Multivariate Analysis of Antioxidant Power and Polyphenolic Composition in Red Wines

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It has been demonstrated that the dietary intake of compounds having antioxidant activity is very important, and various chemical, biological, and electrochemical methods have been proposed to evaluate the antioxidant power of compounds such as polyphenols. Wine, although nonessential, has a high polyphenol content up to 2−3 g/L in red wines obtained by traditional maceration. The polyphenol content of wines is usually evaluated by the Folin reagent, which provides an appropriate response to the requirements of wine manufacturers. Because the presence of individual polyphenols may be evaluated by HPLC, more or less selective methods toward the various chemical classes of polyphenols have been developed. An HPLC method set up recently was applied to evaluate how individual polyphenols contributed to the overall antioxidant power (AOP) of 60 Italian red wines, trying to identify the effect that individual compounds may have on the total AOP. Application of the multivariate analysis allowed us to detect some determining compounds such as gallic acid and some flavonols. On the basis of the correlation between two traditional chemical methods, namely the total polyphenol determination by the Folin reagent and the flavanol determination by the condensation reaction with *p*-(dimethylamino)-cinnamaldehyde, it was shown that the use of these two merely chemical methods is well correlated ($r = 0.83$ and 0.87) to an AOP evaluation of red wines.

Keywords: *Wine; polyphenol; antioxidant power; principal component analysis; partial least square analysis*

INTRODUCTION

Recent studies (*1*−*3*) have widely demonstrated that the intake of antioxidants with our diet can carry out an effective protective action toward the oxidative stress created by imbalance between oxidative and strongly reactive species in the body and its defense mechanisms (*4*). It has been discovered that a series of illness such as cancer, atherosclerosis, diabetes, and arthritis, can be linked to the damaging action of extremely reactive forms of oxygen called reactive oxygen substances (ROSs). These are also implicated, more in general, in the processes that are responsible for body aging.

Endogenous mechanisms of defense (redox mediators such as superoxide dismutase, catalase, peroxidase, and metal binding proteins) can be supported with very good results by external antioxidants introduced with diet. These antioxidants are part of a heterogeneous group of molecules, which, when present at a low concentration as compared to that of an oxidizable substrate, can suppress, delay, or prevent oxidation of that substrate, and which are all able to oxidize in a very easy way to give stable products, acting therefore against the damaging action of oxygen and other reactive species such as radicals (*5*).

Vegetables and fruits, such as tomato, cabbage, onion, tea, bean, and citrus fruits, are the food products with the largest amounts of natural antioxidants (*6*, *7*). Grapes, and grape products such as wine, also contain large amounts of antioxidants. In particular, red wine, having an extremely high polyphenol content, plays a very important role. There are many beneficial aspects, reported in other papers, regarding the consumption of antioxidants and, in particular, polyphenols: cardiovascular protective effect (*8*), anti-cancer action (*9*), and cellular membrane protection (*10*, *11*).

In this work, multivariate analysis was applied to the data obtained by analyzing several samples of Italian red wines with a new HPLC method, which made use of a coupled revelation system (*12*) and a new potentiometric assay of their antioxidant power (AOP) (*13*).

MATERIALS AND METHODS

Samples. Sixty red wines, from Oltrepò Pavese and Lake Garda regions, of vintage 1997 (the most part) and vintage 1996 were analyzed.

Chemical Standards. Phenolic acids, catechin, epicatechin, trans-resveratrol, rutin, quercetin, and myricetin were purchased from the local representative of Aldrich, Fluka and Sigma (Sigma-Aldrich, Milan, Italy). Cyanidin 3-*O*-glucoside hydrochloride and malvidin 3-*O*-glucoside hydrochloride were obtained from Extrasynthese (Genay Cedex, France). Other compounds were matched by their retention time and the spectra of compound(s) extracted from selected sources (*14*): quercetin 3-*O*-glucoside and 3-*O*-glucuronide were extracted from *Pastinaca sativa*, L. var. *sativa* and *Foeniculum vulgare*, Mill. var. *azoricum*; kaempferol was obtained by acid hydrolysis of a methanolic extract obtained from *Chicorium endivia*, L. var. *crispum*; petunidin 3-*O*-glucoside was from *Atropa belladonna* L.; and 2-*S*-glutathionyl caffeoyl tartaric acid was prepared by adding reduced glutathione to white wine (*15*).

