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ARTICLE

Antioxidant Activity of Wines Determined by a Polarographic Assay Based on Hydrogen Peroxide Scavenge

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Antioxidant (AO) activity of various red and white wines of different origin as well as some individual phenolic compounds present in wine has been assessed using a polarographic assay. Direct current polarography has been used to survey hydrogen peroxide scavenge (HPS) upon gradual addition of tested samples. Results expressed as reciprocal value of wine volume required for 50% decrease of anodic limiting current of hydrogen peroxide have been validated through correlation with Folin−Ciocalteau and DPPH assays. All wines exhibit HPS activity analogous with total phenolic content and DPPH scavenge. Reliability and accuracy, low cost, and rapid and direct experimental procedure open a wide area for application of this assay, making it a good alternative to standard, widely accepted AO assays.

KEYWORDS: Antioxidant activity; DPPH; hydrogen peroxide scavenge; polarography; total phenolics; wine

INTRODUCTION

Phenolic compounds present in wine vary in amount and composition markedly depending on the grape cultivar, soil, nutrition, climatic conditions and winemaking procedure (*1*−*3*). Diverse analytical methods have been used to evaluate the phenolic content of red and white wines (*4*). There are essential differences in qualitative and quantitative compositions of red and white wines. Red wines outperform significantly white ones with respect to phenolic content and AO activity (*5*−*10*). Polyphenols from red wine are assumed to prevent low-density lipoprotein (LDL) oxidation. Beneficial effects of moderate consumption of red wine either toward chronic diseases such as coronary heart disease or toward colorectal cancer have been reported (*11*). It has been demonstrated recently that white wine may have significant anticarcinogenic potential as well (*12*). The low incidence of heart disease among the French population despite a high fat intake, known as the "French paradox", is attributed to high consumption of red wine (*13*). White wine, if rich in tyrosol and hydroxytyrosol, can provide cardioprotection similar to red wine (*14*).

Biodistribution, absorption and metabolism of polyphenols are still under investigation (*15*−*20*). Polyphenols are absorbed from the gut in their native or modified form and subsequently metabolized to products detected in the plasma (*15*, *16*). Metabolic transformations of polyphenolic compounds in the organism probably modify their activities (*16*, *17*). Polyphenolic metabolites could be a key for understanding health effects of wine (*18*, *19*). The AO activity of some phenolic metabolites was found to be similar to those of phenolic themselves (*20*).

Antioxidative activity of wines, as an important nutritional topic, represents an active research area. There is not yet a standard method accepted for the evaluation of the AO activity of wines. Although numerous AO assays have been applied in wine analysis (*21*−*25*), there is still a requirement for a more simple, rapid and reliable assay. Thus, improvement of existing or development of novel assays applicable in wine analysis has been still in focus. Electrochemical techniques, based on phenolic OH groups' behavior to act as hydrogen- or electron-donating agents, offer direct determination of the total AO activity, i.e. evaluation of the overall reducing power, without the use of reactive species. Various electrochemical techniques, relying upon direct or indirect oxidation of phenolic compounds, such as cyclic voltammetry (CV) (*26*−*29*), flow-through column electrolysis and flow injection analysis (*30*, *31*), coulometric detection and the combined high-performance liquid chromatography−electron capture detection (*32*), have been employed in wine analysis. The main electrochemical approaches used are CV and flow injection analysis with amperometric detection (FIA-ED) (*33*). Cyclic voltammetry has been used to characterize a range of oxidation potentials of phenolic acids and flavonoids, ascorbic acid, and sodium metabisulfite, making an important contribution to AO properties of wine (*26*). Also, application of potentiometric redox titration is becoming more popular in enology (*34*−*36*).

The feasibility of polarography for studying AO properties has been investigated as well. A rapid polarographic method for lipid peroxidation measurement based on the decrease of dissolved oxygen reduction current has been developed by Hamilton and Tappel (*37*). Bumber et al. reported a polarographic study of AO activity of proteins and amino acids, based on the shift of the first oxygen reduction potential (*38*).

