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Proposal of a method for fluorimetric analysis of malvin in red wines

by

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Summary: A fluorimetric method for the quantitative determination of malvin (malvidin 3,5-diglucoside) in red wines is described. The method is based on previous fractionation of the wine in a Polyclar AT column and later formation of a fluorophore, by oxidation of the malvin. The proposed method has good precision and accuracy and when applied to hybrid red wines affords results significantly comparable with those obtained by HPLC.

Key words: malvin, anthocyanin diglucosides, hybrid vines.

Introduction

Most methods used for the characterization of red wines made from grapes from hybrid or American vines are based on the detection of anthocyanin diglucosides and, in particular, of malvidin 3,5-diglucoside (malvin) by paper chromatography (BOURZEIX 1967; HADORN *et al.* 1967) or thin layer cellulose chromatography (FLANZY and BOURZEIX 1968). More recently, HPLC methods have been described (HEBRERO *et al.* 1989). Nevertheless, the most used methods are based on the observation or measurement of the fluorescence emitted by malvin, when it is oxidized under certain conditions. Among these is the method of DORIER and VERELLE (1966), in which the fluorescence emitted is detected by visual observation when the samples are illuminated with Wood light (λ = 360 nm). Different modifications of this test have been proposed for its quantitative use, carrying out the measurement by fluorimetry (BIEBER 1967; HADORN *et al.* 1967; O.I.V 1990). The results obtained in our laboratory were not reliable, which might have been due to two facts: utilization of reaction conditions insufficiently optimized and interference of the matrix in the fluorimetric reaction. In the present work, we describe a series of experiments designed to optimize the conditions for the fluorimetric reaction and to confirm the existence of a sample matrix effect and to design a simple method for the determination of malvin in red wines by fluorimetry.

Materials and methods

Grape samples from a hybrid variety (*Vitis vinifera* × *V. berlandieri* 41B) were used to obtain, from their skins, standards of mono- and diglucoside anthocyanins according to the procedure described in HEBRERO *et al.* (1989).

Wine samples: a) Red wines from *V. vinifera* of known quality, purchased from commercial sources, in which the absence of malvin was confirmed by HPLC, and b) non commercial red wines, wholly or partially made from hybrid vines, supplied directly by individual wine makers.

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HPLC analysis of anthocyanins: This was conducted as in HEBRERO *et al.* (1989) on a Hewlett-Packard mod. Series 1050 chromatograph. The column was a reverse phase Nucleosil C_{18}, 5 μm (25 × 0.46 cm). The solvents were: A, 10 % formic acid; B, acetonitrile. For elution, a gradient was established with the following conditions: 5 to 9 % B over 5 min, 9 to 11 % B over 10 min, 11 to 15 % B over 25 min, 15 to 20 % B over 10 min, and 20 to 30 % B over 15 min, with a flow rate of 1.0 ml/min. Detection was carried out at 525 nm with an HP 1040 Series II diode array detector, coupled to an HP ChemStation 79994 data treatment station. Malvin content was determined by comparison of its peak area with a curve obtained with solutions of a standard of malvin (Sigma).

Results and discussion

1. Preliminary assays

To optimize the fluorimetric reaction conditions the reaction described by DORIER and VERELLE (1966), modified by HADORN *et al.* (1967) was taken as a basis: 1 ml of wine is mixed with 1 ml of 1N H_2SO_4 and with 1 ml of 1 % $NaNO_2$, this is shaken and left to stand for 3 min; then, 10 ml of a solution of methanol containing 1 % NH_3 (g) are added. The mixture is shaken and centrifuged for 5 min at 3000 rpm. The fluorescence of the supernatant is measured before 15 min have elapsed.

To study whether the duration of the reaction was sufficient to complete the oxidation, malvin solutions of 50 mg/l were prepared in red wine and the oxidation reaction was allowed to run for different times between the addition of 1 % $NaNO_2$ and the incorporation of the methanol solution containing 1 % NH_3 (g). Later, the mixture was centrifuged and the fluorescence of the supernatant was measured. Fluorescence intensity increases as the reaction time progresses (Figure). A time of 30 min was chosen at which at least 95 % of the maximum fluorescence intensity was developed. Another aspect observed was that the most homogenous fluorescence values (coefficient of variation 1.3 %) were obtained when the fluorimetric measurement was performed 30 min after the addition of the methanol-ammonia solution and the centrifuging at 3500 rpm over 10 min. In these conditions, linear outlines were obtained for standard curves obtained with solutions of malvin in red wine up to 100 mg/l.

