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A comparison of analytical methods for measuring the color components of red wines

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Abstract

The monomeric and polymeric pigments of 20 young red wines were analysed using most recent of the approaches available for phenolics measurements in wine, including: (i) HPLC with silica-based reversed-phase, (ii) HPLC with polymeric-based reversed-phase columns, (iii) the spectrophotometric Adams' tannin and polymeric pigments assay, (iv) the Boulton's copigmentation assay, and (v) the Somers' unbleached polymeric color assay. Moreover, a modification of an existing HPLC method, *i.e.* the addition of SO_2 to the mobile phases, allowed the unbleached polymeric pigments to be analysed by HPLC for the first time. The wines displayed a variation in their color density at 520 nm that ranged by 10-fold, and included wines made from Pinot noir, Merlot, Cabernet sauvignon, Cabernet franc, Sangiovese, Cagnulari and Cannonau grapes. The total color of wines was an aggregate number of three components: copigmentation (8–30%), total free anthocyanins (24–35%), and polymeric pigment (35–63%). Cross-comparison between the selected method was performed and discussed. In particular, the polymeric pigments estimated by HPLC with polymeric-based reversed-phase column were in good agreement with the result of the reversed-phase C_{18} column ($R^2 = 0.9703$) and the sum of small and large polymeric pigments estimated by the Adams' assay ($R^2 = 0.9511$). The level of copigmentation can be almost completely described by the levels of monomeric pigments ($R^2 = 0.9464$) and not by the tannin content as has often been suggested (copigmentation vs tannin: $R^2 = 0.4827$).

Keywords: Anthocyanins; Copigmentation; HPLC; Method comparison; Polymeric color; Wine

1. Introduction

Anthocyanins are water-soluble pigments present in red grape skins which partitioned into wine during the vinification. The monomeric forms are responsible for most of the red color of young wines, and they contribute to the development of red polymeric pigments during wine aging. The extent of red color in wines is due to a number of factors including the type and concentration of anthocyanins, pH, free SO_2 level, and the extent of polymerization and copigmentation. The color displayed by a red wine continues to change during its life and can be affected by a number of winemaking practices and environmental conditions (Boulton, Singleton, Bisson, & Kunkee, 1998; Riberau-Gayon, 1985; Somers, 1998).

Red wine is a complex solution and although its color can be measured by spectral techniques, the relationship between the wine color with its chemical compositions is difficult to explain. Both the knowledge of anthocyanin chemistry and the availability of suitable methods of analysis are required for research and quality control in winemaking. A number of analytical methods have been proposed for measuring the polyphenols of wine, including spectrophotometric (Boulton, Neri, Levengood, & Vaadia, 1999; Di Stefano, Cravero, & Gentilini, 1989; Harbertson, Kennedy, & Adams, 2002; Harbertson, Picciotto, & Adams, 2003; Peri & Pompei, 1971; Ribereau-Gayon & Stonestreet, 1965; Somers & Evans, 1977), and chromatographic methods (Baldi & Romani, 1992; De Beer et al., 2004; Hammerstone, Lazarus,

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Mitchell, Rucker, & Schitz, 1999; Kantz & Singleton, 1990; Kennedy & Waterhouse, 2000; Kennedy, Ferrier, Harbertson, & Peyrot des Gachons, 2006; Lea, 1980; Nagel & Wulf, 1979; Oszmianski, Ramos, & Bourzeix, 1988; Peng et al., 2001; Singleton & Trousdale, 1992; Waterhouse, Price, & McCord, 1999; Vrhovsek, Mattivi, & Waterhouse, 2001). Each of these methods presents advantages and limitations, and the cross-comparison of results obtained with different methods is not always fitting. Somers and Evans (1977) developed a spectral method of estimating the extent of total free anthocyanins and the polymeric pigments in red wine after bleaching the anthocyanins with excess of SO_2. Total free anthocyanin content of red wines measured by Somers' method is higher than those estimated by HPLC, the presence of polymeric pigments may account for this discrepancy (Bakker, Preston, & Timberlake, 1986; Rivas-Gonzalo, Gutierrez, Hebrero, & Santos-Buelga, 1992). The so-called Adams' assay (Harbertson et al., 2002; Harbertson et al., 2003) is a new UV–Vis spectrophotometric method to analyse tannins (*i.e.* polymeric flavan-3-ols), total phenolics, and small (SPP) and large (LPP) polymeric pigments in wine. Investigation of polymeric pigments using HPLC with reversed-phase C_{18} column has been limited because these compounds typically elute together as a broad peak (called *envelope*) that can be difficult to evaluate. The recent availability of polystyrene–divinylbenzene reversed-phase column provides a great opportunity to improve the analysis of polymeric pigments in red wine (Peng, Iland, Oberholster, Sefton, & Waters, 2002).

