Food Chemistry 124 (2011) 1208–1216

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Determination of polyphenols content and antioxidant activity of some red wines by differential pulse voltammetry, HPLC and spectrophotometric methods

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ARTICLE INFO

Article history:
Received 26 May 2009
Received in revised form 16 July 2010
Accepted 16 July 2010

Keywords:
Polyphenols
Antioxidant activity
Red wines
DPV
HPLC
Spectrophotometry

ABSTRACT

Red wines produced in three different wine growing regions of Croatia were analysed for total polyphenols (TP) content, concentration of individual polyphenols and antioxidant activity (AA). TP content was measured by differential pulse voltammetry (DPV), Folin–Ciocalteu (FC) and HPLC methods. Individual polyphenols were measured by HPLC and AA was determined by ABTS and DPPH methods. The results showed that DPV is a very sensitive method for the determination of TP. A very high correlation was obtained between the TP content determined by DPV, FC and HPLC. The major polyphenols in the wines were gallic acid and (+)-catechin. TP, individual polyphenols and AA varied among wines from different regions. Wines from the Dalmatia region had the highest TP content, the highest concentration of important antioxidants and significantly higher AA. There was a very high correlation between AA and TP in all of the wines tested.

1. Introduction

Wine, especially red wine, is a very rich source of polyphenols, such as flavanols (catechin, epicatechin, etc.), flavonols (quercetin, rutin, myricetin, etc.), anthocyanins (the most abundant is malvidin-3-*O*-glucoside), oligomeric and polymeric proanthocyanidins, phenolic acids (gallic acid, caffeic acid, *p*-coumaric acid, etc.), stilbenes (*trans*-resveratrol) and many others polyphenols. Many of these compounds (e.g. resveratrol, quercetin, rutin, catechin and their oligomers and polymers proanthocyanidins) have been reported to have multiple biological activities, including cardioprotective, anti-inflammatory, anti-carcinogenic, antiviral and antibacterial properties (King, Bomser, & Min, 2006; Santos-Buelga & Scalbert, 2000). These biological properties are attributed mainly to their powerful antioxidant and antiradical activity.

The results of many published epidemiological studies suggest that regular, moderate consumption of red wine has reduced the incidence of many diseases such as risk of coronary heart disease (CHD), atherosclerosis, cancers, etc. (Cooper, Chopra, & Thurnham, 2004; Teissedre, Frankel, Waterhouse, Peleg, & German, 1996). The most intriguing are the studies which reported the possible association between red wine consumption and decrease in risk, and some suppression and inhibition of cancers (Briviba, Pan, & Rechkemmer, 2002). Currently, chemoprevention is being used in medicine as a new strategy to prevent cancers. Natural phytochemicals, including red wine polyphenols, appear to be very promising substances to block, reverse, retard or prevent the process of carcinogenesis (Russo, 2007).

Many epidemiological studies have found that regular intake of red wine or red wine polyphenols has positive effects on human health. Therefore, determination of the chemical composition, polyphenols content and antioxidant activity of red wine could be very useful for the interpretation of epidemiological studies.

Many different methods, including high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) in combination with different detectors, UV–Vis, photo diode array (PDA), mass spectrometry (MS), and electrochemical (EC) detectors, have been used to investigate the polyphenolic content and chemical composition of wine. Mass spectrometry (MS), especially, is responsible for great progress in the identification and characterisation of polyphenols in wine (Escarpa & Gonzalez, 2001).

In recent times, different electrochemical methods have been proposed for the characterisation of polyphenols in wine on the basis that practically all polyphenolic molecules present in wine are electrochemically active. Cyclic voltammetry (CV) was the first electrochemical method used for characterisation of polyphenols and determination of total polyphenols (TP) content in wines (De Beer et al., 2004; Kilmartin, Zou, & Waterhouse, 2001, 2002). Flow injection analysis with electrochemical detection (FIA–ED) has also been used for determination of TP in wines (Blasko, Rogerio, Gonzalez, & Escarpa, 2005).

Differential pulse voltammetry (DPV) has also been explored in the analytical detection of polyphenols in foods. Thus, Blasko,

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0308-8146/$ - see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.foodchem.2010.07.047

Gonzalez, and Escarpa (2004) used DPV for characterisation of flavonoids and phenolic acids in fruit juices.

There is no report in the literature about the application of DPV for the determination of total polyphenols (TP) content in wines. Therefore, the aims of this study were: (i) to use DPV for the determination of TP content in some Croatian red wines; (ii) to compare the results obtained by DPV with those obtained by spectroscopic Folin–Ciocalteu (FC) method and HPLC method; (iii) to identify and quantify the major polyphenolic compounds in wines by HPLC; (iv) to determine antioxidant activity (AA) of investigated red wines by two different methods: DPPH and ABTS methods; and (v) to evaluate any correlation between the TP content and the AA of the red wines investigated in this study.

2. Materials and methods

2.1. Chemicals

Gallic acid monohydrate (398225), caffeic acid (C0625), *p*-coumaric acid (C9008), (+)-catechin hydrate (C1251), (−)-epicatechin (E1753), quercetin dihydrate (Q0125), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·) (D9132) and 2,2′-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (A1888) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Trolox ((±)-6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid) (56510), ammonium peroxodisulfate (09915) and phosphoric acid (85% HPLC grade) were purchased from Fluka (Buchs, Germany). Methanol (HPLC grade) was obtained from Merck (Darmstadt, Germany). Acetic acid (99.7%) was obtained from Panreac, Barcelona, Spain. Sodium acetate, sodium carbonate, sodium dihydrogen phosphate, disodium hydrogen phosphate and Folin–Ciocalteu reagent were obtained from Kemika (Zagreb, Croatia). Alumina powder was obtained from Buehler (USA).

2.2. Wine samples

Eleven widely consumed, commercially produced Croatian red wines, made from red grape varieties (*Vitis vinifera* L.) grown in three different wine-producing regions of Croatia (Dalmatia, Istria and Slavonia), were chosen as follows: (i) five brands (Plavac Hvar, Ivan Dolac, Dingač, Babić and Kaštelet) from the Dalmatia region (south and central Adriatic coast); (ii) two brands (Teran and Merlot) from the Istria region (north Adriatic coast); and (iii) four brands (Klikun Noir, Pinot Noir, Zweigelt and Frankovka) from the Slavonia region (continental part of Croatia). The wines, packed in glass bottles and made in the vintage year 2005–2006, were purchased from local supermarkets and stored at room temperature until analysed. Before analysis, wine samples were filtered through 0.45 μm polytetrafluoroethylene (PTFE) filters (Varian, USA).

2.3. Standards

(+)-Catechin and gallic acid were chosen as standards for measurement of the TP content. The stock solutions of (+)-catechin (3 g/L) and gallic acid (5 g/L) were prepared in methanol (HPLC grade). These stock solutions, protected from light with aluminium foil and kept in refrigerator, were stable for at least 1 month. The working standards were prepared freshly from the stock solutions for each new measurement.

