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Radical Scavenging Capacity of Wine Anthocyanins Is Strongly pH-Dependent

TOMASZ BORKOWSKI,† HENRYK SZYMUSIAK,† ANNA GLISZCZYŃSKA-ŚWIGŁO,† IVONNE M. C. M. RIETJENS,‡ AND BOŻENA TYRAKOWSKA*,†

Faculty of Commodity Science, The Poznań University of Economics, Al. Niepodległości 10, 60-967 Poznań, Poland, and Division of Toxicology, Wageningen University, Tuinlaan 5, NL-6703 HE HE Wageningen, The Netherlands

The radical scavenging capacity of red wine anthocyanins was quantified by the so-called TEAC assay with special emphasis on the influence of pH and conjugation on this activity. The pH appears to be a dominant factor in the radical scavenging capacity of wine anthocyanins, with higher pH values increasing this capacity significantly. On the basis of the pK_a values for deprotonation and theoretical calculations, it could be concluded that the effect is due to an increase in intrinsic radical scavenging capacity upon deprotonation. The data also reveal that the reduction in radical scavenging activity of anthocyanins upon their conjugation can, at least in part, be ascribed to an increase in pK_a values upon conjugation. Altogether, the results obtained provide molecular insight into factors that influence radical scavenging potential of anthocyanins and reveal that the radical scavenging-mediated supposed beneficial health effects of these wine pigments will be influenced by the pH of the surrounding matrix or tissue.

KEYWORDS: Anthocyanidins; anthocyanins; radical scavenging capacity; TEAC; pH; body fluids; pK_a

INTRODUCTION

The increasing interest in anthocyanins is due to the appreciation of their broad pharmacological activity (*1*−*5*). They may play an important role in the reduction of lipid peroxidation, LDL oxidation, and enzyme-mediated oxidation and thereby retard the onset and progress of common chronic diseases including atherogenesis, thrombosis, and cancer (*1*−*5*). Anthocyanins may also exert other potential therapeutic effects acting as antineoplastic agents, radiation-protective agents, vasotonic agents, vasoprotective and antiinflammatory agents, chemoprotective agents against platinum toxicity in anticancer therapy, and hepatoprotective agents against carbon tetrachloride damage (*1*−*5*). They have been used to treat various microcirculation diseases resulting from capillary fragility, in the treatment of diabetic retinopathy, and in fibrocystic disease of the breast in humans (*2*). The broad range of biological activities of anthocyanins is often related to their antioxidant properties (*2*−*5*). Atherosclerosis and coronary heart diseases (CHD) have been linked to excessive consumption of dietary saturated fat and elevated levels of cholesterol in low-density lipoproteins (LDLs) circulating in the blood (*6*). Atherosclerosis involves both LDL oxidation and platelet formation, and these processes can affect each other. Red wine polyphenols are believed to contribute to the beneficial health effects of wine (related to the so-called "French paradox") protecting the cardiovascular system against the deleterious consequences of lipid peroxidation and LDL oxidation, induced by reactive oxygen species, thereby decreasing coronary heart disease (CHD) mortality (*6*, *7*).

Anthocyanins are one of the main classes of flavonoids in red wine, and they may contribute significantly to its powerful antioxidant properties (*5*). Anthocyanins are natural pigments widely distributed among flowers, fruits (particularly in berries), and vegetables and are responsible for their bright colors. In red wines, 3-glucosides of malvidin, cyanidin, delphinidin, petunidin, and peonidin (**Figure 1**) are present, but malvidin-3-glucoside, malvidin-3-glucoside acetate, and malvidin-3-glucoside coumarate are the most abundant pigments (*5*).

As members of the flavonoid group, anthocyanins share a $C_6C_3C_6$ carbon skeleton. Anthocyanins exist in an aqueous phase in a mixture of essentially four molecular species (**Figure 2**). The flavylium cation (AH^+) is formed and dominates at acidic pH 1−3; a carbinol pseudobase (B) is formed upon deprotonation and hydration at pH 4−5 and can further undergo ring opening to chalcones (C_E and C_Z) (*5*, *8*, *9*). Finally, the flavylium cation can alternatively be transformed to quinoidal-base isomers (A_1, A_2, and A_3) through deprotonation and proton-transfer reactions and can undergo further conversion to quinoid anions (*8*). The relative amounts of cation (AH^+), quinoidal forms (A_1, A_2, and A_3), carbinol pseudobase (B), and chalcones (C_E and C_z) at equilibrium vary with both pH and the structure of anthocyanins (*9*). There is, at present, no clear molecular insight

* To whom correspondence should be addressed. Telephone: +48618569383. Fax: +48618543993. E-mail: bozena.tyrakowska@ae.poznan.pl.
† The Poznań University of Economics.
‡ Wageningen University.

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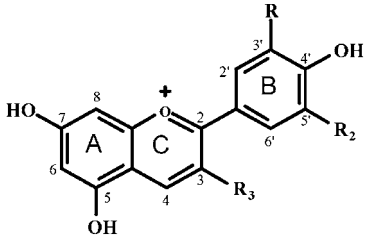

Compound	R1	R2	R3
pelargonidin	H	H	OH
cyanidin	OH	H	OH
peonidin	OCH_3	H	OH
petunidin	OCH_3	OH	OH
delphinidin	OH	OH	OH
malvidin	OCH_3	OCH_3	OH
cyanidin-3-O-β-glucoside	OH	H	O-β-glucose
delphinidin-3-O-β-glucoside	OH	OH	O-β-glucose
malvidin-3-O-β-glucoside	OCH_3	OCH_3	O-β- glucose

Figure 1. Structures of the various anthocyanins of the present study.

into factors that influence radical scavenging capacity of various anthocyanins in biological systems. The literature data on the antioxidant activity of anthocyanins are difficult to interpret because different methods are used to determine antioxidant potency of anthocyanins and a different order of their antioxidant activity is observed in different studies (*2*−*5*). This may in part be related to many different forms in which anthocyanins may exist at different pH values and the fact that the influence of pH on the radical scavenging capacity of anthocyanins has not been taken into account. This possible pH-dependent effect on the radical scavenging ability of anthocyanins is especially of interest because the pH range of different human body fluids is known to vary widely from pH 1 in the stomach, pH 5.3 in the small intestine, pH 6.8 in mouth saliva, pH 7.4 in blood and tissue fluid, pH 8 in the large intestine to pH 7−8.7 in the pancreas and pH 8.3−9.3 in the duodenum (*10*).

Therefore, the objective of the present study was to investigate the pH-dependent radical scavenging capacity of a series of anthocyanin wine pigments. Radical scavenging capacity of anthocyanins was quantified by the modified TEAC assay (*11*). Experimental data for deprotonation and radical scavenging capacities were compared to theoretically calculated parameters for OH deprotonation and radical scavenging ability. The results obtained show significant pH-dependent influences on the antioxidant action of anthocyanins and reveal that these effects occur at pH ranges relevant for human body fluids.