Generally, assays based on HPS have attracted increased attention, tending to be more often applied in determination of total AO activity. In comparison to artificial reactive species often used in many standard AO assays, hydrogen peroxide has an

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advantage as naturally occurring, reactive oxygen species able to reproduce *in vivo* state conditions. In contrast to radicals present in the cell, peroxide is stable enough. Besides assays based on photometric or fluorometric determination of hydrogen peroxide (*39*−*42*), electrochemical ones have been developed. Kinetics of hydrogen peroxide elimination after its injections into white and red wines has been studied by Karyakina et al. (*43*). The application of polarographic assay in beer analysis has been demonstrated (*44*). Using a dropping mercury electrode (DME) with renewable surface as main advantage of polarographic approach, a fast and reliable HPS assay that can be efficiently used in determination of total AO activity has been developed.

In this study, application of HPS assay in determination of total AO activity of red and white wines of different origin has been demonstrated. Capacity of wine to scavenge hydrogen peroxide has been correlated with total phenolic contents (Folin−Ciocalteu) (FC) and DPPH (1,1-diphenyl-2-picrylhydrazyl free radical) scavenge and strict correlations obtained discussed.

MATERIALS AND METHODS

Chemicals and Wines. Hydrogen peroxide was from Merck (Darmstadt, Germany). (+)-Catechine, quercetine, resveratrol and ellagic acid were from Sigma (St. Loius, MO). Folin−Ciocalteu reagent was from Merck (Darmstadt, Germany). DPPH reagent was from Aldrich (Milwaukee, WI). Various white and red wines of different origin, either domestic or imported, were purchased from Serbian markets.

Polarographic Instrumentation and Procedure. Direct current (DC) polarography with a dropping mercury electrode (DME) as working electrode was used. The counter electrode was a large-area platinum electrode, and the reference one was a saturated calomel electrode (SCE) (Radiometer Analytical SAS). The current−potential ($i-E$) curves were recorded using the polarographic analyzer PAR (Princeton Applied Research Instrument), model 174A, equipped with X-Y recorder (Houston Instruments, Omnigraphic 2000). The volume of the experimental solution in the electrolytic cell was 20 mL. Borate buffer (pH 9.8) was prepared from 50 mL of mixture containing 0.2 M H_3BO_3 and 0.2 M KCl and 40.8 mL of 0.2 M NaOH. Starting H_2O_2 concentration of 5.7 mM was obtained by addition of 10 μL of 35% H_2O_2 into 20 mL of borate buffer. Before each $i-E$ curve recording, the stream of the pure nitrogen was passed through the cell solution. During curve recording, the inert atmosphere was kept by passing the nitrogen above the cell solution. Autooxidation of phenolics has been prevented by keeping the atmosphere inert. The initial potential was 0.1 V. The potential was scanned toward more negative values with a scan rate was 10 mV s^{-1}. A programmed drop time was 1 s. The DME current oscillations were filtered out with low pass filter positioned at 3 s. All experiments were done at room temperature.

Decrease of H_2O_2 limiting anodic current value (i_l) in the presence of AO species was noticed in slightly acidic, neutral or slightly alkaline solution, but more alkaline values of pH (above pH 9) have been chosen for better reproducibility. Initial i_l value (i_{l0}), obtained by recording 5.7 mM H_2O_2 solution, has always the same intensity. Standard deviation of about 2% was calculated upon repetitive recording of the initial H_2O_2 solution, indicating satisfactory reproducibility. Examined wine samples, without any pretreatment, were gradually added (in equal aliquots of 100 μL) into the electrolytic cell with a buffered H_2O_2 solution. Red wines were added in five aliquots and white wines in ten.

Measurement of AO Activity Using DPPH. Free radical scavenging activity of wine samples was analyzed by DPPH assay (*45*), based on measurement of the loss of 1,1-diphenyl-2-picrylhydrazyl free radical violet color in reaction with potential AOs, monitored by decrease of absorbance on 517 nm. A volume of 200 μL of diluted wine was mixed with 1800 μL of a methanolic solution of DPPH (0.1 mM). After shaking and standing 30 min in dark, absorbance on 517 nm was measured. Appropriate water dilutions of wines (1:60 to 1:25 for red, 1:5 to 3:1 for white wines) were experimentally found to satisfy the linear dependence concentration vs absorbance. Since some samples of white wines have shown weak antioxidant activity, dilution 3:1 or 2:1 (wine to water ratio) was prepared. All samples were prepared in triplicate, and four dilutions of each wine were used to get the EC_{50} value from the graph $I\% = f(c)$. Percentage of inhibition of DPPH radical ($I\%$) of each wine sample was calculated according to eq **1**:

$$I\,(\%) = \left[\frac{(A_{\text{blank}} - A_{\text{sample}})}{A_{\text{blank}}}\right] \times 100 \qquad (1)$$

where A_{blank} is absorbance of DPPH with water instead of the sample and A_{sample} is absorbance of DPPH after reaction with tested wine sample. Results are expressed as reciprocal value of I (%) multiplied by 100.

Measurement of Total Phenolic Content. Total phenolic content of wines was determined by the Folin−Ciocalteau (FC) assay (*46*). A volume of 200 μL of diluted wines was mixed with 1000 μL of FC reagent diluted with distilled water in proportion 1:10. After 6 min standing in the dark, 800 μL of sodium carbonate solution (7.5%) was added. After shaking and standing for an additional two hours in the dark, absorbance at 740 nm was measured. Distilled water was used as blank. All samples were measured in triplicate. Four dilutions of each wine were used and results averaged. Appropriate dilutions of each wine were experimentally found (1:70 to 1:35 for red, 1:7 to 1:2 for white wines) to give absorbance between 0.2−0.7 on 740 nm. Each absorbance was adjusted for the value of blank probe. The same procedure was used for four concentrations of gallic acid standards (10, 25, 50, and 100 μg mL^{-1}), and a calibration curve was calculated. The total phenolic content was expressed in gallic acid equivalents (GAE), as concentration of gallic acid (μg mL^{-1}) that corresponds to the dilution of wine with the same value of absorbance at 740 nm.

Determination of HPS Activity of Individual Phenolics. Polarograms of individual phenolics known to be present in wine such as (+)-catechin, quercetin, resveratrol and ellagic acid are recorded. (+)-Catechin, quercetin and resveratrol were dissolved in alcohol while ellagic acid was dissolved in borate buffer (pH 9.8). Aliquots of 100 μL of AOs at a concentration of 5 mM were gradually added into the working solution of H_2O_2. HPS activity of individual phenolics has been expressed as concentration required for 50% of peroxide anodic current decrease.

Statistic Analysis. Results were expressed as the mean value ± SD. Concerning the HPS assay, SD was obtained by linear regression analysis from dose-dependence curves in the vicinity of 50% decrease of hydrogen peroxide limiting current by using OriginPro 6.1. In the FC and DPPH assays, SD was obtained from triplicates by using PASS 2008 statistical analysis software package.

RESULTS AND DISCUSSION

HPS Activity of Red and White Wine. Mechanism of anodic oxidation of H_2O_2 at the DME in alkaline solutions has been studied. Anodic dissolution of electrode mercury and formation of the mixed mercury complex [$(Hg(O_2H)(OH)$] more probably corresponded with polarographic wave of H_2O_2 (*47*). At concentrations higher than 0.15 mM a peak of anodic limiting current has been developed instead of wave. The phenomenon of DC polarographic current attributed to the formation of the mixed mercury complex and its decrease that is proportional to the total AO activity of analyzed samples enabled HPS assay development (*44*).

Here, the fate of the hydrogen peroxide DC anodic wave was used to evaluate and compare total AO activity of red and white wines of different origin. For the object of this study we have taken 11 wines (5 white and 6 red). Red wines were added in five equal aliquots of 100 μL while white wines were added in ten aliquots. Polarograms of H_2O_2 before and after addition of red and white wine (volume range 100−500 μL) are shown in **Figure 1**.