2.4. Differential pulse voltammetry (DPV)

The DPV measurements were performed using a potentiostat (model 273A, Princeton Applied Research EG&G, USA) running with electrochemical software (model 270/250, EG&G PAR, USA). Voltammetric measurements were carried out in a standard three-electrode electrochemical cell with a glassy-carbon (GC) electrode of 3 mm diameter (model MF-2012, Bioanalytical Systems, USA) as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and platinum gauze as the counter electrode. Before each measurement, the surface of the GC working electrode was carefully polished with 0.3–0.05 μm alumina powder and then thoroughly rinsed with doubly distilled water. Immediately before the DPV measurements, the GC electrode was cleaned electrochemically by cyclic voltammetry, scanning 5–10 cycles in the potential range between −0.2 and 1.0 V at a scan rate of 50 mV s^{-1}, in supporting electrolyte (0.1 mol dm^{-3} sodium acetate–acetic acid buffer pH 3.6). The samples in the electrochemical cell were de-aerated by purging with high purity argon during the electrochemical measurements.

After electrochemical cleaning of the GC electrode, the required volume of sample (solution of catechin or wine) was added by micropipette into the supporting electrolyte in the electrochemical cell and the differential pulse voltammograms were recorded immediately to minimise adsorption of polyphenols (or others electrochemical active species) onto the GC electrode surface. DPV conditions were: scans range, from 0.0 to +1.0 V; pulse amplitude, 50 mV; pulse width, 70 ms; scan rate, 5 mV s^{-1}. All measurements were done in, at least, triplicate.

Wine samples were diluted 1/100, 1/200, 1/400, 1/600 and 1/800 (v/v) by 0.1 mol dm^{-3} sodium acetate–acetic acid buffer (pH 3.6) in order to obtain a linear correlation between anodic peak current density and volume fraction of wine. The 400-fold dilution was found to be the optimum and electrochemical parameters obtained for this dilution were used for quantification of TP content in wine.

The catechin standard calibration curve was constructed by plotting the peak current density of the first anodic peak (P1) of catechin *vs.* the corresponding concentration of catechin. TP_{DPV}, the TP content in wine determined by the DPV method, was calculated using the catechin calibration curve and expressed as mg catechin equivalents per litre of undiluted wine (mg CE/L).

2.5. Spectrophotometric determination of TP content

Spectrophotometric determination of the TP content was done with the Folin–Ciocalteu micro method as adapted for wine analysis (Waterhouse, 2009) using gallic acid or catechin as the standard. The calibration curve of absorbance *vs.* concentration of standard was used to quantify TP_{FC} content. Results were expressed as mg catechin equivalents per litre of wine (mg CE/L) or mg gallic acid equivalents per litre of wine (mg GAE/L).

2.6. HPLC analysis of wine composition and polyphenols content

Polyphenol standards (gallic acid, (+)-catechin, (−)-epicatechin, caffeic acid, *p*-coumaric acid and quercetin) were used for characterisation of the phenolics in wine. Calibration curves were made by diluting stock solutions with methanol to give concentration of the standard in the range 1–100 mg/L for gallic acid, (−)-epicatechin), caffeic acid, *p*-coumaric acid and quercetin, and 1–125 mg/L for (+)-catechin. The calibration curves were constructed from chromatograms as peak area *vs.* concentration of standard. All phenolic standards gave linear calibration curves within the concentration range studied ($r = 0.9988–0.9990$). The limits of detections (LOD) were calculated from the parameters obtained from calibration curves, using the formula $LOD = 3\ s_a/b$, where s_a is the standard deviation of the *y*-intercept of the regression line and *b* is the slope of the calibration curve (Ribani, Collins, & Bottoli, 2007). The calculated LOD was 1.3 mg/L for gallic acid, 0.3 mg/L for

caffeic acid, 0.6 mg/L for *p*-coumaric acid, 0.5 mg/L for quercetin, 0.6 mg/L for (+)-catechin and 0.4 mg/L for (−) epicatechin.

The content of individual wine polyphenols (flavonols, flavan-3-ols and phenolic acids), responsible for the anodic voltammetric peaks of wines in DPV measurements (see Figs. 2 and 3.), were determined by the RP-HPLC method. The HPLC apparatus consisted of a Varian LC system (USA) equipped with a ProStar 230 solvent delivery module, and a ProStar 330 PDA detector. The separation of phenolic compounds was performed on an OmniSpher C18 column (25 cm × 4.6 mm, Varian, USA) equipped with ChromSep guard-column (1 cm × 3 mm, Varian, USA).

The flavonols, flavan-3-ols and phenolic acids present in wines were separated using phosphoric acid (0.1%) as solvent A and 100% methanol (HPLC grade) as solvent B. The elution conditions were: 0–30 min from 5% B to 80% B; 30–33 min 80% B; 33–35 min from 80% B to 5% B; flow rate = 0.8 mL/min. The operating conditions were: column temperature, 20 °C; injection volume, 10 μL. A re-equilibration period of 10 min was used between individual runs. UV–Vis spectra were recorded in the wavelength range 190–600 nm. The detection wavelength was 280 nm for gallic acid, (+)-catechin and (−)-epicatechin, 320 nm for caffeic acid and *p*-coumaric acid and 360 nm for quercetin.

The identification and peak assignment of flavonols, flavan-3-ols and phenolic acids in wine was based on comparison of retention times and spectral data in the wavelength range 190–600 nm with those of authentic standards. Spiking the wine samples with phenolic standards was done for additional identification of individual polyphenols. Identified flavonols, flavan-3-ols and phenolic acids were quantified using calibration curves of authentic standards. The TP content was determined from the total area of the RP-HPLC chromatograms at 280 nm and expressed as mg GAE/L of wine (TP_{HPLC}).

2.7. Determination of antioxidant activity (AA)

The AA of wines was investigated by the ABTS and DPPH methods. The ABTS assay is based on the scavenging ability of antioxidants to the long-life $ABTS^{\cdot+}$ radical cation (Villano, Fernandez-Pachon, Troncoso, & Garcia-Parrilla, 2004). The DPPH method is based on the measurements of the scavenging ability of antioxidants towards the stable free radical $DPPH^{\cdot}$ (Brand-Williams, Cuvelier, & Berset, 1995).

