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# pH-Dependent Radical Scavenging Capacity of Green Tea Catechins

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The effect of pH on the radical scavenging capacity of green tea catechins was investigated using experimental as well as theoretical methods. It was shown that the radical scavenging capacity of the catechins, quantified by the TEAC value, increases with increasing pH of the medium. Comparison of the p$K_a$ values to theoretically calculated parameters for the neutral and deprotonated forms indicates that the pH-dependent increase in radical scavenging activity of the catechins is due to an increase of electron-donating ability upon deprotonation. The data also reveal that the radical scavenging activity of the catechins containing the pyrogallol (or catechol) and the galloyl moiety over the whole pH range is due to an additive effect of these two independent radical scavenging structural elements. Altogether, the results obtained provide better insight into the factors determining the radical scavenging activity of the catechins and reveal that the biological activity of green tea catechins will be influenced by the pH of the surrounding medium or tissues.

**KEYWORDS: Catechins, DFT calculations, radical scavenging capacity, TEAC, p$K_a$**

## INTRODUCTION

Catechins (flavan-3-ols) belong to the group of flavonoids and are currently the subject of considerable investigation because of their possible beneficial health effects and abundance in the human diet (*1–3*). The daily intake of catechins and proanthocyanidins has been estimated to be 18–50 mg, with the main sources being tea, chocolate, fruit, and red wine (*4*). A relatively high level of catechins in human daily diet has been reported to be correlated with the reduction of common chronic diseases and the promotion of health (*2, 5–10*). Catechins may play an important role in the reduction of lipid oxidation and accumulation of cholesterol, and thus they have been suggested to partly counteract atherosclerosis and decrease the risk of other cardiovascular diseases. These polyphenolic compounds have also been reported to exhibit a positive influence on our health acting as cytoprotective, antiproliferative, and antimutagenic agents. They may protect against long-term complications in type-2 diabetes, influencing glucose and lipid metabolism. Furthermore, catechins show antibacterial, antiviral, and/or antibiotic activities (*11–13*).

The broad range of catechin-mediated biological activities is often ascribed to their antioxidant properties. Mechanisms of such antioxidant action can include (1) scavenging of highly reactive oxygen (ROS) and nitrogen species (RNS), and (2) suppression of the formation of ROS either by inhibition of enzymes or chelation of transition metal ions (*1*). Furthermore they may act as antioxidants indirectly through (3) regeneration of α-tocopherol, (4) stimulation of phase II and "antioxidant" enzymes, and/or (5) inhibition of the redox-sensitive transcription factors such as nuclear factor κB and activator protein AP-1 (*9*).

Catechins are present in plants and in foods of plant origin including beverages such as tea and wine. Especially green tea contains a considerable amount of catechins (**Figure 1**), namely, catechin (C), epicatechin (EC), epigallocatechin (EGC), and their gallate esters: epicatechin gallate (ECg), and epigallocatechin gallate (EGCg), among which the latter is the most abundant. The unique set of these polyphenolic compounds contributes significantly to the beneficial health effects ascribed to green tea (*2, 7–9, 14*).

As members of the flavonoids group, catechins (flavan-3-ols) contain the diphenylpropane skeleton ($C_6C_3C_6$), but they do not possess a 4-oxo function, and they do have a saturated heterocyclic ring. These structural features cause a lack of electron delocalization between the A and B rings, enabling stabilization of the phenoxyl radical formed upon electron-donating action. This delocalization is generally considered a factor that enhances the antioxidant activity of flavonoids. Therefore, it is concluded that the potent radical scavenging antioxidant capacity of catechins is due to a high number of OH groups in their structures (*1*). Structure–activity relationships with the most biologically active flavan-3-ols, such as EGCg, indicate that a linear increase of the rate constants for the

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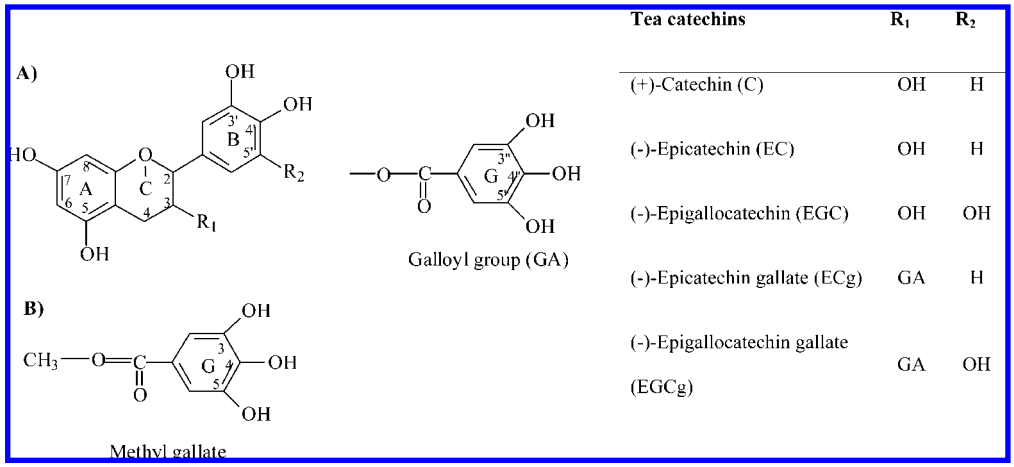


| Tea catechins | $R_1$ | $R_2$ |
|---|---|---|
| (+)-Catechin (C) | OH | H |
| (-)-Epicatechin (EC) | OH | H |
| (-)-Epigallocatechin (EGC) | OH | OH |
| (-)-Epicatechin gallate (ECg) | GA | H |
| (-)-Epigallocatechin gallate (EGCg) | GA | OH |

**Figure 1.** Structures of (**A**) the various tea catechins of the present study and (**B**) methyl gallate.

reaction of EGCg with OH$^{\bullet}$ radicals correlates with the number of reactive hydroxyl groups, suggesting that the galloyl moiety attached to the flavan-3-ol is important for antioxidant activity of EGCg (*15*).