The objective of this study was to compare selected established and innovative methods of analysis of color components in red wine, including HPLC, chemical assays and UV–Vis spectral methods, and to understand the relationship between the methods.

2. Materials and methods

2.1. Wines

Twenty young red wines were selected to cover a wide range of color density ranging from almost zero to 10 Absorbance Units (AU) at 520 nm. These were two Sangiovese (Atlas Peak Winery, CA), four Merlot (Robert Mondavi Winery, CA; Trefethen Vineyards, CA), two Cabernet sauvignon (Robert Mondavi Winery, Trefethen Vineyards), one Cabernet franc (Trefethen Vineyards), nine Pinot noir (Robert Mondavi Winery, Saintsbury, CA; UCDavis Winery, CA), one Cagnulari, and one Cannonau red wines (Santa Maria la Palma, Alghero, Italy). At the time of these analyses 18 wines were 11 months old, and two wines were 23 months old (the two Sangiovese wines from Atlas Peak Winery).

2.2. Analytical determinations

Before analysis the wine pH of each was adjusted to 3.6 and filtered through a 0.45 μm membrane filter (Acrodisc CR, Pall Corporation, Corvina, CA). For each wine the following parameters were measured according to the methods described in the literature:

- *spectrophotometric assays*: total phenolics, total color, total free anthocyanins, copigmentation (Boulton et al., 1999), polymeric color (Somers & Evans, 1977), small and large polymeric pigments, and tannins (Harbertson et al., 2002; Harbertson et al., 2003);
- *chromatographic assays*: HPLC analysis of phenolic compounds by reversed-phase (RP-C_{18}) column (Donovan, Meyer, & Waterhouse, 1988), and by polystyrene–divinylbenzene reversed-phase (PLRP) column (Peng et al., 2002). Besides using the original HPLC conditions, the method of Peng et al. (2002) was slightly modified with the introduction of SO_2 (100 mg/l) to the mobile phases to maintain the bleaching effect on wine anthocyanins throughout the run. All the others analytical conditions were kept as described by Peng et al. (2002). Separation was carried out at 30 °C on a polystyrene–divinylbenzene reversed-phase column (PLRP-S, 250 × 4.6 mm, 100 Å, 5 μm particle size) with a precolumn cartridge of the same material (Polymer Laboratories, Amherst, MA). Samples were analysed using a HP1100 HPLC system (Palo Alto, CA) equipped with four pumps, diode array detector, autosampler with an injection valve with a 20-μl loop, and data acquisition software (ChemStation). The diode array detection signal from 200 to 800 nm was stored and two wavelengths were monitored at 280 and 520 nm. The absorbance at 280 nm provides a measure for all phenolic compounds, whereas at 520 nm the colored compounds are detected.

All samples were analysed in duplicate. Compounds detected in HPLC were quantified by integration as peak area.

2.3. Statistical analysis

The mean, the standard deviation, and the linear regression analysis were computed with the Statistica software 6.0 (StatSoft, Tulsa, OK).

3. Results and discussion

3.1. HPLC vs HPLC (each other)

Two HPLC methods using RP-C_{18} column (Donovan et al., 1988) and PLRP column (Peng et al., 2002), respectively, were compared for their ability to analyse the polymeric pigments in red wines. Many simple phenolics (e.g. flavanols, phenolic acids, etc.) were detected and eluted as a single peak in both columns (Fig. 1). On the other hand, polymeric phenolics eluted in RP-C_{18} column as a broad envelope-peak from 40 to 63 min, whereas a single large peak (t_R 66.0 min) resolved at baseline was observed

