COLOUR AND ANTIOXIDANT PROPERTIES
OF MALVIDIN-3-GLUCOSIDE AND VITISIN A

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This study presents findings from comparing the properties of anthocyanin pigments: i. malvidin-3-glucoside (Mal-3-G), the most significant anthocyanin present in young red wine; ii. Vitisin A (5-carboxypyranomalvidin-3-glucoside), a major product from a reaction induced in red wine between Mal-3-G and pyruvic acid during its ageing. These pigments were determined and compared to the absorption spectrum in 200–770 nm at various pH values, such results including the ability of cation Al^{3+} to change the values of absorbance. Changes in the colour parameters L* a* b* were determined in the CIELAB colour space of Mal-3-G and Vitisin A in relation to pH and the addition of $AlCl_3$. Both coloured compounds were investigated for their resistance to discoloration by sulphur dioxide. Total antioxidant activity was evaluated by two methods (FRAP, DPPH), and the values were compared to other phenols.
Keywords: anthocyanins, pyranoanthocyanins, phenols, antioxidant activity, pigment(s), red wine

Anthocyanins belong to a large group of secondary plant metabolites and occur in all forms of plant tissue. Anthocyanin pigments consist of either two or three chemical units, these being aglycon bases or flavylium rings (anthocyanidin), sugars or (potentially) acylating groups. In fact, anthocyanins and other phenols have been the focus of ever greater attention in health and medicine due to their anti-carcinogenic, -allergic, -ulceric, -arthritic, -inflammatory, and anti-microbial properties (BENVENUTI et al., 2004; SHI et al., 2005; LIANG et al., 2008; BUENO et al., 2012).

Malvidin-3-glucoside (Mal-3-G; Fig. 1A) is the most common anthocyanin found in *Vitis vinifera* L., its colour being dependent on the pH of the wine. It is bleached by SO_2 and also readily oxidises (SOMERS & EVANS, 1977). Recently, a particular subset of anthocyanin derivatives – pyranoanthocyanins are not actually present in grapes, but arise during wine production through condensation reactions. They are so-named due to the presence of a fourth pyrane ring. BAKKER and TIMBERLAKE (1997) called these products Vitisin A (Fig. 1B), which contribute to the orange-red colour of wine that develops over the course of ageing. Pyranoanthocyanins as Vitisin A, hydroxyphenyl-pyranomalvidin-3-glucoside, dihydroxy-phenyl-pyranomalvidin-3-glucoside, and methoxy-hydroxyphenyl-pyranomalvidin-3-gluco-side formed through a reaction Mal-3-G and pyruvic acid, hydroxycinnamic acids, or acetaldehyde were described by FULCRAND and co-workers (1998) and by RENTZSCH and co-workers (2007). Artificially enriching wine with pyranoanthocyanins has also been formerly studied by ROMERO and BAKKER (2000) and by MORATA and co-workers (2007). Furthermore,

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the antioxidant radical scavenging capacity of the pyranoanthocyanins present in aged wine, stemming from the chemical transformation undergone by anthocyanins, has been theoretically explained and tested (García-Alonso et al., 2004; Leopoldini et al., 2010; Azevedo et al., 2014). Aim of experiments was to compare colour and antioxidant properties of Mal-3-G with Vitisin A and to evaluate the absorption spectra and CIELAB parameters in dependence on pH values, addition of $AlCl_3$ or sulphur dioxide.
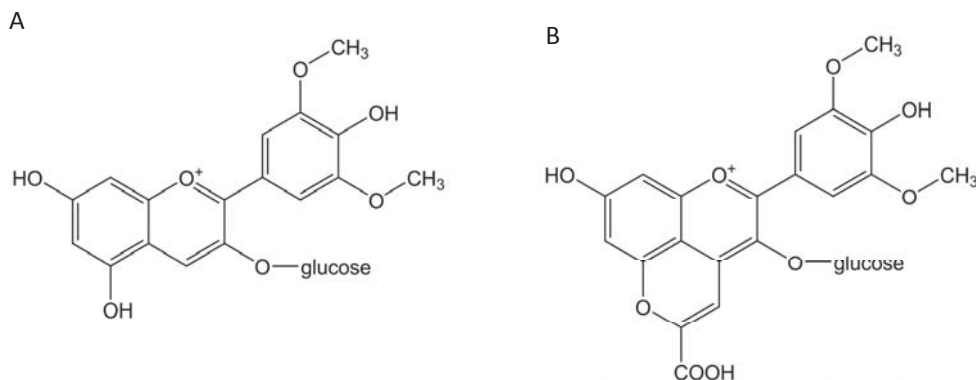