In all investigated cases, a prominent difference can be observed between polarograms of red and white wine. As seen in **Figure 1**, height of initial hydrogen peroxide limiting current (i_{l0}) has been the same while residual hydrogen peroxide limiting current (i_{lr}) obtained upon gradual addition of tested samples indicates significantly higher AO activity of red wine. Percentage of i_l decrease was calculated upon each addition of tested wines

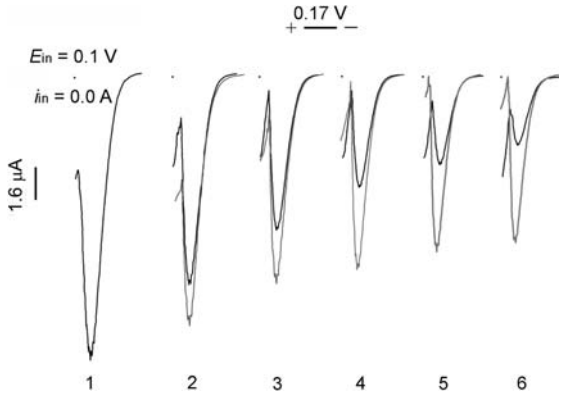

Figure 1. Anodic polarographic curves of H_2O_2 before (1) and after addition of red (dark line) and white wine (gray line): (2) 100, (3) 200, (4) 300, (5) 400 and (6) 500 μL.

according to eq **2**:

$$\%\ \text{scavenged}\ [H_2O_2] = \left(\frac{i_{lr}}{i_{l0}} - 1\right) \times 100 \qquad (2)$$

Percentage of i_l decrease has been plotted against the volume of added samples. Dose−response curves (percentage of i_l decrease vs volume of added sample) for red (R1−R6) and white wines (W1−W5) are shown in **Figure 2**. The lack of linearity between the percentage of decrease of the anodic limiting current and concentration of assayed samples has been observed.

From linear part of dose−response curves volume of sample required for 50% decrease of i_{l0}, assigned as AO_{50}, has been determined. AO_{50} is expressed in μL. As a measure of AO activity it has the disadvantage that the higher the AO activity, the lower the value of AO_{50}. Thus, reciprocal value of AO_{50} has been introduced as criterion for AO activity. AO_{50}^{-1} is calculated according to eq **3**:

$$AO_{50}^{-1} = \left(\frac{1}{AO_{50}}\right) \times 10^3 \qquad (3)$$

AO_{50}^{-1} is expressed in mL^{-1}. Red wines reached 50% at volumes between 200 and 300 μL while white wines did so between 600 and 1000 μL. Results expressed as AO_{50} and AO_{50}^{-1} are summarized in **Table 1**. Ranking order of wine AO activity has been based on AO_{50}^{-1} values. Differentiation between red and white wines on the basis of both those values is obvious.

Validation of the HPS Assay. HPS activity of tested wine samples has been compared with total phenolic content (FC) and DPPH scavenge activity in order to validate the HPS assay. Although cyclic voltammetry has been efficiently used to measure wine total phenols, (*1*), Folin−Ciocalteau assay has been used in this study as the most commonly applied. Spectroscopic assay employed artificial radical species DPPH has been chosen as widely applied in analysis of different grape product, including wines, polyphenolic compounds present in wine and their metabolites (*20*, *48*). Surveying of vinification process and influence of enological practice on AO activity of wines has been investigated using DPPH as well (*49*). More over, the DPPH method was found comparable with other standard methods such as ABTS, lipid peroxidation etc. There are different ways to express results of the DPPH test, depending on analyzed samples. Total H atom donating capacity can be expressed either as concentration of pure compounds or as volume of complex samples required to reduce 50% of DPPH, i.e. EC_{50} index. Values of EC_{50}, the same as AO_{50}, are inversely proportional to AO activity. In this report,