MATERIALS AND METHODS

Chemicals. Pelargonidin (4′,3,5,7-tetrahydroxyanthocyanidin)-chloride, cyanidin (3′,4′,3,5,7-penthahydroxyanthocyanidin)chloride, peonidin (3′-*O*-methyl-4′,3,5,7-tetrahydroxyanthocyanidin)chloride, delphinidin (3′,4′,5′,3,5,7-hexahydroxyanthocyanidin)chloride, and malvidin (3′,5′-di-*O*-methyl-4′,3,5,7-tetrahydroxyanthocyanidin)chloride were purchased from Extrasynthese (Genay, France). Cyanidin-, delphinidin-, and malvidin-3-*O*-*β*-glucosides were purchased from Polyphenols (Sandnes, Norway). 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and microperoxidase-8 were purchased from Sigma−Aldrich (Steinheim, Germany). Hydrogen peroxide (30%) was purchased from Merck (Darmstadt, Germany).

Determination of pK_a. The pK_a values of pelargonidin, peonidin, malvidin, cyanidin, delphinidin, cyanidin-3-*O*-*β*-glucoside, delphinidin-3-*O*-*β*-glucoside, and malvidin-3-*O*-*β*-glucoside were determined from absorption spectra as a function of pH as described by Sauerwald et al. (*12*).

TEAC Assay. The antioxidant activity of anthocyanins was measured by the modified TEAC assay performed essentially as described previously (*3*), with some modifications (*11*). The major advantage of the modified TEAC assay is that it permits studies of radical scavenging activity over a wide pH range (2−9.5). The TEAC assay is based on the ability of the antioxidant to scavenge the blue-green-colored $ABTS^{\cdot+}$ (2,2′-azinobis(3-ethylbenzothiozoline-6-sulfonic acid) diammonium salt) radical cation relative to the $ABTS^{\cdot+}$ scavenging ability of the water-soluble vitamin E analogue, Trolox (*3*).

In the present study, microperoxidase-8 (MP8) instead of metmyoglobin was used to generate the $ABTS^{\cdot+}$ in PBS (phosphate-buffered saline) pH 7.4. MP8 (final concentration of 0.2 μM) and ABTS (final concentration of 3.0 mM) in PBS were mixed, and the reaction was initiated by the addition of hydrogen peroxide (final concentration of 0.1 mM).

The $ABTS^{\cdot+}$ solution thus obtained was diluted 1:1 (v/v) to give an absorption of about 0.6 at 734 nm. This dilution was carried out using 0.2 M sodium acetate or potassium phosphate buffers of various pH values to give $ABTS^{\cdot+}$ solutions at pH values varying between 2 and 9.5. The $ABTS^{\cdot+}$ solutions thus obtained were used for determination of the TEAC values. Antioxidants (Trolox or anthocyanins) were added as 1% (v/v) of a 100 times concentrated stock solution in 0.01 M HCl in methanol to give the final concentration required. The decrease in absorption caused by the antioxidant compound, measured at 6 min, is reflecting the $ABTS^{\cdot+}$ radical scavenging capacity and was plotted against the concentration of the antioxidant. The linear correlation obtained for this plot allows the assumption that this decrease in absorbance reflects especially the reaction between $ABTS^{\cdot+}$ and the anthocyanin in its nonassociated form. The TEAC value represents the ratio between the slope of this plot for scavenging of $ABTS^{\cdot+}$ by the antioxidant under investigation, compared to the slope of this plot for $ABTS^{\cdot+}$ scavenging by Trolox, used as an antioxidant standard. Solvent controls containing the $ABTS^{\cdot+}$ solutions in the absence of the anthocyanins revealed that the $ABTS^{\cdot+}$ solutions were stable over the whole pH range tested under the conditions applied.

Quantum Mechanical Calculations. The geometries of all anthocyanins molecules studied in their different forms (flavylium cation, carbinol pseudobase, C_E chalcone, and quinoidal-bases) were fully optimized with the B3LYP hybrid density functional theory (DFT) by using a 6-31G(d) basis set as implemented in the Gaussian 98 computational package. Single-point energies were then evaluated by using a higher 6-311G(2d,2p) basis set. The calculated deprotonation energies (DEs), ionization potentials (IPs), and bond dissociation energies (BDEs) were not corrected for zero-point energy and other thermal contributions assuming a negligible error and thus considerably saving computer time.

The DE values were calculated as the electronic energy of the deprotonated molecule minus the electronic energy of the parent molecule. The BDE for homolytic OH bond cleavage in the neutral molecule [BDE(N)] was calculated as the electronic energy of the radical resulting from the hydrogen atom abstraction minus the electronic energy of the neutral parent molecule. The IP for the neutral molecule [IP(N)] was calculated as the electronic energy of the radical cation resulting from the electron abstraction minus the electronic energy of the neutral parent molecule.

Next, the structure of the most stable monoanion of the different forms of the anthocyanins studied was found by comparison of the energy of the monoanions resulting from proton abstraction from each of the individual OH groups present in the molecule.

The BDE for homolytic OH bond cleavage in the deprotonated monoanionic (MA) molecule [BDE(MA)] was calculated as the electronic energy of the most stable radical anion that is formed by an abstraction of the most weekly bonded hydrogen atom from the most stable monoanion minus the electronic energy of this most stable

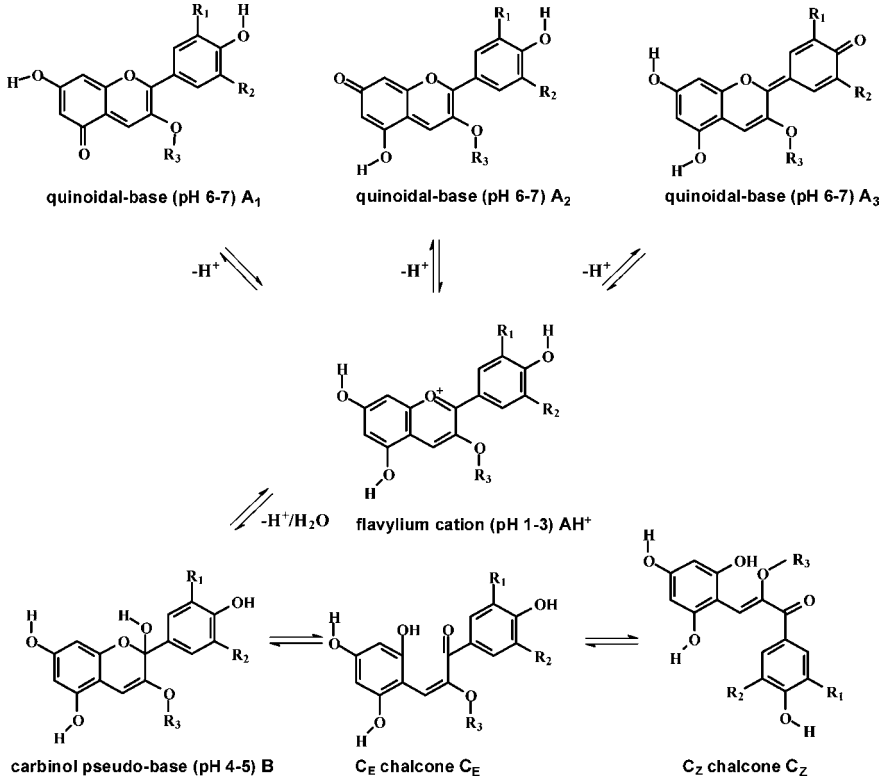

Figure 2. Structural changes of anthocyanins in aqueous solutions based on transformations of red malvidin-3-*O*-β-glucoside flavylium cation into possible purple quinone methides (A_1, A_2, and A_3), colorless carbinol pseudobase (B), and yellow chalcone forms (C_E and C_Z), at different pH (R_1, R_2 = OMe, R_3 = glucosyl) (*5*).