HPLC Analysis. The chromatographic equipment consisted of a 996 diode array detector (DAD) (Waters, Milan, Italy), a

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600 E multisolvent delivery system (Waters) equipped with a 20-μL loop and an EG & G model 400 amperometric detector equipped with two glassy carbon working electrodes and a reference Ag/AgCl electrode. The chromatographic system was controlled by Millennium 2010 software (Waters). The apparatus and the chromatographic conditions are better-described elsewhere (*12*).

Flow Injection Analysis. To measure the antioxidant power of wines, an FIA apparatus working with a Jasco 880 PU pump (Jasco, Milan, Italy) and an EG & G PAR model 400 electrochemical detector equipped with a single glassy carbon electrode operating at a potential of 400 mV, a reference (Ag/AgCl, saturated) electrode, and a platinum counter electrode were used (*13*). The mobile phase was a 50 mM solution of tartaric acid; the pumping rate was 0.6 mL/min.

The operating potential was set at 400 mV. Catechin was used as a reference compound to determine the antioxidant power of wine. The electrochemical behavior of individual species was investigated by coupling the electrochemical detector to the HPLC system. At each peak separated by HPLC, a peak whose surface area was directly proportional to the μA of current released by oxidation of the molecule on the electrode appeared on the recorder connected to the electrochemical detector.

Spectrophotometric Analysis. The total polyphenol (TPP) content was determined according to Scalbert et al. (*16*), and flavanols (FLA) were determined by the reaction with *p*-(dimethylamino)-cynnamaldehyde (DACA) (*17*).

Data Analysis. Principal component analysis (PCA) and partial least square analysis (PLS) were used to classify samples by Unscrambler 7.5 software package (Camo As., Trondheim, Norway). PCA is aimed at finding out the simplest mathematical model able to describe the data set satisfactorily. It is the most appropriate statistical approach when the goal is to detect the relative importance of individual variables for determining the data structure. PLS is aimed at detecting cause−effect relationships. Comparison of regression lines was carried out by applying the least significant difference test by Statgraphics Plus 1.0 software package (Manugest KS Inc., Rockville, MD).

RESULTS AND DISCUSSION

The electrochemical behavior of polyphenols depended on structural features. At the lowest potential established (400 mV vs Ag/AgCl) some polyphenolic compounds having particular structural features, such as three-phenol groups or two-phenol groups in *o*- or *p*-position, were the only ones detected. At the highest potential established (800 mV vs Ag/AgCl) all polyphenols were detected. This value, however, is of no practical use in vivo.

On the basis of an estimation of the peak dimensions and their electrochemical behaviors, the parameters useful for studying the correlation between the antioxidant power of wine samples and their polyphenolic composition were chosen.

The 24 parameters included gallic acid, caffeic acid (measured out as a sum of different existing forms), catechin, epicatechin, procyanidin, myricetin 3-glucoside (MyG), quercetin 3-glucoside (QuG), quercetin 3-glucuronide (QuGL), rutin, myricetin, quercetin, kaempferol, isorhamnetin, delphinidin 3-glucoside (DeG), cyanidin 3-glucoside (CyG), petunidin + peonidin glucosides (PsG), malvidin-3-glucoside (MaG), four unidentified acylated anthocyanins AA_{1-4} (eluted following malvidin and measured at 540 nm), TPP, FLA, and AOP.

A matrix containing all of the 60 wine samples (*18*), each with the 24 above-mentioned parameters (in duplicate), was used to obtain an explanation of the correlation, if any, between the polyphenolic composition of wine and its antioxidant power.

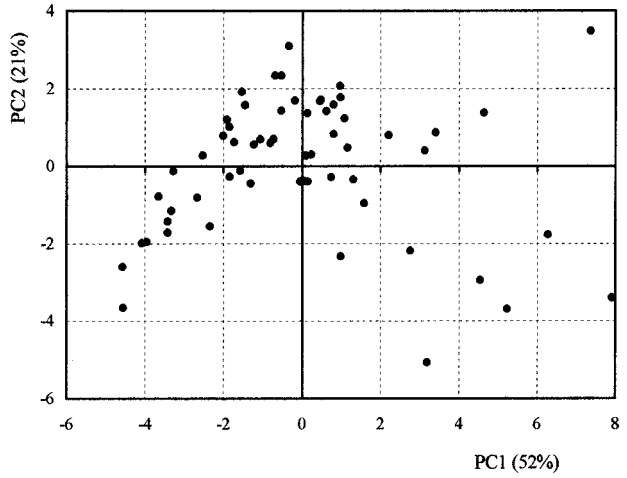

Figure 1. Principal component scores from polyphenolic composition of 60 red wines. Axes *x*: PC1; *y*: PC2.