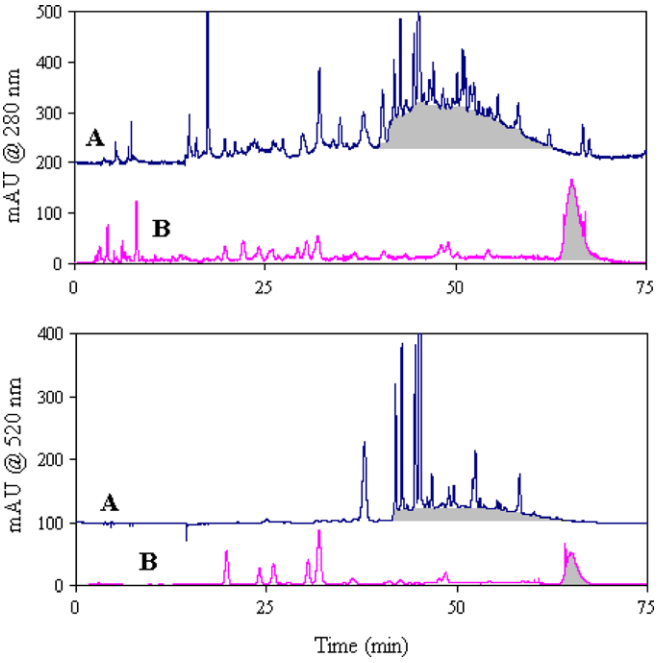

Fig. 1. HPLC chromatograms of a red wine monitored at 280 nm (top) and 520 nm (bottom) using reversed-phase RP-C_{18} (trace A) and polystyrene–divinylbenzene reversed-phase PLRP column (trace B). The shaded area represents the polymeric phenolics.

using PLRP column. Most of the peaks eluting on the envelope-peak when using RP-C_{18} column were simple anthocyanins detectable at 280 and 520 nm. The polymeric and polyphenolic nature of the peak eluting at t_R 66.0 using PLPR column has already been elucidated (Peng et al., 2001; Peng et al., 2002). Comparison between RP-C_{18} and PLRP column to estimate the polymeric polyphenols (expressed as peak area) provided fitting results, the variance attributed to the linear combination between the two variables being explained by the coefficient of determination: $R^2 = 0.9703$ (Fig. 2).

It is well known that polymeric pigments are more stable to SO_2 bleaching than monomeric pigments. The bleaching effect of SO_2 on polymeric pigments of wines was verified by HPLC using PLRP column monitoring at 520 nm. Unbleached wines and samples bleached with excess SO_2 (Somers & Evans, 1977) were analysed using two methods: (i) the one proposed by Peng et al. (2002) and (ii) with the in-house modified Peng's method by addition of 100 mg/l SO_2 to the mobile phases. The bleaching of wine in combination with the addition of 100 mg/l SO_2 to the mobile phases achieved a complete bleaching of monomeric anthocyanins (Fig. 3). In the wines used in this study the polymeric colored peak (t_R 66.0 min) was partially bleached from 5% to 22% and a good correlation ($R^2 = 0.992$) was found between the peak area of polymeric pigments before (*i.e.* total polymeric pigments) and after the SO_2 bleaching (*i.e.* unbleached polymeric pigments) (Fig. 4). The amount of unbleached polymers was strictly proportional to the amount of total polymers irrespectively of the cultivar considered. This suggests that the

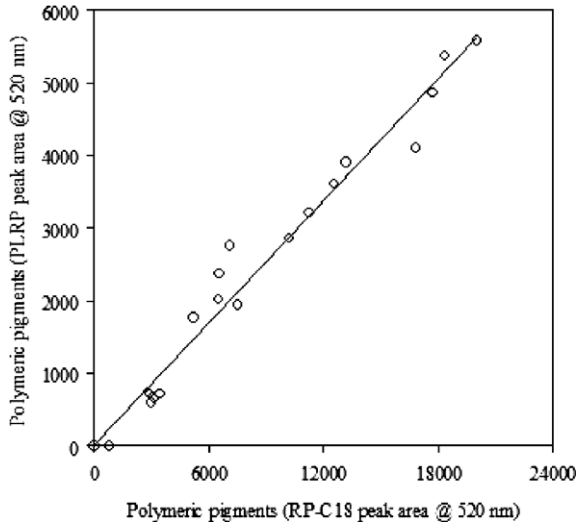

Fig. 2. Correlation between polymeric pigments in red wines using HPLC with RP-C_{18} (shaded area from 40 to 63 min) and PLRP columns (peak at 66.0 min), evaluated at 520 nm and reported as peak area.