Because catechins possess various dissociable OH groups in their structure, it could be expected that the pH of the surrounding medium will influence the radical scavenging capacity of these polyphenolic compounds as it was observed previously for hydroxyflavones and anthocyanins (*16, 17*). This possible pH-dependent effect on the radical scavenging ability of catechins is especially of interest because the pH range of different human body fluids varies widely, from pH 1 in the stomach, through 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, pH 7–8.7 in pancreas, and pH 8.3–9.3 in duodenum (*18*).

Therefore, the aim of the present study was to investigate the influence of pH on the radical scavenging capacity of common tea catechins. Radical scavenging capacity of catechins was quantified by the modified TEAC (trolox equivalent antioxidant capacity) assay and was expressed in the TEAC values. Experimental data for OH deprotonation (p$K_a$) and radical scavenging activities (TEAC) were compared to the theoretically calculated parameters for OH deprotonation, reflected by the calculated deprotonation energy (DE), for hydrogen abstraction, reflected by the calculated bond dissociation energy (BDE), and for electron donation, reflected by the calculated ionization potential (IP). The results reveal that the radical scavenging capacity of tea catechins is strongly pH-dependent and that the effect occurs in the pH range relevant to human body fluids.

## MATERIALS AND METHODS

**Chemicals.** (+)-Catechin hydrate (C), (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECg), (−)-epigallocatechin gallate (EGCg) from green tea, methyl gallate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and microperoxidase-8 (MP8) were purchased from Sigma-Aldrich (Steinheim, Germany). 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was obtained from Roche (Mannheim, Germany). Hydrogen peroxide (30%) was purchased from Merck (Darmstadt, Germany).

**Determination of p$K_a$.** The p$K_a$ values of ECg, EGCg, and methyl gallate were determined from their absorption spectra as a function of pH as described by Sauerwald et al. (*19*).

**TEAC Assay.** The antioxidant activity of catechins was measured by the modified TEAC assay performed essentially as previously described (*20, 21*), with some modifications (*22*). The major advantage of the modified TEAC assay is that it permits studying of the 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$^{\bullet+}$ radical cation relative to the ABTS$^{\bullet+}$ scavenging ability of the water-soluble vitamin E analogue, Trolox (*20, 21*).

In the present study, microperoxidase-8 (MP8), instead of metmyoglobin, was used to generate the ABTS$^{\bullet+}$ in PBS (phosphate buffered saline) pH 7.4. MP8 (final concentration of 0.2 $\mu$M) 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 incubation of ABTS with MP8/$H_2O_2$ was carried out for an hour in a water bath at 30 °C. The ABTS$^{\bullet+}$ solution thus obtained was diluted 1:1 (v/v) using 0.2 M potassium phosphate buffers of various pH values to give ABTS$^{\bullet+}$ solutions at pH values varying between 2 and 9.5. The absorption of the ABTS$^{\bullet+}$ solutions was about 0.6. The ABTS$^{\bullet+}$ solutions thus obtained were used for determination of the TEAC values. During the TEAC assay measurements, the antioxidants (Trolox or catechins) were added as 1% (v/v) of a 100× concentrated stock solution in ethanol to give the final concentration required. The decrease in absorption caused by the antioxidant compound, measured at 6 min, is reflecting the ABTS $^{\bullet+}$ radical scavenging capacity and was plotted against the concentration of the antioxidant. The linear correlation obtained for the plot of the increasing concentrations of antioxidant to the absorbance at 734 nm allows the assumption that the decrease in absorbance reflects the reaction between the ABTS$^{\bullet+}$ radical cation and the antioxidant and is not significantly affected by possible side reactions. The TEAC value represents the ratio of the slope of the plot for scavenging of ABTS$^{\bullet+}$ by the antioxidant under investigation to the slope of the plot for ABTS$^{\bullet+}$ scavenging by Trolox, used as an antioxidant standard (*20, 21*). The TEAC value is expressed in millimolar concentrations (mM) according to the definition of the TEAC value introduced by Miller et al. (*20*). The TEAC value is defined as the concentration of a Trolox solution with equivalent antioxidant potential to a 1 mM concentration of the compound under investigation (*20, 21*).

**Quantum Mechanical Calculations.** The geometries of catechins studied were fully optimized with the B3LYP hybrid density functional theory (DFT) using a 6–31G(d,p) basis set as implemented in the Gaussian 98 computational package. Single-point energies were then evaluated using a higher 6–311+G(d,p) basis set. The calculated deprotonation energies (DE), ionization potentials (IP), and bond dissociation energies (BDE) were not corrected for zero-point-energy and other thermal contributions, assuming a negligible error, thus considerably saving computer-time, especially in the case of larger molecules such as ECg and EGCg.

