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# The Effect of Catechol *O*-methylation on Radical Scavenging Characteristics of Quercetin and Luteolin—A Mechanistic Insight

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The biological effect of flavonoids can be modulated *in vivo* due to metabolism. The *O*-methylation of the catechol group in the molecule by catechol *O*-methyl transferase is one of the important metabolic pathways of flavonoids. In the present study, the consequences of catechol *O*-methylation for the pH-dependent radical scavenging properties of quercetin and luteolin were characterized both experimentally and theoretically. Comparison of the pKa values to the pH-dependent TEAC profiles reveals that *O*-methylation not only affects the TEAC as such but also modulates the effect of changing pH on this radical scavenging activity due to an effect on the pKa for deprotonation. The pH-dependent TEAC curves and computer calculated electronic parameters: bond dissociation energy (BDE) and ionisation potential (IP) even indicate that *O*-methylation of the luteolin catechol group affects the radical scavenging potential only because it shifts the pKa for deprotonation. *O*-Methylation of the quercetin catechol moiety affects radical scavenging capacity by both an effect on the pKa, and also by an effect on the electron and hydrogen atom donating properties of the neutral (N) and the anionic (A) form of the molecule. Moreover, *O*-methylation of a catechol OH-group in quercetin and luteolin has a similar effect on their TEAC profiles and on calculated parameters as replacement of the OH-group by a hydrogen atom. Altogether, the results presented provide new mechanistic insight in the effect of catechol *O*-methylation on the radical scavenging characteristics of quercetin and luteolin.

*Keywords*: Flavonoids; *O*-methylation; Radical scavenging activity; TEAC; pH; pKa

*Abbreviations*: ABTS, 2,2′-azinobis(3-ethylbenzothiozoline-6-sulphonic acid) diammonium salt; BDE, bond dissociation energy; COMT, catechol *O*-methyl transferase; DE, deprotonation energy; DFT, density functional theory; IP, ionisation potential; TEAC, Trolox equivalent antioxidant capacity; Trolox, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

## INTRODUCTION

Natural polyphenols, especially flavonoids and their corresponding glycosides are important constituents of fruits, vegetables, nuts, tea, olive oil and red wine.[1,2] Quercetin (Fig. 1), the main flavonol in our diet, is particularly abundant in onions and apples.[3] Luteolin (Fig. 1) was identified in red sweet pepper and celery.[4] The daily consumption of polyphenols is variable according to food habits and may range from 25 to 1 g/day.[5,6]

The antioxidant properties of these compounds are often claimed to be, at least in part, responsible for the protective effects of these food components against cardiovascular diseases, certain forms of cancer and photosensitivity diseases.[7,8] The antioxidant activity of flavonoids is known to be highly dependent on their structure, particularly the availability of free hydroxyl groups.[8,9] Therefore, conjugation of the free hydroxyl moieties by phase II metabolism may influence the biological activity, including the antioxidant potential of

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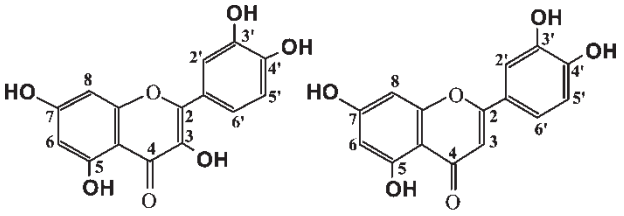


FIGURE 1 Structural formulas and numbering of the model flavonoids, quercetin and luteolin.

the flavonoids. Both quercetin and luteolin are known to be extensively metabolised by the phase II biotransformation system, resulting in glucuronidated, sulfated and methylated derivatives.[2,10] The present study focuses on the consequences of catechol *O*-methylation for the radical scavenging capacity of luteolin and quercetin, as the model flavonoids to be investigated. For long, it has been known that catechol-*O*-methyl transferases (COMT) are capable of the methylation of the catechol groups of flavonoids, especially quercetin.[11–13] There are two different COMT isoforms: soluble COMT (s-COMT) in the cytoplasm, mainly present in periferal tissues,[14] and membrane-bound COMT (mb-COMT),[15] mainly present in the brain area.[14] Several reports showed the important contribution of methylation to the phase II metabolism of quercetin *in vivo*. In humans, 21% of quercetin circulating in plasma after consumption of a quercetin-rich meal was present as 3′-*O*-methyl-quercetin (isorhamnetin), partly conjugated as glucuronide- and/or sulfate conjugate.[16] In rats, the contribution of *O*-methylation is even higher, since up to 86% of the total amount of circulating quercetin has been reported to be methylated at the 3′-hydroxyl group.[1,13,17] Contrary to 3′-*O*-methyl-quercetin, 4′-*O*-methyl-quercetin is hardly recovered from plasma.[1,13,17–19] This has been ascribed to a very efficient renal clearance, because 4′-*O*-methyl-quercetin can be detected in considerable amounts in bile and urine of rats fed quercetin.[13]

