JOC Article

Acidity of Hydroxyl Groups: An Overlooked Influence on Antiradical Properties of Flavonoids

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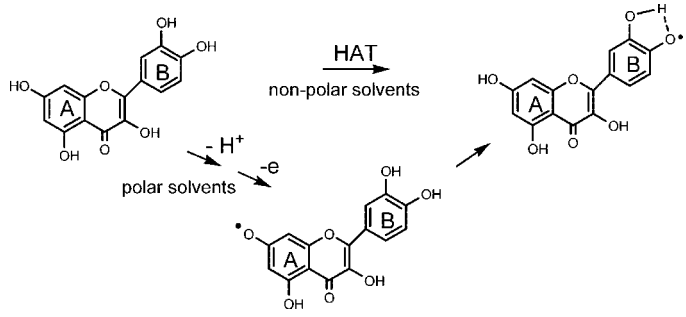

The reactions of 10 flavonoids with 2,2-diphenyl-1-picrylhydrazyl radical (**dpph**•) carried out in alcohols always occur significantly faster than in acidified alcohols or in dioxane. These fast kinetics benefit from the contribution of the electron transfer from a flavonoid anion to a radical, a mechanism known as Sequential Proton-Loss Electron-Transfer (SPLET), which adds to the kinetics of single-step Hydrogen Atom Transfer (HAT)/Proton Coupled Electron Transfer (PCET) processes (see *Acc. Chem. Res.* **2007**, *40*, 222.). The domination of SPLET over HAT/PCET in case of a flavonoid reacting with electron-deficient radicals such as peroxyls or **dpph**• in polar solvents explains the enhancement of antioxidant activity of 3-hydroxyflavone. It also elucidates the great acceleration in the reactions of **dpph**• with quercetin, morin, galangin, and 7,8-dihydroxyflavone. The analysis of structure−acidity and structure−activity relationships for 10 flavonoids clearly indicates that hydroxyl group at position 7 is the most acidic site. Thus, in polar solvents this group can participate in radical reaction via SPLET. In nonpolar solvents the most active site in quercetin (a flavonoid antioxidant commonly found in plants) is 3′,4′-dihydroxyl moiety and HAT/PCET occurs. However, in ionization-supporting solvents an anion formed at position 7 is responsible for very fast kinetics of quercetin/**dpph**• reaction because both mechanisms participate: HAT (from catechol moiety in ring B) and SPLET (from ionized 7-hydroxyl in ring A). Because of conjugation of rings A, B, and C the final structure of the formed quercetin radical (or quercetin anion radical) is the same for the SPLET and HAT/PCET mechanisms.

Introduction

Flavonoids are common components of plants. More than 4000 chemically unique flavonoids identified in various plant species[1] have been isolated from almost all parts of the plant such as leaves, stems, roots, fruits, or seeds. The general structure of these compounds containing three rings designated A, B, and C is depicted in Scheme 1. A presence of a double bond, a carbonyl, and a hydroxyl group in the pyranyl ring C serves as a basis for their classification into several classes and subclasses. Substitution of A and B rings by hydroxyl groups distinguishes individual members of each class.

Flavonoids are among the major antioxidant constituents of our diet. Their daily intake (reported as above 100 mg)[2] is almost at the same level as the sum of the daily doses of other antioxidants including β-carotene (2−3 mg), vitamin C (70−100

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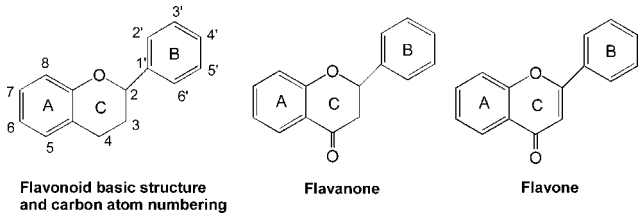
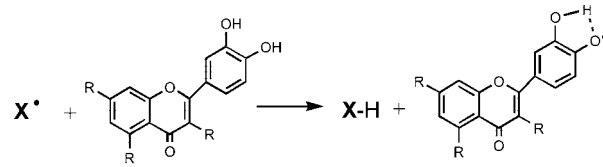
SCHEME 1



mg), and vitamin E (7 −10 mg).[3] These data correspond to the reports of many health benefits coming from dietary intake of flavonoids. Among the most significant profits are the protection against oxidative diseases,[4] ability to modulate the activity of various enzymes,[5] and interactions with specific receptors.[6,7] Since the mechanisms of action of flavonoids on human cells and organism is not completely understood, extensive multidisciplinary studies covering the kinetics of antioxidant action and enzyme binding in vitro, as well as their distribution, absorption, excretion, and metabolism in vivo, still attract great attention.[7−10] In general, the ability of flavonoids to be effective antioxidants depends on three factors: (i) the metal-chelating potential that is strongly dependent on the arrangement of hydroxyls and carbonyl group around the molecule, (ii) the presence of hydrogen-/electron-donating substituents able to reduce free radicals, and (iii) the ability of the flavonoid to delocalize the unpaired electron leading to formation of a stable phenoxyl radical. Both known modes of the antioxidant action, i.e., preventive mechanism and chain-breaking mechanism, are postulated to be responsible for the high activity of flavonoids. However, although the first function, i.e., the ability to chelate the iron and copper cations, is well understood, another one, i.e., the ability of polyphenols to scavenge reactive oxygen species such as peroxyl and hydroxyl radicals, is still far from being fully understood.

As a result of their low redox potentials (E_{pH7} < 0.75 V vs normal hydrogen electrode, NHE) flavonoids are able to reduce free radicals with redox potentials higher than 0.8 V, such as superoxide (ca. 0.9 V vs NHE for $O_2^{\bullet -}$, H^+/H_2O_2 pair), alkoxyl (1.6 V vs NHE for $RO^{\bullet},H^+/ROH$), peroxyl (0.77−1.44 V vs NHE for $ROO^{\bullet},H^+/ROOH$), and hydroxyl radicals (2.3 V vs NHE for $HO^{\bullet},H^+/H_2O$).[11] There are numerous studies devoted to the importance of flavonoid structure for their antiradical activity as chain-breaking antioxidants.[9,10,12−15] It is generally accepted that the excellent antioxidant properties of flavonoids can be explained by the presence of catechol hydroxylic groups in the B ring:[10,12,15]



(1)

The stability of the radical generated from catechol moiety relies on the hydrogen bond (HB) formed between the hydroxyl and oxygen possessing unpaired electron, reaction 1. The presence of a C═C double bond in ring C conjugated with a 4-oxo group is also of great importance for the delocalization of an unpaired electron. Additional antioxidant activity is assigned to the presence of a hydroxyl group at the 3- and 5-positions, and according to Bors et al.,[10] an internal hydrogen bond to a 4-oxo group (if present) makes these positions kinetically equivalent. The present knowledge does not explain, however, the exact role of hydroxyl groups in rings A and C (Scheme 1) during the scavenging of radicals. Glycosylation of the 3-OH group greatly reduces the antioxidant activity of 3-hydroxyflavones.[15] On the other side, it has been shown that hydroxyl groups at positions 5 and 7 are not as potent as 3′,4′-dihydroxyl (in the B ring).[10]

The term "antioxidant activity" is not clearly defined, and there are several definitions based on the nature of the oxidation and reactive intermediates taking part in this process. For lipid peroxidation the activity of an antioxidant can be considered as the ability to suppress the propagation of the kinetic chain of hydroperoxide formation:

$$LOO^{\bullet} + L\text{-}H \rightarrow LOOH + L^{\bullet} \quad (2)$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad (3)$$

by the competitive reaction with phenolic/flavonoid antioxidant, FlavOH:

$$LOO^{\bullet} + FlavOH \rightarrow LOOH + FlavO^{\bullet} \quad (4)$$

As a result, assessment of a flavonoid as a potential radical scavenger is frequently based on measurements of the rate constants for its reaction with reactive oxygen species, i.e., oxygen-centered free radicals such as superoxide, hydroxyl, or peroxyl radicals. Since the measurements of absolute rate constants for reaction 4 are complicated and time-consuming, some simple tests with model radicals such as 2,2-diphenyl-1-picrylhydrazyl radical (**dpph**$^{\bullet}$) or $ABTS^{\bullet+}$ radical cation are commonly applied.

