LC/MS analysis of orange fraction, performed with an ion-spray source, in the negative-ion mode with the orifice voltage set at −90V. Mass spectrum obtained from the TIC chromatogram between 14 and 15 min, corresponding to the coelution of peonidin-3-monoglucoside and malvidin-3-monoglucoside. Note the presence of ion fragments, corresponding to losses of (CO_2) group (−44 mu) and of the glucoside residue (162 mu).

as presented on the mass spectrum of coeluted malvidin-3-monoglucoside- and peonidin-3-monoglucoside-derived pigments (Fig. 4). The loss of 44 mu was attributed to the loss of a carboxyl residue, the presence of which may explain the better response in the negative ion mode. The loss of 162 mu was ascribed to cleavage of the glucosyl moiety. As the aglycone of each derived-pigment is heavier than that of its anthocyanin precursor, it could be deduced that the reaction had modified this part of the molecule, as already observed in the mass spectrum of vinyl phenol adducts [17]. Since the loss of the carboxyl residue is unlikely to result from the glucosyl moiety, the mass excess of the derivative aglycone after fragmentation equal to 24 mu indicated the presence of two additional carbons compared with its anthocyanidin precursor. Moreover, the spectral similarities between the new pigments and malvidin-vinyl phenol adducts suggested that they were based on the same skeleton, the phenol group being replaced, in the former, by a carboxylic function. Therefore, the new pigments were also probably formed by a cycloaddition reaction, as was demonstrated for the vinyl phenol adducts [17]. The reagent which could yield this structure was believed to be pyruvic acid, an end-product of the glycolysis cycle during fermentation. This assumption was first checked by mixing pyruvic acid with a crude extract of grape anthocyanins. The formation of the corresponding pyruvic adducts was confirmed both by UV-visible and mass detections, following HPLC separation. Then, the pyruvic derivative related to the major grape pigment (malvidin 3-monoglucoside) was synthesized using the same procedure and purified for NMR investigations. Monodimensional (^1H) and bidimensional heteronuclear experiments (HSQC and HMBC) were performed in acidified DMSO at 303 K. The structure of compound **1** was deduced from ^1H and ^{13}C chemical shift assignments (Table 2). Proton resonances could be fully assigned after a 1D NOE experiment, allowing unambiguous attributions of H-6 and H-8. Thus, a negative NOE effect could be observed between H-8 and H-2′ (H-6′). At the same time, another similar effect was produced between H-β and H-2′ (H-6′) indicating the respective location of the hydrogen and carboxyl residue on the ethylenic bond of the new pyran ring. From a mechanistic point of view, this result indicated regioselectivity towards the pyruvate reagent in the course of cycloaddition process. In an acidic medium, such as grape pomace, the enolic form of pyruvic acid should be the form involved in the reaction with malvidin-3-monoglucoside. The latter may react both by its nucleophilic

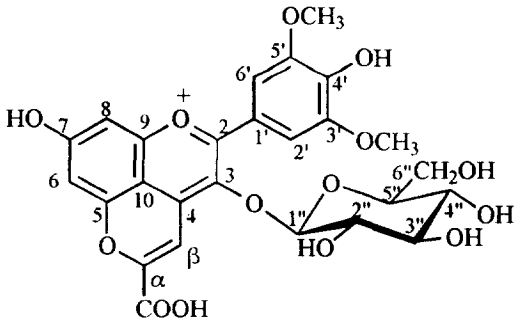

Table 2. ^{1}H and ^{13}C assignements of compound **1** in TFA–DMSO-d_6 (1:10)

Position	$\delta^{1}H$; J(Hz)	$\delta^{13}C$
2		163.4
3		134.8
4		109.3
5		152.7
6	7.20; unresolved	100.8
7		168.2
8	7.42; unresolved	101.1
9		152.7
10		109.5
COOH		160.2
α		154.5
β	7.96; s	106.2
1′		118.4
2′, 6′	7.80; unresolved	109.4
3′, 5′		148.2
OCH_3	3.90	56.5
4′		143.9
1″	4.71; $J = 7.0$	104.5
2″	3.44	74.4
3″	3.22	76.4
4″	3.10	70.0
5″	3.05	77.9
6″A	3.48	61.1
6″B	3.26	

