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Red Wine Phenolic Complexes and Their in Vitro Antioxidant Activity

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Phenolic complexes are a major group of polyphenols in aged red wine. The objective of this work was to evaluate the in vitro antioxidant activity of the phenolic complexes. Thus, red wine polyphenols were fractionated into various fractions including monomers, oligomers, polymers, anthocyanins, and complexes. The in vitro antioxidant activities of these fractions and other phenolic standards (catechin, epicatechin, quercetin, and malvidin 3-glucoside) as well as ascorbic acid were verified by DPPH• test. On the other hand, the variation of antioxidant activities during the reaction between epicatechin and malvidin 3-glucoside mediated by acetaldehyde in a model wine solution was also monitored. The results showed that both the phenolic complex fraction and newly formed condensation products between epicatechin and malvidin 3-glucoside maintain antioxidant activities as strong as those of their compositional phenolics. This work provides, for the first time, direct evidence about the in vitro antioxidant activities of red wine phenolic complexes.

KEYWORDS: Red wine; phenolic complexes; fractionation; in vitro antioxidant activities; DPPH test

INTRODUCTION

Grape polyphenols mainly consist of anthocyanins, catechins, oligomeric and polymeric proanthocyanidins, phenolic acids, stilbenes (including resveratrol), flavonols, flavanonols, and flavones. Red wine polyphenols include both grape polyphenols and new phenolic products formed from them during the winemaking process. The enzymatic and nonenzymatic reactions start as soon as the beginning of winemaking (crushing) and continue throughout fermentation and aging (*1*). This leads to a great diversity of new polyphenols and makes wine polyphenol composition more complex. Due to various chemical reactions during aging, the polyphenol composition of young red wine is also different from that of old red wine (*2*). The newly formed compounds often show specific sensory properties, distinct from those of their precursors. Because one of the most important reactions during red wine aging is the condensation reaction between anthocyanins and proanthocyanidins (*3*), red wine polyphenols largely consist of these condensed compounds. Our earlier works have confirmed that polyphenols in red wine are presented mainly in polymeric forms (*4*−*6*).

On the other hand, it is well documented that moderate consumption of red wine has been associated with reduced risk of heart diseases (*7*−*9*), and the key compounds responsible for this beneficial effect were confirmed to be polyphenols (*10*−*12*). Such findings have prompted numerous research works toward the evaluation of the antioxidant activity and polyphenol levels of red wines (*2*, *4*, *5*, *13*−*17*). Zafrilla et al. (*17*) reported no correlation between the total concentrations of phenolic compounds and the antioxidant activity of conventional and ecological red and white wines, but other authors have observed positive correlation between the phenolic content of a wine and its antioxidant activity (*13*, *19*).

Moreover, various authors speculated that as wine ages its antioxidant activity would be reduced due to the phenolic complexation and reduction of HPLC-detectable phenolic compounds. Pellegrini et al. (*14*) reported that young red wine had higher in vitro antioxidant activity than aged red wine. However, the work realized by Larrauri et al. (*20*) showed that the older wines had higher antioxidant activity than younger wines.

It should be mentioned that the published works generally provided only total antioxidant activity of red wine together with its total polyphenols or several well-known phenolics levels. Furthermore, there have been no available data about the biological activity of newly formed polyphenols during wine storage and aging, phenolic complexes. Because the phenolic complexes are, from a quantitative viewpoint, the major polyphenols in aged red wine, the objective of this work was to evaluate the antioxidant activities of such complexes. For this purpose, the aged red wine polyphenols were separated into various distinct fractions using combined techniques of solid-phase extraction and liquid chromatography according to the method established in our laboratory (*21*). The in vitro antioxidant activity of the isolated phenolic complexes was measured and compared with those of other wine phenolic fractions and phenolic standards. Moreover, a kinetic reaction between epicatechin and malvidin 3-glucoside mediated by acetaldehyde in model solution was performed, which permitted verification of