Given the importance of catechol-*O*-methylation in the phase II biotransformation of flavonoids, the objective of the present study is to characterize the consequences of catechol-*O*-methylation for the radical scavenging capacity of quercetin and luteolin, model flavonoids known to be present in significant amounts in our daily diets.[2,5]

By investigating the consequences of catechol-*O*-methylation of luteolin and quercetin both experimentally, taking into account the effect of pKa and pH on the antioxidant activity,[20,21] and by theoretical quantum mechanical calculations, new mechanistic insight in the effect of catechol-*O*-methylation on the radical scavenging characteristics of these two important model flavonoids was obtained.

## MATERIALS AND METHODS

### Materials

Isorhamnetin (3′-*O*-methyl-quercetin; 3′-*O*Me-Q), tamarixetin (4′-*O*-methyl-quercetin; 4-*O*Me-Q), chrysoeriol (3′-*O*-methyl-luteolin; 3′-*O*Me-L), diosmetin (4′-*O*-methyl-luteolin; 4′-*O*Me-L) were purchased from Indofine Chemical (Somerville, NJ, USA). Quercetin, luteolin, kaempferol and apigenin were obtained from Fluka (Buchs, Switzerland). 3′,5,7-Trihydroxyflavone was synthesised according to the method of Gaydou and Bianchini[22]. Microperoxidase-8 (MP-8) was obtained from Sigma (Steinheim, Germany). 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and ascorbic acid were purchased from Aldrich (Steinheim, Germany). Hydrogen peroxide (30%), methanol (HPLC grade) and acetonitrile (HPLC grade) were purchased form Merck (Darmstadt, Germany).

### Determination of pKa

The pKa values of quercetin, 3′-*O*Me-Q, 4′-*O*Me-Q, luteolin, 3′-*O*Me-L, 4′-*O*Me-L and 3′, 5, 7-hydroxyflavone was determined from absorption spectra as a function of pH as described by Sauerwald *et al.*[23]

### TEAC Assay

The Trolox® equivalent antioxidant capacity (TEAC) was measured by the method of Miller *et al.*[24] with some modifications.[20] The major advantage of the modified TEAC assay is that it permits studies of radical scavenging antioxidant activity over a wide pH range (i.e. pH 4.5–9.5). This reaction between the antioxidant and the $ABTS^{\cdot+}$ cation radical may in theory be influenced by a second electron oxidation of the flavonoid radical, giving rise to quinoid-type flavonoid reaction products, as well as by other side reactions of the antioxidant radical formed. The decrease in absorbance at 734 nm after 6 min caused by the antioxidant compound, is reflecting the $ABTS^{\cdot+}$ radical scavenging capacity and was plotted against the concentration of the antioxidant. The linear correlation obtained for this plot (data not shown) allows the assumption that the decrease in absorbance reflects especially the reaction between the $ABTS^{\cdot+}$ cation radical and the parent antioxidant molecule, and is not significantly affected by secondary side reactions. If side reactions would

play a significant role, deviations from a linear relationship between the decrease in $A_{734}$ after 6 min and the concentration of the flavonoid would be expected.

Solubility of the 3′-*O*Me-L and 4′-*O*Me-L was checked at pH 4.5 and 7.5 and a linear relationship between absorbance and concentration was obtained up to 10 μM. Therefore, pH dependent TEAC profiles were measured at concentrations not exceeding 10 μM.

### Quantum Mechanical Calculations

All geometries of molecules studied were optimised with the B3LYP hybrid density functional theory (DFT) by using a 6–31G(d) basis set as implemented in the Gaussian 98 computational package (Gaussian Inc., Pittsburgh, PA, USA). Single-point energies were then evaluated by using a higher 6–311 G(d,p) basis set. The calculated deprotonation energies (DE), ionisation potentials (IP) and bond dissociation energies (BDE) were not corrected for zero-point-energy assuming a negligible error and thus saving computer-time.

The DE was calculated as the electronic energy of the deprotonated anion minus the electronic energy of the parent molecule. The BDE for homolytic O–H bond cleavage in the neutral flavonoid (BDE(N)) was calculated as the energy of the radical resulting from the hydrogen atom abstraction minus the energy of the N molecule. The IP for the neutral flavonoid (IP(N)) was calculated as the energy of the radical cation resulting from the electron abstraction minus the energy of the N parent molecule.

Similarly the BDE for homolytic O–H bond cleavage in the deprotonated anionic flavonoid (BDE(A)) was calculated as the energy of the radical of the most stable flavonoid anion minus the energy of this most stable anion. The IP of the most stable anion (IP(A)) was calculated as the energy of phenoxyl radical formed by electron abstraction from the most stable monoanion minus the energy of this most stable monoanion.

