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Electrochemical and Density Functional Theory Study on the Reactivity of Fisetin and Its Radicals: Implications on in Vitro Antioxidant Activity

Zoran S. Marković,[†] Slavko V. Mentus,[‡] and Jasmina M. Dimitrić Marković*[,‡]

Department of Biochemical and Medical Sciences, State University of Novi Pazar, Vuka Karadžića bb, Novi Pazar 36300, Republic of Serbia, and Faculty of Physical Chemistry, University of Belgrade, Studentski trg 12-16, 11000 Belgrade, Republic of Serbia

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Antioxidative properties of naturally occurring flavon-3-ol, fisetin, were examined by both cyclic voltammetry and quantum-chemical based calculations. The three voltametrically detectable consecutive steps, reflected the fisetin molecular structure, catecholic structural unit in the ring B, C3-OH, and C7-OH groups in the rings C and A. Oxidation potential values, used as quantitative parameter in determining its oxidation capability, indicated good antioxidative properties found with this molecule. Oxidation of the C3′C4′ dixydroxy moiety at the B ring occurred first at the lowest positive potentials. The first oxidation step induced fast intramolecular transformations in which the C3 hydroxy group disappeared and the product of this transformation participated in the second oxidation step. The highest potential of oxidation was attributed to the oxidation of C7 hydroxy group. The structural and electronic features of fisetin were investigated at the B3LYP/6-311++G** level of theory. Particularly, the interest was focused on the C3′ and C4′-OH sites in the B ring and on C3-OH site in the C ring. The calculated bond dissociation enthalpy values for all OH sites of fisetin clearly indicated the importance of the B ring and C3′ and C4′-OH group. The importance of keto−enol tautomerism has also been considered. The analysis also included the Mulliken spin density distribution for the radicals formed after H removal on each OH site. The results showed the higher values of the BDE on the C7-OH and C3-OH sites.

Introduction

A variety of naturally occurring and synthetic antioxidant molecules have been shown to quench free radicals, reduce lipid peroxidation, and detoxify hydrogen peroxide through nonenzymatic defense mechanisms. These compounds include both dietary factors, such as flavonoids, vitamins C and E, hydroquinones and various sulfhydryl compounds, as well as sodium benzoate, butylated hydroxytoluene, butylated hydroxyanisole, and others. Because of their beneficial actions there has been an increased interest in the application of antioxidants to medical treatment since they can significantly inhibit or postpone oxidative processes with products that are often very toxic.[1−4]

In the past years the chemical behavior of flavonoids as antioxidants has become the subject of intense experimental and theoretical research. Flavonoid family is the vast and major group among the phenolics with more than several thousands known compounds. The numerous investigations provided some circumstantial evidence that flavonoids exhibit biological activity, including antiallergic, antiviral, antimutagenic, anti-inflammatory, vasodilatory, and inhibitory actions.[1] They also play multiple roles in the ecology of plants.[5−8] They are responsible for the colors of fruits (e.g., the red or blue of grape and berry skins) and vegetables; they act as attractors for pollinating insects and as catalysts in the light phase of the photosynthesis. They are involved in the UV protection of plants and protection of plants against microbial invasion;[9,10] they are chelating agents of transition metal ions and also reducing agents.

Overwhelmingly, all the mentioned activities of flavonoids are generally related to their pronounced antioxidant activity, which arises from their ability to scavenge free radicals.[1,4,11−19] Potentially, oxygen radical species can damage almost all types of biologically important molecules: lipids (causing lipid peroxidation), amino acids, carbohydrates, and nucleic acids (causing mutations). An imbalance between antioxidants and reactive oxygen species results in oxidative stress which is implicated in many diseases suggesting that free radicals participate as fundamental components in large majority, if not all, human diseases.[1,12] Antioxidant molecules present in those systems maintain an important balance between the formation of reactive oxygen species and their removal. There are two possible reaction pathways through which flavonoids and other phenolic compounds scavenge free radicals: (1) rapid donation of the hydrogen atom to a radical form, forming a new radical, more stable than the initial one (mechanism leading to the direct O−H bond breaking)

$$\mathrm{ROH} + \mathrm{HO}^{\bullet} \rightarrow \mathrm{RO}^{\bullet} + \mathrm{HOH} \qquad (1)$$

and (2) the chain-breaking mechanism, by which the primary antioxidant donates an electron to the free radical present in the system (e.g., lipid or some other radical), leading to indirect H abstraction

$$\mathrm{ROH} + \mathrm{HO}^{\bullet} \rightarrow \mathrm{ROH}^{+\bullet} + \mathrm{OH} \rightarrow \mathrm{RO}^{\bullet} + \mathrm{HOH} \qquad (2)$$