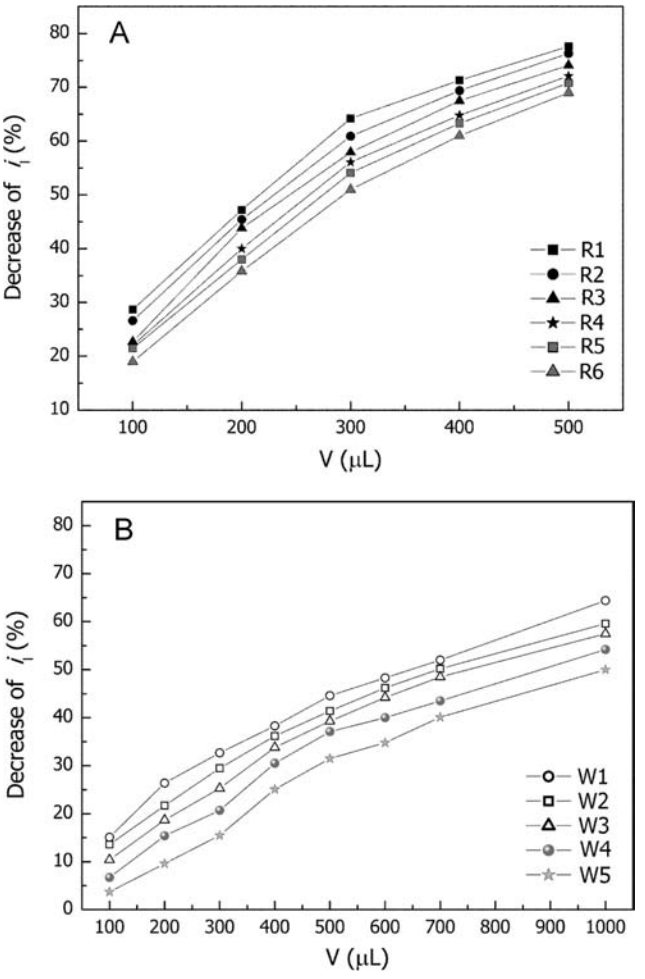

Figure 2. Dose dependence of red (R1−R6) (**A**) and white (W1−W5) (**B**) wines effects on anodic limiting current of H_2O_2, i_l, decrease, i.e. % of scavenged H_2O_2 vs volume of wine samples (μL).

reciprocal value of EC_{50} has been used to make correlation with total phenolics and AO_{50}^{-1}.

As seen in **Table 1**, the red wines' HPS activity values, expressed either as AO_{50} or as AO_{50}^{-1}, are at least two times higher than the white wines' values. HPS activity of wines has been found analogous with content of phenolics. Variation in phenolic content among tested red wines, from 1700 to 2300 GAE, is in agreement with literature data (*50*). Total phenolic content is closely related to AO activity, independent of the method used to determine AO activity (*51*−*53*). Tested red wines scavenged DPPH to a much greater degree than whites, in proportion to their phenolic contents, as observed previously (*54*). The high values of HPS obtained for red wines are accompanied with high content of phenolics and high DPPH scavenge, while low values of HPS activity, DPPH scavenge and total phenolics are obtained for white wines (**Table 1**).

Correlation between HPS, DPPH and FC Assays. The correlation between HPS activity and total phenolic content (FC) is given in **Figure 3**. A strong correlation has been obtained (r^2 = 0.997). Correlation between HPS and DPPH scavenge (r^2 = 0.988) is shown in **Figure 4**, while correlation between DPPH scavenge and phenolic content (FC) (r^2 = 0.989) is shown in **Figure 5**. It is worth noting that the correlation coefficient is higher for HPS vs FC than for DPPH vs FC. Higher correlation between the HPS and FC assay may suggest that the HPS assay covers a wider range of polyphenolic antioxidants if compared with the DPPH assay. This observation confirms previous finding

Table 1. Hydrogen Peroxide Scavenge (HPS), Expressed as AO_{50} and AO_{50}^{-1}, DPPH Scavenge and Total Phenolic Content (FC) of Red and White Wines[a]

	HPS		DPPH		
sample	AO_{50}(mL)	AO_{50}^{-1}(mL^{-1})	EC_{50} (%)[b]	EC_{50}^{-1} ($\%^{-1}$)	FC: GAE (mg mL^{-1})
R1	221 ± 3	4.52 ± 0.06	1.78 ± 0.03	0.555	2314 ± 34
R2	230 ± 4	4.35 ± 0.08	1.57 ± 0.06	0.625	2164 ± 17
R3	244 ± 6	4.10 ± 0.09	2.02 ± 0.04	0.500	2158 ± 38
R4	264 ± 4	3.79 ± 0.06	2.59 ± 0.07	0.385	1802 ± 8
R5	276 ± 1	3.62 ± 0.01	2.23 ± 0.25	0.454	1772 ± 1
R6	294 ± 1	3.40 ± 0.01	2.39 ± 0.07	0.417	1708 ± 9
W1	644 ± 14	1.55 ± 0.03	17.9 ± 1.2	0.056	346 ± 10
W2	694 ± 18	1.44 ± 0.04	31.5 ± 0.6	0.032	242 ± 6
W3	750 ± 19	1.33 ± 0.03	46.5 ± 2.3	0.021	185 ± 2
W4	881 ± 25	1.13 ± 0.03	63.8 ± 1.6	0.016	154 ± 1
W5	1000 ± 24	1.00 ± 0.02	70.2 ± 2.5	0.016	164 ± 3

