Vitis **40** (2), 87–91 (2001)

Analysis of red wine phenolics: Comparison of HPLC and spectrophotometric methods

URSKA VRHOVSEK[1)], F. MATTIVI[2)] and A. L. WATERHOUSE[3)]

[1)] Agricultural Institute of Slovenia, Ljubljana, Slovenia
[2)] Dip. Laboratorio Analisi e Ricerche, Istituto Agrario di San Michele, San Michele all'Adige, Italia
[3)] Dept. of Viticulture and Enology, University of California, Davis, USA

Summary

A recently developed ion-pair normal phase HPLC method which allows a precise chromatographic evaluation of the whole class of high-molecular-mass phenolics of wine was used in order to check the performance of spectrophotometric methods. Thirty-two monovarietal red wines (vintages 1993 and 1998) were analysed for total high-molecular-mass phenolics, proanthocyanidins with 2-4 units, and proanthocyanidins formed by 5 or more units, by means of the normal phase HPLC method. In addition the following spectrophotometric assays were performed: total phenols by Folin-Ciocalteu, Bate-Smith transformation of proanthocyanidins into cyanidin and catechins and proanthocyanidins reactive to vanillin.

K e y w o r d s : polymeric polyphenols, spectrophotometric methods, HPLC.

Introduction

Proanthocyanidins are important for wine, since they are responsible for bitterness and astringency (ARNOLD and NOBLE 1978; ARNOLD *et al.* 1980), moreover they play a very important role in oxidation and browning reactions (CHEYNIER and RICARDO DA SILVA 1991). Proanthocyanidins can also react with anthocyanins to produce pigmented proanthocyanidin products which are important for color stability in red wine during aging (TIMBERLAKE and BRIDLE 1976; SINGLETON and TROUSDALE 1992; DALLAS *et al.* 1996; ESCRIBANO-BAILON *et al.* 1996; REMY *et al.* 2000). These substances have been shown to have beneficial effects on health (RICARDO DA SILVA *et al.* 1991; TEISSEDRE *et al.* 1996). Proanthocyanidins are reported to have a potent radical-scavenging ability and this may impart a protective role against arteriosclerosis (RICARDO DA SILVA *et al.* 1991; RIGO *et al.* 2000).

All these properties largely depend on their level, structure and in particular on their degree of polymerization (PORTER and WOODRUFFE 1984). Proanthocyanidins change in size during wine production and aging and the majority of red wine phenolics are constituents of a very complex mixture of proanthocyanidins, which take part both, in the depolymerization reaction via acid cleavage of interflavan bonds, and in condensation reactions with themselves and with anthocyanins during winemaking and aging (REMY *et al.* 2000).

While a number of HPLC methods have been proposed to precisely evaluate the main monomers and dimers (JAWORSKI and LEE 1987; OSZMIANSKI *et al.* 1988, LAMUELA-RAVENTOS and WATERHOUSE 1994), determination of whole classes of phenolics has usually been achieved by means of spectrophometric indices. However, many methods have been used to characterize proanthocyanidins according to their degree of polymerisation: thin-layer chromatography with a silica adsorbent (LEA and ARNOLD 1978; ESCRIBANO-BAILON *et al.* 1992), spectrophotometric methods (SOMERS and EVANS 1977), spectrophotometric methods after precipitation with salt (HAGERMAN and BUTLER 1978; ITTAH 1991; PERI and POMPEI 1971 a, b), precipitation methods with proteins (ADAMS and HARBERSON 1999), size exclusion chromatography (KANTZ and SINGLETON 1990, 1991; CACHO and CASTELLS 1991), gel permeation method on their peracetate derivatives (WILLIAMS *et al.* 1983), normal phase HPLC methods (RIGAUD *et al.* 1993; PRIEUR *et al.* 1994; SOUQUET *et al.* 1996; HAMMERSTONE *et al.* 1999). In the last few years some new normal phase HPLC methods with various detection systems, including mass spectrometry, UV and fluorescence (FLD), have been employed (FULCRAND *et al.* 1999; HAMMERSTONE *et al.* 1999; LAZARUS *et al.* 1999). Most of these methods can only be used to analyse grape seed proanthocyanidins since they have not been optimized for grape skin or wine samples. Recently, a new ion-pair; normal phase HPLC method has been developed, which allows the precise chromatographic evaluation of the whole class of high-molecular-mass phenolics - including pigmented proanthocyanidins - in grape and wine (KENNEDY and WATERHOUSE 2000).