The first mechanism is governed by the O−H bond dissociation enthalpy (BDE), which is the molecular property used in the assessment of possible radical scavenging potential of the molecule. It is calculated as the difference in heat of formation between the molecule (fisetin in this case) and its radicals,

* To whom correspondence should be addressed. E-mail: markovich@ffh.bg.ac.rs. Phone/fax: + 381 11 187 133.
[†] State University of Novi Pazar.
[‡] University of Belgrade.

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implicating its correspondence to the OH bond-breaking energy. The second mechanism is governed by a one-electron transfer process with both the ionization potential and reactivity of the radical−cation $ROH^{+\bullet}$ playing important roles. Whatever mechanism is involved the formed radical species ($RO^{\bullet}$, phenoxide radical in the case of flavonoids) needs to be relatively stable, so that reactions 1 and 2 could be thermodynamically favorable in the sense that it is easier to remove a hydrogen atom from ROH than from HOH and should slowly react with other molecules without toxic effects such is the oxidative stress.

Fisetin is a naturally occurring flavone-3-ol commonly found in strawberries and other fruits and vegetables. Because of its specific structural features it is considered as a potent antioxidant capable of effective free radical scavenging under in vivo. Medical interest in it arises from its numerous beneficial effects, among which the most striking are involved with stimulating signaling pathways that enhance long-term memory.[20,21]

The objective of this study was to experimentally and theoretically investigate the antioxidant activity of fisetin. Because of its electrochemical activity, antioxidant properties were correlated with redox potential values[22,23] and evaluated by applying cyclic voltammetry (CV). For theoretical calculations of the BDE, density functional (DFT) and semiempirical methods are used. Those methods often give good results with relatively reasonable computational cost still leaving the most reliable methodology to be established.[24−34] In this paper the results of bond order, BDE, highest occupied molecular orbital (HOMO), and Mulliken spin density for fisetin are presented. The structure−activity relationship is also examined in the light of these results. Unlike most theoretical investigations which are focused only on the B-ring, particularly the catechol moiety, in this paper the attention is focused to the DFT interpretation of the reactivity of all OH groups in fisetin and the radicals formed after H-removal from this molecule. Keto−enol tautomerism before H-abstraction is also discussed for explaining the role of 3-OH group.

Experimental Section

Chemicals. The following substances were used: fisetin (Merck), sodium chloride (Merck, USA), acetic acid (Merck, USA), ethanol (Uvasol, Merck, USA), sodium hydroxide (Merck, USA). All the chemicals were used as received.

Supporting Electrolytes. Acetate buffered solutions (pH 2.0−7.0) of constant ionic strength, adjusted by sodium chloride (5×10^{-2} M), were used. The solutions were obtained by mixing acetic acid (5×10^{-2} M) and sodium hydroxide (1.5 M). Stock solution of fisetin (1×10^{-2} M) was prepared in ethanol. This solution was diluted to the concentration of 1×10^{-3}M by addition of the buffers.

CV Measurements. CV measurements were carried out by PAR EG&G Model 273 potentiostat/galvanostat. A three-electrode system, consisting of a rotating disk shaped measuring glassy carbon electrode ($A = 0.196$ cm^2), a saturated calomel electrode (SCE) as reference one, and a platinum foil as an auxiliary electrode, was used. Glassy carbon was a suitable electrode material for electrochemical investigations inside a potential range −0.6 to 1.2 V vs SCE because of its poor catalytic activity for water oxidation and reduction. Nevertheless, at potentials close to 1.2 V vs SCE the current of water oxidation became measurable part of the background current. To make the difference between the background current and the current of fisetin electrochemical reactions simultaneous plotting of those two currents was performed. Another possible source of complications, peroxide formation by oxygen reduction, was eliminated by purging electrolyte solution by nitrogen stream.