The DE values were calculated as the electronic energy of the deprotonated molecule minus the electronic energy of the neutral 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

**Table 1.** Antioxidant Activities of Various Catechins and Methyl Gallate in Different Assays

| catechins | $TEAC_{exp.}$ pH 7.4 | TEAC[a] pH 7.4 | DPPH[b] pH 7.0 | SRSA[c] pH 7.2 | LPO[d] pH 7.4 |
|---|---|---|---|---|---|
| (+)-catechin (C) | 3.22 | 2.40 | 2.4 | | 51.0 |
| (−)-epicatechin (EC) | 3.52[e] | 2.50 | 2.2 | 20.60 | 30.0 |
| (−)-epigallocatechin (EGC) | 3.61[e] | 3.82 | 1.1 | 3.22 | 16.0 |
| (−)-epicatechin gallate (ECg) | 6.12[e] | 4.93 | 0.7 | 2.29 | 10.0 |
| (−)-epigallocatechin gallate (EGCg) | 6.01[e] | 4.75 | 0.6 | 1.45 | 11.0 |
| methyl gallate | 2.20 | 2.44 | | | |

[a] TEAC values were taken from ref *1*. [b] Concentration of catechins required to give a 50% decrease in the signal intensity of DPPH radical ($SC_{50}$) is from ref *23*. [c] Concentration of catechins for 25% inhibition of superoxide radical $O_2^{\cdot -}$ in an enzymatic system ($IC_{25}$) is from ref *24*. [d] Concentration of catechins for 50% inhibition of lipid peroxidation ($IC_{50}$) was taken from ref *25*. [e] See ref *34*.

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.

Similarly, the BDE for homolytic OH bond cleavage in the deprotonated, monoanionic molecule (BDE(A)) was calculated as the electronic energy of the radical formed by hydrogen atom abstraction from the most stable phenoxylate monoanion minus the electronic energy of this most-stable monoanion molecule. Additionally, the BDE values for OH bonds were computed for galloyl moiety-containing derivatives (ECg and EGCg) that were assumed to be protonated at the carbonyl oxygen, thus representing BDE values for protonated molecules. These values were calculated as the electronic energy of the radical cation formed by hydrogen atom abstraction from the most stable conformer of the protonated molecule minus the electronic energy of the protonated parent molecule. The IP of the most stable mononanion (IP(A)) was calculated as the electronic energy of the phenoxyl radical formed by electron abstraction from the most stable phenoxylate monoanion minus the electronic energy of this parent most stable monoanion. In this paper, only results related to the most stable phenoxylate monoanions and phenoxyl radicals are given. No solvent effects are included in the calculations.

## RESULTS

**Radicals Scavenging Capacity of Catechins. Table 1** presents the TEAC values (at the pH values indicated) for the series of catechins studied, including C, EC, EGC, ECg, and EGCg, and for methyl gallate, also included for comparison in the present study (**Figure 1**). For comparison, **Table 1** also presents the literature data on the antioxidant activity of tea catechins and methyl gallate, as determined by the TEAC assay (*1*), the DPPH method (*23*), the SRSA (superoxide radical scavenging activity) method (*24*), and the LPO (lipid peroxidation) assay (*25*). On the basis of the data presented, it can be concluded that the two most effective radical scavengers are the gallate esters ECg and EGCg. The TEAC values of C, EC, and EGC are markedly reduced by at least about 40% in comparison to the TEAC values of the gallate esters. The $ABTS^{\bullet +}$ radical scavenging capacity of catechins tested decreases in the order ECg ≥ EGCg > EGC ≥ EC ≥ C. A similar relative order of the TEAC activity was obtained by Rice-Evans et al. (**Table 1**) (*1*). However, the TEAC values of catechins reported in the literature are lower than the experimental data obtained in the present study with the exception of EGC, which has a comparable radical scavenging ability in both studies. Nevertheless, the TEAC values obtained in the present study correlate well ($r = 0.91$) with the TEAC values reported previously by Rice-Evans et al. at pH 7.4 (*1*). There is no significant correlation between experimental TEAC values and $SC_{50}$ values from the DPPH assay (*23*), the $IC_{25}$ values from the SRSA assay (*24*), and $IC_{50}$ values from the LPO assay (*25*).

**Table 2.** Literature, Experimental, and Theoretically Predicted p$K_a$ Values and Calculated Relative Deprotonation Energies (DE) for Catechins and Methyl Gallate