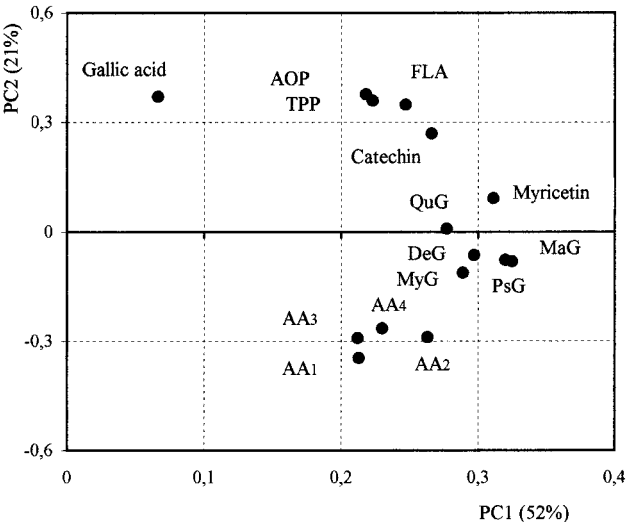

Figure 2. Principal component loading plot from polyphenolic composition of 60 red wines. Axes *x* and *y* loading values are as in Table 1.

Data processing was developed in two steps as follows. Means were processed by PCA in order to select the variables most significant to the determination of the mathematical model. After the variables with low statistical loading had been removed, the new matrix obtained was subjected to PCA again and then to multiple regression analysis (PLS) in order to identify the variable most significant to the determination of the antioxidant power.

Final data processing by PCA was obtained by subtracting nine nonsignificant variables (i.e., caffeic acid, epicatechin, procyanidin, quercetin, quercetin-3-glucuronide (QuGL), rutin, isorhamnetin, kaempferol, and cyanidin-3-glucoside (CyG)) from the initial matrix. Results are reported in Figures 1 (score plot) and 2 (loading plot) with an explained variance of 73%, of which 52% was along PC_1 and 21% was along PC_2.

Results showed that TPP, FLA, and AOP contents (upper region), together with flavonols, myricetin, and QuG, are good discriminating elements. On the basis of the distribution of the wine samples analyzed, those obtained from the Oltrepò Pavese region were described well by the above-mentioned variables. The flavonol content of Oltrepò wines, for instance, was 9.18 mg/L (sample 31), 10.5 mg/L (sample 40), 13.7 mg/L (sample

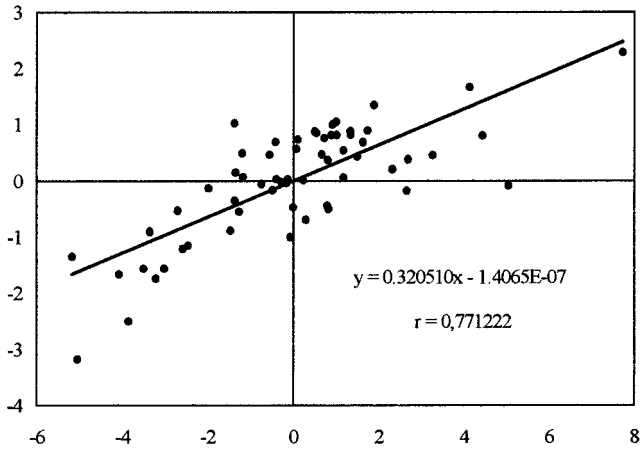

Figure 3. PLS analysis between the independent variable (AOP) and the other dependent variables.

43), 12.6 mg/L (sample 53), 8.68 mg/L (sample 55), and 18.5 mg/L (sample 60), as compared to an average flavonol content of 5.16 mg/L as calculated on all samples.