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Table 1. In Vitro Antioxidant Activities Measured by $DPPH^•$ Test[a]

red wine phenolic fraction/phenolic standard	$DPPH^•$ test: EC_{50} (mg/mg of DPPH) mean	SD	ARP ($1/EC_{50}$) mean	SD
red wine phenolic complexes	0.126d	0.006	7.945c	0.357
red wine oligomer proanthocyanidins	0.182b	0.022	5.552b	0.694
red wine polymer proanthocyanidins	0.149c	0.008	6.743bc	0.353
red wine anthocyanins	0.272a	0.009	3.685a	0.125
red wine total polyphenols	0.155c	0.006	6.478bc	0.267
catechin	0.074e	0.006	13.553e	1.036
epicatechin	0.054f	0.006	18.491f	2.050
quercetin	0.088e	0.001	11.387d	0.138
malvidin 3-glucoside	0.195b	0.004	5.143ab	0.130
ascorbic acid	0.093e	0.001	10.811d	0.083

[a] Mean values followed by the same letter in a column are not significantly different (LSD, 95%).

the variation in antioxidant activities during the formation of phenolic complexes.

MATERIALS AND METHODS

Chemical Reagents. All organic solvents were of HPLC or analytical grade quality. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma-Aldrich (Steinhem, Germany). (+)-Catechin, (−)-epicatechin, quercetin, and ascorbic acid were purchased from Fluka A.G. (Buchs, Switzerland). Acetaldehyde (analytical grade) was obtained from Merck (Darmstadt, Germany). Malvidin 3-glucoside was isolated from Pinot Noir grape skins as described previously (*22*). Ultrapure water was obtained from a Seralpur PRO 90 CN system (Ransbach-Baumbach, Germany).

Isolation of Phenolic Complex and Other Phenolic Fractions from Red Wine. Phenolic compounds of a 1-year-aged red wine made by classic vinification method (*21*) with *Vitis vinifera* varieties (Castelão/Tinta Miúda, 60:40, w/w) were separated into various distinct fractions using combined techniques of solid-phase extraction and liquid chromatography according to the method established in our laboratory (*21*). Briefly, red wine were dealcoholized under vacuum at < 30 °C, neutralized by 0.1 N NaOH solution until pH 7, and then loaded onto a LiChroprep RP 18 column preconditioned by pH 7.0 phosphate buffer. Elution began with pH 7.0 phosphate buffer to elute phenolic acids. The column was then washed by distilled water and dried under enhanced vacuum for several seconds. Elution with ethyl acetate permitted isolating catechins and oligomer procyanidins fraction ($F_{cat} + F_{olig}$) and methanol acidified by 0.1% HCl to a fraction containing anthocyanins, polymeric proanthocyanidins, and other pigmented complexes (F_{pigmt}). The $F_{cat} + F_{olig}$ fraction can be further separated into a catechin fraction (F_{cat}) and an oligomer procyanidin fraction (F_{olig}) using another LiChroprep RP 18 column previously preconditioned by distilled water, by sequential elution first with diethyl ether and then methanol. The F_{pigmt} can be further fractionated into anthocyanins and its derivatives (F_{ant}) and higher polymer proanthocyanidins and pigmented complexes ($F_{poly} + F_{complex}$) using a Toyopearl 40 (F) column previously preconditioned by distilled water, by elution with methanol and 75% acetone in water acidified by 0.1% HCl, respectively. The fraction ($F_{poly} + F_{complex}$), after evaporation to dryness and recovery by methanol/water (20:80, v/v), was injected into the HPLC to separate polymer proanthocyanidins (F_{poly}) from phenolic complex ($F_{complex}$) under the conditions described (*21*). Fractions F_{cat}, F_{olig}, F_{poly}, F_{ant}, and $F_{complex}$ were lyophilized, and the respective powders obtained were stored at −20 °C and under darkness until used.

Imitation of Anthocyanin−Procyanidin Condensation Reaction in Model Solution. The model solution used for the reaction between anthocyanins and procyanidins was prepared as described previously (*22*). Furthermore, 12% ethanol in water (v/v) was acidified with L-tartaric acid at a concentration of 5 g/L. The model solution was adjusted, by 1 N HCl or 1 N NaOH, to pH 1.7, at which anthocyanins are presented essentially in flavylium form. The reaction medium was composed of malvidin-3-*O*-glucoside, epicatechin, and acetaldehyde in a molar ratio of 1:5:11. The molar ratio of anthocyanins/procyanidins (1:5) used in this study represents a red wine rich in these compounds (*22*). The reaction solution was kept at 30 °C in brown glass vials. No special attention to oxygen exposure was paid during the reaction. The formation of reaction products and variation of in vitro antioxidant activity of the reaction solution were monitored periodically by reverse-phase HPLC-DAD/ESI-MS analysis and by DPPH test, respectively, under the conditions described below.