Table 1. Antioxidant Activities of the Various Anthocyanins

anthocyanins (substituent pattern)	TEAC (pH 7.4)	TEAC[a] (pH 7.4)	ORAC[b] (pH 7.0)	DPPH[c]
pelargonidin (4′,3,5,7-tetra OH)	1.23	1.30	1.54	–4.63
cyanidin (3′,4′,3,5,7-penta OH)	4.12	4.20	2.24	–7.40
peonidin (3′-OMe,4′,3,5,7-tetra OH)	1.98	2.22	1.69	–4.05
cyanidin-3-*O*-β-glucoside	2.94		3.49	–6.81
delphinidin (3′,4′,5′,3,5,7-hexa OH)	5.11	4.40	1.81	–8.86
malvidin (3′,5′-diOMe,4′3,5,7-tetra OH)	2.71	2.06	2.01	–4.49
delphinidin-3-*O*-β-glucoside	2.61			
malvidin-3-*O*-β-glucoside	1.89	1.80	1.40	–4.29
ascorbic acid		0.99		–1.83
α-tocopherol		0.97		–1.95

[a] TEAC values were taken from Rice-Evans et al. (*3*, *4*). [b] ORAC values were taken from Wang et al. (*2*). [c] DPPH results are from Fukumoto et al. (*13*).

monoanion molecule. The IP of the most stable mononanion [IP(MA)] was calculated as the electronic energy of the phenoxyl radical formed by electron abstraction from the most stable monoanion minus the electronic energy of this parent most stable monoanion. The cyanidin-3-*O*-β-glucoside, delphinidin-3-*O*-β-glucoside, and malvidin-3-*O*-β-glucoside were modeled taking a methoxyl substituent instead of the glucosyl group.

RESULTS

Radicals Scavenging Capacity of Various Wine Anthocyanins. Table 1 presents the TEAC values at the pH values indicated of the series of anthocyanins studied as model compounds in the present study (**Figure 1**). For comparison, **Table 1** also presents the literature data on the radical scavenging antioxidant activity of anthocyanins tested using the ORAC assay (*2*), the TEAC assay (*3*, *4*), and the DPPH method (*13*). The TEAC values obtained in the present study correlate with TEAC values reported previously by Rice-Evans et al. at pH 7.4 (*3*, *4*) (r = 0.97). The correlation coefficient between experimental TEAC values and DPPH (*13*) values is 0.90. There is no correlation between experimental TEAC at pH 7.4 and ORAC (*2*) values at pH 7.0 (r = 0.45).

pK_a Values of the Anthocyanin Model Compounds. Only a few studies exist presenting the pK_a values of anthocyanins, including a pK_a of 3.62 mentioned for delphinidin-3-*O*-β-glucoside and a pK_a of 4.25 for malvidin-3-*O*-β-glucoside (*14*, *15*). However, these values were not linked to (de)protonation of a specific form of the many that may exist in aqueous solution (**Figure 2**). This coexistence of the anthocyanin forms makes it difficult to experimentally determine the pK_a of specific deprotonation steps, except for the first pK_{a_1} representing deprotonation of the flavylium cations. **Table 2** presents this pK_{a_1} for the model anthocyanins of the present study. From the data presented, it follows that pK_{a_1} values for deprotonation of hydroxyl groups from the flavylium cation (at C4′ or C5) are within the physiological pH range (5.30−6.02, **Table 2**). Methylation and glycosylation generally increase the pK_{a_1} of anthocyanidins with the exception of malvidin glycosylation (**Table 2**). Because anthocyanins exist in an aqueous solution

Table 2. Experimental pK_{a_1} Values for Deprotonation of Flavylium Cation Form (AH^+) and Theoretically Predicted pK_{a_2} Values for Deprotonation of the Most Stable Quinoidal-Base Structure (A_1, A_2, or A_3)[a], Carbinol Pseudobase (B), and *E*-Chalcone (C_E)[b]

anthocyanins	anthocyanin form	AH^+	A_1	A_2	A_3	B	C_E	mean pK_{a_2}
pelargonidin	experimental pK_{a_1}	5.79						
(4′,3,5,7-tetra OH)	predicted pK_{a_2}[c]		7.05[a]			8.35	6.73	7.38
	calculated DE	258.0(5)	332.1(4′)	332.3(4′)	328.1(5)	340.6(3)	330.0(4′)	
cyanidin	experimental pK_{a_1}	5.48						
(3′,4′,3,5,7-penta OH)	predicted pK_{a_2}				6.39[a]	8.04	5.68	6.70
	calculated DE	254.4(4′)	324.2(4′)	324.8(4′)	327.8(5)	338.6(4′)	323.1(4′)	
peonidin	experimental pK_{a_1}	5.93						
(3′-OMe,4′,3,5,7-tetra OH)	predicted pK_{a_2}		7.37[a]			8.36	7.23	7.65
	calculated DE	259.0(5)	334.2(4′)	335.1(4′)	328.0(5)	340.7(3)	333.3(4′)	
cyanidin-3-*O*-β-glucoside	experimental pK_{a_1}	5.88						
	predicted pK_{a_2}				6.73[a]	9.20	6.26	7.40
	calculated DE	256.2(4′)[d]	325.1(4′)	325.7(4′)	330.0(5)	346.2(4′)	326.9(4′)	
delphinidin	experimental pK_{a_1}	5.30						
(3′,4′,5′,3,5,7-hexa OH)	predicted pK_{a_2}				6.30[a]	7.25	4.90	6.15
	calculated DE	249.8(4′)	319.1(4′)	320.1(4′)	327.2(5)	333.4(4′)	318.0(4′)	
malvidin	experimental pK_{a_1}	6.02						
(3′,5′-diOMe,4′,3,5,7-tetra OH)	predicted pK_{a_2}		7.40[a]			8.32	6.97	7.56
	calculated DE	260.2(5)	334.4(4′)	333.4(4′)	327.8(5)	340.4(3)	331.6(4′)	
delphinidin-3-*O*-β-glucoside	experimental pK_{a_1}	5.35						
	predicted pK_{a_2}				6.88[a]	8.33	5.46	6.90
	calculated DE	251.3(4′)[d]	321.8(4′)	321.2(4′)	331.0(5)	340.5(4′)	321.7(4′)	
malvidin-3-*O*-β-glucoside	experimental pK_{a_1}	5.57						
	predicted pK_{a_2}		7.28[a]			9.84	7.54	8.22
	calculated DE	262.4(5)[d]	333.6(4′)	334.3(4′)	330.2(5)	350.4(5)	335.3(4′)	