## RESULTS

### pKa Values of Quercein and Luteolin *O*-methyl Derivatives

Table I presents the experimental pKa values of the various model compounds, as determined in the present study or obtained from the literature. Table I lists calculated relative DEs of various hydroxyl groups of querectin and luteolin as well as of their catechol *O*-methyl metabolites. These calculated DE values reflect the ease of deprotonation. In addition, Table I presents the pKa values of the various model compounds predicted using the calculated DE and the quantitative structure–activity relationship (QSAR) obtained in our previous study[21] for the relationship between the calculated DE and the pKa of a series of hydroxyflavones. Comparison of the experimental pKa values to those predicted using the previously defined QSAR illustrates that this recently developed QSAR model predicts the actual pKa values of the *O*-methyl metabolites relatively well. The results also illustrate that *O*-methylation of the catechol moiety significantly affects the ease of deprotonation, reflected by a significant increase in the calculated DE for deprotonation and a marked increase in the experimental as well as the predicted pKa values. Comparison of the pKa values of the *O*-methylated metabolites and the corresponding aglycones indicates deprotonation to be dependent on the position of *O*-methylation; 4′-*O*-methylation increasing the pKa to a somewhat larger extent than 3′-*O*-methylation. Calculations of the DE values for deprotonation of the C4′-*O*- or C3′-*O*-methylated metabolites theoretically corroborates that the effect

TABLE I Experimental pKa values, calculated deprotonation energies (DE) and theoretically predicted pKa values of the hydroxyl moiety of quercetin and luteolin as well as of their *O*-methylated metabolites

| Compound | pKa observed | DE (kcal/mol) | pKa predicted* |
|---|---|---|---|
| Quercetin (3′,4′,3,5,7-hydroxyflavone) | 7.65<br>7.03‡ | 331.6 (4′)†<br>338.1 (7) | 7.00 |
| 3′-*O*Me-Q (Isorhamnetin) | 7.80 | 338.7 (7)<br>342.0 (4′) | 8.06 |
| 4′-*O*Me-Q (Tamarixetin) | 8.17 | 339.7 (7)<br>350.9 (3′) | 8.21 |
| Luteolin (3′,4′,5,7-hydroxyflavone) | 7.06 | 327.9 (4′)<br>341.7 (7) | 6.37 |
| 3′-*O*Me-L (Chryoseriol) | 7.70 | 338.1 (4′)<br>342.3 (7) | 7.96 |
| 4′-*O*Me-L (Diosmetin) | 7.90 | 343.7 (7)<br>346.9 (3′) | 8.82 |

* Predictions of pKa values was done using the calculated DE and the QSAR obtained in our previous studies;[21] pKa = 0.1525 DE − 43.596; $r = 0.9808$ (DFT).
† The number between brackets refers to the position of OH moiety. ‡ pKa Value taken from Ref. [23].

on the ease of deprotonation is more pronounced upon C4′-*O*- than upon C3′-*O*-methylation. The pKa values obtained also indicate that, as for many hydroxyflavones, also for the *O*-methylated derivatives deprotonation equilibria are expected to occur within the physiological pH range.

Finally, the DE values presented in Table I for deprotonation of the various moieties also indicate that the *O*-methylation of the catechol moiety at both C4′–OH and C3′–OH changes the preferential site of OH deprotonation from C4′–OH to C7–OH. This holds for the 4′-*O*-methyl derivatives of both quercetin and luteolin as well as for the 3′-*O*-methyl derivative of quercetin. For luteolin, however, C3′–OH-methylation does not affect the order of preference for deprotonation and C4′–OH remains the site that can be more easily deprotonated than C7–OH, although the relative difference between the DE for 4′–OH and 7–OH deprotonation reduces in the 3′-*O*Me-L as compared to luteolin itself.

## pH-Dependent Radical Scavenging Activity of Luteolin and Quercetin and their Catechol *O*-methyl Metabolites

Previous studies revealed a significant influence of pH on radical scavenging behaviour of polyphenols, showing an increase in TEAC value around the pKa of the compounds.[20,21] The results presented in Fig. 2, which show the pH dependent TEAC profiles of quercetin and luteolin aglycones as well as their *O*-methylated metabolites, reveal that the quercetin and luteolin *O*-methyl metabolites behave in a similar way. The antioxidant action of Trolox C (standard) was previously shown to be unaffected over the whole pH range tested.[20] Thus, the TEAC values for all compounds increase with increasing pH. Comparison of the pKa values (Table I) to the pH-dependent TEAC profiles leads to the conclusion that this increase in TEAC value occurs around the pKa, which suggests that it is related to deprotonation of the flavonoid under study. Upon deprotonation quercetin and luteolin, but also their *O*-methylated derivatives become better radical scavengers reflected in a significant pH-dependent increase in the TEAC values around their pKa.