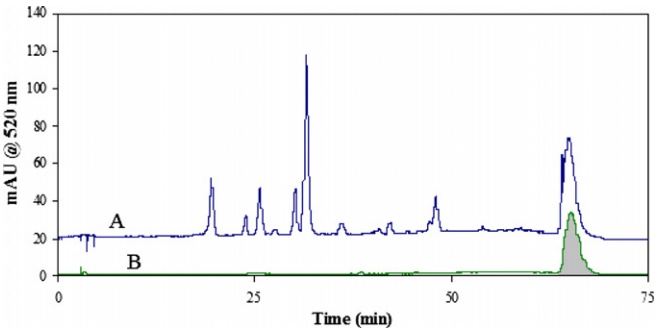

Fig. 3. HPLC chromatograms of a red wine monitored at 520 nm using polystyrene–divinylbenzene reversed-phase PLRP column. Legend: (A) without SO_2 in the mobile phase (as in Pengs' method); (B) addition of 100 mg/l SO_2 in the mobile phases. The shaded area represents the unbleached polymeric pigments.

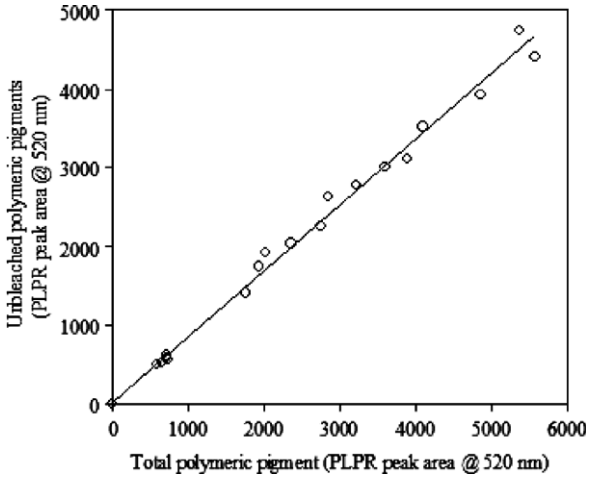

Fig. 4. Correlation between total and SO_2 resistant polymeric pigments in red wines using HPLC with PLRP column, evaluated at 520 nm and reported as peak area. Note. Total polymeric pigments were analysed without SO_2, whereas the unbleached polymeric pigments were analysed by addition of SO_2 to wines and mobile phases.

polymerization of colored phenolics may occur in a similar way in the different cultivar, the age of wine probably being the factor making more difference.

3.2. HPLC vs Adams' assay

The area of the broad envelope-peak (t_R 40–63 min) eluted with the RP-C_{18} column and monitored at 280 nm was consistent with the tannin value estimated with the Adams' assay ($R^2 = 0.922$). This finding confirmed the polymeric nature of the late eluting peak, whereas the negative intercept of the regression equation ($y = 0.0192x - 85$) suggested the additional presence of low molecular weight tannins, less than four flavan-3-ol subunits, that are not detected by the Adams' assay.

The sum of SPP and LPP estimated by the Adams' assay showed a good correlation ($R^2 = 0.9511$) when compared to the total polymeric pigments measured by HPLC with PLRP column. This finding confirmed the pigmented nature of the polymers assayed by Adams' method, and showed the improved performance of the PLRP compared to the RP-C_{18} column. However, in HPLC and Adams' assay the tannins are separated on different basis, the latter method only measures the phenolics that can bind to bovine serum albumine and precipitate along with protein. Thus, to investigate selected topics related to tannin properties, e.g. astringency of red wine, a careful comparison of results from the two methods must be taken.

3.3. HPLC vs Somers' assay

The Somers' assay (Somers & Evans, 1977) measures only unbleached polymeric pigments, whereas the HPLC with PLRP column measures total polymeric and unbleached polymeric pigments, the latter when SO_2 is added to wines and mobile phases. Despite a satisfactory correlation ($R^2 = 0.9854$) between the unbleached polymeric pigments measured by Somers' assay and the total polymeric pigments by PLRP-HPLC (without SO_2) was found, it is more appropriate to compare the same components, *i.e.* the level of unbleached polymers, by the two methods. The PLRP-HPLC with SO_2 showed a good correlation ($R^2 = 0.992$) with the classical Somers' spectrophotometric bleaching method, and the constant error disclosed from the intercept value of 0.3 AU implies an overestimation of polymeric pigments by the Somers' assay (Fig. 5). The classic spectrophotometric assay developed by Somers and Evans (1977) for measuring the polymeric pigments in red wines is based upon two assumptions: (i) bisulfite bleaches only the monomeric anthocyanins; and (ii) the color of the polymeric pigments increase by a factor of 5/3 on acidification. Harbertson et al. (2003) found the Somers' assay to overestimate the polymeric pigments in red wines compared to the sum of LPP and SPP estimated by the Adams' assay. The improved specificity of the latter method for polymeric pigments is most likely due to the pH value of analysis (pH 4.9) and the lack of