Table 3. ^{1}H,^{13}C correlations found in the long-range (HMBC) heteronuclear correlation experiment

Proton	^{13}C correlation
β	COOH, α, 3, 10, 4
6	7, 5, 10, 8
8	7, 9, 10, 6
2′, 6′	2, 3′, 5′, 4′, 1′, 2′, 6′
CH_3O	3′, 5′
1″	3

hydroxyl at C-5 and its electrophilic C-4 upon the double bond of enolisated pyruvic acid (Fig. 5). This cycloaddition step should be followed by dehydration and re-aromatisation processes to yield the isolated malvidin-3-monoglucoside pyruvic adduct.

After all the protons had been attributed, all the corresponding carbons were assigned from a short-range HSQC experiment. The assignment of the quaternary carbons was obtained from a long-range HMBC experiment (Table 3). Given the small quantity of sample, a long experiment (5 h) was required, leading to some product degradation. In fact, the five aromatic protons were quite stable, whereas partial sugar degradation appeared to occur. Consequently, all expected cross-peaks between aromatic protons and their $^{2}J,^{3}J$ coupling carbons could be detected, whereas ^{3}J(C,H) correlations were hardly visible for the glucoside protons. The assignment of carbons introduced by the cycloaddition reaction and coupling with H-β was the delicate point. Among the five expected ^{2}J and ^{3}J (C,H) correlations, four were clearly visible. However, the cross-peak corresponding to the signal at δ 109.48 was larger than the other ones and deformed, so that two overlapping carbon resonances could be reasonably assumed, the second one being slightly shifted at δ 109.30. Moreover, the signal at δ 109.48 could be definitely assigned to C-10 by its other ^{3}J (C,H) couplings to H-6 and H-8, while the assignment of C-3 at δ 134.85 was deduced via its other ^{3}J (C,H) coupling to the sugar anomeric proton. On the other hand, the signals at δ 154.51 ppm and 160.24 were logically attributed to C-α and the carboxyl carbon, respectively, although opposite assignments would be also consistent with such signals and with ^{2}J, ^{3}J (C, H) correlations to H-β. Finally, the remaining carbon, C-4, was assigned to the signal at 109.30, which seems a reasonable shift value compared with those of similar carbons within the structure, such as C-10.

Bakker *et al.* [21, 22] recently reported the characterization of new colour-stable anthocyanins occuring in red port wine, which appear very similar. In particular, one of them referred to as vitisin A shows polarity, as well as UV-visible, mass and ^{1}H NMR spectra similar to those of the malvidin-3-monoglucoside pyruvic acid adduct, but the structure proposed is slightly different. Thus, the flavylium cation possesses an aldehyde and hydroxyl substituents on the ethylenic bond of the new pyran ring, instead of the hydrogen and carboxyl groups that we have ascribed. Their identification achieved by mono- and two dimensional NMR experiments was carried out on a mixture of the different forms of the pigment in equilibrium, after 2 to 16 days [22]. Although the signal at δ 7.96 can be assigned to an aldehyde proton, ^{1}J, ^{2}J and ^{3}J (C,H) correlations found in our heteronuclear experiments are not consistent with the aldehyde structure proposed [22] but fit the flavylium cation presented herein. This is further supported on one hand, by the characteristic mass spectral fragmentation indicating the loss of a carboxyl group and, on the other hand, by the synthetic pathway starting from pyruvic acid (Fig. 5).