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In most papers on antioxidant activity of natural compounds, the mechanism of reaction 4 is not discussed, thus giving the impression that single-step hydrogen atom transfer (HAT) occurs, which is not always true, because the kinetics and mechanism of reaction 4 depends on the medium. In general, the rate of HAT (including PCET) is governed by two major factors. The first one is the strength of the phenolic O−H bond. The higher the value of Bond Dissociation Enthalpy (BDE_{O-H}), the slower is the HAT process. The second factor is the magnitude of the Kinetic Solvent Effect (KSE), i.e., the ability of a phenol to be a HB donor and ability of a given solvent to be a HB acceptor.[16,17] A phenol molecule that is hydrogen-bonded to a solvent molecule does not react with radicals (for steric reasons). Thus, the rate constant k^s for HAT from a H atom donor to any attacking radical in all solvents can be correlated with the rate constant in a non-HB solvent, k^0, via eq I:[18]

$$\log k^s = \log k^0 - 8.3\alpha_2^H\beta_2^H \qquad \text{(I)}$$

where α_2^H represents the relative ability of the substrate to donate HB (range 0 to ca. 1),[19] and β_2^H represents the relative ability of the solvent to accept HB (range 0 to 1.00).[20]

In polar solvents that can support ionization of a phenol, the HAT/PCET process still operates; however, some (sometimes very significant) deviations from eq I can occur. These divergences are caused by another mechanism affecting the kinetics of reaction. According to this process a deprotonation of a phenol in solvent S:

$$PhOH + S \rightleftarrows PhO^- + HS^+ \qquad (5)$$

is followed by fast electron transfer from phenolate to an electron-deficient radical (Y$^\bullet$, such as peroxyl or **dpph**$^\bullet$):

$$PhO^- + Y^\bullet \rightarrow PhO^\bullet + Y^- \qquad (6)$$

$$Y^- + HS^+ \rightarrow YH + S \qquad (7)$$

This mechanism was named Sequential Proton-Loss Electron-Transfer (SPLET).[21,22] The rate of SPLET mechanism depends on the amount of phenolate anion. Thus, the ion-solvating ability of a solvent (termed solvent acity and basity[23]), as well as the acidity of a phenol, play a crucial role in HAT/SPLET competition/cooperation.[21,22,24] SPLET is favored for reactions of phenols having low pK_a's with electron-deficient radicals to yield product molecules having high pK_a's, e.g., **dpph**$^\bullet$/**dpph**-H and peroxyls ROO$^\bullet$/ROOH.[21]

Previous studies of the SPLET mechanism allowed us to successfully resolve a controversy over the mechanism of curcumin antioxidant action[25] and also to explain some anomalies in the kinetics of the reaction of synthetic phenolic antioxidant.[24] Moreover, using this approach it has been proved that kinetics of reaction occurring in alcohols can be affected by participation of phenolate anion for weak acids such as α-tocopherol,[26] which is characterized by a pK_a two units greater than that of phenol itself. Importantly, other groups of researchers also applied this reasoning in their studies. For example, the occurrence of SPLET in methanol and ethanol has been clearly demonstrated by Foti et al. [27] and later confirmed in a number of experimental [28,29] and theoretical studies.[30]

It is known that deprotonated flavonoids are more potent electron donors and are better radical scavengers than neutral molecules.[14,31,32] Thus, the exploration of the role of acidity and acid/base equilibria in the kinetics of phenol/radical reactions can be helpful in the understanding of the structure−activity relationship of natural antioxidants. In the current paper we provide evidence supporting a hypothesis that the acidity of OH groups in flavonoids has capital meaning for the kinetics of their reactions with **dpph**$^\bullet$. We decided to study 10 flavonoids of various hydroxylation pattern (Chart 1) to explain the mechanism of the antiradical action of quercetin, an example of a flavonoid possessing a catechol group in ring B and other hydroxyl groups in rings A and C. Quercetin modifies eicosanoid biosynthesis, protects low-density lipoprotein from oxidation, prevents platelet aggregation, and prevents or delays the occurrence of age-related cognitive deficits and neurodegenerative diseases. Many of these benefits are correlated with anti-inflammatory and antioxidant properties of that flavonoid.[33] During the period from 2002 to the end of 2007 the SCOPUS database lists more than 4900 scientific publications with keyword "quercetin", and in almost 30% of them the additional keyword is "antioxidant". The role of antioxidant action in therapy is still discussed (redox-independent mechanisms can be more important), and antiradical properties of quercetin attract great attention. Therefore, the relationship between the acidity and antiradical activity of quercetin, as well as of other flavonoids, is particularly important.

Results and Discussion

Acidity Constants of the Flavonoids. Figure 1 presents absorbance plots for two monohydroxyflavones and two dihy-

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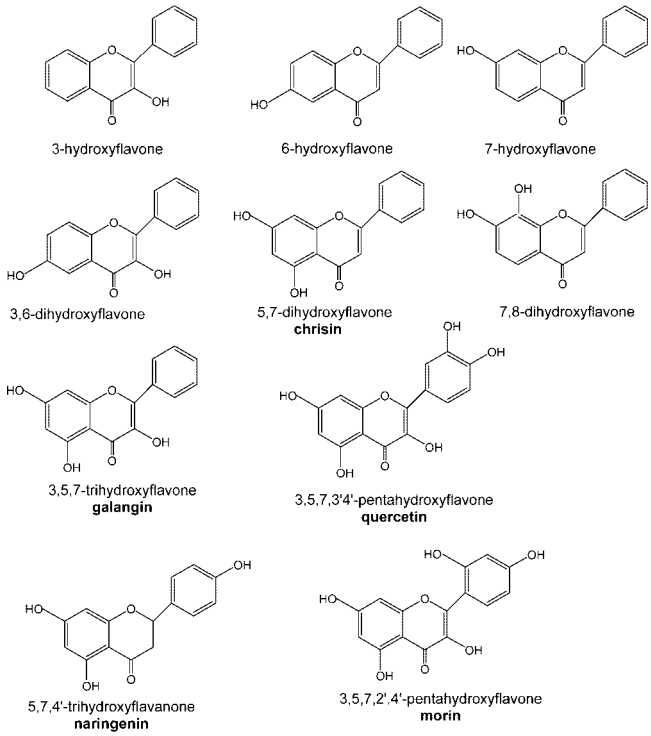
CHART 1. Structures of the Flavonoids Studied in This Work



droxyflavones at pH range from 3 to 12. As can be seen, there are two main bands: (i) a band with maximum at 320−400 nm attributed to the ring B absorbance and (ii) a band with maximum in the range 250−285 nm representing absorbance of the A ring.[34] Acid−base equilibria have a strong effect on the UV−vis spectra of phenols and flavonoids, manifested by the considerable changes of the band shapes and intensities with changing pH values. For monohydroxylated compounds one or more isosbestic points can be found corresponding to a neutral form being in equilibrium with the deprotonated molecule. In contrast, for polyhydroxylated phenols, when more than two forms are present in the solution at the same pH, the clear isosbestic points are not observed; see Figure 1D. Even small changes in protonation status of any OH group are accompanied by a strong increase or decrease in the intensity of the bands.

