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Influence of Solvent Composition on Antioxidant Potential of Model Polyphenols and Red Wines Determined with 2,2-Diphenyl-1-picrylhydrazyl

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Supporting Information

ABSTRACT: Solvent composition has a large influence on measured antioxidant potential (AOP) of model polyphenols and red wines with 2,2-diphenyl-1-picrylhydrazyl (DPPH). We have shown that incorporation of aqueous buffer in the assay medium results in higher reactivity of catechin and caftaric acid, which are among major polyphenolic constituents of wines. Consequently, AOPs of red wines determined after 60 min of incubation at 25 °C in buffered methanol are 1.5−1.6-fold of values determined in methanol. Even in buffered methanol, wine polyphenols are not fully oxidized after 60 min. Only about half of Trolox equivalents were determined in comparison to the Folin−Ciocalteu assay. Buffer composition, pH, time of incubation, temperature, and concentration of antioxidants and DPPH all contribute to the resulting value of the AOP being standardized or at least strictly reported because minor differences in experimental procedures can account for large variations in determined AOP with DPPH for the same samples.

KEYWORDS: *Antioxidant capacity, DPPH radical, Folin−Ciocalteu, wine, polyphenols, solvent composition, Trolox equivalent*

INTRODUCTION

Antioxidants are important components of fruits and vegetables. They can scavenge reactive oxygen and nitrogen species and bind redox active metal ions, resulting in the stabilization of the food against oxidative changes. Consumption of foods with high concentrations of polyphenols has been shown to correlate positively with a lower incidence of several human diseases.[1]

Chromatographic separation followed by various modes of detection is the method of choice for assaying the composition of polyphenols in complex matrices.[2] The results obtained by such analyses often lack an understanding of the reactivity of polyphenols in radical and redox reactions.

The ease of the experimental procedures coupled with the inexpensive equipment required has resulted in widespread application of a variety of methods based on spectrophotometric detection for evaluating antioxidant potential (AOP). Folin−Ciocalteu (FC), ferric reducing ability of plasma (FRAP), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays are those most commonly used.[3]

The DPPH method is based on electron transfer (ET) from the ionized antioxidant to the radical oxidant,[3] resulting in the formation of products with lower absorbance in the visible range. Hydrogen atom transfer from non-ionized antioxidants is the other mechanism involved in the reaction of polyphenolic antioxidants with free radicals.[4−6] It contributes significantly to the overall reaction rate only in aprotic organic solvents. Kinetic solvent effects were attributed to the differential solvation of the antioxidants and are independent of the nature of the radical.[7] In hydrogen-bonding solvents, which stimulate the ionization, ET prevails.[8] Consequently, the rate of reaction of polyphenols with DPPH is higher in alcohols than in aprotic organic solvents.[6] The reaction rates of antioxidants with DPPH in alcohols still differ by a few orders of magnitude.[6,9] Some, such as ascorbic acid, react completely in the range of seconds, whereas certain polyphenols do not reach completion even after a few hours.[10] The rate of reaction can be further increased by incorporation of water into the reaction mixture with alcohol, as shown for α-tocopherol[11] and some polyphenols.[12] The mixture of water and alcohol is nevertheless rarely applied for evaluation of AOP of food samples with DPPH, despite the fact that water is the main solvent for polar antioxidants in biological systems.[13] A much more common practice is to perform the DPPH assay in alcoholic solution, where samples are incubated at room temperature for 15−120 min, before the absorbance in the range of 515−520 nm is measured.[14] Under such conditions, the reaction is far from completion when complex food matrices are analyzed.

We have used red wine and model antioxidants to show that solvent composition has a large influence on determined AOP with DPPH. Red wine is a rich and complex source of polyphenols.[15] Monomeric (e.g., catechin) and oligomeric flavanols make the most abundant class of polyphenolic compounds in red wines.[16] Typically, the AOP of wine is determined in MeOH.[17] The obtained results can be expressed as DPPH equivalents in wine,[18] Trolox equivalents (TE) in wine,[19] the percent of inhibition of DPPH at fixed dilution of wine into the assay solution,[20] or a dilution of wine into the

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assay solution that is needed for 50% reduction of the absorbance (IC_{50}) of DPPH.[21] The extensive survey of over 100 Spanish red wines revealed that average AOP determined by DPPH is 14 mM TE and total polyphenol content determined by the FC assay 2400 mg of gallic acid equivalents/L of wine.[22] Interestingly, FC and DPPH assays are newer normalized to the same antioxidant; therefore, it is practically impossible to compare determined AOP by both methods. The lack of standardized experimental protocols and the kinetic nature of the assay, coupled even with different methods of evaluating the results,[14] makes the results of the DPPH assay difficult to interpret and sometimes confusing in comparison to the FC assay, which is the standard procedure for the assay of total antioxidants.