Both acylated anthocyanins (AA_{1-4}) and monoglucoside monomers were placed in the lower PCA region although they had a low statistical loading for the determination of the overall AOP. A limited fraction oxidized at 400 mV potential because the overall anthocyanin content of wine during bottling mainly depends on MaG, which could oxidize only at a potential higher than 800 mV and was present in some samples at concentrations higher than 100 mg/L, as compared to other anthocyanin concentrations as low as a few mg/L. Apart from MaG, as mentioned above, the monomeric anthocyanin fraction did not contribute to the overall AOP because CyG, PsG, and DeG were highly unstable and tended to disappear rapidly. Therefore, they had a sort of antioxidant activity on the other antioxidant compounds by preventing them from disappearing during wine storage.

The distribution of variables was rather anomalous: they tended to distribute along the axis of the first component, probably because of the lack of variables unrelated to the polyphenol composition, such as acidity and alcoholic content, which were able to further differentiate the samples. Hence, the matrix obtained was subjected to the PLS analysis to determine the degree of correlation between the independent variables and the dependent variable. The AOP was assigned as independent variable and the remaining variables were the dependent variables. Samples were thus distributed along a line with a quite good correlation coefficient of 0.771, as shown in Figure 3.

The most significant variables were found to be the spectrophotometric readings of TPP and FLA, gallic acid, catechin, and myricetin determined by HPLC, as shown in Table 1.

No individual compound or compound family was identified as the main cause of the overall antioxidant activity because of the highly complex polyphenolic set of red wines. Hence, the correlation between the spectrophotometric determinations of TPP and FLA and the electrochemical response was investigated.

The relationship between AOP and the TPP content followed the equation

$$\text{AOP} = 0.345\ \text{TPP} + 173\ (r = 0.83,\ p < 0.01)$$

Table 1. Loading Values by PLS Analysis.

variable	X loading	Y loading
gallic acid	0.301	0.329
catechin	0.383	0.056
MyG	0.177	−0.277
QuG	0.232	−0.186
myricetin	0.315	−0.124
DeG	0.216	−0.232
PsG	0.221	−0.277
MaG	0.231	−0.262
AA_1	0.008	−0.354
AA_2	0.071	−0.368
AA_3	0.044	−0.296
AA_4	0.084	−0.261
TPP	0.446	0.272
FLA	0.466	0.269

where AOP was expressed in mg/L catechin and TPP was expressed in mg/L gallic acid.

Because the chemical determination of total polyphenols with the Folin reagent is a nonselective method of analysis, its response is determined by all oxidizable compounds of wine. It would thus appear that there cannot be a correlation between the above-mentioned determination and the response obtained by the electrode, characterized by its selectivity.

Unlike the general response provided by the TPP measurement, the spectrophotometric determination of FLA is a selective method, at least with respect to flavan-3-ols and their derivatives. A good correlation was also obtained between this spectrophotometric index and the electrochemical response of samples. In this case, an expected result was obtained: as shown by Vivas and Glories (*19*), the reaction with DACA was performed only by molecules that, because of their chemical structure, had an antioxidant activity. Polymers (procyanidins), due to their hydroxylic groups in *o*-position, were reactive when subjected to 400 mV potential.

The relationship between the antioxidant power and the flavanol content followed the equation

$$\text{AOP} = 1.151\ \text{FLA} + 341\ (r = 0.87,\ p < 0.01)$$

where AOP was expressed in mg/L catechin and FLA was expressed in mg/L catechin.

The linear models proposed are considered reliable as they are supported by a number of samples falling within a wide range of the variables taken into account: 357 to 3510 mg/L for TPP; 56.7 to 940 mg/L for FLA; and 167 to 1563 mg/L of catechin for the AOP.

CONCLUSIONS

From the study of the correlation between the polyphenolic composition and the antioxidant power, a linear correlation was obtained only between the colorimetrically estimated total phenolics and total flavanols, which were low specific protocols. Results showed flavanols to be a family of extremely important compounds for determining the redox balance of wine. From a practical point of view, this function may be used to protect wine from oxidation by adding condensed tannins (procyanidins) during fermentation. Both relationships provided the same information. According to Simonetti et al. (*20*), it can be concluded that the evaluation of the antioxidant power of wine requires only the determination of the total phenolics by the Folin−Ciocalteau method,

which is simpler and faster than the method used for total flavanol evaluation.

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