Fig. 1. Chemical structures of (A) Mal-3-G (malvidin-3-glucoside) and (B) Vitisin A
(5-carboxypyranomalvidin-3-glucoside)

1. Materials and methods

1.1. Chemicals and standards

Methanol, citric, hydrochloric, gallic, and pyruvic acids, $K_2S_2O_5$ (potassium metabisulphite), TPTZ [2,4,6-tris(2-pyridyl)-*s*-triazine], DPPH (2,2-diphenyl-1-picrylhydrazyl), $FeCl_3$, $AlCl_3$, Trolox, catechin, rutin, and quercetin were from Sigma-Aldrich (Prague, Czech Republic), Mal-3-G (malvidin-3-glucoside): from Extrasynthese (Genay, France).

Vitisin A (5-carboxypyranomalvidin-3-glucoside) was prepared by adding pyruvic acid (4 g l⁻¹) into red wine of the Cabernet Moravia cultivar (from Němčičky, wine subregion Velké Pavlovice, wine region Moravia, Czech Republic). The enriched wine was then stored in 50-litre glass demijohns at a stable temperature of 9 °C in dark for 8 months.

Isolating and purifying Vitisin A has been previously detailed in a study by Marhol and co-workers (2013). In brief, preparative flash chromatography on silica gel was used to fractionate 5-carboxypyranoanthocyanins from the crude lyophilizate of wine. The fraction thus created, rich in Vitisin A (checked via UPLC-DAD-MS), was further purified using a preparative C18 column (250 × 20 mm i.d., 10 μm, Labio, Prague, CZ). The composition of such fractions obtained and the final product was checked via UPLC-DAD-MS (Acquity UPLC/Q-TOF Premier, Waters, Milford, USA). Finally, NMR analysis of the purified Vitisin A was performed to confirm its identity and purity.

1.2. Preparation of samples for colourimetric measurements

Vitisin A and Mal-3-G were dissolved at a concentration of 1 g l⁻¹ in methanol with 0.01%
HCl. These solutions were diluted using buffers of pH 1.30, 2.35, 3.41, 4.40, 5.65, and 6.90
(prepared from 0.1 M of citric acid in deionised water). The pigment concentrations of every
sample equalled 100 mg l⁻¹.

In order to study the influence of Al^{3+} on the absorption spectrum, 60 µl of 10% solution
of $AlCl_3$ was added to the pigment samples thus prepared.

SO_2 was added at a dose of 0.5 g l⁻¹, in the form of 50 µl comprising 10 µl of 20%
$K_2S_2O_5$ to 2 ml of each pigment sample; this at pH 3.41 and at the pigment concentration of
100 mg l⁻¹.

1.3. Colourimetric measurements

The absorption spectra of the pigment samples were measured in a 10 mm silica cuvette
against deionised water at 200–770 nm using a Helios β spectrometer (Spectronic Unicam,
Cambridge). The influence of adding SO_2 was estimated by measuring absorbance values
against a blank containing SO_2, at a wavelength corresponding with the absorption maximum
of a pigment. The chromaticity of pigment samples in the CIE Lab colour space was measured
using a Lovibond RT850i colorimeter (The Tintometer Ltd, Lovibond House, Amesbury).
The CIE standard illuminant C was used to simulate daylight (2 mm absorption cell against
deionised water).