[a] The pK_{a_2} value for the most stable neutral structure of quinoidal base. [b] Predictions were made on the basis of the presented calculated deprotonation energies (DE) and the QSAR reported previously (*16*). The number in parentheses refers to the position of the OH moiety. [c] Predictions of pK_{a_2} values for some products of flavylium cation deprotonation and transformation (A_1, A_2, A_3, B, and *E*-chalcone) (**Figure 2**) were done using the calculated DE and the QSAR obtained in previous studies (*16*); equation of regression line used, $pK_a = 0.1525DE - 43.596$, $r = 0.9808$. [d] The calculated parameters obtained for glycosylated compounds were determined on the basis of their model 3-methoxy derivatives.

as a mixture of different forms, the pK_{a_2} determination can only be determined on the basis of theoretical calculations.

An estimation of the pK_{a_2} values of the different neutral anthocyanin forms (**Figure 2**) was made based on the following consideration. Recently, a quantitative structure activity relationship (QSAR) was obtained relating experimental pK_a values of the OH groups of a series of hydroxyflavones to the theoretically calculated deprotonation energies (*16*). The correlation coefficient of this QSAR was 0.98. Anthocyanins of the present study are structurally related to the hydroxyflavones for which the QSAR was defined. This QSAR was used to predict pK_{a_2} values. To this end, **Table 2** lists the relative deprotonation energies (DE) of various hydroxyl moieties of the different neutral forms of anthocyanins, including the most stable quinoidal-base (A), carbinol pseudobase (B), and *E*-chalcone (C_E) forms (**Figure 2**). Using these DE values and the QSAR described for the hydroxyflavones, pK_{a_2} values were calculated (**Table 2**). The results thus obtained illustrate that for many of anthocyanins deprotonation equilibria are expected to occur at physiological pH values. The calculated deprotonation energies of the OH moieties confirm that the C4′ and C5 OH moieties are the ones that preferably deprotonate. The pK_{a_2} value, predicted for deprotonation of quinoidal-base, carbinol pseudobase, and *E*-chalcone, vary from 4.90 (for delphinidin *E*-chalcone form) to 9.84 (for malvidin-3-*O*-β-glucoside carbinol pseudobase form). From all predicted pK_{a_2} values, only the pK_{a_2} for deprotonation of the hydroxyl group at C4′ in cyanidin-3-*O*-β-glucoside carbinol pseudobase and the pK_{a_2} for deprotonation of the hydroxyl group at C5 in malvidin-3-*O*-β-glucoside carbinol pseudobase are above the physiological pH range (i.e., pH > 9).

Altogether these pK_a values illustrate that hydroxyl moieties in the anthocyanin model compounds, especially those at C4′ and C5, are sensitive to deprotonation at physiological pH. Thus, the pK_a of the hydroxyl moieties at C4′ and C5 may be a factor to be taken into account when studying the pH-dependent radical scavenging activity of anthocyanins in the physiological pH range.

Furthermore, the results obtained indicate that the position and number of hydroxyl substituents in the B ring affect the pK_a values. Introduction of additional ortho OH group(s) results in a decrease in the pK_{a_1} and pK_{a_2} values. As for the pK_{a_1} of the flavylium cation, the data show that for the pK_{a_2} an additional ortho OH substituent increases the ease of deprotonation, whereas methylation and glycosylation show the opposite effect.

pH-Dependent Radical Scavenging Capacity of Anthocyanins Aglycons (Anthocyanidins). Figure 3 presents the pH-dependent TEAC values for the three unconjugated anthocyanidin model compounds of the present study, namely, pelargonidin, cyanidin, and delphinidin. The antioxidant action of Trolox was previously shown to be unaffected over the whole pH range tested (*11*).

From the plots presented, it follows that additional hydroxyl groups at the ortho position strongly increase radical scavenging capacity of anthocyanins especially at pH values above 4 for the first additional OH and above 6 for the second additional OH moiety. Arrows in **Figure 3** indicate the position of the experimental pK_{a_1} and predicted pK_{a_2} values with respect to the pH-dependent TEAC profile. This illustrates that the increase in TEAC values is likely to result from deprotonation equilibria expected to occur in the aqueous solution with increasing pH.

Figure 4 presents the pH-dependent TEAC profile of cyanidin compared to that previously reported for quercetin (*16*), a flavonol with similar substituent pattern. These curves reveal that cyanidin shows generally similar pH-dependent radical scavenging behavior as quercetin.

pH-Dependent Radical Scavenging Capacity of O-Methylated Anthocyanidins. Figure 5 presents the pH-dependent TEAC profiles of some methylated anthocyanidins known to be present in red wines, namely, the O-methylated (3′-OCH_3

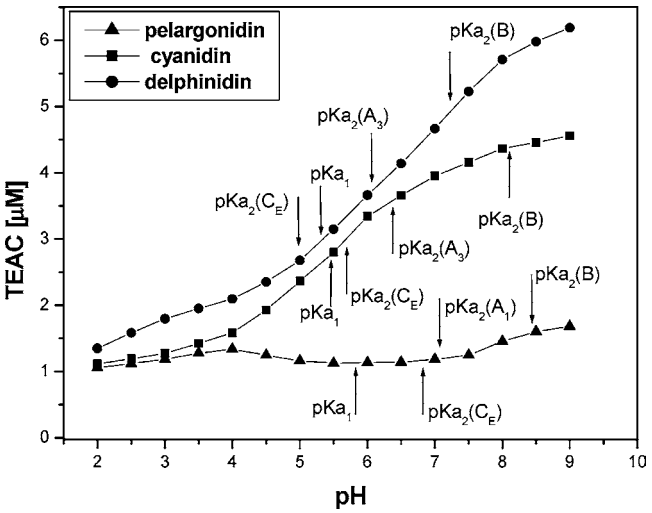

Figure 3. pH-dependent TEAC profile of anthocyanins aglycons: pelargonidin (4′,3,5,7-tetrahydroxyanthocyanidin), cyanidin (3′,4′,3,5,7-pentahydroxyanthocyanidin), and delphinidin (3′,4′,5′,3,5,7-hexahydroxyanthocyanidin), representing anthocyanins with increasing number of hydroxyl moieties in the B ring. Arrows indicate the experimental pK_{a_1} value of the preferably deprotonated OH group in the flavylium cation, as well as theoretically predicted pK_{a_2} values for deprotonation of a carbinol pseudobase (B), most stable quinoidal-base forms (A_1 or A_3), and *E*-chalcone (C_E).