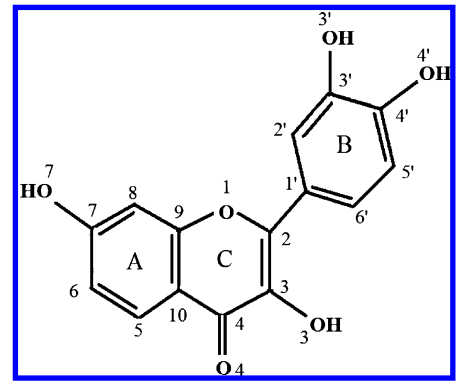


Figure 1. Atomic numbering of fisetin.

Because of the ability of fisetin oxidation products to adsorb on the electrode surface, prior to each measurement the glassy carbon electrode was polished by alumina (0.05 μm) and carefully rinsed with bidistilled water. The equilibration period was at least 10 s. The CV measurements were performed without stirring. Cyclovoltammograms were recorded in a range of potentials from −0.600 V to + 1.200 V, at a potential sweep rate ranging from 20 to 200 mV/s. The cell volume was 10 cm^3. The voltammograms of the supporting electrolyte buffer solutions were recorded first, after which an aliquot of the fisetin solutions were added, and the voltammograms recorded under the same conditions.

Computational Method. All calculations were conducted using Gaussian 03[35] with the B3LYP hybrid functional[36−38] The triple split valence basis set 6-311+G (d,p) was used. This polarized basis set adds p functions to hydrogen atoms in addition to the d functions and diffuse functions on heavy atoms. The suitability of this level of theory for studies of bond dissociation enthalpy involving quercetin and taxifolin has already been evidenced elswhere.[39] The geometrical parameters for fisetin and its 3-OH, 3′-OH, 4′-OH, and 7-OH radical species were optimized in vacuum. Vibrational analysis and natural bond orbital (NBO) analysis were performed for all structures. Obtained zero-point energies were used to correct all energetic terms. All calculated structures were verified to be local minima (all positive eigenvalues for ground-state structures) by frequency calculations. The bond dissociation enthalpy (BDE) for fisetin was calculated using the equation

$$\mathrm{BDE} = H_{\mathrm{fis}} - H_{\mathrm{fis}\bullet} - H_{\mathrm{H}\bullet}$$

where H_{fis}, $H_{\mathrm{fis}\bullet}$, and $H_{\mathrm{H}\bullet}$ present the enthalpies of fisetin, fisetin radical, and hydrogen, respectively. The ionization potential (IP) of fisetin was determined by optimizing neutral and cationic species.

Potential energy surfaces were obtained in relation with the torsion angle τ between the rings B and C, defined by the C3−C2−C1′−C2′ atoms (Figure 1). The torsion angle τ was scanned in steps of 15° without constraints on all other geometrical parameters. The effects of the following torsion angles rotations were also studied: C2−C3−OH, C6−C7−OH, C2′−C3′−OH, and C3′-C4′−OH. Afterward, the structures were further optimized without any constrain around each potential minimum.

Results and Discussion

Electrochemical Activity of Fisetin. Fisetin is a tetrahydroxyflavone molecule possessing structural features known as

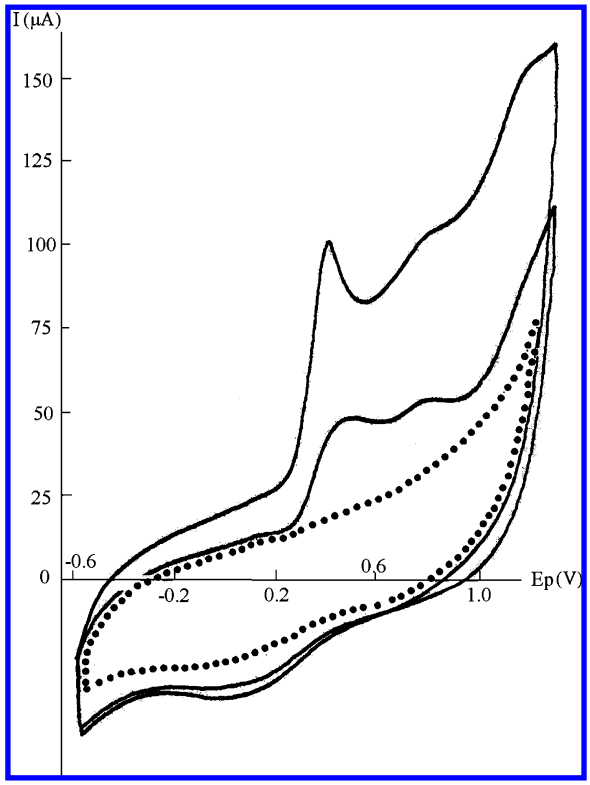


Figure 2. Two consecutive cyclovoltammograms of fisetin ($c = 1 \times 10^{-3}$ M) at pH 4.0 (polarization rate 100 mV/s).