1.4. Determination of antioxidant activity

Phenols (Mal-3-G, Vitisin A, gallic acid, catechin, rutin, and quercetin) were dissolved in
methanol (500 mg l⁻¹). The procedures are described by MARHOL and co-workers (2013).
Briefly, FRAP: solution of 25 ml sample with 2 ml mixture (12 mM $FeCl_3$, 10 mM TPTZ and
3.6 pH acetate buffer; ratio 1:1:10) was measured after 10 min at 593 nm; DPPH: solution of
0.1 ml sample with 1.9 ml DPPH (in 0.1 mM methanol) was measured after 30 min at 515
nm; both as Trolox equivalent.

1.5. Statistical methods

Each sample and measurement was repeated three times. Averages and standard deviations are
shown in relevant figures. Data of antioxidant activity were analysed by ANOVA, applying
the Tukey's multiple range test for making comparisons with Unistat, v. 6.1 (Table 1).

Table 1. Comparison of antioxidant activity of Mal-3-G and Vitisin A to other phenolic compounds

Compound	FRAP		DPPH	
Antioxidant activity	(mM Trolox/g phenol)	(mM Trolox/mM phenol)	(mM Trolox/g phenol)	(mM Trolox/mM phenol)
Mal-3-G	3.77±0.13[b]	2.00±0.13[b]	2.14±0.07[a]	1.13±0.07[b]
Vitisin A	2.38±0.09[a]	1.34±0.10[a]	1.24±0.06[a]	0.70±0.06[a]
Gallic acid	18.10±0.83[d]	3.08±0.28 [c]	21.81±2.37[a]	3.71±0.80[d]
Catechin	4.13±0.22[b]	1.20±0.13[a]	5.48±0.3[a]	1.59±0.18[b]
Rutin	2.22±0.06[a]	1.36±0.08[a]	3.82±0.32[a]	2.33±0.39[c]
Quercetin	9.30±0.53[c]	2.81±0.32[c]	8.27±0.63[a]	2.50±0.38[cd]

Different superscripts in each column indicate the significant differences in the mean at P<0.05.

2. Results and discussion

The pH of a solution greatly influences the absorption of Mal-3-G in the VIS spectrum (Fig. 2A). The authors found that the rise in pH values from 1.30 to 6.90 significantly diminished the absorbance of Mal-3-G where wavelengths corresponded to the absorption maximum (515–525 nm). This was connected with the equilibrium distribution of four protolytic forms of Mal-3-G with differing spectral properties, as described by Brouillard and Delaporte (1977). At pH 1.30, red flavylium cation prevailed in the solution and the measured absorbance value reached 4.55. Increasing the pH triggered a gradual decrease in the absorbance of the Mal-3-G solution; indeed, a 90.4% reduction in its value was measured at pH 4.40. A significant proportion of Mal-3-G was then present in the colourless form of a pseudo-base. Raising the pH value further resulted in the gradual appearance of small amounts of yellow chalcone of Mal-3-G and the blue quinoic base form of Mal-3-G; these manifested as two new absorption maxima (recorded at pH 6.9). Asenstorfer and co-workers (2003) determined the absorption maximum for chalcone anion and quinonoidal anion at 443 nm and 578 nm, respectively.

The same concentration of Vitisin A, in the portion of wavelength indicating its absorption maximum, exhibited significantly lower absorbance compared to Mal-3-G. At pH 1.30, the maximum measured absorbance was 1.34, i.e. only 29.5% of the absorbance of Mal-3-G measured at the same pH. However, the absorbance values for Vitisin A altered more subtly, depending on changes in pH. A more visible decrease occurred only at pH 5.65. Compared to the maximum absorbance value at pH 1.30, this was equivalent to merely 38.8% (Fig. 2B). Simultaneously significant shift of absorption maximum at pH 6.9 was observed. It corresponded to the purple quinonoidal dianion formation of Vitisin A (Asenstorfer & Jones, 2007).
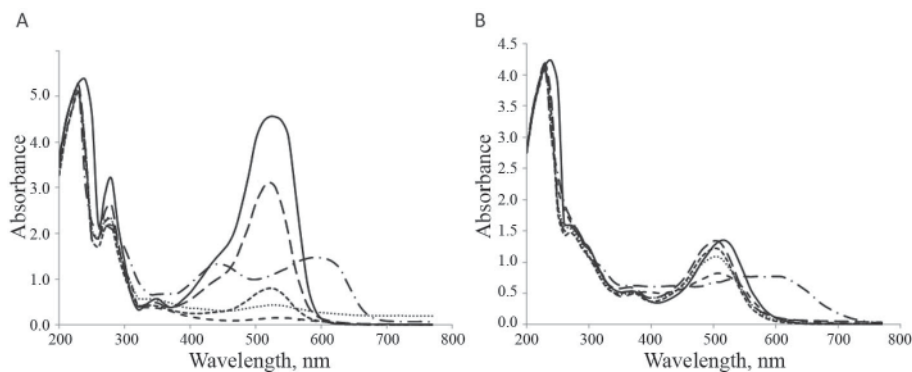