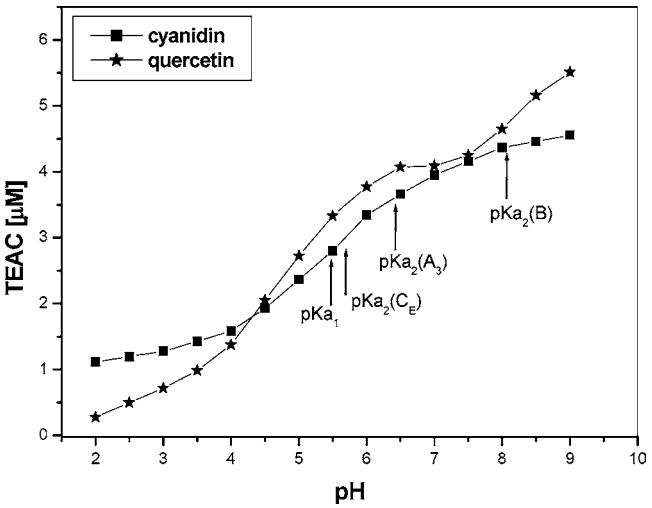

Figure 4. pH-dependent TEAC profile of cyanidin (3′,4′,3,5,7-pentahydroxyanthocyanidin) and quercetin (3′,4′,3,5,7-pentahydroxyflavonol). Arrows indicate the experimental pK_{a_1} value of the preferably deprotonated OH group in flavylium cation as well as theoretically predicted pK_{a_2} values for deprotonation of a carbinol pseudobase (B), most stable quinoidal-base form (A_3), and *E*-chalcone (C_E).

or 3′,5′-di-OCH_3) derivatives of cyanidin and delphinidin. The results obtained reveal that also for these methylated anthocyanins there is a marked increase in TEAC antioxidant capacity with increasing pH. A comparison of all of the pK_a values (**Table 2**) to the pH-dependent TEAC profiles leads to the conclusion that this increase in the TEAC value occurs around the pK_a values, which suggest that it is related to deprotonation of the anthocyanins under study. Upon deprotonation, cyanidin and delphinidin but also their methylated derivatives become better radical scavengers reflected in a significant pH-dependent increase in the TEAC value with an increasing pH. Important to note is the fact that the pH at which the increase in TEAC value is observed is shifted to a higher pH for the O-methylated

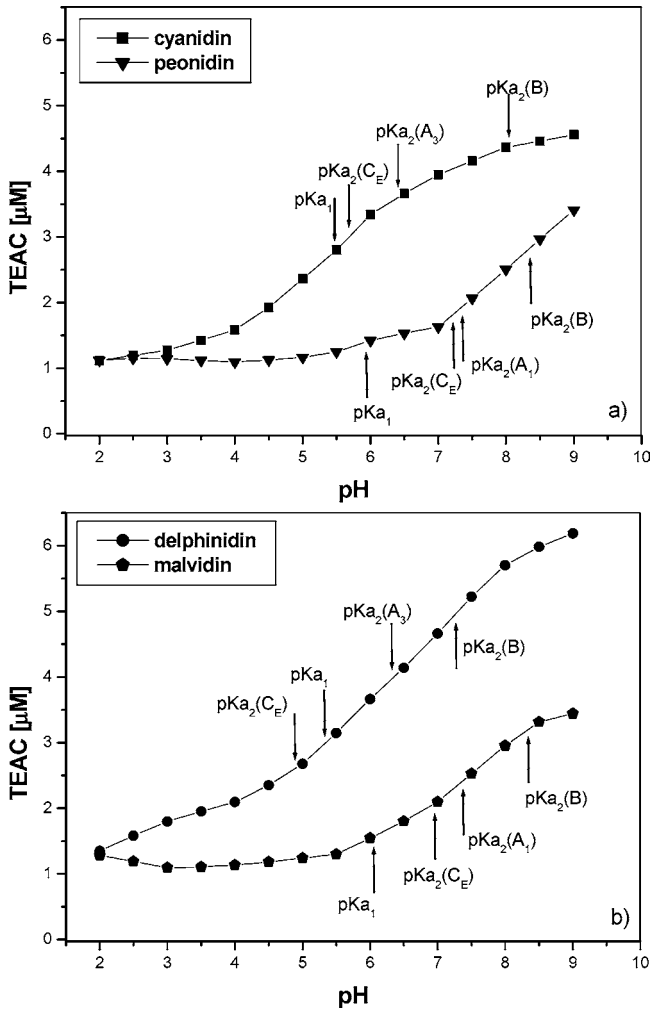

Figure 5. pH-dependent TEAC profile of (a) cyanidin and its 3′-O-methylated derivative (peonidin) and (b) delphinidin and its 3′,5′-O-dimethylated derivative (malvidin). Arrows indicate the experimental pK_{a_1} value of the preferably deprotonated OH group in flavylium cation as well as theoretically predicted pK_{a_2} values for deprotonation of a carbinol pseudobase (B), most stable quinoidal-base forms (A_1 or A_3), and *E*-chalcone (C_E).

forms as compared to the unconjugated parent molecule, mainly because of a higher pK_a value upon methylation.

pH-Dependent Radical Scavenging Capacity of Glycosylated Anthocyanidins. Figure 6 presents the pH-dependent TEAC profile of two 3-O-glycosylated anthocyanidins compared to the profile obtained for the corresponding aglycons. For both model compounds, glycosylation at the 3-OH reduces the radical scavenging capacity at physiological pH values but still shows a pH-dependent increase in the TEAC value. The pK_a values are generally higher than those observed for the corresponding aglycons, shifting the overall rise of the TEAC activity with an increasing pH by about 2−3 pH units as a result of glycosylation.