very important for high antioxidant activity and scavenging radical activity.[11,12] Those activities reside mainly in its hydroxyl groups at C3, C3′, C4′, and C7 and are enhanced with carbonyl group at C4, a double bond between C2 and C3 conjugated with the 4-oxo group enabling higher electron delocalization (Figure 1).

Previously, the electrochemical oxidation of fisetin is studied by Golabi and Irannejad[40] with the aim to modify GC electrode for determination of NADH and ascorbic acid. They confirmed that the oxidation products kept adsorbed on the GC surface modifiing its electrocatalytic behavior.

By the obtained cyclic voltammograms it is possible to suppose that the oxidation of fisetin proceeds in three poorly resolved steps. The final oxidation products obviously stay on the electrode surface and block its electrochemical activity toward fisetin dissolved in the electroyte, since the peak heights decrease with the repetition of polarization cycles (Figure 2). Therefore, the electrode surface is polished before each recording, and the first voltammogram was only considered.

By enlarging steply the anodic potential values, from 0.6 V upward, only the first oxidation step shows reversible cathodic peak, which is significantly reduced in surface compared to anodic one. This confirms conclusions published previously[41] that one deals with the oxidation of catehcholic group, followed by intramolecular rearrangement.

The oxidation peak potentials and peak currents are pH dependent (Table 1). The pattern of fisetin oxidation potential change at different pH values is shown in Figure 3. Starting from the low pH values (Table 1) and proceeding upward, the peak potentials shift toward more negative values. This indicates the participation of protons in the electrochemical steps, i.e., that the deprotonation acompanies the deelectronation. Deprotonation facilitates oxidation due to the shift of the molecular charge to negative value.[42] The plot of the E_p vs pH for the firts oxidation peak (Figure 4) has the slope of 52 mV per pH

TABLE 1: Potential (E_p) and Current (I_p) Values of Fisetin ($c = 1 \times 10^{-3}$ M) Oxidation Peaks in Different Buffers

pH	υ (mV/s)	E_{p_1}(V)	I_{p_1} (μA)	E_{p_2} (V)	I_{p_2} (μA)
2	100	0.516	117.5	0.853	75.0
4	100	0.364	102.5	0.763	45.5
6	100	0.310	89. 7	0.634	37.5
7	100	0.248	54.5	0.686	12.5

unit, which closely corresponds to the same number of protons and electrons involved in electrode process. Obtained results indicate two-electron oxidation of fisetin in the pH range observed, following the mechanism of the eHHe type established for hydroquinones by Driebergen.[43] According to numerous substantial and exstensive investigations on antioxidant activities of different flavonoids[42] the first oxidation wave (E_p (pH 4) $\approx$ 0.40 V) is possible to correlate to the oxidation of the catecholic group in the B ring, which involves the elimination of two electrons.

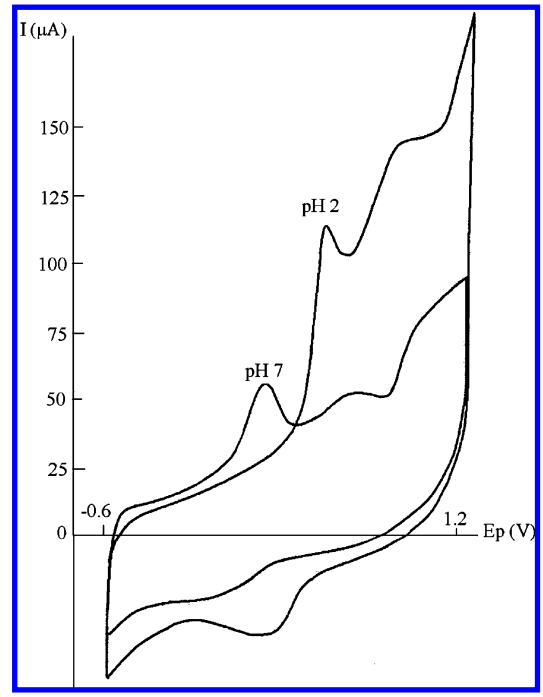


Figure 3. CV curves of fisetin ($c = 1 \times 10^{-3}$ M) at pH 2 and pH 7 (polarization rate 100 mV/s).