| catechins | p$K_a$[a] experimental | ref | p$K_a$[b] predicted | DE[c] [kcal/mol] |
|---|---|---|---|---|
| (+)-catechin (C) | 8.97 (B) | *26* | 8.58 | 335.7 (4′) |
| | 9.26 (A) | *26* | | 336.9 (3′) |
| | 11.18 (A) | *26* | | |
| | 13.25 (B) | *26* | | |
| | 8.64 (p$K_{a1}$) | *27* | | |
| | 9.41 (p$K_{a2}$) | *27* | | |
| | 11.26 (p$K_{a3}$) | *27* | | |
| | 13.26 (p$K_{a4}$) | *27* | | |
| | 8.68 (p$K_{a1}$) | *28* | | |
| | 9.70 (p$K_{a2}$) | *28* | | |
| | 11.50 (p$K_{a3}$) | *28* | | |
| (−)-epicatechin (EC) | 8.72 (p$K_{a1}$) | *27* | 8.62 | 336.4 (3′) |
| | 9.49 (p$K_{a2}$) | *27* | | 336.9 (4′) |
| | 11.23 (p$K_{a3}$) | *27* | | |
| | 13.40 (p$K_{a4}$) | *27* | | |
| (−)-epigallocatechin (EGC) | 7.73 | | 8.01 | 327.2 (4′) |
| | | | | 331.4 (3′) |
| (−)-epicatechin gallate (ECg) | 7.76 | | 7.64 | 321.6 (3′) |
| | | | | 321.8 (4″) |
| (−)-epigallocatechin gallate (EGCg) | 7.68 | | 7.58 | 320.6 (3′) |
| | | | | 321.6 (4″) |
| methyl gallate | 8.03 | *29* | 8.06 | 327.9 (4) |
| | 7.92 | | | |

[a] Capital letters in parentheses refer to the identification of A and B ring, respectively. [b] Prediction of p$K_a$ was done using calculated DE and the QSAR of catechins; the equation of the QSAR defined was $pK_a = 0.0662DE - 13.648$; $r = 0.9451$ (DFT). [c] The number in parentheses refers to the position of the most easily deprotonated OH moiety.

**The p$K_a$ Values of the Catechins.** There are some studies presenting p$K_a$ values of C, EC, and methyl gallate measured by use of different methods (**Table 2**) (*26–29*). However, there are no literature data on p$K_a$ values of EGC, ECg, and EGCg. Therefore, the p$K_{a1}$ values of these tea catechins and that of methyl gallate, which was used as model compound, were determined spectrophotometrically (**Table 2**). **Table 2** also lists the calculated relative deprotonation energies (DE) of various hydroxyl groups of the catechins, reflecting their ease of deprotonation. The DE values indicate that the preferential site of OH deprotonation in C, EC, and EGC is the C3′-OH and/or C4′-OH moiety. Introduction of the galloyl moiety into a molecule results in a change of preferential site of OH deprotonation from C4′-OH to C4″-OH.

The experimental p$K_a$ values for tea catechins were plotted against the calculated deprotonation energies of the most acidic OH group of these catechins, and this resulted in a quantitative structure–activity relationship (QSAR) (**Figure 2**). The correlation coefficient of the QSAR obtained was 0.945. This correlation validates the p$K_a$ values for OH deprotonation determined in the present study as well as the DFT method used for theoretical calculations on the catechins. Moreover, the QSAR thus obtained may be used to predict the p$K_a$ values of OH

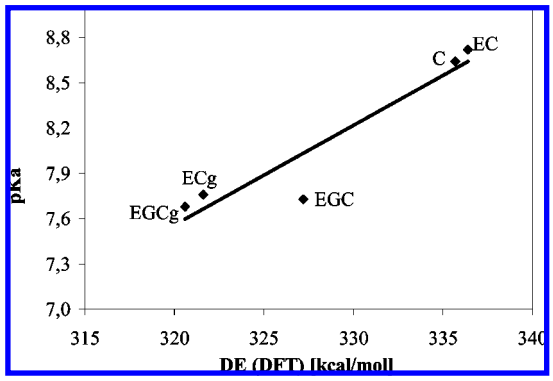


**Figure 2.** Plot of the p$K_a$ values of tea catechins (experimental and literature) against the calculated deprotonation energies (DE). The equation of the QSAR obtained is p$K_a$ = 0.0662DE − 13.648; $r$ = 0.945 (DFT). The p$K_a$ value of catechin (C) was taken from ref *27*.

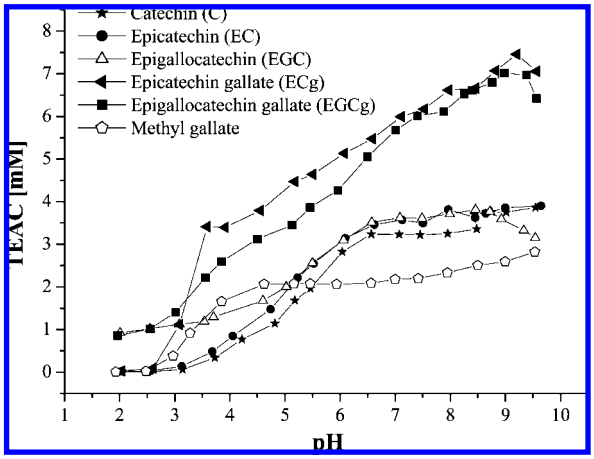


**Figure 3.** pH-Dependent TEAC profile of green tea catechins: catechin, epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate, and methyl gallate. The data are the mean of at least three independent measurements obtained within the experimental error.

moieties for other compounds belonging to the flavan-3-ols group (e.g., gallocatechin or catechin gallate).

Altogether, the p$K_a$ values indicate that deprotonation of the most acidic OH groups in catechins could occur within the physiological pH range. In particular, the p$K_a$ of the most acidic OH group may be a factor to be taken into account in studies on pH-dependent radical scavenging capacity of catechins in the physiological pH range.