2.7.1. ABTS method

Five different dilutions of each wine sample were analysed by the ABTS method. A stock solution of the $ABTS^{\cdot+}$ radical cation was generated chemically by mixing 0.2 mL of ammonium peroxodisulfate solution (65 mmol dm^{-3}) with 50 mL of ABTS solution (1 mmol dm^{-3}, prepared in 0.1 mol dm^{-3} phosphate buffer pH 7.4). The mixture was left overnight and then 0.5 mL of $ABTS^{\cdot+}$ radical cation stock solution was mixed with 2 mL of phosphate buffer (pH 7.4) in a cuvette and the absorbance at 734 nm (A_{ABTS}) was measured. Subsequently, 0.1 mL of diluted wine was added in the cuvette, the solution was mixed quickly, and the absorbance at 734 nm (A_{wine}) was measured after 60 s. The decrease in absorbance ($\Delta A = A_{ABTS} - A_{wine}$) after 60 s was calculated for each diluted wine sample. The decrease in absorbance caused by the addition of Trolox as the standard was measured by the same procedure for each concentration of Trolox (50–400 μmol/L) and the calibration curve for the decrease in absorbance ($\Delta A = A_{ABTS} - A_{Trolox}$) of Trolox *vs*. Trolox concentration was constructed by linear regression. AA of each diluted wine sample was calculated on the basis of the Trolox calibration curve and expressed in mmol/L of Trolox equivalents (mmol TE/L); i.e. as TEAC values. Additionally, the percentage inhibition of $ABTS^{\cdot+}$ radical cation caused by the addition of each diluted wine sample was calculated as:

$$\%\text{Inhibition} = [(A_{ABTS} - A_{wine})/A_{ABTS})] \times 100$$

The percentage of inhibition of $ABTS^{\cdot+}$ radical caused by each diluted wine sample was plotted against the volume of undiluted wine in the reaction mixture. Using the curve obtained, AA was expressed as the volume (μL) of wine needed to reduce the initial $ABTS^{\cdot+}$ radical concentration by 50% (i.e. as the Efficient Concentration, EC_{50}).

2.7.2. DPPH method

Five dilutions of each wine were analysed by the DPPH method. A 50 μL of each diluted wine was mixed with 120 μL of methanolic $DPPH^{\cdot}$ solution (1 mmol dm^{-3}) and 1880 μL of methanol to attain a final concentration of $DPPH^{\cdot}$ in the reaction mixture of 5.85×10^{-5} mol dm^{-3}. The reaction mixture was kept in the dark at room temperature for 15 min and then the absorbance at 517 nm of this mixture (A_{wine}) was measured against the blank sample (50 μL of diluted wine, 2000 μL of methanol). The $DPPH^{\cdot}$ blank solution was prepared fresh each day (120 μL of 1 mmol dm^{-3} $DPPH^{\cdot}$, 1930 μL of methanol) and its absorbance at 517 nm (A_{DPPH}) was measured daily. Trolox standards with final concentration 0–2550 μmol/L in methanol were assayed under the same conditions as those used for the diluted wine samples; i.e. 50 μL of Trolox was mixed with 120 μL of methanolic 1 mmol/L $DPPH^{\cdot}$ solution and 1880 μL of methanol. After 15 min, the absorbance at 517 nm (A_{Trolox}) against the prepared blank sample was measured. The calibration curve for Trolox, constructed by linear regression of absorbance value (A_{Trolox}) *vs*. Trolox concentration, was used to calculate the AA of each diluted wine sample and to express their AA in mmol of Trolox equivalents (mmol TE/L); i.e. as TEAC values. TEAC values were determined as described above for the ABTS method. The percentage inhibition of the initial concentration of $DPPH^{\cdot}$ radical caused by each diluted wine sample was calculated as:

$$\%\text{Inhibition} = [(A_{DPPH} - A_{wine})/A_{DPPH})] \times 100$$

The percentage inhibition of $DPPH^{\cdot}$ radical caused by each diluted wine sample was plotted against the volume of undiluted wine in the reaction mixture. Using the curve obtained, AA was expressed as the volume (μL) of wine needed to reduce the initial concentration of $DPPH^{\cdot}$ radical by 50% (i.e. as Efficient Concentration, EC_{50}).

2.8. Statistical analysis

All measurements were done in triplicate and the results were presented as mean value ± standard error (standard deviation, SD). Correlation and regression analyses were done with the Statistica 7.1 (StatSoft Inc., Tulsa, USA) and OriginPro 7.5 (OriginLab Corporation, Northampton, USA) software packages. The direction and magnitude of the correlation between the variables was quantified by the correlation coefficient, *r*. Correlations were considered statistically significant at $p < 0.05$.

3. Results and discussion

3.1. Differential pulse voltammetry measurements

Differential pulse voltammetry (DPV) was used to characterise polyphenols in red wines and (+)-catechin was used as the standard to express the TP content.

The differential pulse voltammograms of (+)-catechin exhibited two well-defined anodic oxidation peaks (Fig. 1). The first oxidation peak (P1) was observed in the potential range 0.440–−0.475 V *vs*. SCE and is shifted to more positive values with increasing concentration of catechin. According to the literature (Janeiro & Oliveira Brett, 2004; Kilmartin et al., 2001, 2002) this

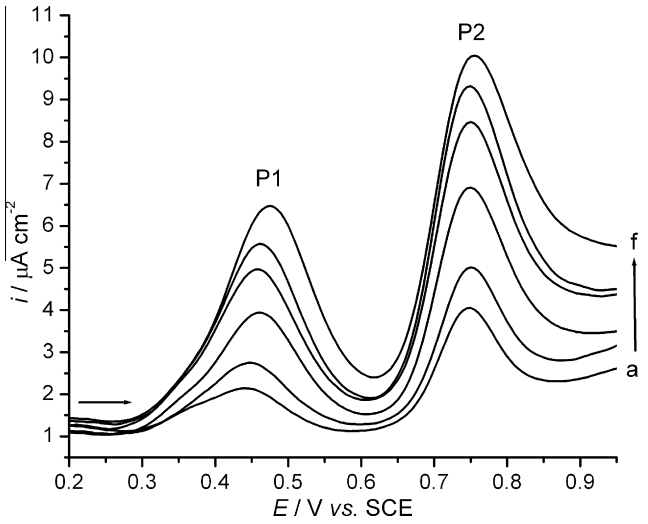


Fig. 1. Differential pulse voltammograms of catechin concentration of: (a) 3, (b) 5, (c) 8, (d) 10, (e) 12, (f) 15 mg L^{-1}, measured at the GC electrode in acetate buffer solution pH 3.6. Scan rate, 5 mV s^{-1}.

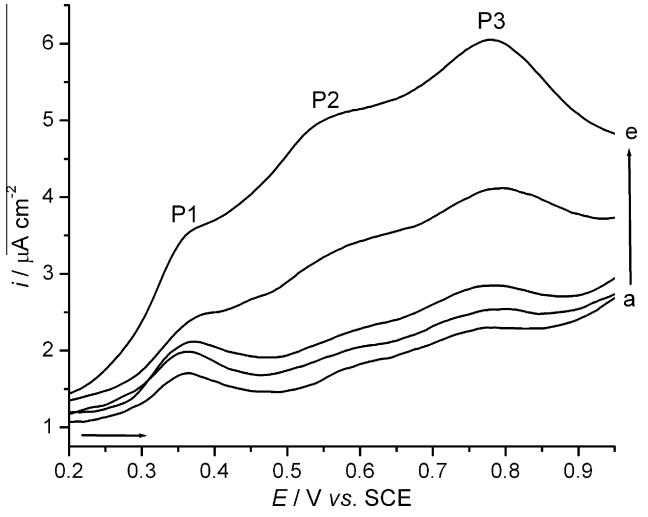


Fig. 2. Differential pulse voltammograms of red wine Klikun diluted: (a) 1/800, (b) 1/600, (c) 1/400, (d) 1/200, (e) 1/100 in acetate buffer solution pH 3.6, measured at the GC electrode. Scan rate, 5 mV s^{-1}.