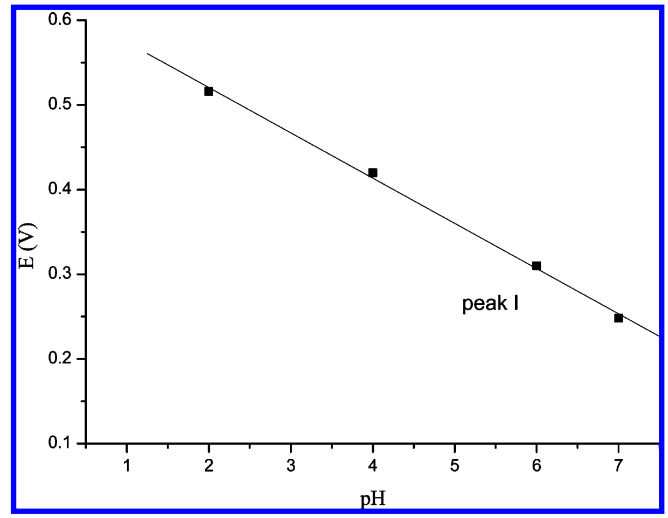
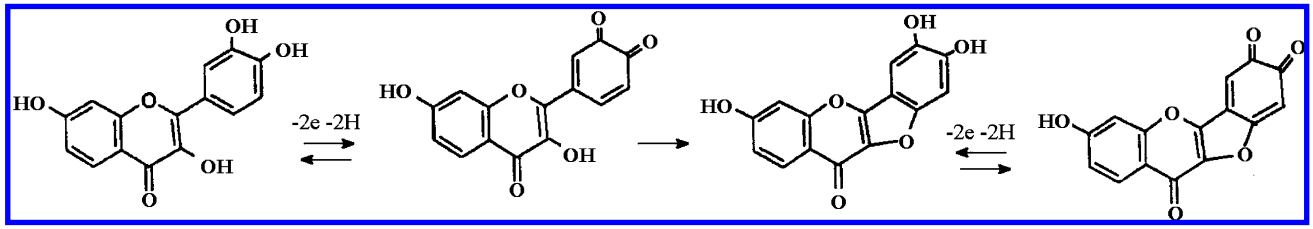


Figure 4. Plot of E_p vs pH for 1×10^{-3} M fisetin.

SCHEME 1: Proposed Oxidation Pathway of Fisetin in Aqueous Buffered Solutions



The product of the first oxidation step, *o*-quinone, undergoes fast intramolecular rearrangement (EC mechanism) giving a new oxidizable species, with renewed catehol unit, in the B ring, on the account of transformation of C3 hydroxyl group into keto group. From the fact that reverse cathodic peak is much smaller than the corresponding first anodic peak, one may conclude that the intramolecular rearrangement is very fast. The ratio anodic vs cathodic peak height tends to reach unity on enlarging the scaning rate, confirming that one deals here with the EC type of electrochemical processes.

In the second anodic oxidation step (E_p (pH 4) $\approx$ 0.80 V) primarily participates the catechol formed by intramolecular rearrangement of *o*-quinone. It is not excluded that a part of *o*-quinone, which does not catch up to undergo rearrangement, participates with its C3 hydroxyl group in the second oxidation step too, but the similarity in energies of these two molecular states does not allow the two mutually resolved corresponding peaks in the cyclovoltammogram.

The third oxidation step observed at a potential of nearly 1 V may be attributed to the oxidation of C7 hydroxyl group. This may be confirmed by the similarity of this oxidation potential with the oxidation potential of flavone containing hydroxyl group only in the A ring: either 5,5-dihydroxyflvone or 7-hydroxyflavone.[42] This step is difficult to examine electrochemically because it occurs at potentials adjacent to the potential of water oxidation.