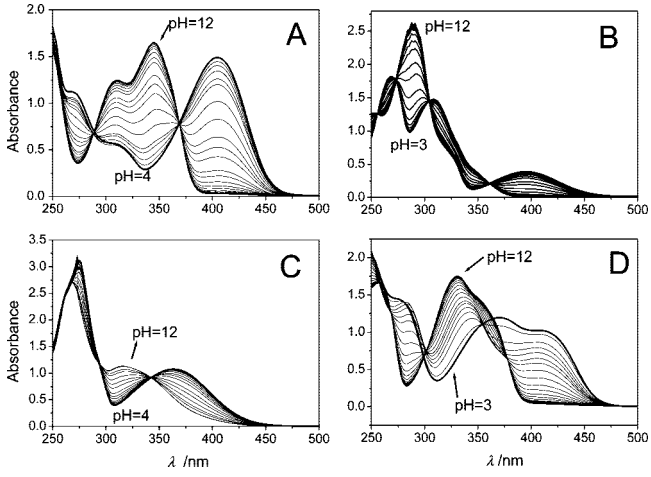


FIGURE 1. Absorption spectra for 1.0×10^{-4} M flavonoids at various pH values (water/methanol 1:1): (A) 3-hydroxyflavone, (B) 6-hydroxyflavone, (C) 5,7-dihydroxyflavone, (D) 3,6-dihydroxyflavone.

As a result of this fact, the spectrophotometric titration is frequently applied for the determination of pK_a. Although this method is more time-consuming than potentiometric titrations, it can be successfully applied for compounds of low solubility (below millimolar concentration) and for compounds having extremely low or extremely high pK_a's.

To validate our method, we started our measurements with the analysis of simple model phenols, obtaining values in excellent agreement with literature ones.[35] For example, for 4-methylphenol we obtained pK_a = 10.13 ± 0.06 (lit. 10.10 and 10.02), for 2-methoxyphenol pK_a = 10.03 ± 0.05 (lit. 9.98 and 9.90), and for 3-nitrophenol pK_a = 8.40 ± 0.02 (lit. 8.23 and 8.36).[35]After such validation of our methodology we measured the pK_a's of the selected flavonoids. A typical plot of the distribution of the neutral form of a flavonoid versus its mono-, di-, and trianionic species within the entire pH interval is presented in Supporting Information (Figure S1). The pK_a values for 10 flavonoids calculated on the basis of spectrophotometric titrations are collected in Table 1, listed together with the literature pK_a's for these flavonoids. With some exceptions (discussed below), the presented parameters that describe the first step of deprotonation are in rather good agreement with the previously published values. The less satisfying agreement with literature data was obtained for second and third pK_a's. Ionized flavonoids are unstable when exposed to air, and we suppose that literature pK_a values for second, third, and higher steps of deprotonation are contaminated by pK_a's of oxidation products. To be sure that our results are free from such errors, a continuous flow of nitrogen through the titration flask was sustained to eliminate the traces of air.

Analysis of literature acidity constants reveals significant variation among the published values. The most striking examples are quercetin (differences for pK_{a1} are about 2.5 units) and morin (the pK_{a1} values are within the range from 3.5 to 8.2); see Table 1. The value obtained in our analyses for morin (pK_a = 5.2) is located in the middle of that scattered range. In the series of 10 compounds from Table 1 morin is the only flavonoid with a 2′-hydroxyl group. Agrawal and Schneider[36] applied ^{13}C NMR to follow the stepwise deprotonation of 14

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(37) Our results confirmed that morin is more acidic than other flavonoids with a 7-OH group. The possible explanation of such exceptionally strong acidity and unusual deprotonation site of this flavone is that deprotonation of the 2′-hydroxyl group gives an anion additionally stabilized by a hydrogen bond with the hydroxyl at position 3:

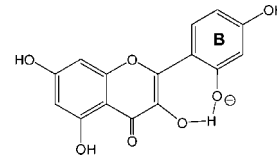

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TABLE 1. pK_a Values from the Literature and Measured in the Present Work,[a] O−H Bond Dissociation Enthapies (BDE), and Ionization Potentials (IP) for Neutral Molecules and for Anions[b]

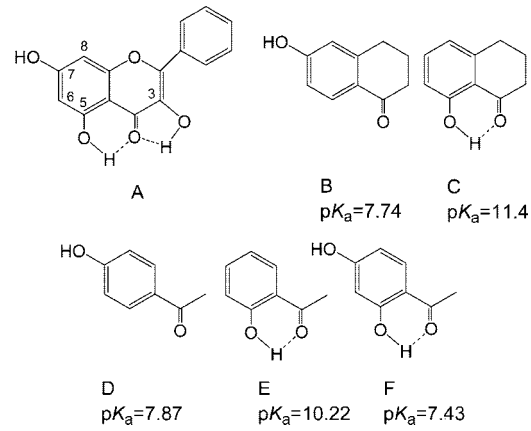
	pK_a			IP (eV)	
compound	lit. values	this work	BDE[d] (kcal/mol)	neutral	from anion
3-hydroxyflavone	10.34[38] 9.6[39] 9.25[40] 9.99[41]	9.4 ± 0.2	89.9	7.48	2.36
6-hydroxyflavone		9.0 ± 0.2			
7-hydroxyflavone	8.48[38] 7.39[39]	7.7 ± 0.1	92.1	8.02	2.85
3,6-dihydroxyflavone		9.0 ± 0.2; 10.7 ± 0.3			
5,7-dihydroxyflavone (chrysin)	8.37; 12.37[38] 9.14[40] 7.9; 11.40[41]	8.0 ± 0.2; 11.9 ± 0.4	94.9 (7)	7.74	2.96
7,8-dihydroxyflavone	7.6[32,c]	7.4 ± 0.1; 11.4 ± 0.1	81.9 (7)	7.73	2.67
3,5,7-trihydroxyflavone (galangin)	6.8; 9.4[14]	7.6 ± 0.1; 9.5 ± 0.1; 10.9 ± 0.3	88.1 (3)	7.36	3.13
5,7,4′-trihydroxyflavanone (naringenin)	8.83[40]	7.5 ± 0.1; 8.4 ± 0.1; 9.8 ± 0.1	89.5 (4′)		
3,5,7,2′,4′-pentahydroxyflavone (morin)	8.04[40] 3.46; 8.1[14]	5.2 ± 0.1; 8.2 ± 0.2; 9.9 ± 0.2			
3,5,7,3′,4′-pentahydroxyflavone (quercetin)	8.21[40] 5.7; 7.1; 8.0; 9.9; 11.0[42,e] 7.03; 9.15[43] 7.7; 8.77; 9.8[44]	8.45 ± 0.1; 9.31 ± 0.2; 11.12 ± 0.2	78.6 (4′)	7.03	2.69

[a] In water/methanol, (1:1 v/v), measured by spectrophotometric titration. [b] BDE and IP values in gas phase are predicted on the basis of DFT calculations by Lemanska et al.[32] [c] Predicted on the basis of QSAR, see ref 32. [d] Values in parentheses refer to position of OH group. [e] These values were measured spectrophotometrically. Other pK_a's measured potentiometrically for quercetin are 5.54, 6.95, 8.21, 9.9, and 11.0; see ref 42.

phenols and 4 flavonoids (chrysin, apigenin, naringenin, morin) in methanol or in DMSO mixtures with D_2O. They concluded that in chrysin, apigenin, and naringenin the hydroxyl groups are deprotonated in the range 7-OH > 4′-OH > 5-OH and, exceptionally, deprotonation of morin starts at position 2′. Such unusual deprotonation sequence in morin has never been explained.[37]