Reverse-Phase HPLC-DAD Analysis. Reverse-phase HPLC-DAD analysis was used for monitoring the phenolic compositional changes in the model solution during the reaction between malvidin-3-*O*-glucoside and epicatechin mediated by acetaldehyde. The HPLC apparatus was a Waters system, equipped with a quaternary pump (Waters 600), a controller (Waters 600), a thermostat controlling the column temperature and an autosampler (Waters 717 plus), and a photodiode array detector (Waters 996) coupled to a data processing computer (Millennium 32). The column (250 × 4 mm) was a cartridge of 4 μm Superspher 100 RP18 (Merck). The mobile phase flow rate was fixed at 0.7 mL/min. The detection ranged from 250 to 650 nm, 525 nm being used for the detection of anthocyanins and their derivatives and 280 nm for the detection of all types of polyphenols. The column temperature was set at 30 °C. Elution conditions were similar to those of previous work (*23*), with slight modification as follows: solvents A (formic acid/water, 5:95, v/v) and B (acetonitrile/water/formic acid, 30:65:5, v/v/v) were used; gradient elution was from 25 to 85% B in 70 min, isocratic elution with 85% B in 15 min, followed by washing and re-equilibration of the column to the initial conditions.

Direct Infusion−Electrospray Ionization−Mass Spectrometry (ESI-MS) Analysis. Direct infusion−ESI-MS was used for verifying the chemical composition of red wine phenolic fractions and the reaction products in the model solution. The equipment of ESI-MS was an Esquire 3000^{plus} ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). A Bruker Daltonics DataAnalysis 3.0 was used for data analysis. The samples were infused directly into the ESI source with a syringe pump (74900 Series, Cole-Parmer Instrument) at a constant flow rate of 180 μL/h. Mass spectra were recorded from m/z 150 to 2000 in a positive ionization mode when anthocyanins or their derivatives were analyzed or in negative ionization mode when procyanidins were analyzed. Normal scan resolution (13000 m/z/s) was selected. Voltages for the skimmer and the capillary were, respectively, −40 and +4000 V for negative ion mode or 40 to −4000 V for positive ion mode. Other MS analysis conditions are as follows: compound stability, 100%; nebulizer gas (N_2), 10 psi; drying gas (N_2), 10 L/min; dry temperature 300 °C.

Scavenging Activity on 1,1-Diphenyl-2-picrylhydrazyl Radical ($DPPH^•$). The scavenging effects of the isolated red wine phenolic fractions on $DPPH^•$ were evaluated as previously described (*24*), with slight modification. Briefly, an 0.08 mL aliquot of the isolated wine polyphenol fractions in methanol (different concentrations) and 3.12 mL of $DPPH^•$ solution in methanol (60 μM) were added directly to a 10 mm cell with stopper. The mixture was immediately shaken vigorously for about 10 s by a Vortex mixer. Absorbance at 515 nm (A_{515}) was recorded continuously against methanol as blank reference, using Cary 100 Bio UV−vis spectrophotometer (Varian, Australia), during 60 min (until the reaction reached steady state). The initial concentration of $DPPH^•$ was calculated for every experiment from a calibration curve made