**pH-Dependent Radical Scavenging Capacity of Catechins.** **Figure 3** presents the pH-dependent TEAC profiles for the catechins studied and, for comparison, also the effect of pH on the radical scavenging capacity of methyl gallate. The antioxidant action of Trolox is unaffected over the whole pH range tested (data not shown). From the plots presented, it follows that the radical scavenging capacity of catechins significantly increases with increasing pH of the medium. It also appears that the radical scavenging capacities of catechin gallate esters are higher than the radical scavenging activities of C, EC, and EGC over almost the whole pH range.

Generally, pH-dependent TEAC profiles of C and EC are very similar. The lack of essential differences between the radical scavenging capacities of these two catechins may result from their structures, which differ only in stereochemistry at the C2-position in the C ring. An additional OH group in the B ring, creating the so-called pyrogallol moiety as in EGC, causes an increase of the radical scavenging ability of EGC, especially at acidic pH (pH from 2.0 to 4.5) as compared to the radical scavenging capacity of EC and/or C. This difference is no longer observed at higher pH ranges (pH 5–9.5). Comparing the pH-dependent TEAC profiles of gallate esters, it can be seen that EGCg, with a pyrogallol moiety, also shows higher antioxidant activity at acidic pH (pH range from 2.0 to 3.0) than ECg. Therefore, it can be concluded that the additional OH group in the B ring at the 5′ position forming the pyrogallol moiety, increases the radical scavenging ability at acidic pH values. Comparison of the profile of EC to that of ECg and the profile of EGC to that of EGCg (**Figure 3**) indicates that the galloyl containing ECg and EGCg show a significantly higher radical scavenging capacity over the whole pH range above 2.5. Moreover, especially for ECg and to a less extent also for EGCg, there is a relatively strong increase in TEAC values between pH 2.5 and 3.5. A similar effect of pH on the radical scavenging ability of the galloyl moiety can also be derived from the pH-dependent TEAC profile for methyl gallate. In this curve the TEAC values increase sharply from pH 2.5 to 4.0. Additional experiments were performed to check the influence of pH on catechin solubility, especially at this acid pH region. This was done because it was suggested previously that antioxidant assays on flavonoids could be affected by the low solubility of these compounds (*16*). However, no effect of the pH on the solubility of ECg, EGCg, or methyl gallate was observed (data not shown), and such an effect of pH on the solubility of the gallate-containing molecules can therefore not explain the relative strong reduction of radical scavenging capacity of these molecules observed at low pH values. Alternatively, the effect may be caused by protonation of the O−C=O carbonyl moiety, causing the molecules to be protonated at very low pH values, which can be expected to significantly reduce their electron- and hydrogen-donating ability. As far as we know, no experimental data related to basicity (p$K_b$ values) of gallic acid esters are published. However, the effect of such a protonation on the radical scavenging activity of galloyl-containing catechins, which can not be excluded to take place in relatively acidic solution, will be discussed below. Because such a protonation can not occur in the nongalloyl-containing catechins of the present study, for which the sharp change in radical scavenging ability at low pH values was not observed, further comparison must be done between the different catechin model compounds focused on the region at pH values higher than 3.5, where the different molecules were expected to behave in a chemically similar way.

From the pH dependent TEAC profiles at pH values above 3.5, it can be concluded that a galloyl moiety attached to a flavan-3-ol at the C3-position contributes significantly to an increase of radical scavenging capacity of catechins over the whole pH range.

Furthermore, **Figure 4A** presents the pH-dependent TEAC profile for EGCg compared to the theoretical curve representing the sum of the pH-dependent TEAC profiles of EGC and methyl gallate. From the comparison of the TEAC profile of EGCg and the theoretically calculated curve, it can be concluded that the TEAC values for EGCg can be modeled over the whole pH range by taking the sum of the TEAC values of EGC and methyl gallate. This indicates that in EGCg two independent radical scavenging moieties are present, which together result in an additive radical scavenging effect. A similar conclusion can be derived from the results presented in **Figure 4B**, which reveals that the pH dependent TEAC profile of ECg can be adequately modeled over the whole pH range by taking the sum of the curves for EC and methyl gallate.