Most Acidic Site in Quercetin. Experimental data for quercetin allow us to state that this pentahydroxyflavone is not as acidic as reported previously by Escandar et al.[42] Its acidity is similar to that determined by Georgievskii,[40] and the kinetic data (discussed below) confirmed that observation. A correct determination of the site of primary deprotonation of quercetin is of great importance if reaction of quercetin anion with a radical is considered. Spectrophotometric or potentiometric titrations do not make it possible to distinguish the range of acidity of particular OH groups in polyhydroxylated phenols. Moreover, according to our knowledge, a limited number of works actually discuss the site of primary dissociation. Harborne et al. in their book[34] noticed that the addition of sodium methanolate caused ionization of hydroxyls at all positions, but the addition of weaker base, i.e. sodium acetate, caused ionization of hydroxyls only at positions 3, 7, and 4′. Values of pK_a determined for monohydroxylated flavonoids can be helpful for assignment of the acidity of particular hydroxyl groups in polyhydroxylated flavonoids. The lower acidity of the 3-hydroxyl within the acidity range 7 > 6 > 3 (see Table 1) originates from the formation of an intramolecular hydrogen bond. This observation is in good agreement with the large difference in acidities reported more than 40 years ago for 6-hydroxy-1-tetralone (Chart 2, structure B) and 8-hydroxy-1-tetralone (Chart 2, structure C) by Magnusson et al.,[45] as well as known differences in acidities of 4-hydroxyacetophenone and 2-hydroxyacetophenone (Chart 2, structures D and E, respectively).[35]

CHART 2. Structures of 3,5,7-Trihydroxyflavone (Galangin), Hydroxytetralones, and Hydroxyacetophenones



The presence of OH group at position 5 causes a rather slight change of the acidity of the 7-OH group, and this observation is in agreement with similar acidities of acetophenones (Chart 2, compounds D and F). In general, with the exception of morin (discussed previously[37]), all of the compounds with a 7-OH group have similar acidity (pK_a between 7.5 and 8.5) and this group is the most acidic, despite the hydroxylation pattern in other positions. Such observation corroborates with the results of the work by Agrawal and Schneider,[36] who ranged decreasing acidities of OH groups in quercetin in the order 7 > 4′ > 3.

(45) Magnusson, L. B.; Postmus, C. J.; Craig, C. A. *J. Am. Chem. Soc.* **1963**, *85*, 1711–1715.

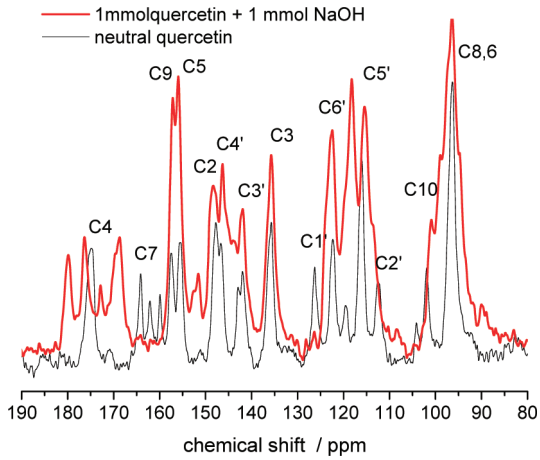


FIGURE 2. ^{13}C CP/MAS NMR spectra of quercetin and its monosodium salt: neutral (black line, with peak assignments on the basis of values from Table 3) and monosodium (red line). The spectra in color and other spectra of neutralized quercetin (1 mmol of quercetin + 0.5 and 2 mmol of NaOH, respectively) are in Supporting Information.

Recently, that range of acidity has been questioned by Milane et al., who claimed that "the pK_{a1} = 7.7 could be attributed to the deprotonation of hydroxyl on the 3-position, the pK_{a2} = 8.77 to the hydroxyl on the 7-position (...). Finally, the pK_{a3} = 9.8 corresponded to the hydroxyl in the 4′-position instead of the 7-position."[44,46] However, our results for monohydroxylated and dihydroxylated flavones collected in Table 1 clearly show that pK_a for 3-OH group is almost two units higher than that for 7-OH. Combination of two or three hydroxylic groups does not increase the acidity of 3-OH, as was shown for 3,6-dihydroxyflavone and galangin (Table 1). Thus, 3-OH group, *although more acidic than phenolic OH*, cannot be more acidic than 7-OH. The hypothesis that 3-OH is more acidic than 7-OH is in surprising contradiction with other experimental results.[36] Importantly, it also contradicts with the results of our structure−acidity relationship studies. To solve this contradiction we decided to perform additional NMR measurements. Solid-state ^{13}C CP/MAS NMR technique is a valuable method of analysis of powder solids that do not form crystals, so we decided to apply this technique for quercetin and its partially neutralized salts. Spectra of quercetin and its monosodium salt are presented in Figure 2. Other ^{13}C CP/MAS NMR spectra for quercetin/NaOH ratios of 1:0.5 and 1:2, can be found in Supporting Information. ^{13}C NMR signals for neutral flavonoids were carefully interpreted by Agrawal in his monograph.[47] Recently, detailed interpretation of ^{13}C CP/MAS for a number of flavonoids have been published by Wawer and Zielinska.[48] Analyzing these data, we were able to assign the solid-state NMR signals for quercetin; see Table 2.

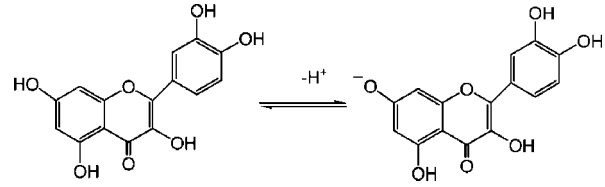
Comparison of the spectra shown in Figure 2 indicates that the signals of carbon atoms C1′−C6′ in ring B of neutral quercetin overlap with the C1′−C6′ signals for the monosodium salt of quercetin (Figure 2). The signal for C-3 also remains in the same field after neutralization of one OH group. In contrast,

TABLE 2. Assignments of the ^{13}C CP/MAS Signals and Chemical Shifts of Neutral Quercetin[a]

atom	δ, ppm	atom	δ, ppm
C-2	146.5 (146.2)	C-1′	126.3 (125.0)
C-3	135.7 (135.3)	C-2′	112.5 (112.3)
C-4	174.9 (174.3)	C-3′	142.0 (141.6)
C-5	157.5 (157.1)	C-4′	147.8 (148.0)
C-6	96.3 (96.1)	C-5′	116.0 (115.7)
C-7	164.1 (164.0)	C-6′	122.3 (121.9)
C-8	96.3 (96.0)		
C-9	155.6 (155.0)		
C-10	101.9 (101.6)		

[a] Literature ^{13}C CP/MAS chemical shifts from ref 48 are in parentheses.

we observed that signal for C-7 (164 ppm) was considerably shifted toward higher frequency field and reached δ = 168.7 ppm for the monosodium salt, Figure 2. Similar strong shifts were also reported by Agrawal and Schneider for carbon atoms adjacent to OH groups being deprotonated during ^{13}C NMR titration of phenols and flavonoids in solution.[36] Deprotonation of 7-OH produces significant shielding of C-4 and also some changes in the ability of the carbonyl group to accept an intramolecular hydrogen bond from hydroxyl groups at C3 and C5. As a result, new split signals for C-4 appear in the range 172−180 ppm. Taking into account the results of NMR experiments together with our experimental pK_a data for the studied flavonoids, we can draw a conclusion that quercetin initially deprotonates from the 7-OH group:



Our experimental results are in line with theoretical calculations for quercetin deprotonation in liquid phase.[49,50]

Summarizing this part of our work, we can order the investigated flavonoids with respect to their increasing acidity: 3-hydroxyflavone < 3,6-dihydroxyflavone ≈ 6-hydroxyflavone < 3,5,7,3′,4′-pentahydroxyflavone (quercetin) < 5,7-dihydroxyflavone (chrisin) < 7-hydroxyflavone ≈ 3,5,7-trihydroxyflavone (galangin) < 7,8-dihydroxyflavone < 5,7,4′-trihydroxyflavone (naringenin) < 3,5,7,2′,4′-pentahydroxyflavone (morin). A comparison of the structures of these flavonoids with their acidity leads to the following conclusions: (i) formation of an intramolecular hydrogen bond causes a decrease in acidity of the OH group being a HB-donor (5-OH and 3-OH), (ii) acidity of the OH group being a HB-acceptor in catechol group does not significantly increase in comparison to that of a non-H-bonded group (acidity of 7,8-dihydroxyflavone is almost the same as the acidity of 7-hydroxyflavone; the acidity of the 3′,4′-dihydroxyl group is not stronger than the acidity of the 7-OH in quercetin), (iii) with the exception of morin, the 7-OH group

(46) It seems that Milane and collaborators misinterpreted their results of ^{1}H NMR analysis of partially neutralized quercetin. They reported that the first deprotonation step is manifested by a decrease of the chemical shifts for H atoms at C6 and C8 and (to a smaller extent) at the 2′ and 6′ positions, and they concluded that the only explanation is that 3-OH was deprotonated first. In our opinion these data do not entirely support this interpretation.