peak corresponds to the reversible oxidation of the 3′, 4′-dihydroxyl moiety (–OH groups) at the B-ring (catechol moiety) of (+)-catechin (Scheme 1.). This oxidation reaction is pH- and concentration-dependent and included a two-electron (2e)-two-proton (2H) oxidation reaction mechanism. The interpretation of the second oxidation peak of catechin, observed at 0.750 V (Fig. 1, P2), in not so uniform in the literature. It is possible that less oxidizable –OH groups in the structure of catechin at the C and A rings might become oxidised at higher anodic potentials. According to Janeiro et al. (2004) this second oxidation peak of catechin corresponds to the oxidation of –OH group at position 3 on the C-ring of catechin (Scheme 1.). The oxidation process at the potential of P2 is irreversible and not concentration-dependent (see Fig. 1).

The first anodic peak (P1) of catechin was chosen for quantification of polyphenols due to its reversibility, very good reproducibility and linear dependence on current density of this peak *vs.* the concentration of catechin. The calibration curve was constructed by plotting the current density of peak P1 (μA cm^{-2}) *vs.* the concentration of (+)-catechin. The resulting calibration plot is linear in the catechin concentration range from 1–15 mg/L and is described by the equation: i_p (μA cm^{-2}) = 0.3637 x + 1.1025, where i_p is the current density of P1 and x is the concentration of catechin expressed in mg/L (correlation coefficient, r = 0.9977; p < 0.0001). The limit of detection (LOD) was calculated from the parameters obtained from calibration curve using the formula LOD = 3 s_a/b, where s_a is the standard deviation of the y-intercept of the regression line and b is the slope of the calibration curve (Ribani et al., 2007). Under the given conditions, LOD for catechin determination was 0.53 mg/L. Also, the limit of quantification (LOQ) was calculated as LOQ = 10 s_a/b and was 1.77 mg/L.

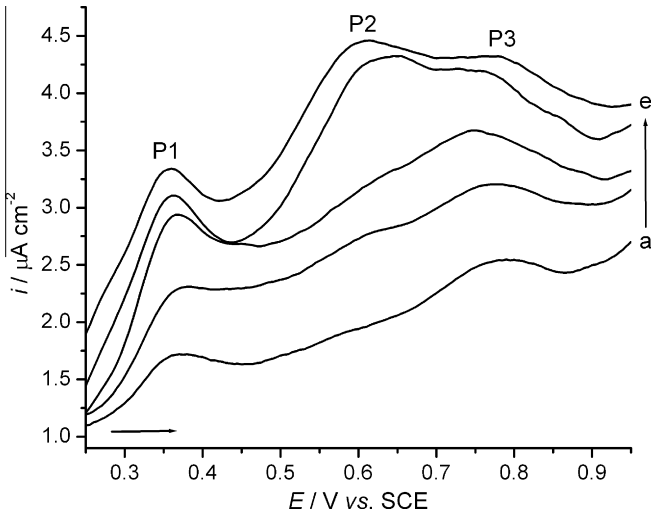
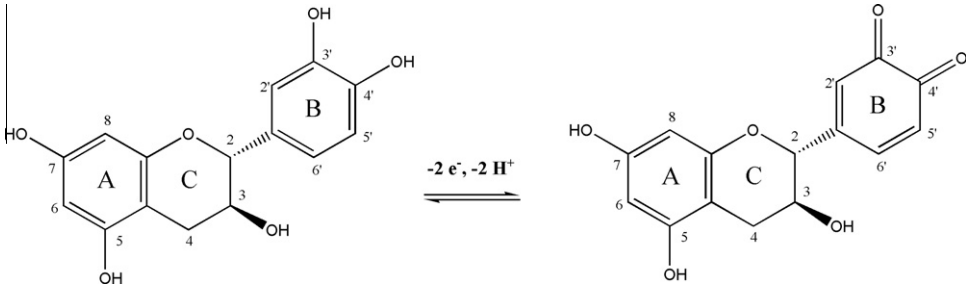


Fig. 3. Differential pulse voltammograms of red wines: (a) Frankovka, (b) Pinot Noir, (c) Zweigelt, (d) Plavac Hvar, (e) Ivan Dolac, diluted 1/400 in acetate buffer solution pH 3.6, measured at the GC electrode. Scan rate, 5 mV s^{-1}.

The differential pulse voltammograms of wines were recorded in order to investigate the analytical potential of the electrochemical oxidation of electroactive polyphenolics species present in wines in determining the TP content. During a differential pulse, the wine polyphenolics were oxidised as the electrode potential is scanned in the positive direction and the overall current response was the sum of the oxidation of the various polyphenolics species present in wine. Differential pulse voltammograms were recorded for different dilutions of wines (1/100, 1/200, 1/400, 1/600 and 1/800, v/v) in order to: (i) determine the range of dilution in which the current density of P1 was linearly (directly) proportional to the "concentration" of wine phenolics; (ii) find the dilution factor of wine required to reach a current density for P1 of wine <6.5 μA cm^{-2}, i.e. to be in a linear part of the calibration curve for catechin (see Fig. 1). It was found that the current density of P1 of all wine samples was only fully linear with concentration corresponding to 400-fold dilution or greater. At the same time the peak current densities of P1 for 400-fold dilution (or greater) have values of <6.5 μA cm^{-2}. We found that 400-fold dilution best met all of the above-mentioned criteria for the quantitative determination of polyphenols content (and gave the best reproducibility of DPV measurements). Therefore, the DPV results for a dilution of 1/400 were used for quantification of the TP content in the wines in this study.

The typical differential pulse voltammograms for different dilutions of the wines are shown in Fig. 2. The DPV curves for the Klikun wine represent the behaviour of all other wines. All wine samples had peak P1 in the potential range 0.360–0.370 V *vs.* SCE. This peak was more pronounced if dilution of wines is higher and for 400-fold dilution or greater the current of this peak is directly proportional to dilution factor. The oxidation current



Scheme 1. Chemical structure and reaction of oxidation of (+)-catechin.

generated in this potential range can be ascribed to wine polyphenolics with high reducing capacity, i.e. with low oxidation potential. According to the literature (Kilmartin et al., 2001, 2002), peak P1 could be ascribed to the oxidation of different polyphenolic compounds present in wines that have a structure with *ortho*-diphenol (catechol) groups at B-ring, like flavonoids catechin, epicatechin, quercetin, and some phenolic acids like gallic, caffeic, and tannic acids.