The availability of a reference method can be used to investigate the accuracy of information obtained by the spectrophotometric methods which are commonly applied to characterize wine phenolics in the context of quality control of red wines. The new HPLC method allows the direct measure of the oligomeric and polymeric flavonoids, or different fraction of them, instead of measuring the colour produced by a more or less selective reaction.

For routine quality control, spectrophotometric methods such as the Folin-Ciocalteu, the Bate-Smith and the vanillin assays, which are well understood in terms of their

Correspondence to: Dr. URSKA VRHOVSEK, Agricultural Institute of Slovenia, Hacquetova 17, 1000 Ljubljana, Slovenia. Fax: +386-1-2805255. E-mail: Urska.Vrhovsek@KIS-H2.sl

mechanism, are considered very valuable because they cause low cost and are quick and reproducible.

The purpose of the present work was to compare the performance of some well established spectrophotometric methods for the analysis of different classes of polyphenols with a newly developed normal phase HPLC method (KENNEDY and WATERHOUSE 2000), in order to estimate the level of agreement between these different assays for the analysis of red wine components and to evaluate the methods.

Material and Methods

Chemicals: All chromatographic solvents (methylene chloride, methanol, formic acid) were HPLC grade and were purchased from Fisher Scientific (Santa Clara, CA, USA); heptanesulfonic acid (HPLC grade) from Alltech (Deerfield, IL, USA); (-)-epicatechin from Sigma (St.Louis, MO, USA); (+)-catechin from Fluka (Switzerland). A cacao bean proanthocyanidin extract was prepared as previously described (RIGAUD *et al.* 1993).

Wine samples: In order to study the effects due to condensation reactions taking place during the aging of wine, two different wines from 8 grape cultivars (Cabernet Sauvignon, Enantio, Lagrein, Marzemino, Merlot, Pinot noir, Schiava, Teroldego) with different amounts and types of polyphenols (MATTIVI and NICOLINI 1997) were produced from the vintages 1993 and 1998. Thirty-two experimental monovarietal red wines were produced in the experimental winery of the Istituto Agrario di San Michele all'Adige using a traditional skin-contact technique. After natural malolactic fermentation, settling, filtering and bottling wines were stored at cellar temperature until the analyses were started in February 2000.

Analytical methods: HPLC method. The high-molecular-mass phenolics of wine (P) were measured by integrating two areas of the chromatogram at 280 nm separately, the values corresponding to proanthocyanidins from 2-4 units (LMWP) and to proanthocyanidins formed by ≥5 units (HMWP). A cacao bean proanthocyanidin extract was injected at the beginning and at the end of each sequence – consisting of about 8 wine samples – in order to calibrate the retention times of different oligomers. Quantitative data were calculated on the basis of a calibration curve with the external standard method and expressed as epicatechin ($mg \cdot l^{-1}$). P is the sum of LMWP and HMWP.

Wine samples were prepared following a modified method (KENNEDY and WATERHOUSE 2000). To remove ethanol from the wine 5 ml of the sample were evaporated in a 50 ml pear-shaped flask under reduced pressure at 35 °C. Then the sample was applied to a C18-SPE column (1 g, Alltech), which had been activated with methanol (2 ml) and was rinsed with water (5 ml) before. The applied sample was washed with 18 ml water and then eluted with 18 ml methanol into a 50 ml pear-shaped flask. The methanol was evaporated under reduced pressure and the sample was solved again in 2 ml methanol. The final samples were filtered through 0.45 μm, 13 mm PTFE syringe-tip filters (Millipore, Bedford, MA, USA) into LC vials.

Spectrophotometric methods. The assays tested were some of the most widely used spectrophotometric methods (DI STEFANO *et al.* 1989), carried out under optimized conditions (RIGO *et al.* 2000).

To remove the organic acids, residual sugars, free SO_2, amino acids, proteins, and other hydrophilic compounds which could cause interference, and to convert the phenols from the aqueous to the methanolic solvents before the assay, a preliminary clean-up of the phenols was performed with a Sep-Pak C18, 0.5 g (Waters, Milford, USA) previously conditioned with 2 ml methanol followed by 5 ml of 5 mM H_2SO_4. The loading and washing of the polar compounds were done at low pH with diluted sulphuric acid, in order to improve the recovery of acidic phenols, such as gallic acid.