The application of these new conditions in wine samples showed acceptable precision (coefficients of variation ranging between 1.9 and 4.1 %, in assays carried out with five different hybrid wines), but deficiency in accuracy.

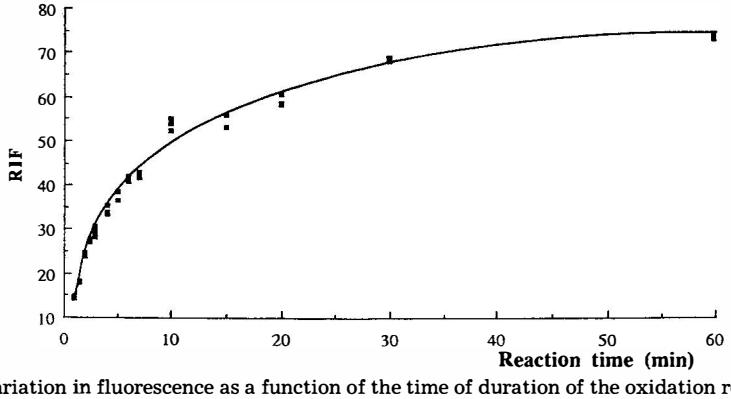

Figure: Variation in fluorescence as a function of the time of duration of the oxidation reaction of malvin (average of 3 replicates).

In the next step, malvin (5, 10 and 20 mg/l) was added to hybrid red wines previously diluted in proportions of 1:2, 1:10 and 1:25 with a red wine free of diglucosides. The recovery of malvin added improved as the dilution increased, however the recoveries were still not satisfactory (dilution 1/2: recoveries 82—149 %; 1/10: 60—140 %). This, together with the fact that the more diluted wines (1:25) showed the most precise and accurate recoveries (82—103 %; standard deviation: 7.4), suggested the influence of a sample matrix effect on the fluorimetric determination of malvin.

In the first instance, we thought that this matrix effect might be due to the interference of other anthocyanins, so this fact was checked working with model anthocyanin solutions extracted from the skins of grapes from a hybrid variety. Solutions (3-monoglucosides of delphinidin, cyanidin, petunidin, paeonidin and malvidin, and 3,5-diglucosides of delphinidin, cyanidin, petunidin and paeonidin) were separately prepared in synthetic wine (solution containing 5 g/l of tartaric acid and 10 % ethanol, adjusted with NaOH at pH 3.2), with concentrations ranging between 2.5 and 100 mg/l. None of the 3-monoglucosides developed fluorescence, whereas the 3,5-diglucosides did and their fluorescence spectra were similar to those of malvin. The fluorescence compared with that obtained for equivalent concentrations of malvin, was, however, some 20-fold lower for the 3,5-diglucosides of cyanidin, petunidin and delphinidin and about 10-fold lower for paeonidin 3,5-diglucoside.

To check whether the presence of other anthocyanins together with malvin would modify the actual fluorescence of this compound, each of the anthocyanins was added in different concentrations (2.5 and 80 mg/l) to model solutions of malvin in synthetic wine (45 mg/l). The addition of monoglucoside anthocyanins did not cause important variations either in fluorescence intensity or in spectra. The 3,5-diglucoside of cyanidin hardly affected the fluorescence, whereas the 3,5-diglucosides of petunidin and of delphinidin increased the fluorescence when they were present in concentrations at least similar to that of malvin (increases in the fluorescence intensities of 15 and 9 %, respectively, as compared with the situation observed in solutions of malvin alone). The paeonidin 3,5-diglucoside elicited an increase in the fluorescence intensity at concentrations 9-fold lower than those of malvin. In solutions containing similar concentrations of both anthocyanins was approximately 28 % higher than in solutions containing only malvin. In no case were modifications observed in the fluorescence spectrum of malvin, caused by the presence of the other diglucosides. Thus it can be assumed that on determining malvin other diglucoside anthocyanins contribute to the fluorescence emitted, although this would only be important at concentrations equal to or higher than those of malvin. This would be uncommon since malvin is always the most important diglucoside in wines from hybrid or American stocks, normally accounting for 64—88 % of the total diglucosides (GUTIÉRREZ-FERNÁNDEZ *et al.* 1992). However, the results obtained in these assays do not seem able to explain the matrix effect that causes the deficiency in accuracy of the fluorimetric analytical method.