Fig. 2. Dependence of VIS-absorption spectrum of 100 mg l^{-1} (A) Mal-3-G and (B) Vitisin A on pH values: 1.30, 2.35, 3.41, 4.40, 5.65, and 6.90
——— : pH=1.30; — — : pH=2.35; - - - - - : pH=3.41; • • • • : pH=4.40; – – – : pH=5.65; -.- : pH=6.90.

Adding Al^{3+} to Mal-3-G caused a slight shift in the absorption maximum – from the red to the blue zone – more significant shifts to the blue zone (bathochromic shift) through utilizing Al^{3+} could be observed for anthocyanins associated with hydroxyl groups in the *ortho* position, i.e. petunidin-glucosides (Markakis, 1974). Adding Al^{3+} caused significant increase in Mal-3-G absorbance at pH 3.41 (from 0.799 to 3.773), and at pH 4.40 (from 0.438

to 2.517) at 515–525 nm. This might be due to stabilization of the red flavilium cation of Mal-3-G or an equilibrium shift to this protolytic form. The addition of Al^{3+} did not trigger major alteration in the absorption maximums of Vitisin A (Fig. 3).
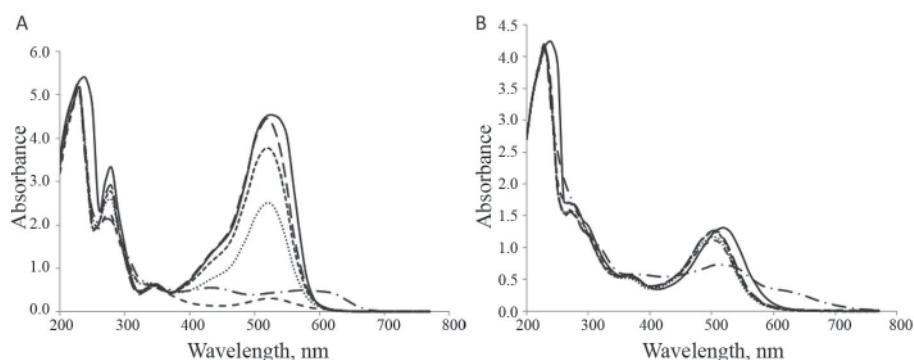

Fig. 3. Dependence of VIS-absorption spectrum of 100 mg l^{-1} (A) Mal-3-G and (B) Vitisin A on pH values: 1.30, 2.35, 3.41, 4.40, 5.65, and 6.90, after adding Al^{3+}
——— : pH=1.30; — — : pH=2.35; - - - - - : pH=3.41; • • • • : pH=4.40; – – – : pH=5.65; -.- : pH=6.90.