(47) Agrawal, P. K. *Carbon-13 NMR of Flavonoids*; Elsevier: Amsterdam, 1989.

(48) Wawer, I.; Zielinska, A. *Magn. Res. Chem.* **2001**, *39*, 374–380.

(49) Recently, Leopoldini et al.[50] calculated the acidity of some phenolic antioxidants in gas and liquid (water) phase. The solvation causes 30−40 kcal/mol decrease of the deprotonation energies and can also change the acidity order in a series of compounds. Importantly, their results showed that the most acidic site in quercetin is 4′ (in the gas phase) and 7 (in water), in agreement with our experimental results in a water/methanol system.

(50) Leopoldini, M.; Russo, N.; Toscano, M. *J. Agric. Food Chem.* **2006**, *54*, 3078–3085.

TABLE 3. Comparison of Measured Bimolecular Rate Constants (k^s in M^{-1} s^{-1}) for Reactions of dpph• with 10 Flavonoids[a] and the Acceleration Ratio[b]

	solvent (β_2^H)								
flavonoid	MeOH (0.41)[c]	MeOH, H^+[e] 10 mM[e]	MeOH, H^+[e] 100 mM[e]	EtOH (0.44)[c]	EtOH, H^+[e] 10 mM[e]	EtOH, H^+[e] 100 mM[e]	ethyl acetate (0.45)[c]	dioxane (0.47)[d] (0.41)[c]	$k^{MeOH}/k^{MeOH/H^+}$
3-hydroxyflavone	47	0.88	0.45	7.0	nd[f]	nd[f]	0.055	0.061	104
6-hydroxyflavone	0.22	0.040	0.012	0.083	nd[f]	nd[f]	0.0067	0.0053	26.7
7-hydroxyflavone	0.12	0.083	0.022	0.43	nd[f]	nd[f]	0.0053	0.0029	5.5
3,6-dihydroxyflavone	23	1.7	1.3	8.0	1.6	1.0	0.21	0.11	17
5,7-dihydroxyflavone (chrisin)	2.7	0.15	nd[f]	0.81	0.46	0.41	0.074	0.0030	18
7,8-dihydroxyflavone	40000	1500	190	14000	450	130	17.9	2.6	210
3,5,7-trihydroxyflavone (galangin)	310	4.6	3.4	260	4.3	2.0	0.62	0.27	91
5,7,4′-trihydroxyflavanone (naringenin)	0.99	0.73	0.61	0.69	0.42	0.32	0.034	0.011	1.6
3,5,7,2′,4′-pentahydroxyflavone (morin)	5200	750	150	5400	1500	150	15.0	8.9	35
3,5,7,3′,4′-pentahydroxyflavone (quercetin)	3000	50	58	5400	41	25	9.3	3.0	51

[a] In methanol, acidified methanol, ethanol, acidified ethanol, ethyl acetate, and dioxane. Full data with errors are given in Supporting Information. [b] Ratio of the rate constants in MeOH to the rate constants in acidified MeOH. [c] Reference 20. [d] Reference 25, statistically not corrected. [e] Alcohols containing acetic acid in 10 and 100 mM concentration. For acetic acid $\beta_2^H = 0.42$ (ref 25). [f] nd = not determined.

is the most acidic site in all of the flavonoids studied in this work, and the presence of other hydroxyls in position 3, 5, and 6 does not considerably change the acidity of 7-OH group.

Kinetic Measurements. Our present study of antiradical abilities of mono- and polyhydroxylated flavonoids is based on the measurements of kinetics of their reactions with **dpph•** radical. The radical has been widely used to measure the hydrogen atom donating abilities of natural antioxidants.[25] The rates of reaction of **dpph•** with flavonoids (Flav-OH)

$$\textbf{dpph}^{\bullet} + \text{Flav-OH} \rightarrow \textbf{dpph}\text{-H} + \text{Flav-O}^{\bullet} \qquad (8)$$

were determined by monitoring the decay of **dpph•** at 517 nm in a stopped flow apparatus, as described previously.[21] Flav-OH was always used in excess as compared with the **dpph•** concentration, which was generally ca. $1-8 \times 10^{-5}$ M. In all measurements excellent pseudo-first-order decays of **dpph•** were observed (with rate constant k_{ex}), and the bimolecular rate constants for reaction 8, k^s, were calculated from the slopes of plots of k^{ex} vs [Flav-OH] as

$$k^{ex} = \text{constant} + k^s[\text{Flav-OH}] \qquad (\text{II})$$

for dilute concentrations of flavonoids to exclude their self-association.

It has to be emphasized that all flavonoids listed in Table 1 are more acidic than phenol ($pK_a = 10.0$).[35] Therefore, in ionization-supporting solvents all of these polyphenols should exhibit increased reactivity toward peroxyls and **dpph•** radicals, as predicted by SPLET mechanism. The participation of SPLET in phenol-radical reaction is kinetically important, and the overall rate of reaction can be hundreds of times faster as compared to the pure HAT process.[22] Equation I applies only to "pure" HAT/PCET reactions. Thus, it can be employed as a valuable tool for quantifying the importance of the SPLET process in different solvents and with different phenols.[21] Unfortunately, k^0 in eq I could not be determined for our **dpph•**/ArOH reactions because flavonoids are insoluble in saturated hydrocarbons ($\beta_2^H = 0.00$). We therefore measured k^s for this reaction in two non-hydroxylic solvents, i.e., 1,4-dioxane ($\beta_2^H = 0.41$, after statistical correction its β_2^H is 0.47, see Table 3), and ethyl acetate ($\beta_2^H = 0.45$) and

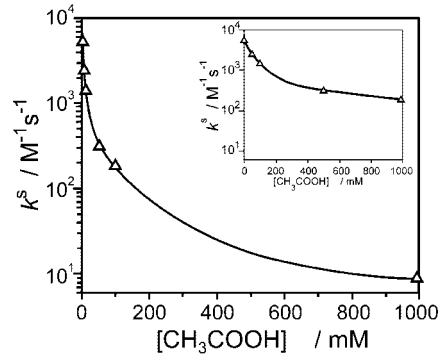


FIGURE 3. Plot of bimolecular rate constant for reaction morin + **dpph•** in methanol versus increasing amount of acetic acid.