The systematic study of the influence of the aqueous phase on determined AOP of food samples with DPPH under typical assay conditions was to our knowledge not performed. In the present study, we have analyzed the influence of aqueous buffer in mixtures with methanol on the value of the AOP obtained for model antioxidants and red wines. The rationale for the study was (1) to define the influence of solvent composition, time of incubation, and pH of buffer used in the DPPH assay on the resulting AOP and (2) to compare the AOP values of model antioxidants and wines determined by DPPH and FC assays.

MATERIALS AND METHODS

Materials. (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox; 238813), gallic acid (G7384), caftaric acid (15029), (+)-catechin (C1251), FC reagent (47641), and DPPH (D9132) were from Sigma-Aldrich (Steinheim, Germany).

Acetic acid (1.00063), NaOH (1.06498), Na_2CO_3 (1.06392), and methanol (1.06009) were obtained from Merck (Darmstadt, Germany).

Slovenian red wines Refosco (dry), Pinot noir (dry), and Blaufränkisch (dry) were obtained from a local supermarket. All water was purified using a Milli-Q system from Millipore (resistivity > 18 MΩ cm).

DPPH Assay. Solutions of 310 μM catechin and 200 μM DPPH were prepared in methanol. Acetate (50 mM) at pH 5.25 was made by titrating the acid with NaOH. The assay solutions were made by mixing 1000 μL of DPPH solution with appropriate volumes of acetate buffer, Milli-Q water, and methanol to give a total volume of 1930 μL. The reaction was started by adding 70 μL of 310 μM catechin dissolved in methanol to the assay solution and thoroughly mixing. All buffers, solvents, and microcentrifuge tubes (2 mL) were incubated at 25 °C prior to the assay. Solutions containing DPPH and catechin were incubated for 60 min in the dark at 25 °C before measuring absorbance at 520 nm (Varian Cary 100 UV−vis spectrophotometer with a temperature controller). The absorbances of the samples (100 μM DPPH and 10.9 μM catechin) were subtracted from blanks (100 μM DPPH) prepared in the appropriate solvents, and results were expressed as dA_{520}. All experiments were performed in triplicate.

The influence of the incubation time was on the value of the antioxidant potential. Model antioxidants were dissolved in water, and wines were diluted with water. A total of 5 mL of 200 μM DPPH was mixed with 4.5 mL of methanol or 55 mM acetate at pH 5.25 in 15 mL centrifuge tubes. The reaction was started by adding 500 μL of the antioxidant solution in water and thoroughly mixing. All solutions were incubated in the dark at 25 °C. At predetermined time intervals, aliquots were transferred into a cuvette and absorbances were read at 520 nm. The absorbances were subtracted from the blanks that were prepared in the same way except that 500 μL of Milli-Q water was added to the assay medium. The effect of buffer pH on quenching of the DPPH radical by antioxidants was determined in 50 mM acetate buffers at pH 3.75, 4.75, 5.25, and 5.75. The results were expressed as dA_{520}. All experiments were performed in triplicate.

The molar ratio of Trolox/antioxidant and TE antioxidant potential of wines were determined in methanol containing 5 vol % Milli-Q water or 50 vol % acetate buffer at pH 5.25. The assay solutions were made by mixing 1000 μL of 200 μM DPPH and 900 μL of methanol or 900 μL of 55 mM acetate buffer. The calibration curves for each antioxidant were prepared by pipetting 10−100 μL of antioxidants diluted in water into the assay solutions and making up to 2000 μL with Milli-Q water. Solutions were incubated in the dark at 25 °C for 60 min, and then absorbances were read at 520 nm. The absorbances were subtracted from the blanks (100 μL of Milli-Q water was added), and the results were expressed as dA_{520}. The molar concentrations of antioxidants or dilutions of wines required to quench 0.55 absorbance at 520 nm (conditions that correspond to IC_{50}) were determined from calibration curves. The molar ratio of Trolox/antioxidant and TE of antioxidants in wines (millimolar Trolox in wine) were determined by normalization to dA_{520} obtained from the calibration curve for Trolox. The molar ratio DPPH/Trolox was determined from the Beer−Lambert law (ε_{DPPH} = 11 000 L mol^{-1} cm^{-1}).

The total volume of the methanol/water mixture is less than the sum of the individual volumes.[23] Because maximal differences in volumes do not exceed 3%, we assume a first approximation that the volumes are additive, which is practically always applied in typical assays of antioxidant activity.

FC Assay. Appropriate volumes of model antioxidants or wines diluted with water were pipetted into a 2 mL microcentrifuge and made up to 1400 μL with Milli-Q water. A total of 300 μL of FC reagent, diluted 3-fold with water, was added, and the solution was mixed. After 5 min, 300 μL of 20% Na_2CO_3 was added and mixed. The absorbances at 765 nm were measured after 90 min of incubation at room temperature. The molar ratio of Trolox/antioxidant was determined from calibration curves for each antioxidant, comparing it to the calibration curve obtained with known concentrations of Trolox. The antioxidant potential of wines was expressed as TE (millimolar Trolox in wine).

Determination of Total SO_2 in Wines. Total SO_2 in wines Pinot noir (55 mg/L), Blaufränkisch (70 mg/L), and Refosco (50 mg/L) was determined by a simplified Ripper method[24], in which alkaline hydrolysis of SO_2 adducts precedes iodometric titration at acidic pH.