The colour schemes for Mal-3-G and Vitisin A are presented in Fig. 4. The values measured highlight the orange-red shade of Vitisin A, in contrast with the purple-red of Mal-3-G. Moreover, the newly formed pyran ring stabilises the colour of pyranoanthocyanin at varying pH. Across the entire pH range monitored, the colour of Mal-3-G was darker (lower value of L*) by 15.1% (pH 5.65) and 22.6% (pH 1.3) than for the same concentration of Vitisin A. Following the reaction with Al^{3+}, Mal-3-G at pH 1.3 through to 4.4 turned darker still, unlike Vitisin A, where a further decrease in colour brightness occurred after adding AlCl$_3$ (a rise in the original value of L* by 9.2% at pH 1.3).
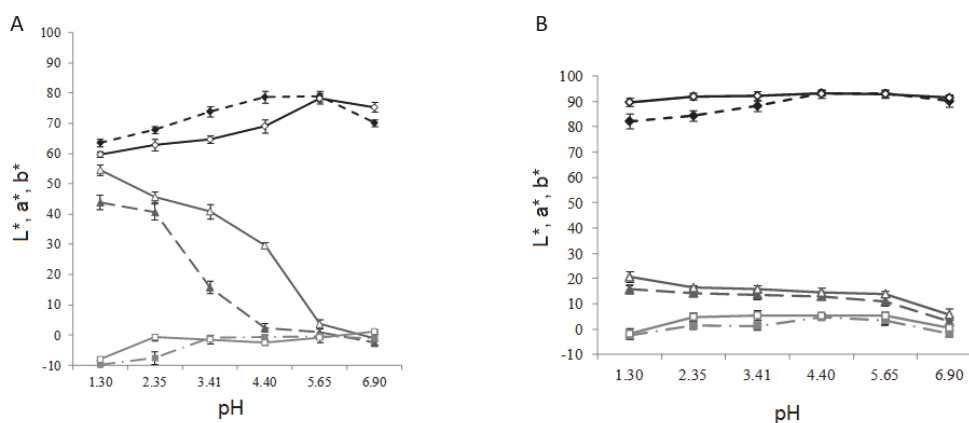

Fig. 4. Changes of colour parameters in the CIELAB colour space as dependent on pH and adding Al^{3+} (averages and standard deviation of: L*, L* + Al^{3+}, a*, a* + Al^{3+}, b* and b* + Al^{3+}) of 100 mg l^{-1} (A): Mal-3-G; (B): Vitisin A
-◆- : L*; —◇— : L*+Al^{3+}; - -▲- - : a*; —▲—:a*+Al^{3+}; – ■ –: b*; —□—:b*+Al^{3+}.

At the pH 1.3, Mal-3-G was a distinct red (a* increased by +43.8), while blue shades actually decreased (b* decreased by –9.7 and –7.54, respectively). Starting from pH 3.41, the proportion of the red shade began to decrease significantly (i.e. to almost 0 at pH 5.65), in addition to which the same decrease was recorded for the blue shades. Reactions with Al^{3+}, taking place after adding $AlCl_3$, increased the percentage of the red shade of Mal-3-G across the entire range of pH values; and, as compared with the value measured at pH 1.30, a significant decrease (by 93.3%) was observed at pH 5.65.

Nevertheless, Vitisin A showed a more stable colour than Mal-3-G. At pH 1.30, the percentage of red shades had diminished by 64%, in comparison with Mal-3-G (a low value +a*). However, as compared to Mal-3-G, the percentage of yellow shades was higher at pH 2.35 through to 5.65 (positive values b*). Consequently, the colour of Vitisin A had more of an orange hue, and adding Al^{3+} did not largely alter its colour manifestation (Fig. 4).