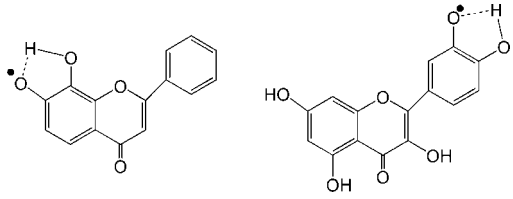
in two hydroxylic solvents, i.e., methanol ($\beta_2^H = 0.41$) and ethanol ($\beta_2^H = 0.45$). These two hydroxylic solvents have much higher dielectric constants, ε, than the two non-hydroxylic ones. Hence, they have much greater abilities to support ionization and, consequently, the SPLET reaction mechanism (eqs 5−7). Comparison of the rate constants for reaction 8 carried out in these four solvents can, therefore, explain the role of phenol ionization in **dpph•**/flavonoid reactions. Results of measurements are summarized in Table 3 together with k^s values measured in two alcohols acidified by the addition of acetic acid, as described in our previous works.[21,24,25]

In all of the solvents listed in Table 3, the **dpph•**/flavonoid reactions followed pseudo-first-order kinetics. Therefore, reaction rate constants can be compared for pairs of solvents characterized by similar HB-basicity. Indeed, the processes carried out in methanol and ethanol are much faster than those in dioxane and in ethyl acetate. For all compounds reacting in methanol the addition of as little as 10 mM acetic acid dramatically decreases the k^s; see Table 3. An increase of the concentrations of acetic acid caused the further decrease of the bimolecular rate constants (see Figure 3).

Because β_2^H parameters for acetic acid, methanol, and ethanol are similar, we exclude additional HB formation with CH_3COOH as a reason for the decreasing rate for flavonoid + **dpph•** reaction. Thus, the decrease of reaction rate after addition

of acid is caused by a declining amount of phenolate anions, which emphasizes the importance of dissociation to this electron-rich form in the overall reactivity. In other words, in neat alcohols all of the flavonoids are partially ionized and their anions react rapidly with the **dpph•** radical. Addition of the acid suppresses ionization and the reaction is now governed only by HAT instead of a combination of accelerated HAT and fast SPLET mechanisms. Our previous experiments with the reaction of simple phenols + **dpph•** in the presence of base showed an enormously high increase of rate constants.[21] Indeed, in the present experiments addition of a few drops of 0.1 mM sodium methanolate to the quercetin solution caused a rate acceleration too large to be measured by the stopped flow apparatus (reaction was completed during the dead time of mixing).

Structure−Reactivity Relationship: Reactivity vs Acidity. As we reported previously,[21,24,25] the pure HAT mechanism occurs in dioxane ($\varepsilon = 2.2$, see Table 3). Assuming that all flavonoids listed in Tables 1 and 2 have similar HB-donating ability and neglecting the higher HB-donating ability of catechols, the same magnitude of KSE should be observed. This assumption seems to be reasonable for unhindered hydroxyl groups in a series of similar compounds (however, α_2^H values are higher for catechols,[51] so catecholic flavonoids should be compared to each other within their class). Therefore, in dioxane the range of the flavonoid/**dpph•** reactivity expressed as $k^{dioxane}$ (collected in Table 3) should be in opposite order to their BDE's. Indeed, quercetin is the most reactive (78.6 kcal/mol) followed by 7,8-dihydroxyflavone (81.9 kcal/mol), which is more reactive than galangin (88.1 kcal/mol) and 3-hydroxyflavone (89.9 kcal/mol), and the least reactive are 7-hydroxyflavone (92.1 kcal/mol) and chrisin (94.9 kcal/mol).[52] *This straightforward relationship clearly shows that in nonpolar solvent a reaction between* ***dpph•*** *and 10 flavonoids is controlled by the HAT mechanism thermodynamically based on BDE values*. However, the rate constants in neat methanol and ethanol are ranged in different manner: 7,8-dihydroxyflavone ≫ morin, quercetin > galangin, followed by considerably less reactive: 3-hydroxyflavone ≫ 3,6-dihydroxyflavone > chrisin > naringenin > 6-hydroxyflavone > 7-hydroxyflavone. If the presence of catechol moiety was the most important factor affecting the rate of reaction, quercetin should be as reactive as 7,8-dihydroxyflavone (and even more reactive, as in dioxane), because both catecholic flavonoids can form stable radicals where the remaining *o*-OH group stabilizes the radical as a result of the intramolecular hydrogen bonding:[53]



The nearly identical reactivity of morin (without catechol moiety) and quercetin shows that the kinetics of the reactions in methanol and ethanol are noticeably affected by the acidity of a phenol and, as a result, by the amount of accessible phenolate anions. The ratio ($k^{MeOH}/k^{MeOH/H^+}$) listed in the last column of Table 3 can be assumed to be a contribution of the ionized form of flavonoid over the nonionized form to its overall antiradical reactivity in methanol. This ratio is similar for quercetin and morin and, surprisingly, two times larger for galangin, a flavonoid that is 10 times less reactive toward **dpph•** but more acidic than quercetin. Since the acidity of some flavonoids resembles the acidity of acetic acid ($pK_a = 4.8$), the more reliable quantity for comparison of the anion participation is the acceleration ratio expressed as $k^{MeOH}/k^{dioxane}$, which can be applied as a parameter indicating the role of phenol dissociation (and SPLET participation) in the overall kinetics of flavonoid + **dpph•** in ionization-supporting solvents; see Table 4.

TABLE 4. Acceleration Ratios[a]

flavonoid	$k^{MeOH}/k^{dioxane}$
7,8-dihydroxyflavone	15384
galangin	1148
quercetin	1000
5,7-dihydroxyflavone	900
3-hydroxyflavone	770
morin	584
3,6-dihydroxyflavone	209
naringenin	90
6-hydroxyflavone	60
7-hydroxyflavone	41

[a] Ratios of rate constants for reaction with dpph• radicals carried out in methanol and in dioxane.

Reaction of Quercetin with dpph• Radical in Polar Solvents. The astonishing value of the acceleration ratio $k^{MeOH}/k^{dioxane}$ for 7,8-dihydroxyflavone shows the importance of the acidity of the OH group in ring A. This is one of the most prominent examples of the SPLET mechanism leading to formation of stable phenoxyl radical. Quercetin, with a less acidic catechol moiety in B ring, is not as efficiently activated as 7,8-dihydroxyflavone. If the hydroxyl at position 3′ or 4′ of quercetin was deprotonated first, the acceleration ratio for that flavonoid should be of the same order as for 7,8-dihydroxyflavone. Instead, the acceleration ratio for quercetin is similar to that for galangin. Both flavones have an identical hydroxylation pattern in ring A and have almost the same $k^{MeOH}/k^{dioxane} \approx 1000$. This is yet another (kinetic) proof showing that the 7-hydroxyl group is the most acidic site in quercetin. However, the absolute rate constant for reaction of **dpph•** with quercetin is 10 times larger than for the reaction of **dpph•** with galangin. The same (10-fold) difference in reactivity is also observed for the rate constants in dioxane. *Thus the 7-OH group in both flavonoids plays an important role as the site of ionization and of electron transfer (according to SPLET)*. A significant difference in absolute rate constants for the quercetin and galangin pair confronted with the same acceleration ratio $k^{MeOH}/k^{dioxane}$ can be explained by the presence of 3′,4′-dihydroxyl moiety in ring B of quercetin. For the reaction of quercetin + **dpph•** we propose the mechanisms shown in Scheme 2.