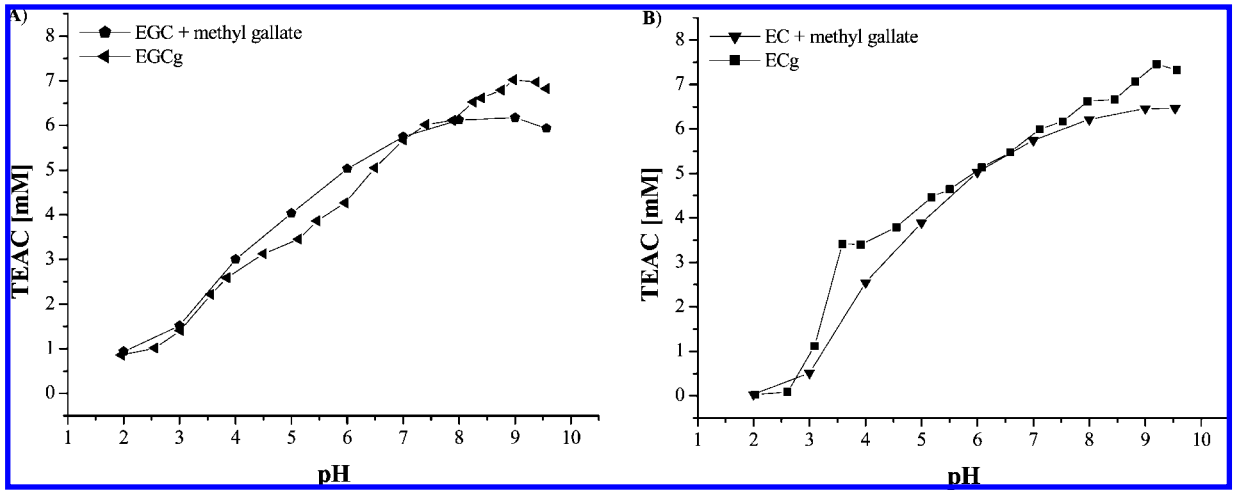


**Figure 4.** pH-Dependent TEAC profiles of (**A**) EGCg and the theoretical curve representing the sum of the EGC and methyl gallate TEAC curves and of (**B**) ECg and the theoretical curve representing the sum of the EC and methyl gallate TEAC curves.

Finally, to explain the pH-dependent increase in the TEAC values, the experimental TEAC values were compared to the p$K_a$ values (**Table 2**). From this comparison it follows that the pH-dependent increase in the TEAC values can, at least in part, be related to the deprotonation of an OH moiety. Upon deprotonation of their most easily dissociable OH group (**Table 2**) the catechins become better antioxidants.

**Calculated Parameters for Radical Scavenging Capacity of Catechins.** To get better molecular insight in the effects of additional OH groups and of deprotonation on the radical scavenging capacity of catechins, the TEAC values, as derived in the present study, were compared to the theoretically calculated electronic parameters including homolytic OH bond dissociation energies (BDE) and ionization potentials (IP), both for the neutral (N) and for the monoanionic (A) forms of the compounds under investigation. All calculated electronic descriptors are presented in **Table 3**. BDE reflects the ease of hydrogen atom donation, whereas IP reflects the ease of electron donation. It is generally assumed that the mechanism for the antioxidant action of polyphenols in their neutral form may be hydrogen-atom donation (*16, 22*), reflected by the BDE(N) value.

The BDE(N) value calculated for the weakest OH bond in ECg is lower than the one calculated for EC. This would qualitatively explain the observed increase in the TEAC antioxidant activity of ECg containing a galloyl moiety in relation to EC over almost the whole pH range. However, this effect does not occur in the cases of EGC and EGCg, and the higher antioxidant activity of EGCg than that of EGC is not reflected in a relatively lower calculated BDE(N) value of EGCg than EGC.

Therefore, it can be concluded that the rise in radical scavenging activity upon addition of a galloyl moiety is not the result of an effect on the intrinsic electronic characteristics of the molecule facilitating hydrogen donation. Moreover, the effect is due to the addition of an independent moiety that can act as a radical scavenger, resulting in an overall radical scavenging activity that equals the sum of the original molecule and that of the added galloyl moiety (**Figure 4**). The fact that the BDE(N) values of methyl gallate are in the same order as those calculated for the different catechins further supports that the galloyl moiety is able to act as an independent radical scavenging moiety.

In addition, **Table 3** presents the calculated parameters for deprotonated forms of the catechins, including BDE(A) an IP(A) values. These data provide more insight into the mechanism underlying the increase in the TEAC value with increasing pH values, that is, upon deprotonation of a catechin. The actual mechanism of antioxidant action of the deprotonated forms can still be either hydrogen atom or electron donation or both. Comparison of the BDE values for the deprotonated forms (BDE(A)) to the neutral forms (BDE(N)) (**Table 3**) indicates that there is no significant decrease in the BDE values upon catechin deprotonation. This implies that, based on the BDE values, the observed increase in radical scavenging capacity of tea catechins upon deprotonation can not be explained. Therefore, it can be concluded that hydrogen atom donation is not the main mechanism of antioxidant action of catechins upon deprotonation. In contrast, the parameter reflecting the ease of electron donation, that is, IP, is much lower for the deprotonated

**Table 3.** Theoretically Calculated Bond Dissociation Energies (BDE) as well as Ionization Potentials (IP) for Neutral (N) and Monoanionic (A) Forms of Catechins and Methyl Gallate