Total phenols (FC). Total phenols were assessed by reducing phosphotungstic-phosphomolybdic acid (Folin-Ciocalteu's reagent, FOLIN and DENIS 1912) to blue pigments by phenols in alkaline solution (DI STEFANO and GUIDONI 1989). Concentrations were determined by means of a calibration curve as (+)-catechin ($mg \cdot l^{-1}$). The relative standard deviation (R.S.D.) of the method based on repeated analysis (N=12) of a red wine with 1180 $mg \cdot l^{-1}$ was 2.45 %.

Proanthocyanidins (PROC). The proanthocyanidin assay is based on a very specific reaction, *i.e.* the conversion to anthocyanidins by acid-catalysed cleavage of the interflavonoid bonds followed by autoxidation (PORTER *et al.* 1986). The proanthocyanidins were evaluated by transformation into cyanidin (DI STEFANO *et al.* 1989); iron salts were used as catalyst to increase the reproducibility of the yield of cyanidin, n-butanol was replaced by an optimal percentage of ethanol. Under these conditions the average yield has been estimated to be 20 % (DI STEFANO *et al.* 1989) and the proanthocyanidin concentration ($mg \cdot l^{-1}$) can be conventionally expressed as 5 times the amount of cyanidin formed, by means of a calibration curve with cyanidin chloride (ε=34.700). The R.S.D. of the method based on repeated analysis (N=12) of a red wine with 1167 $mg \cdot l^{-1}$ was 2.74 %.

Index of vanillin (VAN). The catechins and proanthocyanidins reactive to vanillin were analyzed according to the optimized and controlled vanillin-HCl method of BROADHURST and JONES (1978), following the conditions described by DI STEFANO *et al.* (1989). Concentrations were calculated as (+)-catechin ($mg \cdot l^{-1}$) by means of a calibration curve. The R.S.D. of the method based on repeated analysis (N=12) of a red wine with 475 $mg \cdot l^{-1}$ was 3.54 %.

Statistical treatment: Analysis of variance and regression analysis were computed with Genstat 5 (Lawes Agricultural Trust, IACR-Rothamsted, UK). Linear correlation analyses were computed with Microsoft Excel 97.

Results and Discussion

Previous extensive investigation of a large data set of these spectrophotometric results by means of the Common Principal Component Analysis demonstrated that in general, for all wine varieties, the data of PROC and FC are strictly correlated, and they are orthogonal (*i.e.* independent) to the total anthocyanins (ANT) parameter, while the VAN values

are less strictly correlated to both FC and PROC, and inversely correlated with the concentration of ANT (Mattivi *et al.* 1995).

The normal phase HPLC method of Kennedy and Waterhouse (2000) allowed the direct measure by integrating the whole peak area at 280 nm of the total high-molecular-mass grape phenolics, mostly proanthocyanidins, of wine (P) (Kennedy and Waterhouse 2000), but does not include the monomers. Separate integration of two areas of the chromatogram at 280 nm, the values corresponding approximately to proanthocyanidins with 2-4 units (LMWP) and to proanthocyanidins formed by ≥5 units (HMWP) can also be estimated and expressed as epicatechin (mg·l^{-1}) (Fig. 1). As

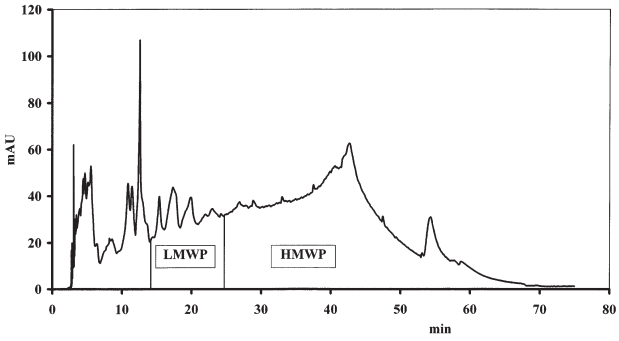

Fig. 1: Normal phase chromatogram of high molecular mass phenolics of wine.

suggested by Kennedy and Waterhouse (2000), the chromatographic signal at 520 nm is related to the concentration of pigmented tannins, again not including the monomers. Preliminary trials (data not shown) indicated that the chromatographic area at 520 nm is proportional to the amount of red pigments for similar wines (e.g. wines of similar age), but it appears to be problematic to associate a quantitative value to this area, since the pH of the eluting solvent optimal for proanthocyanidins is slightly >4.0. Such a pH value is too high to perform an accurate calibration with a common monomeric anthocyanin, and the percentage of red pigments of various structures which are in the flavilium form is unpredictable. Since a method for the direct measurements of total red pigments, known to constitute the large majority of colouring matter in red wines, does not exist, further effort to improve the normal phase HPLC method for the quantitative analysis of pigmented tannins could be a valuable goal for future research.