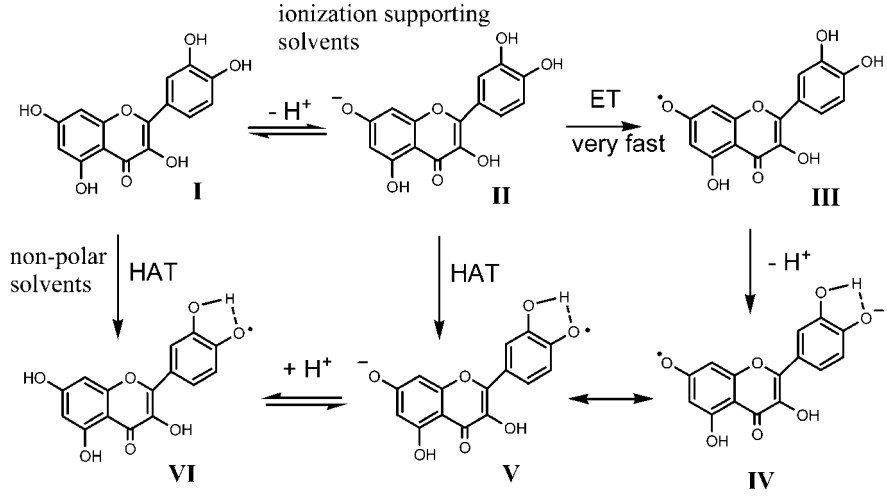
In nonpolar solvents or in solvents that do not support ionization, one-step HAT occurs from nonionized quercetin (structure **I**) to give quercetin radical (**VI**). In methanol (and

(51) Foti, M. C.; Barclay, L. R.; Ingold, K. U. *J. Am. Chem. Soc.* **2002**, *124*, 12881–12888.

(52) The same reactivity of 7-OH-flavone and 5,7-di-OH-flavone in dioxane (their O-H BDEs differ by ca. 3 kcal/mol; see Table 1) can be explained by kinetic solvent effect: 7-OH-flavone is perhaps a slightly better HB donor than 5,7-diOH-flavone. We do not know the HB-donating ability of 7-OH group, but the simple rule "the stronger acid, the better HB donor" should operate in this case, in agreement with the equation $\alpha_2^H = 23.41(pK_a)^{-1.32}$ (see: Mulder, P.; Litwinienko, G.; Lin, S.; MacLean, P. D.; Barclay, L. R. C.; Ingold, K. U. *Chem. Res. Toxicol.* **2006**, *19*, 79–85.

(53) Wright, J. S.; Johnson, E. R.; DiLabio, G. A. *J. Am. Chem. Soc.* **2001**, *123*, 1173–1183.

SCHEME 2. Possible Mechanisms for Quercetin Reaction with dpph• Radicals



other polar solvents that can support ionization) the neutral quercetin is in equilibria with monoanion form (structure **II**) and two reaction pathways are possible. The first one is a HAT from the catechol moiety to form the radical anion (Scheme 2, step **II** → **V**). This reaction is strongly accelerated by increased electron density in rings A and C. Furthermore, the alternative route is a fast electron transfer from phenolate anion (structure **II**) to **dpph•** radical (step **II** → **III**). Since phenoxyl radicals are considerably stronger acids than their parent compounds,[14,25] the acidity of all hydroxyl groups in structure **III** is dramatically increased. Ring A is strongly electron-withdrawing, and as an effect of conjugation, the catechol moiety in ring B is the most probable site of deprotonation (step **III** → **IV**). After deprotonation of catechol the negative charge is immediately transferred to strongly electron-deficient ring A, and finally a radical anion with unpaired electron at ring B (catechol radical) and negative charge at ring A is formed (structure **V**). A similar mechanism was proposed by one of us to resolve the curcumin antioxidant controversy.[25]

In fact, radical **III** can cause deprotonation of any OH group in quercetin, but such path will be not as productive as catechol deprotonation leading to stable catechol radical anion (**V**). This hypothesis is supported by the values of kinetic parameters for galangin and 5,7-dihydroxyflavone. Although parameters $k^{MeOH}/k^{dioxane}$ are about 1000 (proving the SPLET), the absolute rate constants k^{MeOH} are much smaller for these two compounds than for quercetin (10 and 1000 times, respectively; see Table 3), which is a consequence of the inability of galangin and 5,7-dihydroxyflavone to form a stable semiquinone radical like catechols. Another argument for favored fast deprotonation of catechol moiety instead deprotonation of 3- and 5-OH groups is that the latter are H-bonded to the carbonyl group, and thus they are weaker acids than 3′,4′-hydroxyls group.

Comparing the values of $k^{MeOH}/k^{dioxane}$ (listed in Table 4) for quercetin (= 1000) and for more acidic 7-hydroxyflavone (= 41), one can state that the acceleration of reaction rates is not founded on the acidity of flavonoids. However, although quercetin is 0.7 pK_a unit less acidic than 7-hydroxyflavone, the IP value for the anionic form of the latter is 0.15 eV higher (2.85 eV, see Table 1), making the 7-hydroxyflavone anion a worse electron donor than the quercetin anion.

It should be stressed that the SPLET mechanism does not exclude the conventional HAT/PCET process directly from the site of the lowest BDE_{O-H} (Scheme 2, step **I** → **VI** and step **II** → **V**), which still is an efficient pathway of quercetin/radical reaction (see also footnote 55). However, the SPLET starting from deprotonation of the 7-OH group (more acidic than 3′,4′ hydroxyls) is the most reasonable explanation of the 1000-fold acceleration of the reaction rate in methanol. Zielonka et al.[54] reported the acceleration of the rates of $Br_2^{\bullet-}$ and $^{\bullet}N_3$ reactions with deprotonated genistein versus its neutral form. They confirmed their experimental observations with DFT calculations of ΔIP = −95 kcal/mol and ΔBDE = −10 kcal/mol for monoanionic form of genistein in comparison with its neutral form.[54] The results of our experiments (Table 4) show a strong acceleration even for monohydroxylated flavonoids (their anions cannot be a source of H atom donors, and thus SPLET mechanism is responsible for the rate acceleration in this case).[55]

It is not possible to distinguish the reaction pathways **II** → **V** and **II** → **III** → **IV** → **V** on the basis of analysis of reaction products (which is the same for both mechanisms and not stable); however, the kinetic data clearly indicate that ionization increases the phenol reactivity. Ionization, together with other structural features (such as the presence of catechol groups, conjugation, etc.) should be considered as important factors in structure−activity relationship studies.

Role of the 3-Hydroxyl Group. The SPLET mechanism and acid−base equilibria can also be helpful in the explanation of the role of the 3-hydroxyl group in flavonoids. In a few works the hydroxyl group at position 3 was reported as the group that increases the antioxidant activity of flavones.[9,15,57] Glycosylation at 3-OH reduces the antioxidant activity of the compound.

(54) Zielonka, J.; Gebicki, J.; Grynkiewicz, G. *Free Radical Biol. Med.* **2003**, *35*, 958–965.

(55) Values of oxidation-reduction potentials, pK_a's, and BDEs for **dpph•** and several peroxyl radicals have been reviewed in ref.[22] An example, how deprotonation of a phenol can govern the reaction mechanism, was described by Nakanishi et al.:[56] reduction potential of 2,2-bis(4-*tert*-octylphenyl)-1-picrylhydrazyl, dopph•, in acetonitrile E^0_{red} vs SCE = 0.18 V, oxidation potential of 2,2,5,7,8-pentamethylchroman-6-ol (PMHC, α-tocopherol analogue) E^0_{ox} vs SCE = 0.77 V, and HAT process is preferred. However, deprotonation of the OH group causes very large negative shift of E^0_{ox}: for PMHC anion E^0_{ox} = −0.47 V vs SCE, and thus electron transfer reduction becomes thermodynamically possible. Similarly, a large decrease of E^0_{ox} for catechin and deprotonated catechin is observed (1.18 and 0.12 V vs SCE, respectively). All of these data describe processes in acetonitrile.[56] Reduction potentials for peroxyl radicals are higher than for **dpph•**,[22] and thus the driving force for reaction between phenol anion and peroxyl radical is larger.

(56) Nakanishi, I.; Miyazaki, K.; Shimada, T.; Iizuka, Y.; Inami, K.; Mochizuki, M.; Urano, S.; Okuda, H.; Ozawa, T.; Fukuzumi, S.; Ikota, N.; Fukuhara, K. *Org. Biomol. Chem.* **2003**, *1*, 4085–4088.

(57) Ratty, A. K.; Das, N. P. *Biochem. Med. Metab. Biol.* **1988**, *39*, 69–79.