Calculated Parameters for Radical Scavenging Capacity of Anthocyanins and Their Conjugates. To obtain more insight in the effect of 3′-O-methylation and 3-O-glycosylation on the radical scavenging capacity of cyanidin and delphinidin and in the effect of protonation states on the TEAC activity of these compounds, theoretical electronic parameters were calculated. **Tables 3** and **4** present these electronic descriptors for the various forms of the compounds under investigation, in the different protonation states. These electronic parameters include

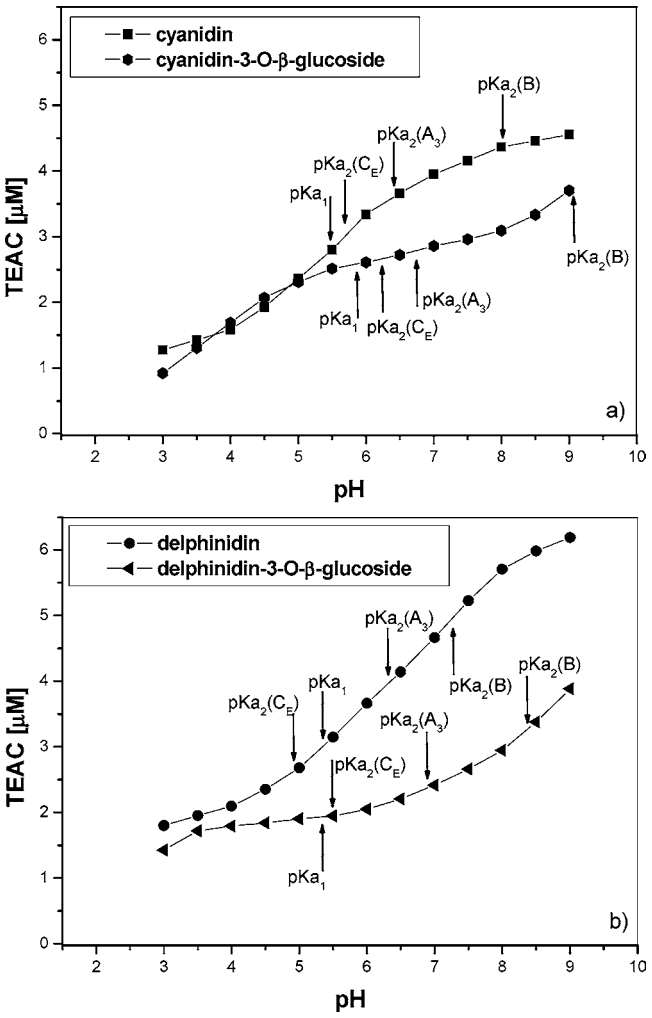

Figure 6. pH-dependent TEAC profile of (a) cyanidin and cyanidin 3-*O*-*β*-glucoside and (b) delphinidin and delphinidin-3-*O*-*β*-glucoside. Arrows indicate the experimental pK_{a_1} value of the preferably deprotonated OH group in flavylium cation as well as theoretically predicted pK_{a_2} values for deprotonation of a carbinol pseudobase (B), most stable quinoidal-base form (A_3), and *E*-chalcone (C_E).

OH bond dissociation energy (BDE), representing the ease of hydrogen atom donation and IP representing the ease of electron donation.

For the flavylium cation, the BDE is not significantly influenced upon 3′-O-methylation or 3-O-glycosylation of cyanidin and the same holds for 3′- and 5′-O-methylation and 3-O-glycosylation of delphinidin. This indicates that conjugation is not affecting the radical scavenging ability of the flavylium cation of anthocyanidins, reflected by generally similar TEAC values for the aglycon and conjugates at low pH values, where the flavylium cation is the dominant form. Upon deprotonation of the flavylium cation to any of the possible neutral forms (see **Figure 2**), the BDE is decreased, reflecting higher radical scavenging capacity, an effect that appears to occur for the aglycons and also for their methylated conjugates. For the 3-O-glycosylated forms, the same effect is observed upon deprotonation of the flavylium cation but to a somewhat lower extent. This implies that upon deprotonation of the flavylium cation the aglycon and methylated compound become better hydrogen donators (lower BDE) than the 3-O-glycosylated compounds from an electronic point of view. Furthermore, because in the various forms there appears to be no significant theoretical difference in electronic characteristics of the aglycons as compared to the corresponding methylated compounds, it can be concluded that differences in the pH-dependent TEAC values for the aglycon and the methylated derivative at a similar pH value are more likely due to the extent of deprotonation at the pH of interest.

The data presented in **Table 3** also allow for a comparison of the calculated BDE values for the monoanionic forms of the aglycons and their conjugates. As for the neutral forms, the BDE of the methylated derivatives are comparable to those for the aglycons, whereas the BDE values for hydrogen-bond cleavage in the glycosylated monoanionic forms generally tend to be somewhat higher than those for the aglycon or methylated analogues.

The other theoretical parameter, IP, representing the ease of electron donation and thus being another parameter reflecting radical scavenging capacity, is not influenced by methylation but also not by 3-O-glycosylation, not in the neutral but also not in the anionic form (**Table 4**). Comparing the calculated BDE and IP values of the neutral forms to those of the corresponding monoanioinc forms indicates that, upon conversion of the neutral forms into the monoanions, both values are lowered, reflecting improved radical scavenging capacity of the monoanions. This is in line with the increase in the TEAC value upon increasing pH, the latter facilitating deprotonation.

DISCUSSION

In the present study, the radical scavenging capacity of common red wine anthocyanins was investigated with special emphasis on the possible influence of pH, able to influence the various (de)protonation equilibria known to exist for these compounds in aqueous solution (*5*, *8*, *9*) (**Figure 2**). This pH-dependent behavior of radical scavenging capacity may be of biological relevance given the pH range of different human body fluids, known to vary widely from pH 1 in the stomach, pH 5.3 in the small intestine, pH 6.8 in mouth saliva, pH 7.4 in blood and tissue fluid, pH 8 in the large intestine to pH 7−8.7 in the pancreas and pH 8.3−9.3 in the duodenum (*10*). Special emphasis was also paid on another factor known to influence the radical scavenging capacity and thus the possible biological activity of flavonoids, namely, the influence of conjugation, either because of their natural occurrence or because of expected mammalian biotransformation, on their radical scavenging capacity (*3*, *17*−*19*).

The pH was shown to be a dominant factor in the ultimate radical scavenging capacity of the wine anthocyanins, with increasing pH significantly increasing the capacity for radical scavenging. Similar effects were reported previously for a series of flavonols, including quercetin (*16*), and a comparison of the data of the present study to the results reported before reveals that for anthocyanins the effect of pH on TEAC values is rather similar. A close comparison of the pH-dependent profile for radical scavenging capacity of the anthocyanin cyanidin to those for the flavonol quercetin with the corresponding substituent pattern illustrates that at low pH (pH 1−4.5) anthocyanidin is a somewhat better radical scavenger than flavonol, whereas at higher pH (pH > 4.5), both classes of flavonoids show similar radical scavenging capacity. This observation, together with the fact that in red wine anthocyanins are known to be present to a higher level than flavonols (*1*), lead to the conclusion that at the pH of red wine, being pH 2−3, anthocyanins contribute to the radical scavenging capacity of the wine relatively more than the corresponding flavonols. Upon consumption and passing the acidic pH of the stomach, however, the pH of the gastrointestinal tract and biological tissues may enhance the radical scavenging