Second oxidation peak (P2) was observed in the potential range 0.550–0.630 V. This peak was more pronounced at lower dilutions (1/100 and 1/200), while at higher dilutions (1/400 and greater) P2 was observed mainly as a shoulder on the DPV curves (see Fig. 2). P2 is more pronounced in wines with a very high TP content (especially in red wines reach in anthocyanins), such as the famous red wines from Dalmatia (Ivan Dolac, Dingač and Plavac Hvar, see Fig. 3). This is in accordance with statements in the literature (Kilmartin et al., 2001, 2002) that the oxidation peak in this potential region can be ascribed to the oxidation of wine anthocyanins; e.g. malvidin anthocyanins. Oxidation of *trans*-resveratrol and some phenolic acids (e.g. ferulic acid) would also contribute to P2 (Kilmartin et al., 2001).

A third oxidation peak (P3) was observed between 0.760 and 0.780 V (Figs. 2 and 3). This peak is relatively well pronounced in all of the wines in this study, but was more pronounced at lower dilution factors. With dilution, the profile of P3 is unchanged but the peak current was decreases (see Fig. 2). According to the literature (Kilmartin et al., 2001, 2002), an oxidation peak in this potential range can be the result of the oxidation of polyphenols with higher oxidation potentials, such as some phenolic acids (*p*-coumaric and vanillic acids), and the second oxidation of some flavonoids, like catechin, epicatechin quercetin, etc. (due to the oxidation of –OH group at position 3 on the C-ring of flavonoid structure). In wine samples, this peak is somewhat broadened compared to the corresponding P2 peak of catechin (Fig. 1), which is understandable because this peak in wine is the result of the combined effects of the oxidation of several wine polyphenolics with slightly different oxidation potentials.

The TP content in the wines was determined by the use of electrochemical parameters obtained from the DPV voltammograms of wines. Some of DPV voltammograms for different wines are shown in Fig. 3. All of the wines in this study showed a very well pronounced first oxidation peak (P1), and the current density of this peak was used for quantification of the TP content. The second oxidation peak (P2) was more pronounced for wines from the Dalmatia region (Dolac, Dingač, Plavac, Babić, etc.), and the third oxidation peak (P3) was more pronounced for wines from the Slavonia and Istria regions (Zweigelt, Pinot, Frankovka, Merlot, etc.).

The electrochemical parameters of DPV measurements were determined for all dilution factors (1/100, 1/200, 1/400, 1/600 and 1/800) of the 11 investigated wines but the electrochemical parameters of DPV measurements for the 400-fold diluted wine samples were used for quantification of polyphenols content. The TP content (TP_{DPV}) was calculated from the current density of the first oxidation peak, i_p (P1), using the calibration curve for catechin as the standard, and expressed as mg catechin equivalents per litre of wine (mg CE/L) (Table 1). The results were expressed as mean ± SD of three independent analyses.

Table 1 shows that each of the wines had a high or very high TP content. The red wines from the Dalmatia region had the highest TP content (Ivan Dolac, Dingač, Plavac Hvar, 2491–2223 mg CE/L), while those from the continental part of Croatia (Slavonia region) had a significantly lower TP content (e.g. Frankovka, Merlot, Klikun Noir, 851–1224 mg CE/L).

We compared our results with TP content of red wines that were also measured by an electrochemical method (CV) and were reported in papers of other authors. Piljac, Martinez, Stipčević, Petrović & Metikoš-Huković (2004) and Piljac-Žegarac, Martinez, Valek, Stipčević, and Kovačević-Ganić (2007) reported the TP

Table 1
Total polyphenols (TP) content of some Croatian red wines measured by differential pulse voltammetry (DPV), Folin–Ciocalteu (FC) and HPLC, and their antioxidant activity (AA) determined by ABTS and DPPH methods.

Wine	TP_{DPV} (mg CE/L)	TP_{FC} (mg CE/L)	TP_{FC} (mg GAE/L)	TP_{HPLC} (mg GAE/L)	AA_{ABTS}-TEAC (mmol TE/L)	AA_{ABTS}-EC_{50} (μL of wine)	AA_{DDPH}-TEAC (mmol TE/L)	AA_{DPPH}-EC_{50} (μL of wine)
Ivan Dolac	2491 ± 99	3013 ± 45	3264 ± 49	2760 ± 93	24.2 ± 0.8	1.6 ± 0.1	37.8 ± 2.8	2.4 ± 0.1
Dingač	2413 ± 105	2872 ± 56	3111 ± 60	2677 ± 42	22.9 ± 0.6	1.9 ± 0.1	32.0 ± 3.1	2.5 ± 0.1
Plavac Hvar	2223 ± 79	2675 ± 59	2898 ± 64	2430 ± 71	22.2 ± 0.5	1.9 ± 0.1	31.5 ± 0.9	2.5 ± 0.1
Zweigelt	1859 ± 102	1971 ± 81	2135 ± 87	1975 ± 48	16.1 ± 0.6	2.6 ± 0.1	19.7 ± 2.5	4.3 ± 0.1
Pinot Noir	1654 ± 87	1685 ± 71	1825 ± 77	1812 ± 20	12.8 ± 0.3	3.2 ± 0.1	19.5 ± 2.7	4.3 ± 0.2
Babić	1606 ± 88	1625 ± 72	1759 ± 73	1746 ± 25	12.5 ± 0.7	3.2 ± 0.2	16.6 ± 0.5	4.9 ± 0.1
Kaštelet	1505 ± 100	1589 ± 74	1721 ± 78	1645 ± 14	11.7 ± 0.4	3.4 ± 0.2	15.3 ± 1.9	5.2 ± 0.1
Teran	1497 ± 71	1530 ± 18	1657 ± 19	1574 ± 17	11.0 ± 0.6	3.9 ± 0.2	14.1 ± 0.3	5.3 ± 0.1
Klikun Noir	1224 ± 94	1343 ± 25	1455 ± 27	1346 ± 93	10.0 ± 0.2	4.1 ± 0.1	12.8 ± 2.7	7.6 ± 0.4
Merlot	1081 ± 65	1163 ± 21	1260 ± 23	1174 ± 41	9.2 ± 0.1	4.6 ± 0.1	10.8 ± 0.3	8.5 ± 0.2
Frankovka	851 ± 76	934 ± 34	1012 ± 37	1002 ± 79	7.9 ± 0.4	5.1 ± 0.2	9.2 ± 0.6	8.9 ± 0.3

Results represent mean values ± standard deviation (SD) of three independent measurements.

content of 449–4884 mg CE/L for some Croatian red wines, and De Berr et al. (2004) reported a TP content of 752–4819 mg CE/L in Californian red wines.

3.2. Folin–Ciocalteu measurements

The TP content of the wines was determined by the conventional spectrophotometric Folin–Ciocalteu (FC) method, which gave TP_{FC} expressed as mg of catechin equivalents (mg CE/L) or mg of gallic acid equivalents (mg GAE/L). In Table 1 were reported TP_{FC} values as mean value ± SD. The TP_{FC} values, which were in the range 934–3013 mg CE/L or 1012–3264 mg GAE/L, were 1–20% higher (dependent on the brand of wines) than those obtained by the DPV method (TP_{DPV}, Table 1). Such differences in the TP content of red wines obtained by FC and some electrochemical methods have also been reported by others, who used CV as the method for characterisation of polyphenols in red wines (Kilmartin et al., 2001, 2002; De Berr et al., 2004; Piljac et al., 2004, 2007) The higher values obtained by FC method can be explained by the fact that FC reagent is not specific only to phenolic compounds; it can be reduced also by non-phenolic compounds, including ascorbate, sulphite and reducing sugars.