Figure 2b show that 3′-*O*Me-L and 4′-*O*Me-L are not active as $ABTS^{\cdot+}$ radical cation scavengers in the pH range between 4.5 and 6.5. pH-Dependent UV-spectroscopy was performed and showed that changes in TEAC observed are not due to solubility effects since a linear relation between absorbance and concentration of the compounds was obtained (data not shown).

*O*-Methylation of the C4′–OH and C3′–OH position in the quercetin and luteolin molecules results in a decrease of their TEAC values compared to their aglycone form over the whole pH range

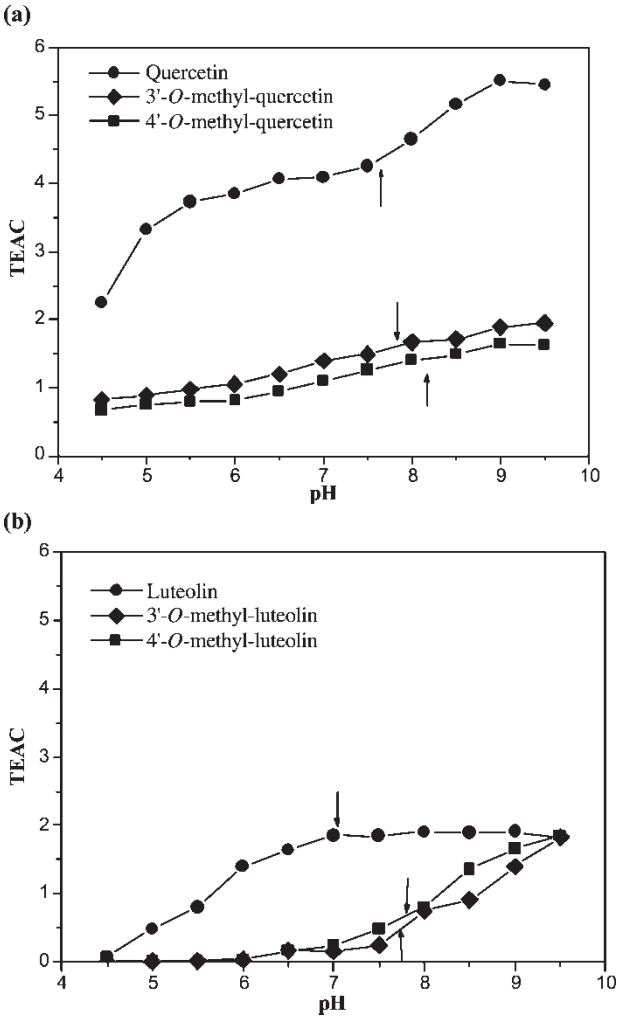


FIGURE 2 Effect of pH on the TEAC value of: (a) quercetin and *O*-methylated metabolites of quercetin and (b) luteolin and *O*-methylated metabolites of luteolin. Arrows indicate the experimental pKa value of preferably deprotonated OH group in molecule.

tested (Fig. 2). This effect is more pronounced for quercetin than for luteolin. For luteolin the effect can even be overcome at high pH, since at pH 9.5 the TEAC values of luteolin and its *O*-methylated analogues is similar.

## pH-Dependent TEAC Profiles of Kaempferol, Apigenin and 3′,5,7-Trihydroxyflavone

Figure 3 shows the pH-dependent TEAC profiles of kaempferol (4′,3,5,7-*tetra*-hydroxyflavone) (Fig. 3d), apigenin (4′,5,7-*tri*-hydroxyflavone) (Fig. 3e), and 3′,5,7-*tri*-hydroxyflavone (Fig. 3f). For comparison, Fig. 3a–c shows the curves for 3′-*O*Me-Q (Fig. 3a), 3′-*O*Me-L (Fig. 3b), and 4′-*O*Me-L (Fig. 3c). Comparison of Fig. 3a–d, 3b–e and 3c–f, respectively, reveals that the pH dependent TEAC profiles of 3′-*O*Me-Q, 3′-*O*Me-L and 4′-*O*Me-L are very similar to the pH-dependent TEAC profiles of