Experimental Error. All experiments were performed in triplicate, including all dilutions and weighing of model antioxidants. The standard deviation of measured absorbance in DPPH and FC assays never exceeded 3%. For FC assays, in which the absorbance of the blank is practically zero, the experimental error is consequently also not greater than 3%. For the DPPH assay, where AOP is determined by subtraction of absorbance of the sample from the blank, experimental error is significantly higher and depends upon dA_{520}. The smaller the difference, the larger the experimental error. For conditions around IC_{50} (dA_{520} = 0.55), the standard deviations of determined AOPs are not greater than 10%. These are the conditions that were used for estimating the AOP values presented in Table 2.

RESULTS

Reactivity of Catechin in the DPPH Assay. Solvent composition has a large influence on the reactivity of catechin in the DPPH assay (Figure 1), which is, together with its derivatives, the most common polyphenolic compound in red wines.[16] Incorporation of 2.5% acetate buffer in the assay medium resulted in 60% greater dA_{520} than that determined in pure methanol for the same concentration of catechin. Higher contents of acetate buffer in the assay medium result in even larger dA_{520}. When the assay was performed in the mixture containing 50 vol % methanol and 50 vol % acetate buffer, the determined value of dA_{520} was more than 2-fold that in pure methanol.

Water has a less pronounced effect on the reaction of catechin than acetate buffer. When the water content is increased up to 50 vol %, dA_{520} is 70% higher than in methanol. If the content of water is 5% or less, dA_{520} does not differ from

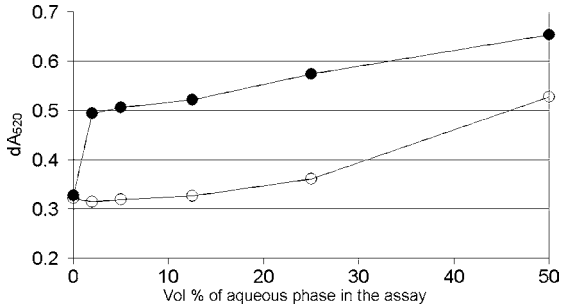

Figure 1. Influence of solvent composition on the quenching of the DPPH radical by catechin. Catechin (10.9 μM) was incubated in solutions containing 100 μM DPPH. Absorbances at 520 nm of the blanks (100 μM DPPH) and samples containing catechin were measured after 60 min of incubation at 25 °C in the dark, and results were expressed as dA_{520}. The assay was performed in solvents containing (○) different volume percentages of Milli-Q water or (●) 50 mM acetate at pH 5.25 in the mixture with methanol.

that in pure methanol. All further experiments on the reaction of antioxidants with DPPH were performed in either a 50:50 mixture of methanol/acetate buffer or in methanol containing 5 vol % Milli-Q water. For simplicity, the solvent containing 5 vol % water in methanol is hereafter referred to as methanol.

Absorbance Spectra and Stability of DPPH. The absorbance of DPPH is red-shifted in 50:50 (v/v) methanol/acetate buffer at pH 5.25 from that in methanol (see the Supporting Information). DPPH (100 μM) had A_{520} = 1.10 in both solvents, when measured in a 1 cm cuvette. This is in accordance with the reported value of approximately 11 000 L mol^{-1} cm^{-1} for ε of DPPH in MeOH in the range from 515 to 520 nm at 25 °C.[25]

1,1-Diphenyl-2-picrylhydrazine ($DPPH_2$), prepared from 100 μM DPPH with 5-fold molar excess of ascorbic acid, also absorbs at 520 nm (see the Supporting Information). The ε value of $DPPH_2$ in methanol is 3% of that of DPPH and 5% in 50:50 (v/v) methanol/acetate buffer at pH 5.25.

The stability of DPPH in solvents determines the applicability of the assay when antioxidants with slow reaction rates are being assessed. Its stability in mixtures of methanol/acetate buffers (pH 3.75−5.75) is lower than in methanol alone (Figure 2). During the shorter incubation of 2 h or less, commonly applied for the routine assessment of antioxidant potential, the decrease of absorbance in all solvents analyzed, except for MeOH at pH 3.75, is less than 3%.

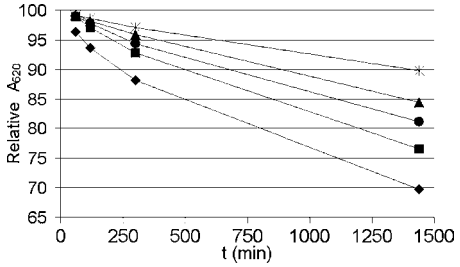

Figure 2. Stability of 100 μM DPPH in (∗) a mixture of 5 vol % Milli-Q water and 95 vol % methanol and mixtures of 50 vol % acetate buffers and 50 vol % methanol at 25 °C. The assay was performed with 50 mM acetate buffers at pH (◆) 3.75, (■) 4.75, (●) 5.25, and (▲) 5.75.