Another aspect that was checked was the possible influence of other matrix factors, such as pH and SO_2 content, which affect the equilibria among the different structural forms of anthocyanins. Solutions of malvin (45 mg/l) were prepared in synthetic wines adjusted to different pH values (ranging between 1.0 and 4.0) by HCl or NaOH. In all the solutions the fluorimetric reaction was carried out in triplicate. The fluorescence intensities measured at the different pH values were fairly close to one another (coefficient of variation 2.2 %), which shows that, under normal circumstances, pH would not be expected to strongly affect the determination.

To study the possible influence of the sulphite, model solutions of malvin (45 mg/l) were prepared in synthetic wine at pH 3.2 containing 0—45 mg/l of total SO_2. The fluorescence reaction was applied before and after treatment with acetalde-

hyde to block the effect of the sulphite. Neither SO_2 nor the addition of acetaldehyde exerted any appreciable effect on the intensity of the fluorescence measured; the small differences observed should be attributed to the precision of the technique.

The next assays consisted in fractionating the samples by column chromatography in order to be able to handle the malvin in less complex media. Polyclar AT was chosen as the stationary phase since it had previously been used by the authors for the separation of free anthocyanins (RIVAS *et al.* 1992). A battery of 20 x 1 cm glass columns loaded with Polyclar AT up to a height of approximately 5 cm was prepared. 1 ml of wine from *V. vinifera* to which 500 mg/l of a malvin standard has been added was placed in each column and eluted with methanol and ethanol of different concentrations, acidified with HCl in proportion ranging between 0.1 and 1 %. The eluates were dried under reduced pressure at low temperature (< 30 °C) and the residues were dissolved in 10 ml of synthetic wine. Using HPLC it was ascertained that with all the eluents employed it was possible to obtain complete elution of the malvin incorporated into the wine deposited in the column; despite this, larger volumes of the ethanolic solutions than those of methanol were required. For later assays, absolute methanol acidified with 0.1 % HCl was chosen, being 10 ml sufficient for completing elution of anthocyanins.

We also checked the possibility of performing the reaction for the formation of the malvin fluorophore directly in the solutions of methanol used for the elution. Solutions of malvin of increasing concentration, ranging between 1 and 20 mg/l, were prepared in methanol containing 0.1 % HCl. The fluorimetric reaction developed satisfactory in this water-alcohol medium and the intensities of fluorescence obtained were linear as a function of the concentration.

2. Proposed fluorimetric method

a) Operating conditions

In a 20 x 1 cm chromatography column fitted with a stopcock and filter, a suspension of Polyclar AT in H_2O, prepared according to GLORIES (1984), is introduced up to a height of approximately 5 cm and this is flushed through with abundant water.

1 ml of wine is carefully added and allowed to filter into the resin. When its level is close to the top of the resin, a volume of methanol acidified with 0.1 % HCl is added. When the coloured band is about to elute from the column, the collection of the eluate is started in a 10 ml flask until this volume is completed.

A 1 ml aliquot of the eluate is taken, adding ½ ml of 1N H_2SO_4 and 1 ml of 1 % (w/v) $NaNO_2$, and allowing the mixture to react for 30 min. Then, 10 ml of a solution of methanol containing 1 % NH_3 (g) is added and the resulting mixture is centrifuged for 10 min at 3500 rpm.

At 30 min after adding the methanol-ammonia mixture, the fluorescence intensity of the supernatant is measured at 384 nm (λ excitation) and 500 nm (λ emission). Prior to this, the sensitivity of the spectrofluorimeter should be checked with a standard solution of quinine sulphate, adjusting to zero with acid methanol subjected to the same reaction conditions.