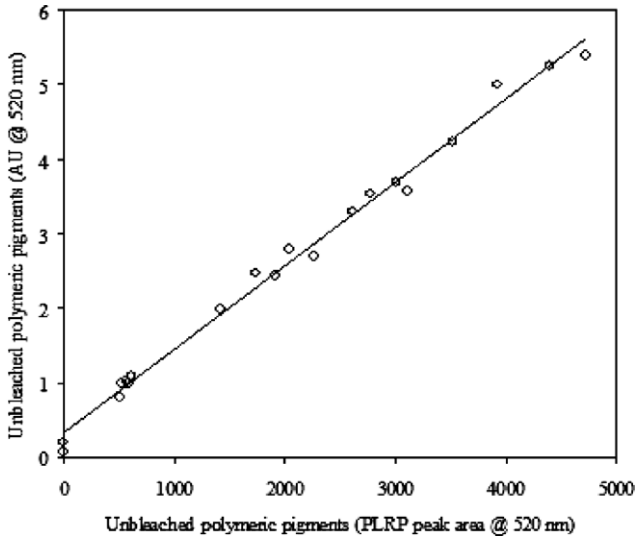

Fig. 5. Correlation between SO_2 resistant polymeric pigments using HPLC analysis with PLRP column (*X*-axis) and evaluated by spectrophotometric Somers' assay at 520 nm (*Y*-axis). Data from HPLC and spectral assay are expressed as peak area and absorbance unit (1 cm length path), respectively.

detection of polymeric pigments with less than four subunits (De Beer et al., 2004).

3.4. HPLC vs Boulton's assay

The extent of red color in wines is due to a number of factors including the type and concentration of anthocyanins, pH, and the extents of polymerization and copigmentation. At the moment of analysis 18 wines were 11 months old, while two of them were 23 months old. The total color of wine was an aggregate number of three components: copigmentation which accounted for about 8–30%, total free anthocyanins (24–35%), and polymeric pigment (35–63%). The sum of total monomeric peaks analysed by RP-C_{18} HPLC showed overall good correlations with total color ($R^2 = 0.8999$), free anthocyanins ($R^2 = 0.9159$), the best relationship being with copigmentation ($R^2 = 0.9464$). The level of copigmentation can be almost completely described by the levels of monomeric pigments and not by the tannin content (copigmentation vs tannin: $R^2 = 0.4827$). The degree of copigmentation is partly cultivar dependent, and significant variation occurs even between wines handled the same way in the same winery (Boulton, 2001).

The pH plays a major role in wine color chemistry. To allow a consistent comparison among wines and methods it is necessary to normalize this value before analysis. By comparing the same wines at two pH values: (i) wine pH, and (ii) adjusted pH at 3.6, a systematic proportional error of color density at AU 520 nm was found (Fig. 6).

In conclusion, the results presented are a comprehensive study of most recent of the approaches available for phenolics measurements in wine. The adopted technique is dependant upon resources available but ideally one would

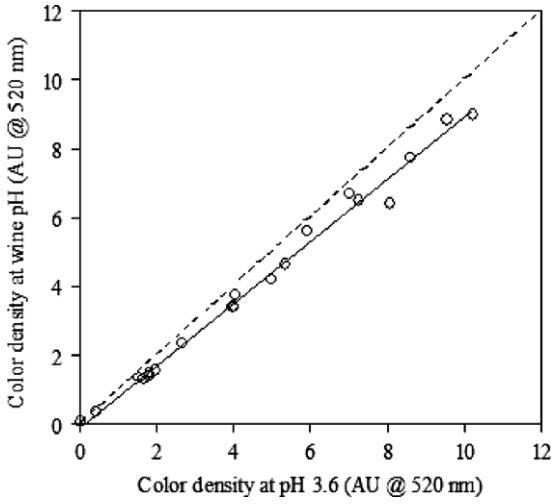

Fig. 6. Correlation between the color density (AU at 520 nm) at wine pH and pH 3.60. Data are expressed as absorbance unit (1 cm length path). The dotted line is the equality line with unit slope ($y = x$).

attempt to use several methods which take advantage of the individual merits herein presented. The addition of SO_2 to the mobile phases allowed the unbleached polymeric pigments to be analysed by HPLC for the first time. In this study the evidence indicating that polymeric pigments are partly bleached by SO_2 was presented. The use of the peak area limits the conclusion that can be drawn in terms of analytical error. The availability of certified polymeric pigments standards is required to get more insight the empirical correlation among the methods.

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