Quenching of the DPPH Radical in the Presence of Antioxidants as a Function of Time. The amount of DPPH reduced to $DPPH_2$ by antioxidants was determined in methanol and in the mixture of methanol/acetate buffer at pH 5.25 for three red wines and model antioxidants. Absorbances of the samples (antioxidant and DPPH) were subtracted from blanks (only DPPH), both measured as a function of time, and results were expressed as dA_{520} (Table 1)

The rate of $DPPH^{\bullet}$ quenching by catechin is strongly affected by the solvent composition (Table 1). dA_{520} determined after 30 min in the mixture of buffer and methanol was almost 2-fold that observed in MeOH, in which 300 min of incubation was needed to achieve the same dA_{520}. Solvent composition has a less pronounced influence on dA_{520} determined after 30 min for caftaric acid, the major polyphenolic antioxidant in white wines.[16] In contrast to catechin, the difference between solvents increased after prolonged incubation. Gallic acid, which is routinely used as a model antioxidant in the FC assay, also gave a higher response in the mixture of buffer and methanol. Trolox is classified as a so-called fast antioxidant,[10] and the reaction reaches a plateau after 30 min only in both solvents.

All three red wines show a similar kinetic profile. The value of dA_{520} is higher in the mixture of buffer and methanol than in methanol for each wine at all time intervals. The largest differences between solvents are observed after 30 min of incubation, where 1.4−1.6-fold dA_{520} is determined in the mixture of buffer and methanol in comparison to methanol. Prolonged incubation results in a higher dA_{520} and smaller difference between values determined in both solvents. Reactions do not reach completion even after 5 h because all dA_{520} values measured after 24 h are higher. To compare our results to works by others, we have chosen 1 h of incubation for further experiments.

Quenching of the DPPH Radical in the Presence of Antioxidants as a Function of pH. The pH of the acetate buffer used in the DPPH assay influences the value of dA_{520} of some model antioxidants and wines. With the exception of Trolox, incorporation of any of the buffers used in the assay results in larger values of dA_{520} after 1 h (Figure 3). The influence of buffer pH on dA_{520} of model antioxidants was most pronounced for caftaric acid, where larger values were determined in buffers with higher pH. Only gallic acid showed the trend that dA_{520} is higher at lower pH. A similar pH dependence for gallic acid was observed when AOP was determined in micelle systems.[26] Catechin did not show a large pH dependence, because less than 10% higher values were observed at pH 5.75 than at pH 3.75. In accordance with this result, values differ by less than 10% in buffers for all of the red wines analyzed. Reactivity of wine polyphenols was not tested at higher pH because of instability of anthocyanins at neutral pH[27] and a high molar extinction coefficient at 520 nm of deprotonated $DPPH_2$ at slightly basic pH.[28]

Comparison of Antioxidant Potentials Determined by DPPH and FC Assays. Higher AOPs were determined with DPPH for most antioxidants and all wines tested after 60 min of incubation in the mixture of acetate buffer and methanol than in the same assay in the absence of acetate (Table 2). AOPs were determined from calibration curves at a concentration of antioxidant that quenched 50 μM DPPH (dA_{520} = 0.55). The calibration curves were not linear for catechin, as also previously observed,[29] and red wines. Therefore, differences in AOP values determined in both

Table 1. Influence of the Incubation Time on the Quenching of 100 μM DPPH by Antioxidants

			dA_{520}				
		solvent	30 min	60 min	120 min	300 min	1440 min
catechin	6.5 μM[a]	buffered MeOH	0.325	0.362	0.393	0.433	0.499
		MeOH	0.166	0.198	0.242	0.323	0.482
caftaric acid	15 μM[a]	buffered MeOH	0.347	0.398	0.481	0.668	0.974
		MeOH	0.301	0.315	0.329	0.369	0.554
gallic acid	8.5 μM[a]	buffered MeOH	0.550	0.551	0.566	0.586	0.636
		MeOH	0.445	0.455	0.487	0.522	0.590
Trolox	24 μM[a]	buffered MeOH	0.483	0.488	0.496	0.502	0.532
		MeOH	0.498	0.503	0.504	0.508	0.523
Pinot noir	1:833[b]	buffered MeOH	0.399	0.439	0.477	0.530	0.648
		MeOH	0.251	0.292	0.327	0.380	0.522
Blaufränkisch	1:667[b]	buffered MeOH	0.418	0.458	0.501	0.568	0.692
		MeOH	0.263	0.306	0.346	0.411	0.546
Refosco	1:1250[b]	buffered MeOH	0.311	0.342	0.375	0.418	0.493
		MeOH	0.218	0.251	0.283	0.328	0.423

[a]Concentration of the antioxidant in the assay medium. [b]Dilution of wine in the assay medium.