| | [kcal/mol] | | | |
|---|---|---|---|---|
| catechins | BDE(N)[a] | IP(N) | BDE(A)[a,b] | IP(A)[b] |
| (+)-catechin (C) | 81.0 (4′) | 171.0 | 80.1 (5) (C4′−O$^-$) | 60.4 (C4′−O$^-$) |
| | 81.3 (3′) | | 80.7 (3′) (C4′−O$^-$) | |
| (−)-epicatechin (EC) | 80.9 (4′) | 168.7 | 79.4 (5) (C4′−O$^-$) | 59.1 (C4′−O$^-$) |
| | 81.5 (3′) | | 79.8 (3′) (C4′−O$^-$) | |
| (−)-epigallocatechin (EGC) | 71.7 (4′) | 164.6 | 77.5 (3′) (C4′−O$^-$) | 59.6 (C4′−O$^-$) |
| | 79.2 (3′) | | 79.9 (5) (C4′−O$^-$) | |
| (−)-epicatechin gallate (ECg) | 77.7 (4″) | 166.8 | 76.1 (3″) (C4″−O$^-$) | 70.9 (C4″−O$^-$) |
| | 80.2 (3′) | | 79.0 (3′) (C4″−O$^-$) | |
| (−)-epigallocatechin gallate (EGCg) | 75.4 (4′) | 166.7 | 75.4 (4′) (C4″−O$^-$) | 71.1 (C4″−O$^-$) |
| | 77.6 (4″) | | 75.8 (3″) (C4″−O$^-$) | |
| methyl gallate | 77.4 (4) | 185.5 | 81.1 (3) (C4−O$^-$) | 64.6 (C4−O$^-$) |
| | 83.9 (3) | | 81.1 (5) (C4−O$^-$) | |

[a] The number in parentheses refers to the position of the OH moiety. [b] The descriptions in parentheses refer to the type of monoanion.

forms of the catechins than for the neutral ones (**Table 3**). Therefore, the increase in electron-donating ability of catechins upon deprotonation could explain the observed increase in the TEAC radical scavenging activity of catechins with increasing pH of the surrounding medium. These results support the conclusion that, upon deprotonation, the radical scavenging capacity of the catechins increases because electron, and not hydrogen atom, donation becomes much easier.

As stated in a previous section, the observed strong reduction of radical scavenging activity at low pH in case of catechins containing the galloyl moiety suggests possible protonation of the carbonyl oxygen within the ester residue. Our calculations have shown that the carbonyl oxygen is indeed the most nucleophilic center in the ECg and EGCg molecules, and therefore most likely becomes protonated in acid media. It is of interest to note that in the most stable conformer of a protonated gallate the proton attached to the carbonyl oxygen forms a strong hydrogen-bond with the O1 atom, which can be derived from the calculated very short interatomic H-O1 distance predicted to be only about 1.5 Å. It is likely that such a protonated, positively charged molecule will become a rather poor electron or hydrogen atom donor as compared to the neutral molecule. Indeed, the BDE value calculated for the homolytic C4′OH bond cleavage in the pyrogallol moiety in protonated EGCg is higher by 4.5 kcal/mol, and for the C4″OH in the galloyl moiety in the same protonated EGCg the BDE value is higher by 6.5 kcal/mol than the corresponding values calculated for the neutral molecule. In the case of ECg, the calculated increase in the C3′OH BDE value upon potonation of the carbonyl oxygen is predicted to be higher by about 5.5 kcal/mol. These theoretically calculated changes in parameters reflecting the ease of H-atom donation (e.g., BDE values) give some explanation for the results of our study showing reduced activity of the catechins containing the carbonyl oxygen of the galloyl moiety in an acidic environment.

## DISCUSSION

In the present study, the effect of pH on the radical scavenging capacity of common tea catechins was investigated using experimental as well as theoretical methods. First, it was investigated whether the deprotonation of the catechins studied occurs at physiological pH, a characteristic determined by their p$K_a$ value. So far, there are only a few studies reporting the p$K_a$ values of catechins (**Table 2**). Therefore, in the present study the p$K_a$ values for several tea catechins were determined spectrophotometrically. The p$K_a$ values indicate that for the common tea catechins deprotonation equilibria occur at physiological pH values. The calculated DE values for deprotonation (**Table 2**) indicate that the C3′−, C4′−, and/or C4″−OH moieties, the latter being present in gallate esters of catechin, are the ones that preferably deprotonate, and their p$K_a$ values were shown to be within the physiological pH range.

In addition, comparison of the p$K_a$ values for the most acidic OH groups to computer-calculated deprotonation energies resulted in a QSAR for the p$K_a$ values of the hydroxyl moieties in the catechins (**Figure 2**). Based on this QSAR, estimation of the p$K_a$ values of OH groups in other catechins (for example in gallocatechin or catechin gallate), for which the data have not been determined experimentally, becomes feasible.

In further studies, the influence of pH on the $ABTS^{\bullet+}$ radical scavenging capacity of the catechins was investigated. The results obtained show that the radical scavenging ability of the catechins increases with increasing pH of the surrounding medium. A similar tendency was observed previously by Nanjo et al. (*23*), who examined the reactivity of selected tea catechins with the DPPH radical at three different pH values (4.0, 7.0, and 10.0).

It is of interest to note that the TEAC values of the catechins with a galloyl moiety are higher than the TEAC values of quercetin and are even higher than those of cyanidin over the whole pH range (*16, 17*). This finding seems to be important when taking into account the fact that catechins are ubiquitous in tea, which is the beverage most commonly consumed worldwide after water. Catechin content in green tea aqueous extract is 221.4 mg/g, which account for 73% of the total polyphenol content in green tea aqueous extract (302.32 mg/g) (*30*). In addition EGCg, EGC, and ECg make up 90% or more of the total catechin content in green tea extract (*23*). Thus, more than 70% of the antioxidant activity of green tea extracts can be accounted for by the catechins and their gallate esters (*1*). Therefore, these results support the conclusion that tea catechins contribute to the antioxidant activity of tea to a much higher extent than other polyphenols (*1, 8, 9, 14*).