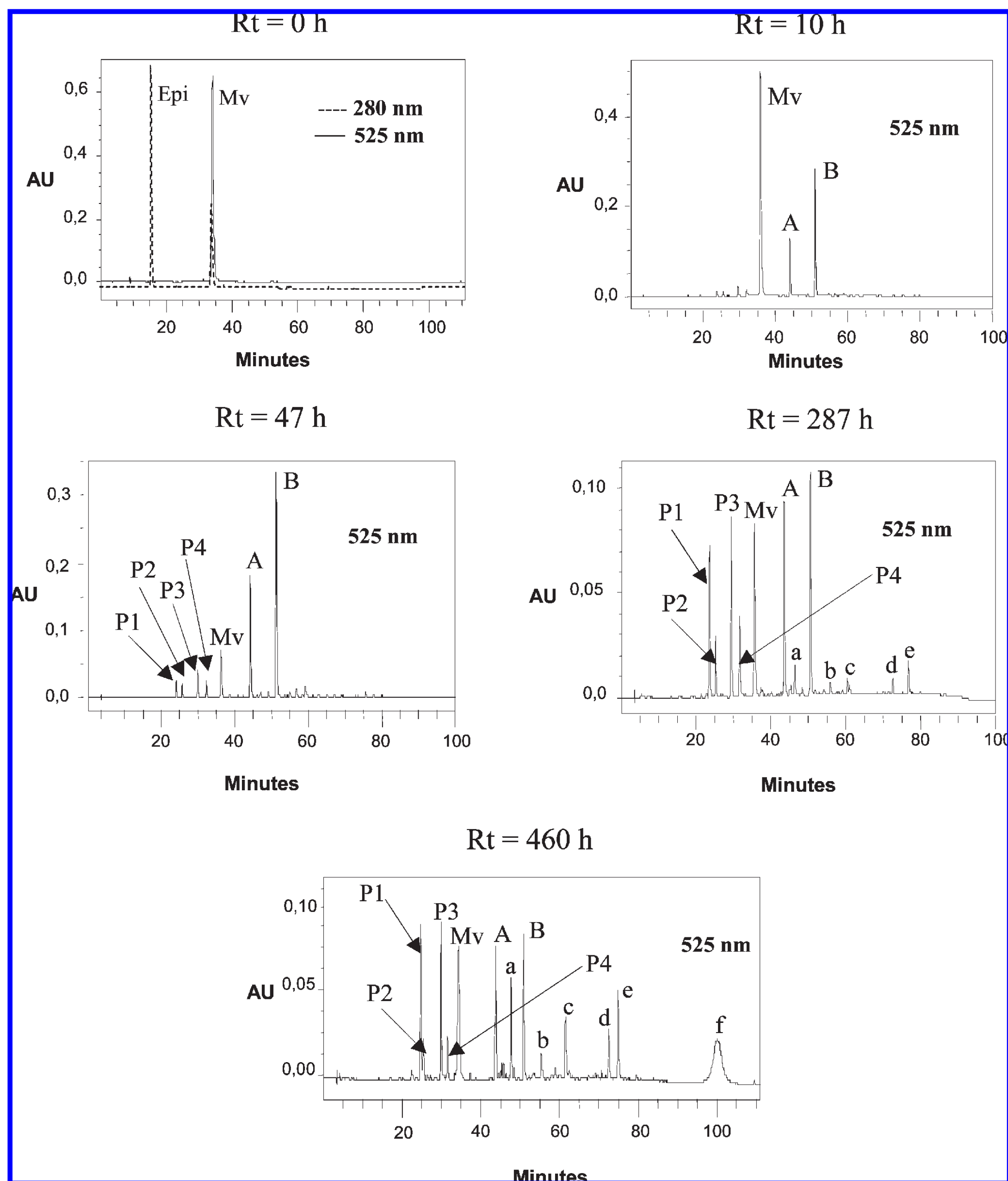

Figure 1. HPLC chromatograms of reaction solution conducted with malvidin 3-glucoside, epicatechin, and acetaldehyde (pH 1.7; 30 °C) at different reaction times. Rt, reaction time; Mv, malvidin 3-glucoside; Epi, epicatechin; A and B are two isomers of epicatechin-ethyl-malvidin 3-glucoside; P1−P4 are four isomers of 1′′-hydroxyethyl-epicatechin-ethyl-malvidin 3-glucoside; e, malvidin 3-glucoside-vinyl-epicatechin; a−d, f, unidentified colored compounds.

by measuring the absorbance at 515 nm of standard samples of DPPH• at different concentrations. The percentage of DPPH• remaining at the steady state, which was calculated as % $DPPH^•_{rem}$ = $100[DPPH^•]_t/[DPPH^•]_{t=0}$, was plotted against the amount of sample divided by the initial concentration of DPPH•. Each point was acquired in triplicate. A dose response curve was obtained for each polyphenol fraction. The antiradical activity is expressed as EC_{50} [(mg/L) of antioxidant/(mg/L) of DPPH•], which is defined as the amount of antioxidant needed to decrease the initial DPPH• concentration by 50% (*25*). The results can also be expressed as antiradical power (ARP = $1/EC_{50}$) (*25*).