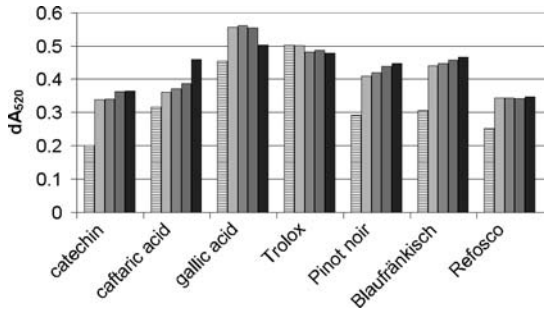

Figure 3. Influence of pH on the quenching of 100 μM DPPH by antioxidants. Concentrations of the antioxidants and dilutions of wines are the same, as shown in Table 1. Experiments were performed in (bar with horizontal lines) a mixture of 5 vol % Milli-Q water and 95 vol % methanol and (gray bars) mixtures of 50 vol % acetate buffers and 50 vol % methanol. Acetate buffers (50 mM) with pH 3.75, 4.75, 5.25, and 5.75 were used. Higher pH is denoted by a darker color. The absorbances of the samples (antioxidants and DPPH) were subtracted from blanks (DPPH). The resulting dA_{520} is proportional to the determined antioxidant potential. All samples and blanks were incubated at 25 °C, and absorbances were measured after 60 min.

Table 2. Influence of the Solvent and Type of Assay on the Determined Antioxidant Potential of Model Antioxidants and Wines

	molar ratio of Trolox/antioxidant		
	DPPH (buffered MeOH)	DPPH (MeOH)	FC
catechin	2.8	0.95	3.7
caftaric acid	1.2	0.9	2.4
gallic acid	3.4	3.0	2.7
Trolox	1.0[a]	1.0[a]	1.0
	TE in wine (mM)		
	DPPH (buffered MeOH)	DPPH (MeOH)	FC
Pinot noir	18	11	31
Blaufränkisch	15	9.5	27
Refosco	20	13	39

[a]The molar ratio of DPPH/Trolox is 2.0.

solvents are larger than anticipated from data shown in Table 1 and Figure 3. The molar ratio DPPH/catechin determined in the presence of acetate is almost 3 times the ratio in methanol. For caftaric acid, the molar ratio is 1.3, and for gallic acid, the molar ratio is 1.1. Two molecules of DPPH are needed to quench Trolox under both conditions tested. Trolox is therefore an ideal standard because it has the same response in both solvents. Determined AOPs of red wines Pinot noir, Blaufränkisch, and Refosco, expressed as TE, in buffered methanol are 1.5−1.6-fold the values determined in methanol (Table 2).

Model antioxidants, with the exception of gallic acid, have higher AOPs (normalized to Trolox) in the FC assay than in the DPPH assay (Table 2). The difference is largest for caftaric acid, where 2-fold AOP was determined by the FC assay in comparison to the DPPH assay in buffered MeOH. Such a difference can be ascribed to a relatively slow reaction rate observed in the DPPH assay (Table 1). AOP of catechin in the FC assay is 1.3-fold of AOP determined with DPPH in buffered MeOH. The difference is much larger (3.9-fold) when AOP determined with FC is compared to the DPPH assay in methanol (Table 2), which can again be ascribed to the slow reaction rate in methanol (Table 1). Red wines show a similar trend to catechin and caftaric acid, which are among major polyphenolic constituents. AOPs of red wines in the FC assay are 1.6−1.9-fold AOP determined in buffered methanol.

DISCUSSION

Antioxidant Potential of Model Polyphenols Determined by the DPPH Assay. Results presented in Figure 1 demonstrate that incorporation of water and particularly water-based buffer into the assay medium results in higher dA_{520} and, thus, a higher apparent AOP of catechin. In the presence of water and, even more importantly, acetate, protolysis increases and the ET mechanism accounts for the higher reactivity. A similar observation that an increase in the concentration of water results in proportionally higher reactivity was observed for butylated hydroxytoluene (BHT) and one of the α-tocopherol analogues.[30] The proportion of water or ionic strength of the buffer cannot be increased further because DPPH precipitates under such conditions.[11]

It was previously shown that oxidation of catechin with DPPH in methanol is a multi-step reaction. A fast initial phase is followed by a slower oxidation step of a partially oxidized molecule.[31] The fast oxidation step results in the formation of semiquinone radicals and quinones. The latter can enter into

Table 3. Comparison of Experimental Conditions of the DPPH Assay and Determined TE in Red Wines by DPPH and FC Assays

	experimental conditions for the DPPH assay			TE in wine		
reference	solvent	incubation time (min)	incubation temperature (°C)	DPPH	FC	DPPH/FC
Rivero-Pérez et al.[22]	98% MeOH	120	room temperature	1.2–25[a] (14)[b]	29–50[a] (40)[b]	0.35
Ginjom et al.[19]	98% MeOH	30	30	11–21[a] (16)[b]	27–59[a] (41)[b]	0.39
Porgali et al.[39]	97% MeOH	25	not reported	5.1–14[a] (8.5)[b]	30–60[a] (40)[b]	0.21
Piljac et al.[38]	99% MeOH	60	20	8.2–11[a] (13)[b]	53–75[a] (62)[b]	0.21
Li et al.[40]	98% MeOH	30	not reported	4.2–17[a] (10)[b]	23–48[a] (34)[b]	0.29
Staško et al.[18]	50% MeOH	10	20	13–18[a] (16)[b]	24–56[a] (38)[b]	0.42
this study	95% MeOH	60	25	9.5–13[a] (11)[b]	27–39[a] (32)[b]	0.34
this study	50% MeOH + 50% acetate	60	25	15–20[a] (18)[b]	27–39[a] (32)[b]	0.56 (0.81)[c]