In spite of the differences between the results obtained by the two methods, a very high correlation was obtained between the TP content (mg CE/L) determined by the DPV method (TP_{DPV}) and that determined by the Folin–Ciocalteu method (TP_{FC}) ($r = 0.9872$, $p < 0.0001$; Fig. 4a).

Comparison of the TP content determined by the FC method in this study with some of the published FC results shows that our values of 1012–3264 mg GAE/L are in general accordance with results determined by the FC method for other Croatian red wines, i.e. values of 1156–2619 mg GAE/L reported by Rastija, Srečnik & Medić-Šarić (2009) and values of 2193–3183 mg GAE/L reported by Katalinić, Miloš, Bodun &Boban (2004). In contrast, Piljac et al. (2004) reported much higher TP contents of 2542–4979 mg GAE/L for some Croatian red wines. TP contents determined by the FC method for red wines from other countries are as follow: De Berr et al. (2004) reported values of 689–2616 mg GAE/L for Californian red wines; Di Majo, La Quardia, Giammanco, Neve & Giammanco (2008) reported 2280–3730 mg GAE/L in some Italian red wines; and Staško, Brezova, Mazur, Čertik, Kalinak & Gescheidt (2008) reported TP content of 1460–3380 mg GAE/L) for some red wines from Slovakia and Austria.

Such a relatively large differences in TP_{FC} content between the investigated red wines from different countries are likely to be the result of using very different grape varieties, different process of viticulture and vinification, different types of soil, different geographical location and many other differences (sometimes unknown) between parameters in the process of grape growing and wine making.

3.3. HPLC measurements

The TP contents of investigated red wines determined from the total area of HPLC chromatograms at 280 nm (De Berr et al., 2004) and expressed as mean value ± SD in mg GAE/L of wine (TP_{HPLC}) are given in Table 1.

As can be seen in Table 1 TP_{HPLC} ranged from 1002 mg GAE/L in Frankovka wine to 2760 mg GAE/L in Ivan Dolac wine.

The values obtained by HPLC were somewhat lower that those obtained by the FC method (see Table 1). This difference in TP content as determined by HPLC and FC methods was 14–17% in wines with a high polyphenol content (Ivan Dolac, Dingač and Hvarski Plavac), whereas this difference was only 1–8% in other red wines. The components that are probably undetected by RP-HPLC method include proanthocyanidins and various polymeric (oligomeric) phenolics and tannins, which are difficult to identify individually by RP-HPLC (De Beer et al., 2004). In spite of this fact, a very good correlation ($r = 0.9923$, $p < 0.0001$, Fig. 4b) was obtained between the results (mg GAE/L) measured by HPLC (TP_{HPLC}) and FC method (TP_{FC}).

On the basis of CV and HPLC analysis of some New Zealand red wines, Kilmartin et al. (2002) reported that a few monomeric phenolics (catechin, epicatechin, quercetin, gallic acid, caffeic acid and *p*-coumaric acid) contained in red wines were responsible for the first anodic oxidation peak (P1) of red wines. These phenolics are well separated and easily determined by RP-HPLC (De Beer et al., 2004).

The content of major catechol contained phenolics responsible for the first DPV anodic peak, along with some other phenolics that contribute at higher oxidation potentials, as determined by the RP-HPLC method are given in Table 2 as mean value ± SD of triplicate determinations. The representative HPLC chromatograms are shown in Fig. 5.

Fig. 5 shows that the major monomeric polyphenols contained in investigated red wines are: (+)-catechin, (−)-epicatechin, quercetin, gallic acid, caffeic acid and *p*-coumaric acid. According to our HPLC measurements, the main phenolic acid was gallic acid (mean values ranged from 51.2 mg/L in Frankovka wine to 179.4 mg/L in Ivan Dolac wine), while the main monomeric flavonoid was (+)-catechin

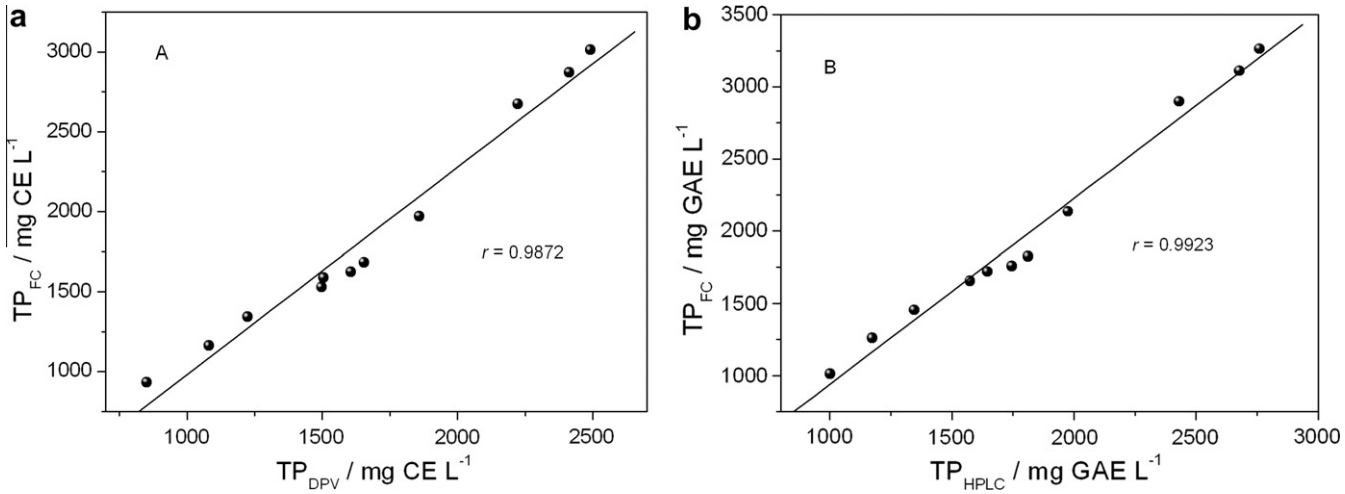


Fig. 4. Correlation between the total polyphenols content (TP) of red wines determined by FC and DPV methods (a) and between TP determined by FC and HPLC methods (b).

Table 2
Content of major monomeric polyphenols (mg L^{-1}) determined in some Croatian red wines by HPLC method.