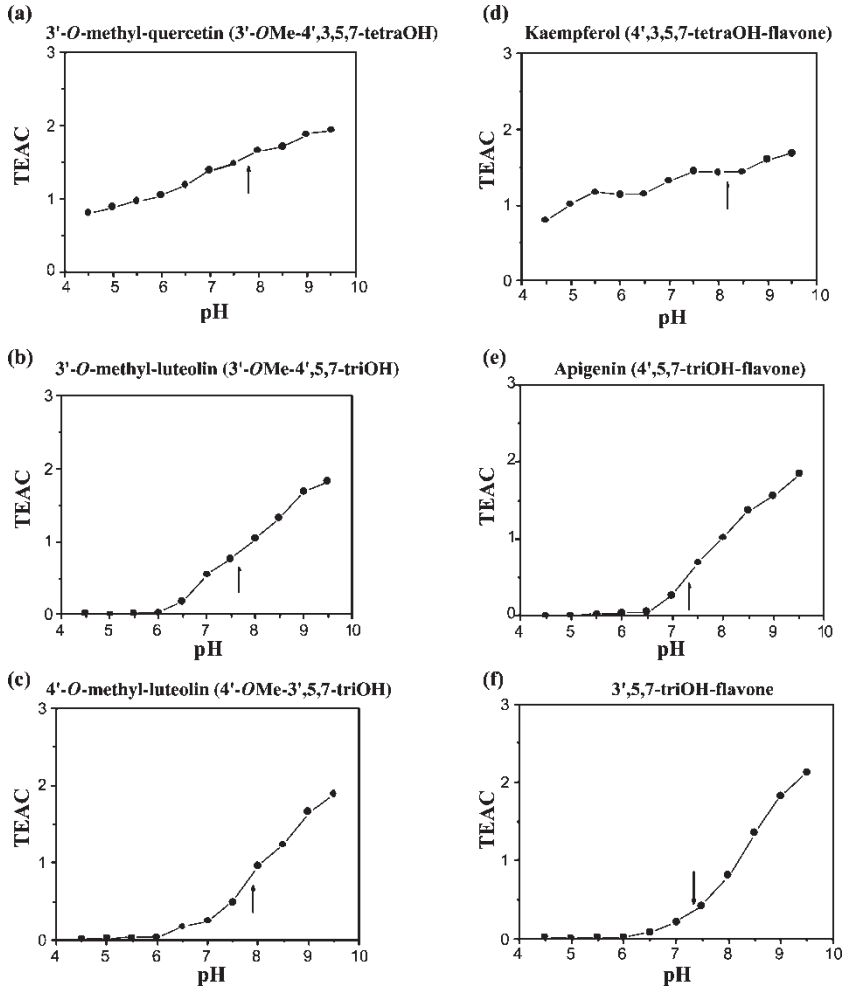


FIGURE 3 pH-Dependent TEAC profiles of selected quercetin and luteolin *O*-methylated metabolites and hydroxyflavone analogues in which OH group is replaced by a hydrogen atom: (a) 3′-*O*-methyl-quercetin, (b) 3′-*O*-methyl luteolin, (c) 4′-*O*-methyl-luteolin, (d) 4′,3,5,7-tetrahydroxyflavone; (e) 4′,5,7-trihydroxyflavone (f) 3′,5,7-trihydroxyflavone. Arrows indicate the experimental pKa value of preferably deprotonated OH group in molecule.

4′,3,5,7-hydroxyflavone, 4′,5,7-hydroxyflavone and 3′,5,7-hydroxyflavone, respectively. This illustrates that *O*-methylation of a catechol OH group effectively results in pH dependent TEAC profiles similar to the pH dependent TEAC profile of the flavonoid analogue in which this OH group is replaced by a hydrogen atom.

### Calculated Parameters for the Ease of Electron and Hydrogen Atom Donation by the Metabolites of Quercetin and Luteolin

To obtain more insight in the effect of *O*-methylation on the radical scavenging antioxidant activity of quercetin and luteolin and in the effect of protonation states on the TEAC activity of quercetin, luteolin, and their *O*-methylated metabolites, the TEAC values, derived in the present study, were compared to the theoretically calculated electronic parameters. Table II presents these electronic descriptors for the neutral (N) and anionic (A) form of the compounds under investigation, including OH bond dissociation energy (BDE), representing the ease of hydrogen atom donation and IP representing the ease of electron donation. The results show that *O*-methylation of the OH group at the C4′ and C3′ positions both in quercetin and luteolin increases the BDE(N) in comparison to the BDE calculated for the parent aglycones. This would provide an explanation why 4′-*O*Me-Q, 3′-*O*Me-Q, 4′-*O*Me-L and 3′-*O*Me-L in their N form can be less effective radical scavengers (reflected in relatively lower TEAC values) than quercetin and luteolin, respectively. Upon *O*-methylation the BDE(N) increases because the phenoxyl radical, resulting from hydrogen atom donation by the neutral forms of *O*-methylated derivatives, cannot be as effectively stabilized by hydrogen bonding within the catechol moiety, making the radical relatively less stable.