However, the primary reason for this phenomenon is unknown, since the BDE for 3-OH group is quite high (see Table 1). Our kinetic data solve this problem. An enolic OH at the position α to the carbonyl group is more acidic than phenol itself. The low electron affinity of the anion of 3-hydroxyflavone makes this compound efficiently activated by ionization: in the alcohols the enolic group is several hundred times more reactive (see Table 4), and surprisingly, for that relatively less acidic compound, the acceleration parameters are larger than for the more acidic 7-hydroxyflavone. However, the IP value for deprotonated 3-hydroxyflavone anion is 0.5 eV lower than for 7-hydroxyflavone anion (see Table 1), and regardless of the smaller fraction of the anion, the driving force for electron transfer is larger in the case of 3-hydroxyflavone than in 7-hydroxyflavone. The same explanation is valid for polyhydroxyflavones containing 3-hydroxyl group. A very small fraction of deprotonated 3-hydroxyl group with its smaller IP is thermodynamically and kinetically important. Therefore, the SPLET mechanism is a possible explanation of the enhancement of antioxidant activity and the role of 3-hydroxyl group.

Naringenin is not as highly reactive in methanol or in dioxane as the flavonoids containing enol group. It can be also rationalized by the lack of a double bond between C-2 and C-3: because ring A is not conjugated with ring B, the radical formed during the HAT/SPLET process is not stable. If other hydroxyl groups and rings A and B are not present, the 7-OH group alone is not a sufficient condition for flavonoid to be a good antioxidant.

Conclusions

Analysis of the reaction rates of 10 flavonoids with **dpph**• radical in solvents of various polarity indicates a significant role of their acidity in the overall reaction kinetics. The reactions carried out in methanol and ethanol are faster than in acidified alcohols or in dioxane. The ability of flavonoid to react quickly and efficiently with electron-deficient radicals such as peroxyls or **dpph**• depends on the acidity of phenolic hydroxyl groups and on the stability of the formed radical. The observed kinetic data are results of the interplay of several factors such as acidity, polarity of the medium, ionization potentials of phenol anions, ability of a flavonoid to be a hydrogen bond donor (and ability of a solvent to be HB acceptor) and cannot be described by the simple rule "the more acidic the flavonoid, the more active radical scavenger". However, the results of our work indicate that the role of phenol acidity should attract greater attention. For example, 3-hydroxyflavone containing an enolic hydroxyl with high O−H BDE reacts very slowly with **dpph**•, but even minute deprotonation of the 3-hydroxyl makes such reaction much faster: in methanol 3-hydroxyflavone is 100 times more reactive than in acidified methanol.

A conjugation of ring A (usually containing more acidic hydroxyls) with ring B improves the antioxidant potency of flavonoids. In an optimal case, as for quercetin, flavones can act via mixed HAT/PCET and SPLET mechanisms. In polar solvents these flavonoids react much faster because the most acidic OH group at position 7 in ring A is deprotonated and phenolate anions react with radicals. Such a mechanism involving deprotonation, electron transfer from anion to a radical, and intramolecular electron transfer to form stable flavonoid radical anion with unpaired electron at catechol moiety (Scheme 2) causes 1000-fold acceleration of the reaction rate in methanol (and perhaps in water) compared to the nonpolar solvent dioxane, where pure HAT occurs from the catecholic hydroxyl to radical. For 7,8-dihydroxyflavone, where the catechol group is the most acidic site, the acceleration of the reaction rate in methanol in comparison to dioxane reaches 4 orders of magnitude. It can be only partially explained by smaller KSE in methanol than in dioxane. Enormously high acceleration is mainly due to flavonoid ionization.

As a result of the presence of hydroxyl groups, many flavonoids (primarily existing in plants in glycosylated form) are mainly located in the water phase of the biological systems,[58] and their solubility in a polar phase is additionally increased by high acidity, which usually is stronger than for unsubstituted phenol. Reactions of flavonoids with electron-deficient radicals can be accelerated by the SPLET mechanism to effectively minimize the accumulation of the reactive oxygen species in the cell.

Experimental Section

Materials. Flavonoids used in the experiments were of highest purity. Their names and structures are listed in Chart 1. Since phenol/**dpph**• reaction kinetics in methanol and ethanol are highly sensitive to traces of acids and bases,[21] prior to the use these solvents were fractionally distilled over a small amount of **dpph**• and a few beads of an acidic ion-exchange resin. Ethyl acetate was fractionally distilled. 1,4-Dioxane was used as received from the supplier. To remove dissolved O_2 and CO_2, solvents were degassed with dry nitrogen prior to use.

pK_a Measurements. Spectrophotometric titrations were performed as described by Albert and Sergeant.[59] Briefly, small volumes of titrant (1 M KOH in water/methanol 1:1 mixture containing a small amount of $BaCl_2$ to remove traces of carbonates) were added to 200 mL of flavonoid solution at a concentration of 10^{-4} M in water/methanol (1:1). A precision pH meter was used with a combined pH glass electrode calibrated on primary pH standards for mixed solvents as recommended by IUPAC. [60] After each addition of titrant and when the pH value was stable, small 0.5 mL samples of titrated solution were transferred to quartz cuvettes (optical path 10 mm), and UV−vis spectra in the range 250−500 nm were recorded. Each time the samples were returned to the main titrated solution. The titrant and titrated solutions were kept in an atmosphere free of carbon dioxide. The pK_a's were calculated by the means of the SPECFIT (Bio-Logic Science) and mainly by DATAN 3.1 (MultiD Analyses). The software analyzes complete spectra (instead of single analytical wavelength) and calculates protolytic equilibria as a function of pH and protolytic constants on the basis of principal component analysis.

^{13}C CP/MAS NMR Measurements. Quercetin and its sodium salts were analyzed by solid-state ^{13}C NMR. The salts were obtained by mixing oxygen-free methanolic solutions containing NaOH and quercetin (the stoichiometric ratio was 0.5, 1.0, and 2.0 mol of NaOH to 1.0 mol of quercetin). The mixture was then evaporated under reduced pressure, and the resulting solid was dried under vacuum. Solid-state cross-polarization under magic angle spinning (^{13}C CP/MAS NMR) spectra were recorded at room temperature on a solid-state NMR spectrometer at the frequency of 100.6 MHz. The experiments were carried out using a proprietary Bruker probehead with 4 mm zirconia rotors driven by dry air at 8.0 kHz. A contact time of 4 ms, repetition time of 30 s, and a spectral width

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of 26 kHz were used for the accumulation of 200−900 scans. Chemical shifts were calibrated indirectly through the adamantan signal recorded at 38.3 ppm relative to TMS.

Kinetic Measurements. These were made following the procedure described previously.[24] Decays of **dpph•** (initial concentrations $1-8 \times 10^{-5}$ M) in the presence of a stoichiometric excess (for less reactive flavonoids at least 5-fold, for the most reactive ones at least 3-fold excess) of a flavonoid at known concentrations were monitored at 517 nm on an Applied Photophysics stopped-flow spectrophotometer, SX 18 MV, equipped with a 150 W xenon lamp at ambient temperature. Some of the measurements were conducted using RX2000 Rapid Kinetic System Stopped Flow Accessory (Applied Photophysics) connected to a UV−vis spectrophotometer. The initial rates of reaction (usually 5−10% of **dpph•** conversion measured as decrease of **dpph•** absorbance at 517nm) were monitored, and pseudo-first-order rate constants, k^{ex}, were calculated as average values from at least two independent sets of measurements. Values of k^s were calculated using eq II; see Results. For additional details see Supporting Information.

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Supporting Information Available: Kinetic data (Tables S1−S52), ionized forms distribution diagram (Figure S1), ^{13}C CP/MAS NMR spectra (Figures S2−S4), and examples of kinetic traces of quercetin + **dpph•** reaction (Figure S5, S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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