[a] Data are presented as means ± SD. [b] % − mL of wine sample per mL of solution × 100.

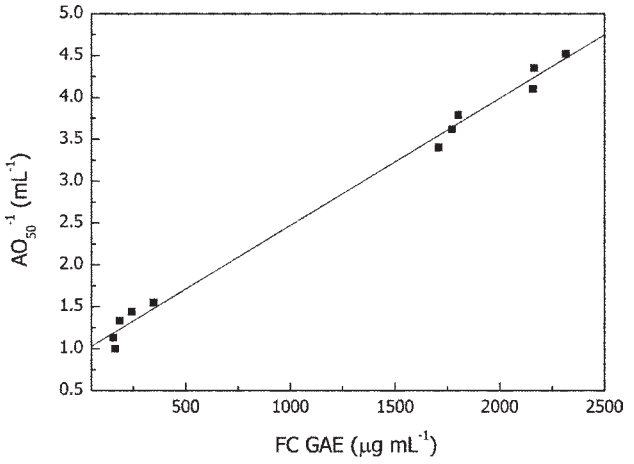

Figure 3. The relationship between HPS scavenge and total phenol content (FC) (r^2 = 0.997).

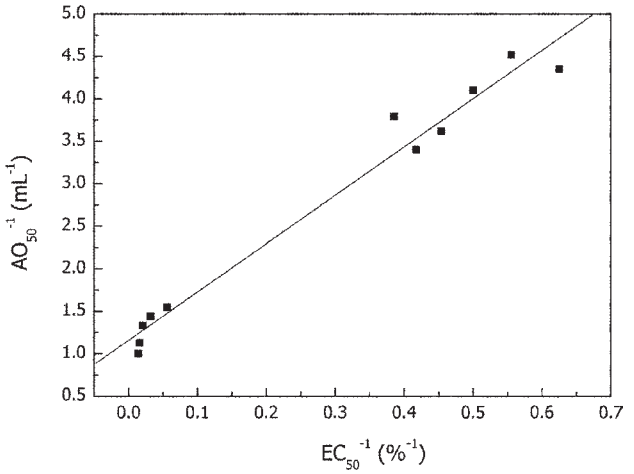

Figure 4. The relationship between HPS scavenge and DPPH scavenge (r^2 = 0.988).

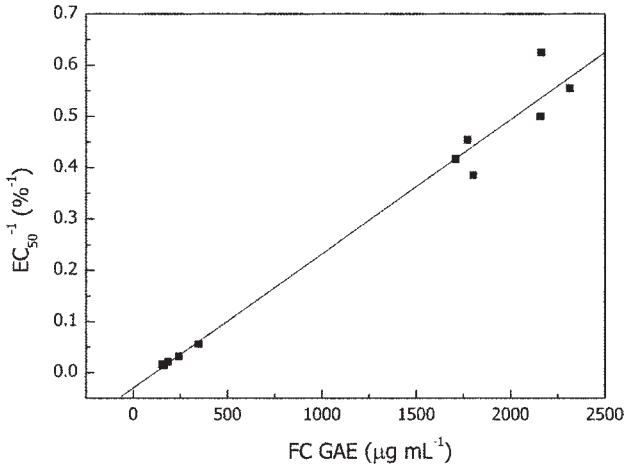

Figure 5. The relationship between DPPH scavenge and total phenolic content (FC GAE) (r^2 = 0.989).