Additionally, Table II presents the calculated electronic parameters of the deprotonated forms of

TABLE II Calculated bond dissociation energies (BDE) as well as ionisation potential (IP) for the neutral (N) and monoanionic (A) form of quercetin, luteolin and their *O*-methylated metabolites

| Compound | BDE(N) [kcal/mol] | IP(N) [eV] | BDE(A) [kcal/mol] | IP(A) [eV] |
|---|---|---|---|---|
| Quercetin (3′,4′,3,5,7-hydroxyflavone) | 78.6 (4′)* | 7.03 | 73.7 (7)* | 2.69 (4′)* |
| 3′-*O*Me-Q (Isorhamnetin) | 86.9 (3) | 6.93 | 77.1 (4′) | 3.04 (7) |
| 4′-*O*Me-Q (Tamarixetin) | 86.8 (3) | 6.92 | 80.2 (3′) | 2.99 (7) |
| Luteolin (3′,4′,5,7-hydroxyflavone) | 80.4 (4′) | 7.42 (7.32)† | 80.7 (7) | 2.93 (4′) |
| 3′-*O*Me-L (Chrysoeriol) | 88.0 (4′) | 7.35 (7.26) | 81.1 (7) | 2.83 (4′) |
| 4′-*O*Me-L (Diosmetin) | 89.0 (3′) | 7.36 (7.25) | 82.0 (3′) | 2.86 (7) |

* The number between brackets refers to the position of OH moiety. † The value between brackets indicates the IP calculated as the energy of the radical cation resulting from electron abstraction and stabilised through proton transfer from C5–OH to C4 = O minus the energy of the neutral parent molecule.



the C4′-*O*-methylated derivatives with a deprotonated 7OH moiety and C3′-*O*-methylated derivatives with deprotonated 4′OH or 7OH moiety, providing BDE(A) and IP(A) values for the A forms of investigated compounds. These data provide more insight in the mechanism underlying the increase in TEAC value with increasing pH, i.e. upon deprotonation, of the C4′-and C3′-*O*-methylated derivatives of quercetin and luteolin. The actual mechanism for the antioxidant action of these deprotonated forms can still be either hydrogen atom or electron donation or both in different rates. Therefore, Table II lists, not only the BDE values for hydrogen atom donation from the weakest remaining OH moieties in the anion (BDE(A)), but also the IP of the deprotonated A molecules (IP(A)). Comparison of the BDE values of the deprotonated forms (BDE(A)) to the BDE values for the neutral forms (BDE(N)) (Table II) reveals that there is a decrease in the BDE values upon hydroxyflavone deprotonation, but the decrease is not significant. This implies that on the basis of BDE values the observed increase in radical scavenging capacity of the antioxidants upon deprotonation cannot be explained. In contrast, the parameter reflecting the ease of electron donation, i.e. IP is greatly influenced by the deprotonation step; the IPs drop significantly. This result is in an agreement with previous findings for other hydroxyflavones[21] and supports the conclusion that upon deprotonation the TEAC value of hydroxyflavones and their metabolites increases (radical scavenging capacity increases) because electron donation becomes the major mechanism for antioxidant action. Moreover, the results show that *O*-methylation of the OH group at the C4′ or C3′ position in quercetin enhances the IP(A) in comparison to the parent aglycone. This would qualitatively explain why C4′- and C3′-*O*-methylated derivatives of quercetin derivatives, even upon deprotonation, can be less effective radical scavengers, reflected in lower TEAC values than the deprotonated form of quercetin itself (Fig. 2a).

In contrast to quercetin, *O*-methylation of the OH group at the C4′ or C3′ position in luteolin does not enhance the IP(A) (Table II). This would explain why 4′-*O*Me-L and C3′-*O*Me-L at high pH values, at which the monoanionic form of the compounds prevails, show nearly the same radical scavenging activity as luteolin (Fig. 2b). This also implies that *O*-methylation has no effect on the radical scavenging activity of luteolin in its deprotonated form. Thus, the pH dependent TEAC curves shown in Fig. 2a and b and the results of the calculations presented in Table II indicate that for luteolin, *O*-methylation of the catechol group affects the antioxidant potential only because it shifts the pKa for deprotonation.

For quercetin however, *O*-methylation of the catechol moiety affects antioxidant activity by both an effect on the pKa for deprotonation, but also by a differential effect on the electron and hydrogen donating properties of the neutral and the anionic form of the molecule.

Finally, to explain the observation that *O*-methylation of a catechol group results in radical scavenging behaviour similar to the analogue in which the OH group is replaced by a hydrogen atom, the calculated molecular parameters BDE and IP of 3′-*O*Me-Q, 3′-*O*Me-L and 4′-*O*Me-L were compared with BDE and IP of their corresponding analogues in which the C3′–OH or C4′–OH group is substituted by a hydrogen atom (Tables II and III).