[a]The range of determined TE for the red wines in the study. [b]The average TE for the red wines in the study. [c]After 24 h of incubation for the DPPH assay.

additional reactions, where solvent adducts, dimers, and degradation products that can also be oxidized are formed.[29,32] The overall kinetics, with the exception of initial fast phases, is very complex, and calculated kinetic constants are often the average of few reactions that all result in the quenching of the DPPH radical. Therefore, in our study, we focused only on the stationary measurements that are applied in routine analysis and not the comparison of apparent kinetic constants.

After 24 h of incubation, only a minor difference between dA_{520} for catechin in methanol and buffered methanol was observed (Table 1). This is a strong indication that only the kinetics are different and not the course of the reaction. The second phase of oxidation of caftaric acid is even slower than that of catechin, and in methanol, the "equilibrium" is not reached after 24 h. Accordingly, differences in dA_{520} are greater after 24 h of incubation than after 30 min of incubation. These slow processes are quantitatively very important and significantly increase the number of exchanged electrons.[32] As with DPPH, two phases were observed in the reaction of polyphenols with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).[33] Slow reaction rates in methanol can be at least partially attributed to the acid impurities in commercially available solvents that drastically reduce the reaction rates of polyphenols with DPPH already in a micromolar range.[6]

Incorporation of Acetate Buffer in the Assay Medium Has a Minor Influence on the Stability of DPPH and Spectral Properties of DPPH and $DPPH_2$ in the Visible Range. The stability of DPPH in hydrocarbon solvents[34] or in mixtures of polar organic solvents and buffer[35] can become a serious problem when prolonged incubations of antioxidant and DPPH are performed. We have shown that DPPH is indeed less stable in buffered methanol than in methanol and that lowering the pH in the range of 5.75–3.75 results in lower stability. Nevertheless, the decrease in absorbance is much slower than previously described. The difference can be attributed to the fact that, in the 75% aqueous phase used in previous experiments,[35] the decrease in absorbance could be the result of precipitation of DPPH[11] and not just DPPH reduction.

Antioxidant Potential of Red Wines Determined by the DPPH and FC Assays. Antioxidant potential of red wines was simultaneously determined by DPPH and FC assays within several studies. The results of the FC assay in those papers were expressed as gallic acid equivalents, and the results of the DPPH assay in those papers were expressed as TE. We have normalized AOP data published in those papers to TE in accordance with the results shown in Table 2 and compared them to the results of our study (Table 3). The average AOPs determined with the DPPH assay for all studies are lower in comparison to the FC assay, ranging from 0.21- to 0.42-fold (Table 3). The large variation between different studies can at least be partially attributed to the variations in the protocols for the DPPH assay, because kinetics are faster in the FC assay[36,37] and the influence of experimental variables is, therefore, smaller. The lowest relative AOPs determined by DPPH can be attributed to the low temperature of the assay[38] and the short incubation time,[39] whereas the highest relative AOP was determined after a relatively short incubation in the presence of 50% water in the assay media,[18] where higher AOP of catechin is determined (Figure 1). The relative AOP determined in 95% methanol in our study is comparable to the large survey of Spanish wines.[22] Incorporation of acetate buffer resulted in larger relative AOP determined by DPPH (0.56) than in other published papers. The differences between both assays are even smaller if calculations were based on 24 h of incubation (Table 1) because only 20% lower AOP of red wines is determined by the DPPH assay in buffered methanol in comparison to the FC assay (Table 3).

There is no magic answer to the question of how to perform the DPPH assay, although application of buffered methanol undoubtedly lead to more reliable results. Incorporation of aqueous buffer into assay media increases the reaction rates (Table 1) and stabilizes the system against the influence of protic solutes in the matrix, because already a small percentage of buffer can have a large influence on determined AOP (Figure 1). Polyphenols are naturally present in the aqueous environment, and it is more relevant to perform the DPPH assay in media that resemble such conditions.[9] Even then, a few hours of incubation would be needed for the reaction of most oxidizable groups. This is nevertheless impractical and prone to experimental error because of the instability of DPPH (Figure 2). The control of experimental variables that influence reaction rates in the DPPH assay and accordingly determined AOP is therefore extremely important. Buffer composition, pH, length of the assay, temperature, and concentration of antioxidants and DPPH should be standardized or at least reported in papers. Because polyphenols are not fully oxidized at "typical" incubation times, differences in experimental procedures can account for large variations in determined AOP with DPPH for the same samples.