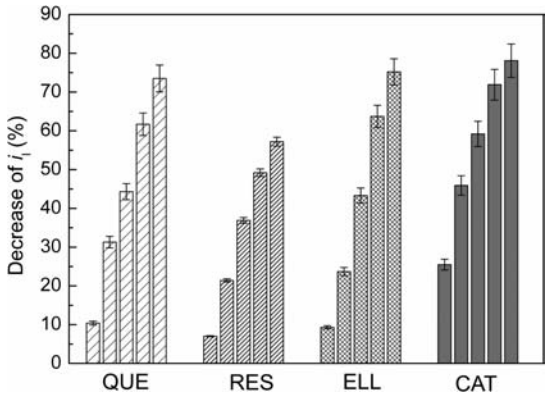

Figure 6. Antioxidative activity of quercetin (QUE), resveratrol (RES), ellagic acid (ELL) and (+)-catechin (CAT) in concentrations 25, 50, 75, 100, and 125 μM. Values are means ± SD.

that DPPH radical is a weak oxidant not able to react rapidly and completely with polyphenols (*55*, *56*). A positive correlation between spectrophotometric HPS assay and lipid peroxidation, ABTS and DPPH tests has been demonstrated by Sroka and Cisowski as well (*40*).

HPS Activity of Individual Phenolics Present in Wine. Different phenolic fractions contribute to the total AO activity of wines in various proportions (*44*). In our study, we have determined HPS activity of four wine polyphenols: (+)-catechin (flavan-3-ol) and quercetin (flavonol), resveratrol (hydroxystilbene) and ellagic acid (phenolic acid). Concentrations of individual compounds tested correspond to the red wine content. All tested individual phenolic components show a significant activity increasing in a dose-dependent manner (**Figure 6**). The ranking order of tested phenolics has been found consistent with structure−AO activity relationship reported previously (*57*, *58*). (+)-Catechin, quercetin and ellagic acid show higher ability to scavenge H_2O_2 than resveratrol. (+)-Catechin exhibited the highest HPS activity

(AO_{50} = 63 μM) while resveratrol exhibited the lowest (AO_{50} = 103 μM). Quercetin and ellagic acid exibit the same HPS activity (AO_{50} = 83 μM).

Multiple OH substitution and conjugation are important determinants of the free radical scavenging activity and electrochemical behavior as well (*59*). Antioxidant activity of phenolics depends on the structure, number and arrangement of the hydroxyl groups and the extent of structure conjugation. Hydrogen atoms from hydroxyl groups are donated and phenoxy radical stabilized by delocalization of the unpaired electron within the aromatic structure is formed. The more stable the phenoxy radical, the higher the antioxidant activity. The highest HPS activity of (+)-catechin can be explained by the presence of a B-ring catechol moiety and 5 hydroxyl groups while resveratrol shows the lowest HPS activity. In our previous study we have reported significantly higher AO activity of (+)-catechin than phenolic acids, such as ferulic, *p*-coumaric, caffeic and chlorogenic (*37*). Ranking order for HPS activity of phenolic acids corroborated well with structure−H_2O_2 scavenging capacity reported by Mansouri et al. (*60*). Relationship structure−HPS activity for wide range of phenolic compounds is under study currently and will be the subject of our next publication.

Conclusion. The polarographic antioxidant assay based on hydrogen peroxide scavenge applicability in enology has been demonstrated. Based on strict correlations with widely accepted methods this assay has been recommended as reliable. It has potential to surpass the main disadvantages of standard AO assays, such as time-consuming experimental procedure, requirement of either fresh biological tissues or artificial reactive species. Polarographic monitoring of hydrogen peroxide decrease allowed colored and turbid samples, usually unacceptable in spectrophotometric assays. Due to simplicity and low cost, this assay can be widely applicable in beverage analysis; a high number of samples can be easily processed.

ABBREVIATIONS USED

AO activity, antioxidant antioxidative activity; AO_{50}, volume of sample required for 50% decrease of H_2O_2 limiting current; AO_{50}^{-1}, reciprocal value of AO_{50}; HPS, hydrogen peroxide scavenge or scavenging; SCE, saturated calomel electrode; DC, direct current; DME, dropping mercury electrode; DPP, differential pulse polarography; i_l, limiting current; DPPH, 1,1-diphenyl-2-picrylhydrazyl free radical; SD, standard deviation.

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