The results show that *O*-methylation of a catechol OH group has a similar effect on the BDE and IP of quercetin and luteolin as OH group removal, that is, replacement of the OH moiety by a hydrogen atom.

## DISCUSSION

In the present study, the effect of catechol-*O*-methylation on the radical scavenging characteristics of two model flavonoids, quercetin and luteolin, was investigated using experimental as well as theoretical methods.

Several *in vivo* studies pointed at catechol-*O*-methylation as a very important phase II metabolism pathway for both quercetin[11–13] and luteolin.[4,10,12] In addition, a few studies

TABLE III Experimental and theoretically predicted pKa values of analogues of quercetin and luteolin *O*-methylated metabolites, in which the 3′- or 4′–OH group is replaced by a hydrogen atom as well as calculated deprotonation energies (DE), bond dissociation energies (BDE) and ionisation potential (IP) for the neutral (N) and the anionic (A) forms of these flavonoids

| Compound | pKa Observed | DE (kcal/mol) | pKa Predicted | BDE(N) [kcal/mol] | IP(N) [eV] | BDE(A) [kcal/mol] | IP(A) [eV] |
|---|---|---|---|---|---|---|---|
| 4′,3,5,7–OH | 8.29* | 338.7 (7)†<br>339.1 (4′) | 8.10 | 86.8 (3)† | 7.08 | 74.1 (4′)† | 2.73 (7)† |
| 4′,5,7–OH | 7.37‡ | 335.5 (4′)<br>342.7 (7) | 7.56 | 89.5 (4′) | 7.52 (7.33)¶ | 81.1 (7) | 3.00 (7) |
| 3′,5,7–OH | 7.25 | 340.6 (7)<br>346.6 (3′) | 8.45 | 91.6 (3′) | 7.67 (7.49) | 85.5 (3′) | 3.01 (7) |

* pKa Value taken from Ref. [25]. † The number between brackets refers to the position of OH moiety. ‡ pKa Value taken from Ref. [26]. ¶ The value between brackets indicates the IP calculated as the energy of the radical cation resulting from electron abstraction and stabilised through proton transfer from C5–OH to C4 = O minus the energy of the neutral parent molecule.



investigated the possible consequences of catechol *O*-methylation for antioxidant activity of flavonoids, generally reporting a reduction in antioxidant efficacy upon methylation,[8,27–30] although others reported the opposite.[31] Because the 4′–OH group is generally suggested to be the hydroxyl moiety primarily involved in both deprotonation, and in hydrogen donation upon radical scavenging action of quercetin and luteolin,[21,32] it was expected that especially *O*-methylation of the 4′–OH moiety may affect deprotonation as well as radical scavenging activities of the flavonoids studied. Results obtained, however, reveal that methylation of the 3′–OH affects both characteristics to almost the same extent as 4′–OH methylation. This supports the view, frequently expressed[8,9] that for optimal antioxidant activity a functional catechol moiety with both 3′–OH and 4′–OH in their unconjugated form, is of importance.

Clearly the reduction in radical scavenging capacity of both luteolin as well as quercetin upon methylation of one of their catechol OH group is in line with other reports indicating that *O*-methylation of the catechol moiety reduces the radical scavenging activity of flavonoids.[8,29,30] The pH dependent TEAC measurements and the theoretical computer calculations of the present study provide new insight into the mechanism behind this effect of *O*-methylation.

First of all *O*-methylation was shown to affect the pKa for deprotonation of the flavonoid molecule. Theoretical calculations corroborated that *O*-methylation of the catechol group results in a significant change in deprotonation characteristics, increasing the DE for deprotonation and shifting the preferential site for deprotonation more in favour of 7–OH deprotonation, the latter because 4′–OH deprotonation either becomes impossible due to methylation of the site, or because 4′–OH deprotonation becomes energetically less favourable upon 3′-*O*-methylation. As a result, the pKa of the flavonoid increases by about 0.2–0.8 pH units upon methylation of its catechol moiety.

These results indicate that the reduction in radical scavenging activity of the flavonoids upon their *O*-methylation can be, at least in part, ascribed to an increase in their pKa values, resulting in a lower extent of deprotonation at physiological pH. The deprotonated forms of the flavonoids are most active in radical scavenging reactions such as in the TEAC assay.[21] Therefore, this shift in the deprotonation equilibrium upon *O*-methylation, resulting in a reduced fraction of deprotonated flavonoid molecules at physiological pH for the *O*-methylated metabolites as compared to the parent flavonoids, results in lower TEAC values at physiological pH. Measurement of the pH dependent TEAC profiles as performed in the present study corroborates this conclusion and even suggests that in the case of luteolin the shift in pKa could almost fully explain the effects of *O*-methylation on radical scavenging activity. This, because at higher pH values the TEAC values of luteolin, 3′-*O*Me-L and 4′-*O*Me-L become similar again. Thus, the pH dependent TEAC profiles of luteolin and its *O*-methylated analogues reveal a shift in pKa of about 0.6–0.8 pH units as the major cause for the differences in radical scavenging activity of luteolin and its *O*-methyl derivatives. Results from DFT calculations supported the conclusion that upon full deprotonation luteolin and its *O*-methylated metabolites have almost equal radical scavenging capacities reflected by almost similar IP(A) and BDE(A) values for the different compounds.