The data set describing the phenolics of 32 wines from two different vintages and 8 different varieties, was considered appropriate to investigate the relationship between the variables under review because the wines had a wide range of values, 3.8-5.6 fold differences, for the assays under consideration. In order to look for differences due to the different age of the wines, the agreement between the different methods was checked (Table) by comparing the data analysis after grouping by year.

As expected, different levels of FC and PROC and to a less extent, of VAN can be explained by the variation of P values (Table). In spite of the different characteristics of the assays, the absolute amount of polyphenols as measured by P, FC and PROC showed rather good agreement,

T a b l e

Regression analysis

Y	X	% variance explained by the model (1)	(2)	t-statistic probability (year)
P	FC	85.4	87.7	0.015
	PROC	79.1	79.1	n.s.
	VAN	62.8	75.9	<0.001
HMWP	PROC	81.5	84.2	0.019
	VAN	53.4	74.6	<0.001
LMWP	VAN	48.1	50.8	n.s.
	PROC	17.2	28.1	0.025
LMWP/HMWP	VAN/PROC	35.3	54.5	0.001
PROC	VAN	62.8	73.0	0.001

(1) linear regression; (2) linear regression grouped by year, two parallel lines. P, FC, etc. see Material and Methods.

providing in most cases quite close values (Fig. 2 a, b). Such agreement was partially unexpected for the FC assay, which, contrary to the two other methods is also measuring the simple phenols, whose amounts are expected to be rather variable in wines of different varietal origin and age. The closeness of the values was even more surprising for the PROC assay, whose numerical values are considered highly debatable being computed assuming a constant yield of the

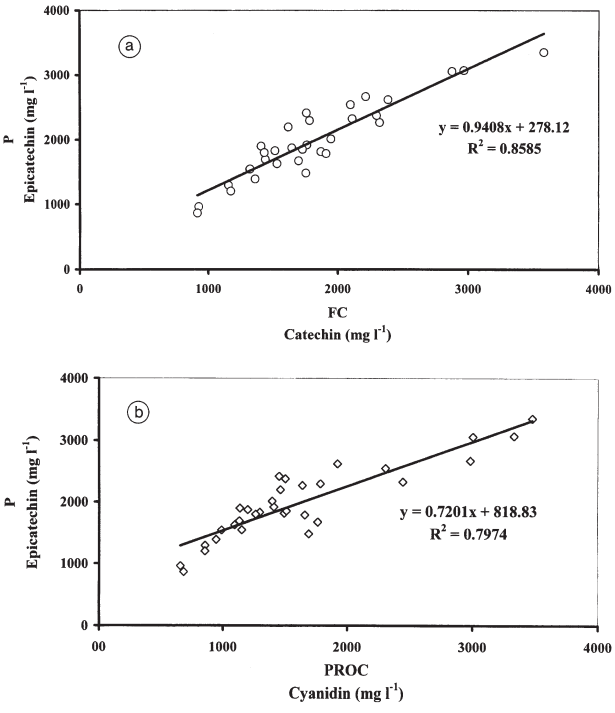

Fig. 2: Correlation between (**a**) the total high molecular mass phenolics (P) and total phenols (FC) and (**b**) P and the proanthocyanidins (PROC).

conversion reaction, equal to 20 %. The values of VAN have some correlation with P ($R^2 = 0.73$), but only if samples of the same age are considered separately (Fig. 3). The model fitting the data, a simple linear regression with data grouped by year, is in very good agreement with the strong decrease of the values of VAN as wines age. This is expected to be caused by the increasing molecular size of phenolics and by the decrease of the monomers, as a consequence of the poly-merization processes during aging. In the light of this result, which was expected on the basis of the different analytes of the assays, a direct comparison between the P and VAN values – even if the values remain proportional for a set of similar wines – is not recommended since VAN is strongly dependent on the chemical age of the wine.