Comparison of the p$K_a$ values for the catechins (**Table 2**) to their pH-dependent TEAC profiles indicates that, for the catechins, this pH-dependent increase in the TEAC values is related to C3′, C4′, or C4″ hydroxyl group deprotonation. A similar effect was observed previously for hydroxyflavones after deprotonation of their C7-OH or C4′-OH (*16*) as well as for 4-hydroxybenzoates upon their C4-OH deprotonation (*22*).

In additional studies, quantum mechanical calculations were performed to obtain more insight in the effect of additional OH moieties and of OH deprotonation on the radical scavenging potential of the catechins. From the comparison of the TEAC values to computer calculated characteristics, it can be derived that neither the calculated OH bond dissociation energy of the neutral form (BDE(N)) nor its ionization potential (IP(N)) provide a parameter to explain the differences in the TEAC values between the different catechins.

Based on the pH dependent TEAC profile for methyl gallate and its comparison to the pH dependent curves for EC and ECg and EGC and EGCg, it can be concluded that the significant increase in the TEAC antioxidant activity of ECg and EGCg as compared to EC and EGC, observed over the whole pH range, can be explained based on an additive effect of the two independent radical scavenging structural elements: the pyrogallol and galloyl moieties present in EGCg and the catechol and galloyl moieties present in ECg. Over the whole pH range tested, both ECg and EGCg show similar TEAC values as the sum of the TEAC values of EC and methyl gallate and the sum of EGC and methyl gallate, respectively (**Figure 4**).

Nanjo et al. (*23*) suggested that the ortho-trihydroxyl group in the B ring and the galloyl moiety attached to flavan-3-ol at the C3 position are the most important features, displaying an excellent scavenging ability of the DPPH radical. Our results showing an additive effect of the two independent antioxidant moieties present in EGCg as well as in ECg provide clear explanation why these structural elements are the most important for the scavenging activity of these catechins. The importance of the ortho-trihydroxyl group and galloyl moiety has also been noted in the case of superoxide anion scavenging (*24, 31*).

Finally, from the comparison of the BDE values for the anionic and neutral forms, it could be concluded that BDE values do not change significantly upon deprotonation (**Table 3**) and this excludes hydrogen atom donation as the main mechanism of the radical scavenging action at higher pH values. In contrast, the parameter reflecting the ease of electron donation, that is, IP, is much lower for the deprotonated forms of the catechins

than for the neutral forms, reflecting easier electron donation upon deprotonation. Therefore, the increase of electron-donating ability upon deprotonation could explain the increase in the TEAC values of catechins with increasing pH value, and it can be concluded that electron donation is the dominant mechanism of antioxidant action of catechins upon their deprotonation. Thus, upon deprotonation the radical scavenging capacity of tea catechins increases because electron donation by the anionic form, rather than hydrogen atom donation by the neutral form, becomes the mechanism of action. This is in agreement with previous findings reported for hydroxyflavones and anthocyanins (*16, 17*).

Our results showing an increase in the TEAC values with increasing pH value are also in accordance with the kinetic study on the radical scavenging action of catechins reported by Mukai et al. (*32*). This study presents the reaction rates of catechins with 5,7-diisopropyl-tocopheroxyl radicals measured spectrophotometrically at pH range 4–12 in aqueous Triton X-100. It was shown that the rate constant obtained for the reaction between catechins and the radicals increased with increasing pH value because of the deprotonation of various phenolic hydroxyl groups in the catechins. In support to the results of our study on the mechanism of antioxidant action of catechins upon deprotonation, it was shown that the reaction rates for catechins increased markedly with increasing anionic character of catechins, that is, the electron-donation capacity of catechins.

The pH-dependent increase in $ABTS^{\bullet+}$ radical scavenging activity of catechins is also in line with the results of a study on the mechanism for the electrochemical oxidation of catechins reported by Janeiro and Bratt (*33*). In this work it was shown that the oxidation potential measured for (+)-catechin using cyclic voltametry was pH dependent and decreases with increasing pH reflecting increased electron donation ability with increasing pH (*33*).

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

## ABBREVIATIONS

ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; $ABTS^{\bullet+}$, the blue-green colored (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) radical cation; BDE, bond dissociation energy; BDE(A), bond dissociation energy for the monoanionic form; BDE(N), bond dissociation energy for the neutral form; C, catechin; DE, deprotonation energy; DFT, density functional theory; DPPH, 2,2′-diphenyl-1-picrylhydrazyl; EC, epicatechin; ECg, epicatechin gallate; EGC, epigallocatechin; EGCg, epigallocatechin gallate; GA, galloyl group; $IC_{25}$, concentration of catechins for 25% inhibition of superoxide radical $O_2^{\bullet-}$ in enzymatic system; $IC_{50}$, concentration of catechins for 50% inhibition of lipid peroxidation; IP, ionization potential; IP(A), ionization potential for the monoanionic form; IP(N), ionization potential for the neutral form; LPO, lipid peroxidation; MP8, microperoxidase-8; QSAR, quantitative structure–activity relationship; RNS, reactive nitrogen species; ROS, reactive oxygen species; $SC_{50}$, concentration of catechins required to give 50% decrease in the signal intensity of DPPH radical; SRSA, superoxide radical scavenging activity; TEAC, trolox equivalent antioxidant capacity.

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