Wine	Gallic acid	Caffeic acid	*p*-Coumaric acid	(+)-Catechin	(−)-Epicatechin	Quercetin
Ivan Dolac	179.4 ± 7.6	18.1 ± 0.6	2.9 ± 0.7	138.0 ± 7.7	29.3 ± 3.6	6.8 ± 0.3
Dingač	143.8 ± 3.9	18.6 ± 0.8	2.5 ± 0.4	130.3 ± 6.0	37.4 ± 2.3	7.0 ± 0.6
Plavac Hvar	128.8 ± 3.1	12.4 ± 0.4	2.5 ± 0.5	135.2 ± 7.5	22.4 ± 1.3	4.6 ± 0.2
Zweigelt	100.1 ± 3.4	4.3 ± 0.2	2.2 ± 0.2	118.7 ± 9.9	37.7 ± 1.0	1.6 ± 0.1
Pinot Noir	97.6 ± 2.8	5.1 ± 0.5	1.8 ± 0.2	117.0 ± 9.5	34.2 ± 1.1	1.3 ± 0.1
Babić	94.8 ± 2.2	4.2 ± 0.3	3.3 ± 0.3	107.7 ± 4.1	10.6 ± 0.7	4.3 ± 0.4
Kaštelet	92.5 ± 1.7	3.2 ± 0.2	2.3 ± 0.3	79.8 ± 8.9	7.9 ± 1.2	1.5 ± 0.2
Teran	86.2 ± 1.3	5.6 ± 0.4	1.8 ± 0.2	62.2 ± 4.2	12.8 ± 1.3	2.8 ± 0.3
Klikun Noir	77.2 ± 1.5	8.2 ± 0.6	2.9 ± 0.4	56.6 ± 7.0	15.3 ± 1.5	1.2 ± 0.1
Merlot	55.5 ± 1.4	5.0 ± 0.2	4.2 ± 0.9	46.2 ± 3.4	7.8 ± 0.4	4.8 ± 0.5
Frankovka	51.2 ± 2.2	5.2 ± 0.4	4.4 ± 0.7	31.0 ± 2.6	9.8 ± 0.5	1.5 ± 0.1

Results represent mean values ± standard deviation (SD) of three independent measurements.

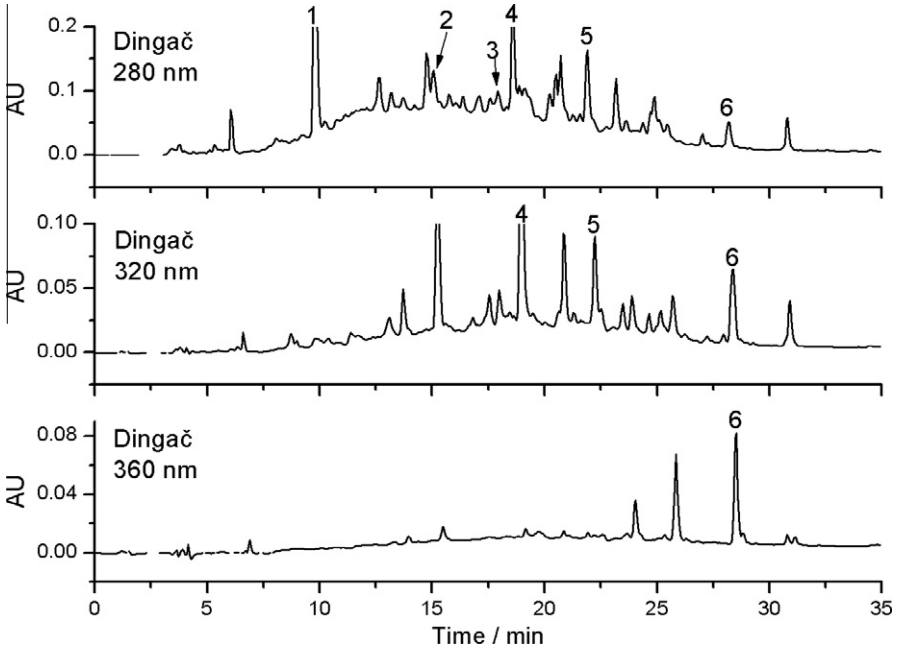


Fig. 5. HPLC chromatograms of red wine Dingač, recorded at 280, 320, and 360 nm. Peak identification: (1) gallic acid, (2) (+)-catechin, (3) (−)-epicatechin, (4) caffeic acid, (5) *p*-coumaric acid, (6) quercetin.

(means ranged from 31.0 mg/L in Frankovka wine to 138.0 mg/L measured in Ivan Dolac wine (see Table 2.). Our results are in accordance with those of many published HPLC analysis of red wines, in which also were reported that the main monomeric phenolics in red wines were gallic acid and (+)-catechin. Thus, Kilmartin et al. (2002) reported that the main monomeric phenolics in some red wines from New Zeland and Australia were gallic acid (37–108 mg/L) and catechin (26–223 mg/L). Similar results have been reported for the HPLC analysis of some Italian red wines (Tarola, Milano, & Giannetti, 2007, 47.21–325.48 mg gallic acid/L and 48.01–179.41 mg catechin/L); and some Hungarian red wines (Pour Nikfardjam, Mark, Avar, Figler, & Ohmacht, 2006, gallic acid content of 29.7–79.2 mg/L and catechin content of 62.1–103 mg/L).

3.4. Antioxidant activity measurements

DPPH and ABTS assays were used to evaluate AA of the wines and the results are given in Table 1 as mean ± SD of triplicate measurements. The values for AA of wines obtained by the DPPH and ABTS assays show the same order of scavenging reactivity (see Table 1), but the absolute values for each wine sample obtained by the two different assays differ for many reasons, including differences in experimental methodology used, e.g. in the free radical that was applied, different reaction times, different absorption wavelength, etc. The values obtained by the DPPH method are somewhat higher then those obtained by the ABTS method, probably due to the longer reaction time, as reported by Fernandez-Pachon, Villano, Garcia-Parrilla, and Troncoso (2004). Staško et al. (2008) also reported that the DPPH method gives higher TEAC values then the ABTS method.

All wines investigated in this study exhibited potent radical scavenging activity. The strongest radical activity (see Table 1) was found in the wines from the Dalmatia region: Ivan Dolac (DPPH method, 37.8 mmol TE/L; ABTS method, 24.2 mmol TE/L), followed by Dingač and Plavac Hvar (DPPH method, 32.0 and 31.5 mmol TE/L, respectively; ABTS method, 22.9 and 22.2 mmol TE/L, respectively). Red wines from the continental part of Croatia showed slightly lower (e.g. Zweigelt, DPPH method, 19.7 mmol TE/L; ABTS method, 16.1 mmol TE/L) or significantly lower AA (e.g. Frankovka: DDPH method, 9.2 mmol TE/L; ABTS method, 7.9 mmol TE/L). Such behaviour is in accordance with slightly lower or significantly lower TP content in red wines from the continental region of Croatia in comparison with the very high TP content in red wines from the Dalmatia (Adriatic coast) region (see Table 1).