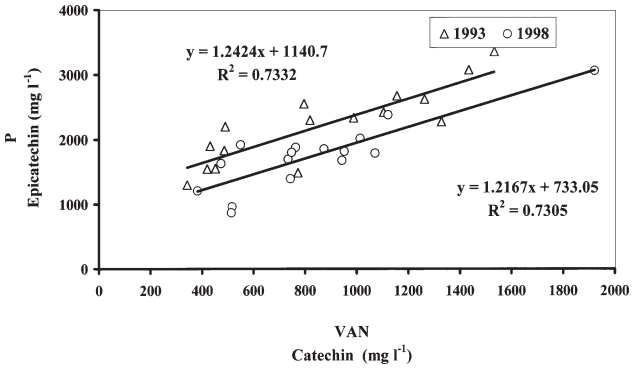

Fig. 3: Correlation between the total high molecular mass phenolics (P) and the index of vanillin (VAN) in 1993 and 1998. Correlations were restricted to wines of similar age, since the VAN values strongly decrease with the age of wine.

In general, for the spectrophotometric parameter VAN, whose values are strongly affected by aging, a simple model with two parallel regression lines leads to a significant improvement of the percentage variance explained (see the regressions P/VAN, HMWP/VAN, PROC/VAN), since the interactions between the explanatory factor and the variate, *i.e.* the differences in slope, were always not significant (Table).

A good agreement was observed between the values of HMWP and PROC (Table), thus confirming the hypothesis that the PROC value provides an estimate of high-molecular-mass proanthocyanidins. The variations of LMWP were only partially explained by the variation of VAN, since the HPLC method is not measuring catechins.

The LMWP/HMWP ratio can be computed to obtain an estimate of the variation of molecular size of the phenolics and this can be compared with the age of wine. Its correlation with the values of the ratio VAN/PROC confirms the latter to be a qualitative polymerization index decreasing with the increase of molecular size of proanthocyanidins. However, due to the differences between the wines in the two years, the differences between the two vintages cannot be analyzed properly. As noted above, the HPLC data do not include the monomeric catechins, so this major factor is missing in the comparison between the two polymerization indices LMWP/HMWP and VAN/PROC (Fig. 4).

While a correlation was apparent and an R^2 value of 0.38 was observed, thus confirming the ratio VAN/PROC to be a qualitative polymerization index decreasing with the increase of molecular size of proanthocyanidins, it appears that there are fundamental differences between these two "tannin" size assessments. (Fig. 4, Table).

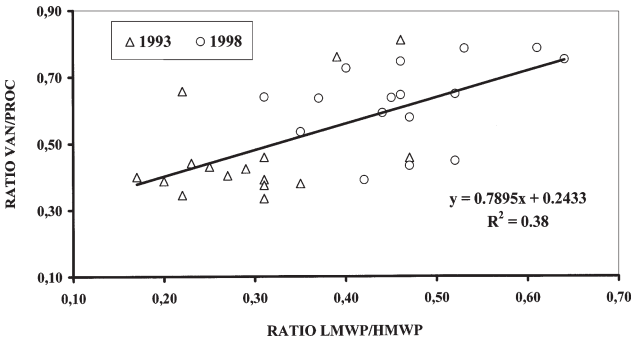

Fig. 4: Correlation between the two polymerization indices LMWP/HMWP and VAN/PROC.

In conclusion, the comparison of results from advanced normal phase HPLC methods and spectrophotometric methods widely used in quality control of wines, provided useful information for the interpretation of the values produced by different spectrophotometric methods, thus helping in the choice of the most appropriate spectrophometric or chromatographic methods. In most cases, a good level of agreement between the reference HPLC and the spectrophotometric values confirmed that traditional spectrophotometric assays provide a good deal of valuable information on the amount and nature of wine phenolics. Total phenols (FC) and proanthocyanidins (PROC) can be used to predict the values of total high-molecular mass phenolics in wine (P). The variations of PROC are mainly linked to variations of phenolics corresponding to ≥5 units (HMWP), while VAN is partially linked to the variation of phenolics corresponding to 2-4 units (LMWP), but obviously the meaning of these parameters remains quite different since only the VAN is sensitive to the catechins. The partially unexpected similarities and differences between the various measures point to the need to better understand the nature of factors producing responses in both the spectrophotometric and chromatographic assays.

Acknowledgements

We thank G. NICOLINI for providing the wines. The excellent technical assistance in spectrophotometric analysis by C. SANCHEZ is gratefully acknowledged. This work was in part supported by bilateral research cooperations between Slovenia and Italy and Slovenia and the USA of the Slovenian Ministry for Science and Technology and by the Provincia Autonoma di Trento and Consiglio Nazionale delle Ricerche.

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Received August 21, 2000