Finally, the full structural elucidation of the mal-

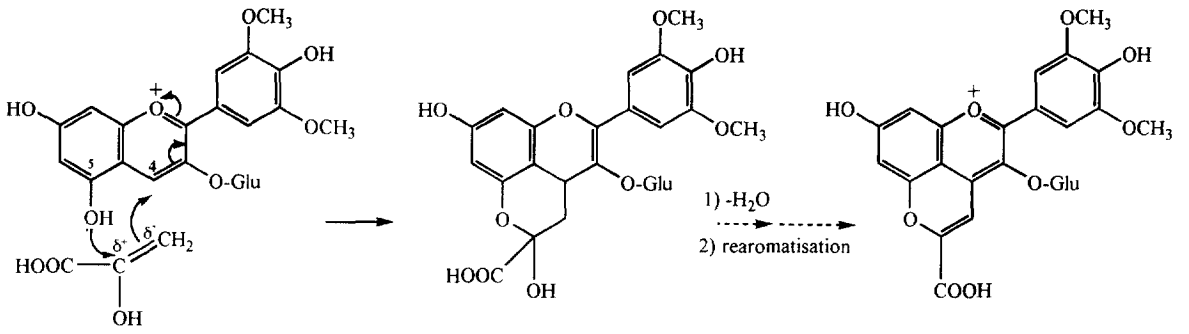

Fig. 5. Mechanism postulated for the reaction between pyruvic acid and malvidin-3-monoglucoside.

vidin-3-monoglucoside pyruvic-adduct suggests that the cycloadditon process is an important way to convert genuine anthocyanins into stable pigments. It appears all the more true since similar reactions involving other yeast secondary metabolites have also been demonstrated [19] and some of the corresponding adducts were detected in wine. These include the structure described as vitisin B [21], which was also synthesized in our laboratory, starting this time from ethanal [19]. Thus, this new anthocyanin reactivity leading to extremely stable tawny pigments appears to be an important route of wine maturation and ageing.

EXPERIMENTAL

Grape pomace

The grape pomace extract (from *V. vinifera* var. Carignane) was provided by the "Société Française de Distillerie". The pigments were extracted starting from 1-ton of red wine marc by soaking in aq. sulfite (0.2%), warmed at 60° during 3 h, with stirring every 30 min. The extract thus provided was concd $\times 5$, then fermented to convert residual sugars into alcohol, again concd $\times 5$ under vacuum and stored at $-5°$ prior to analyses.

Orange fraction

Grape pomace extract (5 ml) dild with 5 ml of H_2O was filtered on a glass fibre filter and deposited on a column filled with 150 ml of Fractogel TSK HW-40(F) (Merck). Elution with 0.05% aq. TFA gave the orange fr.

Crude anthocyanin extract and malvidin-3-monoglucoside

Crude anthocyanin extract and malvidin-3-monoglucoside were isolated from skins of *V. vinifera* var. Grenache Noir and purified as described in Ref. [17].

Partial conversion of crude grape anthocyanins into corresponding pyruvic acid derivatives

The anthocyanin monoglucoside fr. in 0.05% aq. TFA was mixed with excess Na pyruvate ($\times 20$ by wt). The reaction mixt. was kept at 40° for one day, then concd to dryness under vacuum and dissolved in a minimum vol. of 2% aq. HCO_2H prior HPLC/DAD analysis.

Malvidin-3-monoglucoside pyruvic acid adduct

Pure malvidin-3-monoglucoside (10 mg) and 205 mg of Na pyruvate (100 equiv.) were mixed in 10 ml of a 2% solution of HCO_2 in H_2O–EtoH (9:1). The reaction mixt. was maintained at 40° for 48 h and then analysed by HPLC/DAD. Although the reaction was not complete (65% conversion and degradation of initial malvidin-3-monoglucoside peak area), the soln was treated to prevent degradation of the product. Excess pyruvic acid was first removed on a Sep-Pack C-18 cartridge (Waters) by washing with H_2O and the rest of the mixt. eluted with MeoH. After the fr. was evapd to dryness under vacuum by rotary evaporation, the malvidin-3-monoglucoside pyruvic acid adduct was isolated by separation on a Fractogel column as described above. Frs. containing the derived pigment were collected, pooled, concd under vacuum and lyophilised. The amount of purified pigment was ca 2 mg.