Table 3. Theoretically Calculated Bond Dissociation Energies (BDEs) for Flavylium Cation Form (AH^+), as well as for Quinoidal-Base Forms (A_1, A_2, and A_3), Carbinol Pseudobase (B), and *E*-Chalcone Form (C_E) of Studied Anthocyanins in Their Neutral (N) and Monoanionic (MA) Forms[a]

anthocyanins	AH^+	A_1	A_2	A_3	B	C_E
		BDE(N) (kcal/mol)				
cyanidin (3′,4′,3,5,7-penta OH)	86.2(3)	74.2(3)	73.2(3)	75.9(3)	74.2(3)	77.6(3)
peonidin (3′-OMe,4′,3,5,7-tetra OH)	85.4(3)	73.9(3)	73.2(3)	74.4(3)	74.1(3)	77.9(3)
cyanidin-3-*β*-*O*-glucoside	87.0(4′)	78.2(4′)	79.4(4′)	83.2(5)	80.2(4′)	82.7(4′)
delphinidin (3′,4′,5′,3,5,7-hexa OH)	80.2(4′)	71.5(4′)	72.1(4′)	75.7(3)	74.3(3)	76.8(4′)
malvidin (3′,5′-diOMe,4′,3,5,7-tetra OH)	84.9(3)	73.9(3)	72.8(3)	72.8(3)	75.1(3)	77.2(3)
delphinidin-3-*O*-*β*-glucoside	80.4(4′)	71.2(4′)	71.0(4′)	80.3(5)	73.9(4′)	76.6(4′)
malvidin-3-*O*-*β*-glucoside	88.9(4′)	82.0(4′)	82.5(4′)	78.6(5)	82.1(5)	87.9(4′)
		BDE(MA) (kcal/mol)				
cyanidin (3′,4′,3,5,7-penta OH)		68.0(3)	66.7(3)	68.0(3)	75.1(7)	80.9(3)
peonidin (3′-OMe,4′,3,5,7-tetra OH)		68.3(3)	66.7(3)	68.3(3)	65.9(7)	80.6(3)
cyanidin-3-*O*-*β*-glucoside		84.5(7)	82.7(5)	84.5(7)	72.3(5)	84.6(5)
delphinidin (3′,4′,5′,3,5,7-hexa OH)		68.9(3)	67.5(3)	68.9(3)	75.4(7)	80.7(3)
malvidin (3′,5′-diOMe,4′,3,5,7-tetra OH)		68.2(3)	66.7(3)	68.2(3)	65.8(7)	81.6(3)
delphinidin-3-*O*-*β*-glucoside		84.9(7)	84.0(5)	84.9(7)	73.8(5)	84.1(5)
malvidin-3-*O*-*β*-glucoside		85.3(7)	83.6(5)	85.3(7)	76.1(4′)	84.7(5)

[a] The calculated BDE values for glycosylated compounds were determined on the basis of their model 3-methoxy derivatives. The number in parentheses refers to the position of the OH moiety.

Table 4. Theoretically Calculated Ionization Potentials (IPs) for Quinoidal-Base Forms (A_1, A_2, and A_3), Carbinol Pseudobase (B), and *E*-Chalcone Form (C_E) of Studied Anthocyanin Compounds in Their Neutral (N) and Monoanionic (MA) Forms[a]

anthocyanins	A_1	A_2	A_3	B	C_E
		IP(N) (eV)			
cyanidin (3′,4′,3,5,7-penta OH)	150.1	153.1	148.1	149.2	153.4
peonidin (3′-OMe,4′,3,5,7-tetra OH)	148.6	151.1	143.5	148.0	152.1
cyanidin-3-*O*-*β*-glucoside	147.1	149.9	146.6	149.1	154.7
delphinidin (3′,4′,5′,3,5,7-hexa OH)	149.6	153.0	145.5	148.2	152.9
malvidin (3′,5′-diOMe,4′,3,5,7-tetra OH)	146.9	149.0	139.3	147.4	151.5
delphinidin-3-*O*-*β*-glucoside	147.2	149.2	144.2	148.3	154.2
malvidin-3-*O*-*β*-glucoside	144.2	146.2	148.3	147.5	153.3
		IP(MA) (eV)			
cyanidin (3′,4′,3,5,7-penta OH)	68.0	67.8	68.0	58.2	75.2
peonidin (3′-OMe,4′,3,5,7-tetra OH)	66.8	66.5	66.8	48.5	72.7
cyanidin-3-*O*-*β*-glucoside	68.2	68.8	68.2	49.0	70.9
delphinidin (3′,4′,5′,3,5,7-hexa OH)	67.6	67.1	67.6	57.5	74.0
malvidin (3′,5′-diOMe,4′,3,5,7-tetra OH)	65.1	64.7	65.1	49.8	71.4
delphinidin-3-*O*-*β*-glucoside	64.4	64.9	64.4	48.5	70.0
malvidin-3-*O*-*β*-glucoside	63.6	63.3	63.6	46.7	67.7

[a] The calculated IP values for glycosylated compounds were determined on the basis of their model 3-metoxy derivatives.

capacity of both anthocyanins and flavonols significantly, making those with a similar substituent pattern essentially equally effective. On the basis of the actual levels of these two classes of flavonoids in red wine [anthocyanins content being 4 times higher than flavonols in fresh wines and 2 times higher in matured wines (*1*)], it can be concluded that upon consumption anthocyanins may still be the dominant radical scavenging flavonoid species.

The pH-dependent increase in the radical scavenging capacity of hydoxyflavones was previously attributed to an effect on hydroxyl moiety deprotonation (*16*, *20*). For anthocyanins, the problem is more complicated because anthocyanins in aqueous solutions exist as a mixture of different molecular forms (**Figure 2**).

The pH-dependent increase in scavenging capacity of anthocyanins can be explained based on experimental data and theoretically calculated parameters for the main forms of anthocyanins known to exist in aqueous solution. A comparison of the pK_{a_1} values to the TEAC profiles of anthocyanins reveals that part of the pH-dependent increase in radical scavenging capacity is related to the conversion of the flavylium cation, known to be the dominant form at low pH values, to quinoidal-base, carbinol pseudobase, and *E*-chalcone with an increasing pH. Theoretical calculations corroborated that upon deprotonation of the flavylium cation to any of the possible neutral forms the BDE decreases reflecting higher radical scavenging capacity. Upon formation of quinoidal-base, carbinol pseudobase, and *E*-chalcone, the compounds become better radical scavengers, reflected by a significant increase in their TEAC values. The molecular forms of anthocyanins existing at physiological pH of blood and tissue fluid appear to have higher $ABTS^{\cdot+}$ radical cation scavenging antioxidant activity than well-known antioxidants such as vitamin C and vitamin E (**Table 1**). It is of interest to notice that some additional explanations for the observed effects of pH on the TEAC activity of the flavonoids might also be considered. For instance, a pH-dependent effect on possible intermolecular associations between the different anthocyanins present in the solutions may result in a pH-dependent effect on the effective concentrations of relevant species for the overall reaction, thereby influencing the TEAC value. For example, a pH-dependent effect on the dimerization between two anthocyanin molecules may influence their further capacity to scavenge the $ABTS^{\cdot+}$ radical cation and thus influence the TEAC value obtained.