For monitoring the variation in DPPH• scavenging activity of the model wine solution during the reaction between malvidin-3-*O*-glucoside−epicatechin mediated by acetaldehyde, the manipulation was identical to that described above, but the results were expressed directly by percentage of inhibition of DPPH• of the reaction solution at the steady state (% inhibition), that is, % inhibition = $100 \times (A_0 - A_t)/A_0$, where A_t is the absorbance at 515 nm obtained at the steady state and A_0 is the absorbance at 515 nm of the control sample.

Statistical Analysis. All analyses were performed in triplicate, and the data are presented as mean ± standard deviation (SD). One-way analysis of variance and comparison of means (LSD, 99% level) were carried out using Statistica v. '98' edition (StatSoft Inc., Tulsa, OK).

RESULTS AND DISCUSSION

The method for fractionation of red wine polyphenols established in our laboratory very recently (*21*) permitted us to isolate, for the first time, the red wine phenolic complex fraction and thus to verify and compare its antioxidant activity with those of other phenolic fractions or standards. DPPH• is a useful reagent for studying the free radical-scavenging activities of antioxidant compounds. The DPPH• test has been proved to be simple and efficient for evaluation of antioxidant activities of grape polyphenols (*24*). The kinetics of the reaction was dependent on the concentration and structural type of the compound. For each

Table 2. ESI-MS and MS^n Fragmentation of the Major Compounds in the HPLC Chromatograms of **Figure 1**

molecular ion $[M^+]$ (m/z)	MS^n fragment ion[a] (m/z) MS2	MS3	MS4	compound[b]
493	**331**	315, 299		Mv
805	**643**	491		e
809	**647**, 519, 331	357		A
809	**647**, 519, 331	357		B
853	**563**	**401**	357	P1
853	**563**	**401**	357	P2
853	**563**	**401**	357	P3
853	**563**	**401**	357	P4

[a] Fragment ions written in bold were subject to further fragmentation. [b] Abbreviations: Mv, malvidin 3-glucoside; e, malvidin 3-glucoside-vinyl-epicatechin; A and B, two isomers of epicatechin-ethyl-malvidin 3-glucoside, P1−P4, four isomers of 1′′-hydroxyethyl-epicatechin-ethyl-malvidin 3-glucoside.

tested compound, the percentage of reducing DPPH• increased dose-dependently at a given concentration range. According to these plots, the relative concentration (mg/mg of DPPH•) of each tested compound necessary to reduce 50% of DPPH• (EC_{50}) can be determined. EC_{50} is a parameter widely used for the antioxidant activity of one compound (*26*, *27*), sometimes expressed as antiradical power (ARP = $1/EC_{50}$) (*25*). **Table 1** lists the values of EC_{50} and ARP of the tested compounds.

It can be seen, from **Table 1**, that monomer flavanols catechin and epicatechin and the flavonol quercetin present the highest scavenging activity on DPPH• (ARP > 10), followed by red wine phenolic complexes (ARP = 7.95 ± 0.36) and red wine polymeric proanthocyanidin fraction (ARP = 6.74 ± 0.35), whereas the red wine anthocyanin fraction and malvidin 3-glucoside present very low scavenging activity on DPPH• (ARP = 3.69 ± 0.13 and 5.14 ± 0.13, respectively). On the basis of the chemical structural features of these phenolic compounds, the fact that catechin, epicatechin, and quercetin possess strong antioxidant activity may be explained by their B-ring, which has a catechol-type structure acting as reducing agent, whereas the two major anthocyanins in red wine, that is, malvidin 3-*O*-glucoside and peonidin 3-*O*-glucoside, lack such reducing structural features; thus, malvidin 3-*O*-glucoside and the red wine anthocyanins fraction have much lower antioxidant activities. It should be especially mentioned here that, although various authors reported strong antioxidant activities of anthocyanins (*28*) and good correlation between anthocyanins concentration and antioxidant activity (*29*), few published works have verified the antioxidant activity of individual anthocyanins or the purified anthocyanins fraction; anthocyanin extracts obtained by the usual extraction methods contain important amounts of catechins and proanthocyanidins, and thus the latter compounds would contribute significantly to the total antioxidant activity of the extract (*28*).

Red wine phenolic complexes are formed essentially by direct or indirect interaction between catechins or proanthocyanidins with anthocyanins during the wine aging process (*3*). From **Table 1**, it can be noted that the red wine phenolic complexes fraction possesses strong antioxidant activity (7.95 ± 0.36), although it is lower than that of catechins but higher than that of malvidin 3-*O*-glucoside. These results would suggest the major condensation reactions of red wine during the aging process do not change the antioxidant activity of each constitutive phenolic compound.