MS. Analyses were performed on a quadrupole instrument with a mass range of 2400 mu, equipped with an ion-spray source (Sciex). The spectrometer was operated both in the positive- and negative- ion modes. The ion spray was operated at -4kV, the orifice voltage at -60 and -90V.

NMR. Spectra of the sample (ca 1 mg) in [$^2H_6$] DMSO-TFA (9:1, v), were run at 303 K, on a 500 MHz instrument. The solvent signal was used as int. ref. (^1H, δ 2.50).

LC/DAD. Grape pomace and further reaction mixts were analysed by HPLC using a two pump system, a U6K manual injector, an automated gradient controller and a diode-array detector. UV-visible spectra

were recorded from 250 to 600 nm. The column was a reverse-phase Lichrospher 100-RP18 (5 μm packing) (250 × 4 mm i.d.) protected with a guard column of the same material (Merck). Separations were carried out using the following conditions: 1 ml min^{-1} flow rate; oven temperature 30°; solvent A, H_2O–HCO_2H (4:1); solvent B, MeCN–H_2O–H_2CO_2H (4:9:2); elution with linear gradients from 5 to 30% B in 40 min, from 30 to 50% B in 20 min, from 50 to 80% B in 10 min, followed by washing and reconditioning of the column.

LC/MS. HPLC separations were carried out using a narrow-bore reverse-phase column. The column was connected to the ion spray interface via a fused-silica capillary (length 100 cm, 100 μm i.d.). The reaction mixt. was injected through a rotary valve fitted with a 20 μl sample loop. Separation was achieved on a Superspher 100-RP18 (3 μm packing, 125 × 2 mm i.d., Merck) column at a flow rate of 200 μl min^{-1}. Elution was achieved with solvents A and B used in HPLC/DAD analyses and the conditions adapted as follows: 5% of solvent B isocratically for 3 min, then a 3 stage-linear-gradient: from 5 to 20% of solvent B in 7 min, from 20 to 35% in 5 min and from 35 to 50% in 5 min, followed by washing and reconditioning of the column. Absorbance at 280 nm was monitored by a programmable Absorbance detector. After separation, the flow was split so that 50 μl min^{-1} went into the ion-spray source.

LC/MS of orange fraction. Experiments were carried out using the conditions described above. The first one, using an orifice voltage of −60 V, allowed the detection, in the negative-ion mode of $[M^+ - 2H^+]^-$ pseudomolecular ions of the pyruvic acid-derived flavylium cations eluted in the order reported in Table 1.

Other analyses were performed under the same conditions, except that the orifice voltage was set at −90 V, in order to observe some ion fragments. Thus, the following peaks were observed: delphinidin 3-monoglucoside pyruvic acid adduct *m/z* 531.2 with an ion fragment *m/z* 487.1 ($[M^+ - 2H^+]^-$-CO_2); petunidin-3-monoglucoside pyruvic acid adduct *m/z* 545.2, with ions fragments *m/z* 501.1 ($[M^+ - 2H^+]^-$-CO_2) and *m/z* 339.1 ($[M^+ - 2H^+]^-$- CO_2-glucoside); malvidin-3-monoglucoside pyruvic acid adduct *m/z* 559, with ion fragments *m/z* 515.1 ($[M^+ - 2H^+]^-$-CO_2) and *m/z* 353.1 ($[M^+ - 2H^+]^-$-CO_2-glucoside); malvidin-3-(6-acetyl)-monoglucoside pyruvic acid adduct *m/z* 601.2, with ion fragments *m/z* 556.9 ($[M^+ - 2H^+]^-$-CO_2) and *m/z* 352.9 ($[M^+ - 2H^+]^-$-CO_2-6-acetyl-glucoside).

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