The results are quantified by comparison with a calibration curve obtained from solutions of malvin in acid methanol of increasing concentration (1—20 mg/l). The passage of wine through the column affords a dilution of 1:10, a factor that should be taken into account when the calculations are made.

b) Validity assays of method

Linearity: Calibration curves were prepared in methanol containing 0.1 % HCl, at a malvin concentration range between 0 and 20 mg/l by different methods:

— On different days with malvin solutions obtained from the dilution of the same stock solution of malvin.
— From different stock solutions on different days.
— From solutions of malvin of 10, 20, 50, 100 and 200 mg/l, whose aliquots of 1 ml of were passed through Polyclar AT, eluting with 10 ml of acid methanol in each case.

The fluorimetric reaction was always conducted in aliquots of 1 ml. All the curves show good linearity and neither the method of preparation nor the passage of the solutions through the column led to important differences. Tab. 1 shows the mean curve for all different methods (equation: y = 0.4536x–0.01060; r = 0.9999).

Table 1

Calibration curve obtained using the proposed fluorimetric method.

Malvin (mg/l)	R.I.F. mean	Standard deviation
1.0	0.46	0.044
2.0	0.91	0.090
5.0	2.25	0.22
10	4.48	0.41
20	9.08	0.85

Precision : For *replicability* (variability among the measurements obtained with replicates from the same samples) 1 ml of hybrid red wine was passed through the Polyclar AT column and of the 10 ml of eluate obtained 9 aliquots of 1 ml each were taken to perform the fluorimetric reaction. The results obtained afforded a variation coefficient of 2.1 %. For *repeatability* (variability of the measurements carried out at different times on the same sample by different operators), five 1 ml aliquots of the same hybrid red wine were passed through the Polylclar AT and the reaction for the formation of the fluorophore was carried out in triplicate in each of the 5 methanolic eluates obtained. The assay was repeated 5 times on 3 different days by different operators. The method had good precision (mean variation coefficient 1.9 %).

Accuracy — Assay 1 : Three aliquots of 1 ml of a hybrid red wine were passed through Polyclar AT and 1 ml of each eluate was added respectively with 2.5 and 10 mg of malvin per liter. The assays were repeated 3 times on different days. Quantification was made by comparing the intensity of fluorescence obtained with a calibration curve prepared in parallel. Each reaction was carried out in triplicate.

Accuracy — Assay 2 : Different concentrations of malvin (20, 50 and 100 mg/l) were added to a hybrid wine. Aliquots of 1 ml of each of these samples were passed through Polyclar AT. In this way, taking into account the dilution implied by passage through the column, the eluates had malvin additions identical to those of the first assay. As in the previous case, the assay was repeated.

The recoveries obtained ranged between 98 and 109 %, with a mean recovery for all the results obtained in both assays of 102.5 % (standard deviation = 4.9).

3. Comparison between the proposed fluorimetric method and HPLC

Eighteen samples of red wine were analyzed; the hybrid nature of these had previously been confirmed by the qualitative method of Dorier and Verelle (1966). In all samples, we determined the malvin content using the proposed fluriometric

method and HPLC (HEBRERO *et al.* 1989). The following results were obtained (sample: HPLC/proposed method):
1: 27.1/26.7; 2: 12.9/13.0; 3: 121/118; 4: 135/135; 5: 36.9/36.5; 6: 181/191; 7: 172/191; 8: 114/112; 9: 32.2/31.9; 10: 49.6/50.5; 11: 10.3/10.1; 12: 48.0/48.7; 13: 50.7/50.2; 14: 98.4/106; 15: 95.0/97.9; 16: 54.5/53.4; 17: 16.4/16.9; 18: 22.3/21.5. To check whether there were statistically significant differences between the results obtained with each method, the Wilcoxon test was applied (Tab. 2) which pointed to the absence of statistical differences.

Table 2

Results obtained after application of the Wilcoxon test to malvin contents obtained by HPLC and the proposed fluorimetric method.

Wilcoxon signed-rank X_1: HPLC METHOD Y_1: PROPOSED METHOD

	Number:	Σ Rank:	Mean Rank:
- Ranks	8	86.5	10.8
+ Ranks	9	66.5	7.4

note 1 cases eliminated for difference = 0.

Z	-4.7E-1
Z corrected for ties	-4.7E-1
# tied groups	2

$$Z_{0.05} = 1.96 \qquad Z_{exp} < Z_{\alpha}$$

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