■ ASSOCIATED CONTENT

Supporting Information

Absorption spectra of DPPH and $DPPH_2$ in MeOH and buffered MeOH. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.

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■ REFERENCES

(1) Halliwell, B.; Rafter, J.; Jenner, A. Health promotion by flavonoids, tocopherols, tocotrienols, and other phenols: Direct or indirect effects? Antioxidant or not? *Am. J. Clin. Nutr.* **2005**, *81*, 268S−276S.

(2) Kartsova, L. A.; Alekseeva, A. V. Chromatographic and electrophoretic methods for determining polyphenol compounds. *J. Anal. Chem.* **2008**, *63*, 1024−1033.

(3) Huang, D.; Ou, B.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841−1856.

(4) Barclay, L. R. C.; Edwards, C. E.; Vinqvist, M. R. Media effects on antioxidant activities of phenols and catechols. *J. Am. Chem. Soc.* **1999**, *121*, 6226−6231.

(5) Okada, Y.; Tanaka, K.; Sato, E.; Okajima, H. Kinetics and antioxidative sites of capsaicin in homogeneous solution. *J. Am. Oil Chem. Soc.* **2010**, *87*, 1397−1405.

(6) Foti, M. C.; Daquino, C.; Geraci, C. Electron-transfer reaction of cinnamic acids and their methyl esters with the DPPH• radical in alcoholic solutions. *J. Org. Chem.* **2004**, *69*, 2309−2314.

(7) Valgimigli, L.; Banks, J. T.; Ingold, K. U.; Lusztyk, J. Kinetic solvent effects on hydroxylic hydrogen atom abstractions are independent of the nature of the abstracting radical. Two extreme tests using vitamin E and phenol. *J. Am. Chem. Soc.* **1995**, *117*, 9966−9971.

(8) Litwinienko, G.; Ingold, K. U. Abnormal solvent effects on hydrogen atom abstractions. 1. The reactions of phenols with 2,2-diphenyl-1-picrylhydrazyl (DPPH•) in alcohols. *J. Org. Chem.* **2003**, *68*, 3433−3438.

(9) Musialik, M.; Kuzmicz, R.; Pawlowski, T. S.; Litwinienko, G. Acidity of hydroxyl groups: an overlooked influence on antiradical properties of flavonoids. *J. Org. Chem.* **2009**, *74*, 2699−2709.

(10) Brand-Williams, W.; Cuvelier, M. E.; Berset, C. Use of free radical method to evaluate antioxidant activity. *Lebensm.-Wiss. Technol.* **1995**, *28*, 25−30.

(11) Staško, A.; Brezova, V.; Biskupič, S.; Mišik, V. The potential pitfalls of using 1,1-diphenyl-2-picrylhydrazyl to characterize anti-oxidants in mixed water solvents. *Free Radical Res.* **2007**, *41*, 379−390.

(12) Mishra, K.; Ojha, H.; Chaudhury, N. K. Estimation of antiradical properties of antioxidants using DPPH assay: A critical review and results. *Food Chem.* **2012**, *130*, 1036−1043.

(13) Litwinienko, G.; Ingold, K. U. Solvent effects on the rates and mechanisms of reaction of phenols with free radicals. *Acc. Chem. Res.* **2007**, *40*, 222−230.

(14) Magalhães, L. M.; Segundo, M. A.; Reis, S.; Lima, J. L. F. C. Methodological aspects about in vitro evaluation of antioxidant properties. *Anal. Chim. Acta* **2008**, *613*, 1−19.

(15) Monagas, M.; Bartolomé, B.; Gómez-Cordovés, C. Updated knowledge about the presence of phenolic compounds in wine. *Crit. Rev. Food Sci. Nutr.* **2005**, *45*, 85−118.

(16) Waterhouse, A. L. Wine phenolics. *Ann. N. Y. Acad. Sci.* **2002**, *957*, 21−36.

(17) Paixão, N.; Perestrelo, R.; Marques, J. C.; Câmara, J. S. Relationship between antioxidant capacity and total phenolic content of red, rosé and white wines. *Food Chem.* **2007**, *105*, 204−214.

(18) Stasko, A.; Brezova, V.; Mazur, M.; Certik, M.; Kalinak, M.; Gescheidt, G. A comparative study on the antioxidant properties of Slovakian and Austrian wines. *LWT—Food Sci. Technol.* **2008**, *41*, 2126−2135.

(19) Ginjom, I. R.; D'Arcy, B. R.; Caffin, N. A.; Gidley, M. J. Phenolic contents and antioxidant activities of major Australian red wines throughout the winemaking process. *J. Agric. Food Chem.* **2010**, *58*, 10133−10142.

(20) Katalinić, V.; Milos, M.; Modun, D.; Musić, I.; Boban, M. Antioxidant effectiveness of selected wines in comparison with (+)-catechin. *Food Chem.* **2004**, *86*, 593−600.

(21) Šeruga, M.; Novak, I.; Jakobek, L. Determination of polyphenols content and antioxidant activity of some red wines by differential pulse voltammetry, HPLC and spectrophotometric methods. *Food Chem.* **2011**, *124*, 1208−1216.