The decolourisation by SO_2 on both isolated pigments was also tested. In fact, the mechanism of the decolourisation on the monomer anthocyanins was described by Jurd (1964) and by Timberlake and Bridle (1976). Mal-3-G was decolourised completely and at 520 nm, its absorbance decreased by 99.7%. However, Vitisin A only dropped slightly from its original absorbance value (i.e. by 12%) at the wavelength corresponding to its absorption maximum (Fig. 5). This was caused by the absence of binding points for SO_2 on the molecule of Vitisin A (Bakker & Timberlake, 1997). The presence of the fourth ring in Vitisin A makes this pigment more stable than a grape anthocyanin (as Mal-3-G). Furthermore, it not only resists discoloration by SO_2, but also loss in colour due to high pH and oxidative degradation, which can potentially occur in the course of wine production.
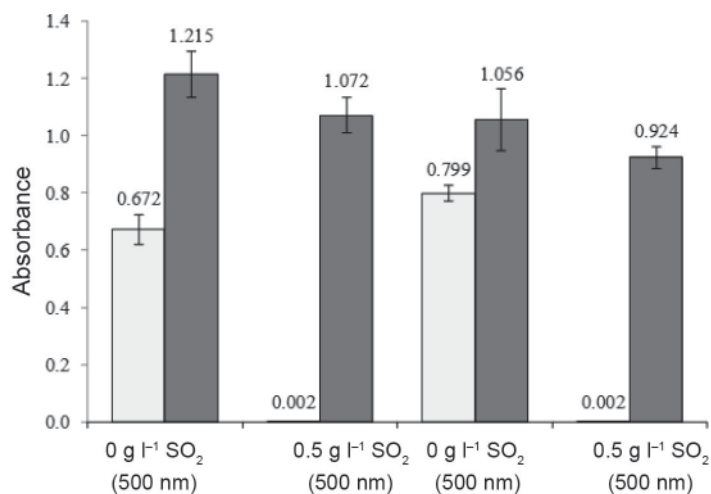

Fig. 5. Influence of adding sulphur dioxide on absorbance of 100 mg l⁻¹ Mal-3-G (malvidin-3-glucoside) and Vitisin A (5-carboxypyranomalvidin-3-glucosid) solutions at pH 3.41.
□: Mal-3-G; ■: Vitisin A

The resulting values for antioxidant activity (Trolox equivalents) related to the same concentration 1 mM of the studied phenols and to the antioxidant activity expressed per 1 g of phenols, which brought about significant difference in interpreting the results (Table 1).

The highest antioxidant activity (DPPH and FRAP) was detected in gallic acid, followed by quercetin. In case of these two phenols, the greatest differences in expression of the resulting values were also recorded. Indeed, the size of the phenol molecule had a tremendous effect on the value of measured antioxidant activity.

Vitisin A is highly stable in colour, resisting change in pH and the addition of SO_2. Concurrently, detection was made of a lower value for the antioxidant activity of Vitisin A in comparison with Mal-3-G. The DPPH method discerned a drop by 37% (mM Trolox/g phenol) and by 33% (mM Trolox/mM phenol), whereas FRAP revealed drops by 42% and 39%, respectively. Therefore, great accord was shown in ratio by both methods. Such results agreed qualitatively with data of García-Alonso and co-workers (2004). The suggestion was that the formation of a 5-carboxypyrano ring brought about decrease in antioxidant activity. Nevertheless, results published by Jordheim and co-workers (2007) showed 5-carboxypyranoanthocyanins with greater antioxidant activity. This confirmed that the antioxidant activity of phenols depends on the method of determination. The purity of the isolated pyranoanthocyanins can also affect the values determined. Azevedo and co-workers (2014) found out that pyranoanthocyanin-phenolics possess a higher antioxidant potential than Mal-3-G, suggesting that adding a catechol or flavanol moiety may heighten antioxidant capacity.

3. Conclusions

Mal-3-G (malvidin-3-glucoside) and pyranoanthocyanins represent the base pigments responsible for the colour of young wine and aged wine, respectively. Their different chemical structures were reasons for finding different colour properties applicable in wine technology or food industry. Unlike Mal-3-G, Vitisin A did not exhibit any significant alteration in absorbance across a wide range of pH. Mal-3-G was redder in colour than Vitisin A at low pH. Vitisin A demonstrated greater colour stability than Mal-3-G. However, at pH 1.30, the proportion of red shade was lower by 64% than for Mal-3-G. The colour of Vitisin A possessed more of an orange hue, and adding Al^{3+} did not change this significantly. Unlike Vitisin A, Mal-3-G was nearly decolourised with tested dose of SO_2. Higher colour stability of Vitisin A can be benefit for organoleptic quality of aging red wine and other common sour food products. The antioxidant activity of this Vitisin A and Mal-3-G as major wine dye was compared with members of common phenols, enabling to reduce radicals in food. A higher antioxidant activity was recorded for Mal-3-G than for Vitisin A. It was related to lower ability of Vitisin A to scavenge of free radicals.

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