However, the fact that the decrease in the absorbance at 734 nm after 6 min caused by the anthocyanins is proportional to

the concentration of the anthocyanins (plot of ΔA_{734} versus the concentration was found to be linear) allows the assumption that it reflects especially the reaction between the $ABTS^{\cdot+}$ radical cation and the anthocyanins in their nonassociated forms. If intermolecular associations between different anthocyanins would play a significant role, deviations from a linear relationship between the decrease in A_{734} after 6 min and the concentration of the anthocyanins would be expected, and this is not what is observed.

The radical scavenging ability of anthocyanins is considered to be related to their oxonium ion structure in the C ring (*21*). Furthermore, several studies have stressed the importance of (i) the catechol moiety in the B ring and (ii) the additional presence of 3- and 5-hydroxyl groups in anthocyanin molecular structure to achieve an efficient antioxidant action (*3*, *22*). In addition, the antioxidant efficiency of anthocyanins has been related to the number of hydroxyl groups in the molecule and also to their hydrogen radical donating abilities (*2*, *3*, *22*). A comparison of the pH-dependent TEAC profiles obtained for pelargonidin, cyanidin, and delphinidin (**Figure 3**) indicates that the antioxidant efficiency of anthocyanins increases upon introduction of an additional OH group at the *ortho* position in the B ring. This is in agreement with previous reports indicating that the presence of the 3′,4′-catechol moiety in especially ring B increases the antioxidant activity of flavonoids (*2*, *3*, *13*, *22*, *23*). The fact that pelargonidin, which has no catechol moiety in the B ring, is not able to result in significant radical scavenging not even at increased pH values additionally supports the importance of the catechol moiety in the B ring in anthocyanin to achieve an efficient radical scaveging action. Noteworthy also is the result of the present study indicating that the pyrogallol moiety in the B ring may enhance the TEAC radical scavenging capacity to a larger extent than the catechol moiety. This is in line with Pannala et. al. (*24*) who reported that the compounds containing the catechol moiety on the B ring and especially those with the pyrogallol moiety are the most potent electron-donating compounds as concluded from the TEAC values of the various flavonoid classes. Glycosylation and methylation of anthocyanidins also may modulate the antioxidant activity (*2*, *3*, *15*). O-Methylation generally reduces radical scavenging activity of anthocyanidins (*3*, *13*). Addition of different sugar molecules either enhances or diminishes (*2*, *3*) radical scavenging efficiency. Glycosylation by glucose reduces the activity of anthocyanidins against the DPPH radical and ABTS radical, whereas the change in antioxidant action against the peroxyl ($ROO^{\cdot}$) radical was shown to depend on the nature of the aglycone (*2*). The results of the present study showing that 3′-O-methylation or/and 3-O-glycosylation decrease the radical scavenging capacity of anthocyanidins (**Table 1**, **Figures 5** and **6**) are in line with results on ABTS and DPPH radical scavenging capacity of anthocyanins reported by others (*3*, *13*).

The molecular characteristics underlying the effect of hydroxyl moiety conjugation on the radical scavenging potential of anthocyanins has not yet been described. The results of the present study reveal that the conjugated anthocyanins show the same pH-dependent behavior as the corresponding aglycons, although at a given pH value, the radical scavenging capacity often appears to be lower than that of the aglycon. Studying the pK_a for deprotonation of the various unconjugated and conjugated forms of anthocyanins known to exist in aqueous solution, as well as the theoretical calculated radical scavenging abilities of these forms provides insight in the mechanism underlying the reduced TEAC values of anthocyanins upon conjugation. There appears to be no significant difference in electronic characteristics of the aglycons as compared to the corresponding O-methylated compounds, whereas an effect on the various pK_a values is readily predicted. From this and the fact that the deprotonated forms appear to be better radical scavengers, it can be concluded that differences in the pH-dependent TEAC values for the aglycons and the corresponding O-methylated derivatives at a similar pH value are mainly due to the effect of methylation on the pK_a values and thus on the extent of deprotonation at the pH of interest. Thus, the reduction in radical scavenging activity of anthocyanins upon their O-methylation can be ascribed to an increase in their pK_a values, resulting in a lower extent of deprotonation at a given pH value and, consequently, a reduced radical scavenging capacity.

Upon deprotonation of the flavylium ion, the aglycon and methylated compounds become better hydrogen donators (lower BDE) than the 3-O-glycosylated compounds from an electronic point of view. This combined with the effects of 3-O-glycosylation on the various pK_a values leads to the conclusion that upon 3-O-glycosylation two effects contribute to the reduced TEAC values as compared to the aglycons. First, similar to the methylation, the pK_a values tend to be higher, resulting in lower relative levels of anionic forms at a given pH value and, given the better radical scavenging capacity of the anionic forms, lower TEAC values at a given pH for the 3-*O*-glucosides than for the aglycons. Second, the neutral as well as the monoanionic forms of the 3-*O*-glucosides are predicted to be somewhat worse radical scavengers than the aglycons reflected by higher BDE values than those obtained for the aglycons. This additional mechanism underlying reduced radical scavenging capacity of the 3-*O*-glucosides as compared to the 3′- or 3′,5′-O-methylated conjugates is due to the position of the conjugation because in all calculations the glucoside moiety was modeled by taking a methyl group, and the modified BDE and IP values upon conjugation at C3 are due to the position of the conjugation rather than the nature of the conjugate.

Altogether, the results of the present study give better insight into factors that influence the radical scavenging capacity of anthocyanins, indicating that the radical scavenging-mediated supposed beneficial effects of the wine pigments on human health will be influenced by the pH of the surrounding matrix, pointing at different levels of the biological activity of anthocyanins in different tissues.

ABBREVATIONS USED

A_1, A_2, and A_3, quinoidal-base forms; ABTS, 2,2′-azinobis-(3-ethylbenzothiozoline-6-sulphonic acid) diammonium salt; $ABTS^{\cdot+}$, the blue-green colored (2,2′-azinobis(3-ethylbenzothiozoline-6-sulphonic acid) diammonium salt) radical cation; AH^+, flavylium cation form; BDE, bond dissociation energy; BDE-(MA), monoanionic molecule bond dissociation energy; BDE-(N), neutral molecule bond dissociation energy; C_E, *E*-chalcon; CHD, coronary heart diseases; C_Z, *Z*-chalcon; DE, deprotonation energy; DFT, density functional therory; DPPH, 2,2-diphenyl-1-picrylhydrazyl; IP, ionization potential; IP(MA), monoanionic molecule ioniztion potential; IP(N), neutral molecule ionization potential; LDL, low-density lipoprotein; MA, monoanionic molecule; MP-8, microperoxidase-8; ORAC, oxygen radical absorbance capacity; PBS, phosphate-buffered saline; QSAR, quantitative structure activity relationship; $ROO^{\cdot-}$, peroxyl radical; TEAC, trolox equivalent antioxidant capacity; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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