To further confirm the antioxidant activities of red wine phenolic complexes, the interaction between epicatechin and malvidin 3-glucoside mediated by acetaldehyde has been evaluated in a model wine solution (12% alcohol, v/v; pH 1.7). Kinetic evolution of malvidin 3-glucoside and formation of new condensation

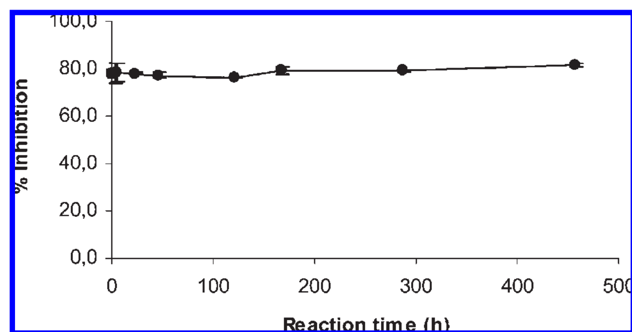

Figure 2. Variation in antioxidant activities of model wine solution during the reaction between epicatechin and malvidin 3-glucoside mediated by acetaldehyde (pH 1.7; 30 °C; ethanol 12%; tartaric acid 5 g/L).

products were monitored by HPLC-DAD and ESI-MS^n, and changes in antioxidant activities of the model wine solution during the reaction period were verified by DPPH• test. These results are presented in **Figure 1**, **Table 2**, and **Figure 2**, respectively.

In **Figure 1**, peaks A and B are two well-known indirect condensation products between epicatechin and malvidin 3-glucoside, that is, two isomers of epicatechin-ethyl-malvidin 3-glucoside (*30*). P1, P2, P3, and P4 are four isomers of 1′′-hydroxyethyl-epicatechin-ethyl-malvidin 3-glucoside identified in our previous works (*22*, *23*). In this work, their identities were confirmed by ESI-MS and ESI-MS^n fragmentation analysis, and the results are presented in **Table 2**.

From **Figure 1**, it can be seen that the two well-known indirect condensation products A and B were formed very quickly, but degraded at the later stage of the reaction. The four isomers of 1′′-hydroxyethyl-epicatechin-ethyl-malvidin 3-glucoside, P1−P4, were formed slowly but were very stable. At the end of the reaction period, the four isomers of 1′′-hydroxyethyl-epicatechin-ethyl-malvidin 3-glucoside became the major pigmented complexes in the reaction solution. Moreover, other colored reaction products, that is, peaks a−f, were also formed during the latter stage of the reaction. Peak e has been identified as malvidin 3-glucoside-vinyl-epicatechin in our previous works (*22*, *23*) and confirmed in this work by ESI-MS and MS^n analysis (**Table 2**), but peaks a−d and f remain to be identified.

Although the phenolic composition of the model wine solution varied markedly during the reaction period due to the formation of new reaction products as described above, it is very important to note, from **Figure 2**, that there was no change in antioxidant activity of the model wine solution throughout the reaction period. These results showed that the condensation products formed during the reaction possess potent antioxidant activities as their constitutive phenolics. In other words, the condensation reactions should not affect the antioxidant activities of each of the phenolic compounds constituting condensation product molecules. The results obtained by this experiment (**Figure 2**) are clearly in agreement with those obtained by in vitro study of antioxidant activities of red wine phenolic fractions (**Table 1**). Although some previous works suggested that phenolic complexes and condensation products might contribute markedly to the overall antioxidant activity of red wine (*31*), the present work provides, for the first time, direct evidence about the in vitro antioxidant activities of red wine phenolic complexes and condensation products.

ABBREVIATIONS USED

DPPH, 1,1-diphenyl-2-picrylhydrazyl; $DPPH^{•}_{rem}$, DPPH• remaining at the steady state; F_{cat}, catechin fraction; F_{olig}, oligomeric proanthocyanidin fraction; F_{pigmt}, fraction containing

anthocyanins, polymeric proanthocyanidins, and other pigmented complexes; F_{ant}, fraction of anthocyanins and their derivatives; F_{poly}, polymeric proanthocyanidin fraction; $F_{complex}$, fraction of phenolic complexes; EC_{50}, antiradical activity; ARP, antiradical power.

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