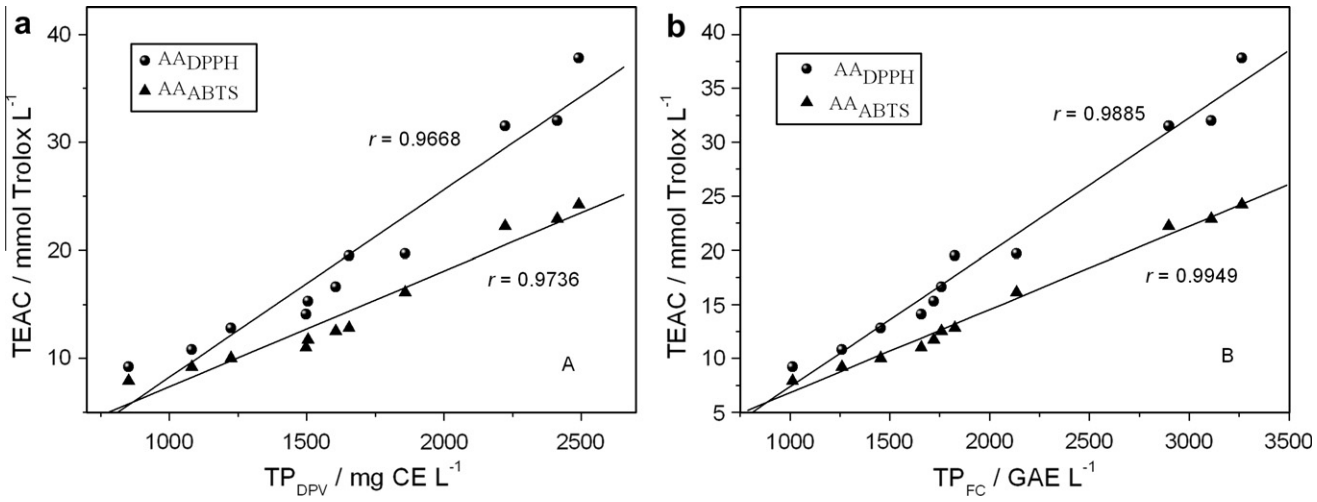


Fig. 6. Correlation between antioxidant activity (AA) of red wines and total polyphenols (TP) content determined by the DPV (a) and FC methods (b).

We compared our results with some of those in the literature. Piljac et al. (2007) reported AA of 8.22–11.43 mmol TE/L as determined by the DPPH method for some Croatian red wines. Fernandez-Pachon et al. (2004) reported TEAC values for 16 Spanish red wines of 4.65–17.41 mmol TE/L (DPPH method) and 2.33–7.85 mmol TE/L (ABTS method). Rivero-Perez, Muniz, and Gonzalez-Sanjose (2008) reported AA of 16.6–41.0 mmol TE/L (ABTS method) and 9.9–25.5 mmol TE/L (DPPH method) for 80 Spanish red wines. Staško et al. (2008) reported values of 13.2–17.8 mmol TE/L (DPPH method) and 7.8–14.5 mmol TE/L (ABTS method) for some Slovak and Austrian red wines. Lugasi and Hovari (2003) reported a range of 6.7–20.1 mmol TE/L (ABTS method) for Hungarian red wines. It is evident that authors have reported very different TEAC values but this is not unexpected because different brands of red wines are produced from very different grapes by different technologies. Also, the experimental conditions for the DPPH and ABTS methods used in the above-mentioned studies differed in many details. Thus, only a general comparison of the AA measured in different red wines with different methods is possible.

AA expressed as EC_{50} ranged from 2.4 to 8.9 μL of wine as measured by the DPPH method and from 1.6 to 5.1 μL of wine as measured by the ABTS method. These EC_{50} values follow the same order of AA as the TEAC values (Table 1). EC_{50} values similar to those we measured by the DPPH method have been reported for some Hungarian red wines (average value of 7.58 μL of wine, Lugasi et al., 2003) and for some Italian red wines (values of 2.06–14.72 μL of wine, Giovanelli, 2005).

A strong correlation was observed between the AA and the TP content of the red wines in this study (Fig. 6).

There is a very strong correlation between the TP content determined by the DPV method (TP_{DPV}) and the AA determined by the DPPH and ABTS methods (AA expressed as TEAC values). The correlation observed with the AA values measured by the ABTS method was even stronger ($r = 0.9736$) than that measured by the DPPH method ($r = 0.9668$) (Fig. 6a). A similar, very high correlation ($r = 0.956$) between the TP content of red wines determined by the electrochemical (CV) method and the AA measured by DPPH method was reported by Piljac et al. (2007).

The AA of the wines in this study showed an even stronger correlation with the TP content determined by FC method (TP_{FC}, GAE/L) (for the ABTS method, $r = 0.9949$; and for the DPPH method, $r = 0.9885$) (Fig. 6b). Many other authors have been also reported a very high degree of correlation between the TP_{FC} content and the AA of red wines. Staško et al. (2008) reported very high correlation coefficients for red wines from Slovakia and Austria (ABTS method, $r = 0.983$; DPPH method, $r = 0.974$). Cimino, Sulfara, Trombetta, Saija & Tomaino (2007) reported for Italian red wines $r = 0.943$ (DPPH method). Katalinić et al. (2004) reported $r = 0.954$ (DPPH method) for some Croatia red wines. Lugasi et al. (2003) reported $r = 0.973$ (DPPH method) for some Hungarian red wines. Paixao, Perestrelo, Marques, and Camara (2007) reported values $r = 0.982$ (DPPH method) and $r = 0.962$ (ABTS method)) for some red wines from Portugal. In contrast, some authors have reported somewhat lower correlation coefficient values. Thus, Villano et al. (2004) reported $r = 0.901$ (ABTS method) for Spanish red wines and Giovanelli (2005) reported for Italian red wines, $r = 0.90$ (DPPH method).

In spite of such differences between the results obtained by different authors, practically all published papers on this subject suggests that the predominant source of the AA of red wines derives from different polyphenolic compounds presented in the wines and depends strongly on their TP content.

4. Conclusion

This study presents the first characterisation of polyphenols in red wines performed by differential pulse voltammetry (DPV). The results showed that DPV is a very sensitive and very selective method for the determination of the total polyphenols (TP) content of red wines and is a notable improvement for TP determination in comparison to the CV and FC methods. A very high correlation was obtained between the TP content as measured by the DPV, FC and HPLC methods. The HPLC analysis showed that the most abundant polyphenolic compounds in the red wines investigated in this study were gallic acid and (+)-catechin. Variations were observed in the TP content and polyphenols composition among wines made from grapes grown in different geographical and wine-producing regions of Croatia. Red wines made in the Dalmatia region, which has a Mediterranean climate, have the highest TP content and a higher concentration of the important wine antioxidants (catechin, epicatechin, quercetin, gallic acid, caffeic acid, *p*-coumaric acid) in comparison to the red wines from the continental wine region (Slavonia). Red wines from Dalmatia region showed also the highest AA. A very high correlation was observed between the AA and TP content, which suggests that the predominant source of the AA of investigated red wines derives from the different polyphenolic compounds present in wines.

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