(22) Rivero-Pérez, M. D.; Muñiz, P.; Gonzalez-Sanjosé, M. L. Antioxidant profile of red wines evaluated by total antioxidant capacity, scavenger activity, and biomarkers of oxidative stress methodologies. *J. Agric. Food Chem.* **2007**, *55*, 5476−5483.

(23) González-Salgadoa, D.; Nezbeda, I. Excess properties of aqueous mixtures of methanol: Simulation versus experiment. *Fluid Phase Equilib.* **2006**, *240*, 161−166.

(24) Luís Moreira Gonçalves, L.; Grosso Pacheco, J.; Magalhães, P. J.; Rodrigues, J. A.; Araújo Barros, A. Determination of free and total sulfites in wine using an automatic flow injection analysis system with voltammetric detection. *Food Addit. Contam., Part A* **2010**, *27*, 175−180.

(25) Kedare, S. B.; Singh, R. P. Genesis and development of DPPH method of antioxidant assay. *J. Food Sci. Tech.* **2011**, *48*, 412−422.

(26) Noipa, T.; Srijaranai, S.; Tuntulani, T.; Ngeontae, W. New approach for evaluation of the antioxidant capacity based on scavenging DPPH free radical in micelle systems. *Food Res. Int.* **2011**, *44*, 798−806.

(27) Fossen, T.; Cabrita, L.; Andersen, O. M. Colour and stability of pure anthocyanins influenced by pH including the alkaline region. *Food Chem.* **1998**, *63*, 435−440.

(28) Friaa, O.; Brault, D. Kinetics of the reaction between the antioxidant Trolox and the free radical DPPH• in semi-aqueous solution. *Org. Biomol. Chem.* **2006**, *4*, 2417−2423.

(29) Goupy, P.; Dufour, C.; Loonis, M.; Dangles, O. Quantitative kinetic analysis of hydrogen transfer reactions from dietary polyphenols to the DPPH radical. *J. Agric. Food Chem.* **2003**, *51*, 615−622.

(30) Musialik, M.; Litwinienko, G. Scavenging of DPPH• radicals by vitamin E is accelerated by its partial ionization: The role of sequential proton loss electron transfer. *Org. Lett.* **2005**, *7*, 4951−4954.

(31) Tsimogiannis, D. I.; Oreopoulou, V. The contribution of flavonoid C-ring on the DPPH free radical scavenging efficiency. A kinetic approach for the 3′,4′-hydroxy substituted members. *Innovative Food Sci. Emerging Technol.* **2006**, *7*, 140−146.

(32) Hotta, H.; Nagano, S.; Ueda, M.; Tsujino, Y.; Koyama, J.; Osakai, T. Higher radical scavenging activities of polyphenolic antioxidants can be ascribed to chemical reactions following their oxidation. *Biochim. Biophys. Acta* **2002**, *1572*, 123−132.

(33) Munoz-Munoz, J. L.; Garcia-Molina, F.; Varon, R.; Tudela, J.; Garcia-Canovas, F.; Rodriguez-Lopez, J. N. Quantification of the antioxidant capacity of different molecules and their kinetic antioxidant efficiencies. *J. Agric. Food Chem.* **2010**, *58*, 2062−2070.

(34) Slangen, H. J. M. Determination of the spin concentration by electron spin resonance. *J. Phys. E: Sci. Instrum.* **1970**, *3*, 775.

(35) Ozcelik, B.; Lee, J. H.; Min, D. B. Effects of light, oxygen, and pH on the absorbance of 2,2-diphenyl-1-picrylhydrazyl. *J. Food Sci.* **2003**, *68*, 487−490.
(36) Everette, J. D.; Bryant, Q. M.; Green, A. M.; Abbey, Y. A.; Wangila, G. W.; Walker, R. B. Thorough study of reactivity of various compound classes toward the Folin−Ciocalteu reagent. *J. Agric. Food Chem.* **2010**, *58*, 8139−8144.
(37) Magalhães, L. M.; Santos, F.; Segundo, M. A.; Reis, S.; Lima, J. L. F. C. Rapid microplate high-throughput methodology for assessment of Folin−Ciocalteu reducing capacity. *Talanta* **2010**, *83*, 441−447.
(38) Piljac, J.; Martinez, S.; Valek, L.; Stipčević, T.; Kovačević Ganić, K. Correlation between the phenolic content and DPPH radical scavenging activity of selected Croatian wines. *Acta Aliment.* **2007**, *36*, 185−193.
(39) Porgalı, E.; Büyüktuncel, E. Determination of phenolic composition and antioxidant capacity of native red wines by high performance liquid chromatography and spectrophotometric methods. *Food Res. Int.* **2012**, *45*, 145−154.
(40) Li, H.; Wang, X. Y.; Li, Y.; Li, P. H.; Wang, H. Polyphenolic compounds and antioxidant properties of selected China wines. *Food Chem.* **2009**, *112*, 454−460.