For quercetin, the situation appeared different. For quercetin, as for luteolin, *O*-methylation of its catechol moiety increases the pKa for its deprotonation, again in line with computer DFT calculations. However, upon full deprotonation the difference in TEAC value between quercetin and its *O*-methylated analogues still exists. Thus for quercetin, the increase in the pKa for deprotonation contributes to the reduction in TEAC value at physiological pH upon *O*-methylation, but it cannot be the only or not even the dominant mechanism causing the reduction in TEAC at physiological pH upon catechol *O*-methylation. For quercetin, in contrast to luteolin, the radical scavenging characteristics of the deprotonated form of the flavonoid

are significantly influenced by *O*-methylation of the catechol moiety. *O*-Methylation of both the 3′–OH and the 4′–OH group of quercetin increases the IP(A) for electron donation and also the BDE(A) for hydrogen donation by the flavonoid anion. This explains why for quercetin, also above the pKa, there is a difference in the TEAC value between the aglycon and its *O*-methylated metabolites resulting in decreased radical scavenging capacity upon *O*-methylation.

Additional results of the present study reveal that *O*-methylation of a catechol OH moiety in luteolin or quercetin, effectively results in removal of the OH moiety as far as radical scavenging behaviour is concerned. This conclusion follows from the observation that the pH dependent TEAC profile of 3′-*O*Me-Q is similar to the pH dependent TEAC profile and TEAC activities of 4′,3,5,7-tetrahydroxyflavone, that of 3′-*O*Me-L similar to that of 4′,5,7-trihydroxyflavone, and, finally, that of 4′-*O*Me-L similar to that of 3′,5,7-trihydroxyflavone. DFT calculations also corroborate this conclusion showing that catechol *O*-methylation has the same electronic effect on the molecule and its DE, IP and BDE characteristics as replacement of the OH group by a hydrogen atom, i.e. removal of the hydroxyl group from the molecule. The mechanistic explanations for these similar effects of *O*-methylation or hydroxyl elimination from the flavonoid molecule on its radical scavenging and deprotonation characteristics were elucidated in the present study. Clearly, there appear to be two mechanisms underlying the reduction in radical scavenging capacities of flavonoids upon *O*-methylation of their catechol moiety, and/or effective elimination of a catechol hydroxyl group. First, *O*-methylation (OH elimination) increases the pKa of the flavonoid resulting in lower levels of deprotonation of the flavonoids at physiological pH and, thus, reduced radical scavenging properties. Second, *O*-methylation (OH elimination) may affect the electronic characteristics of especially the deprotonated form of the flavonoid, reducing its capacity for electron and hydrogen atom donation. Especially of interest is the fact that the results of the present study reveal that the extent to which these two mechanisms are relevant depends on the substituent pattern of the flavonoid. Especially the absence of the 3–OH substituent reduces the consequences of *O*-methylation for the radical scavenging properties of the deprotonated form. This may relate to the fact that the presence of the 3–OH is crucial in generating possibilities for conjugating interactions between the B and the C rings. When absent, modifications in the B ring, including *O*-methylation or OH removal, apparently affect the electron donating characteristics of the molecule to a lesser extent than when the 3–OH is present.

Finally, it is of interest to stress that in theory *O*-methylation may also hamper the radical scavenging capacity of the flavonoids by hampering a possible second electron oxidation. Such a second electron oxidation by the flavonoid would give rise to a flavonoid quinone-type reaction product.[33,34] However, formation of a quinone, upon a second electron oxidation, is expected to be hampered by methylation at the 4′OH but not by methylation at the 3′OH moiety of quercetin or luteolin. This because methylation of the 3′OH leaves the possibility for formation of a quinone methide metabolite from quercetin and luteolin. This would imply that if *O*-methylation would slow down the radical scavenging capacity by preventing quinone formation upon a second electron donation, methylation at the 4′OH should have a larger effect than methylation of the 3′OH. The experimental data obtained in the present study reveal that this is not the case, since methylation of the 3′OH and 4′OH was shown to affect the radical scavenging ability to exactly the same extent. Altogether the results of the present study provide new mechanistic insight in the effect of *O*-methylation of flavonoids, a physiologically relevant phase II modification, on their pH dependent antioxidant characteristics.

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