On the basis of the presented results it is possible to suppose the oxidation pathway of fisetin (Scheme 1) is similar to that of quercetin[44] and other flavonols, 3-OH flavones, with similar hydroxylation pattern.[45]

UV−Vis Spectrochemical Investigation. Structural changes are also evident in electronic spectra of fisetin at different pH values. By increasing the pH and inducing ionization of the OH groups, absorption bands I and II (Figure 5 bands designated on the spectra) belonging to the cinnamoyl (B+C ring) and benzoyl (A+B ring) moieties, respectively, show moderate red shift and broadering. This behavior is a consequence of the sucessive deprotonation as well as the coexisting mixture of ionic and neutral species in the solution.

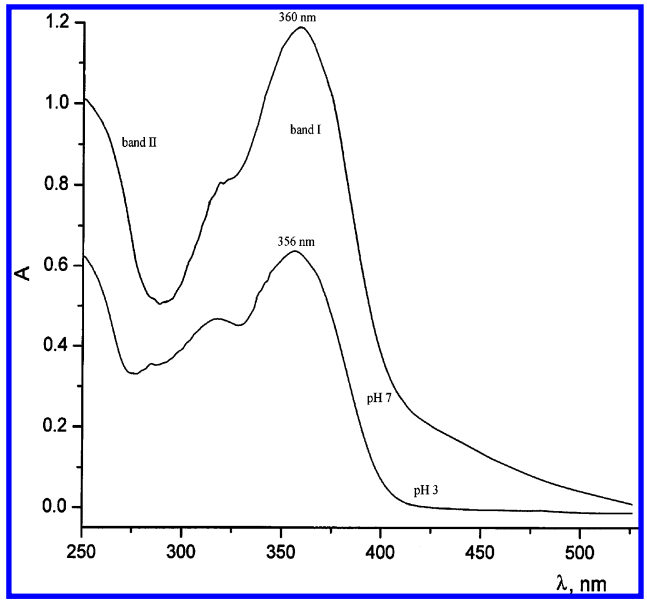


Figure 5. Electronic spectra of fisetin at different pH values.

DFT Study on the Reactivity of Fisetin. To determine the preferred relative positions of the rings B and C, conformational space of the structure 1 (Figure 6) is investigated as a function of torsional angle τ (C3−C2−C1′−C2′) between those rings. The minimization procedure for the fisetin structure, performed at the B3LYP level with the 6-311+G** basis set, yields a planar conformation (τ = 180°) as the more stable one. In this structure, the molecule is completely conjugated and all hydroxyl groups are oriented in a way to form the maximum number of hydrogen bonds. The present results concur with the literature data on theoretical study of quercetin structure obtained using RHF/STO-3G[46] and B3LYP/6-311++G**[25] methods, which both report the planar structure of quercetin molecule.

The DFT results are presented in Figure 7. By removal of the torsional angle constraint, the conformational absolute minimum is found at τ = 180.0° followed by a relative minimum at τ = 0° with energy difference of only 0.91 kcal mol^{-1}. The potential energy maximum lies at τ = 90°, and the intercorvesion barrier between the two minima is about 5.53 kcal mol^{-1}, which is almost equal to that for quercetin.[25]

The ionization potential value of 166.55 kcal mol^{-1} for the structure 1a of fisetin (Figure 5) is very similar to that of the quercetin molecule.[25] On the basis of the ionization potential values and structural similarities between these two molecules one can also expect similar parameters of their antioxidant activities (AA). The experimental values of the half-wave potentials of the first oxidation wave, $E_{1/2}$, confirm this assumption.[47] In favor to this also goes somewhat higher dipole moment of the fisetin structure 1a (3.89 D) in comparison to the value for quercetin.[25]

The results of fisetin NBO analysis indicate strongly localized double bonds at the C2−C3 and C4−O positions of the ring C. The bond order values suggest a highly independent electronic delocalization in the rings B and A, which is also one of the main structural features implicated in antioxidant activity of flavon-3-ols and other flavonoids. The molecular planar structure 1 presented in Figure 6 enables such electronic delocalization in fisetin molecule. The C2−C1′ bond lies in the chromone plane, because the C4−C3−C2−C1′ torsional angle is 180°, and its length is 1.465 Å. The order of this bond, obtained by the NBO analysis, is close to 1 (Table 2) indicating very small conjugation across all the rings of the π system. The double bonds in the ring C around the carbonyl group indicate a cross-conjugated system[48] in which the delocalization is allowed only between C and A or C and B rings but not between rings A and B. This fact is indirectly confirmed by the investigation in

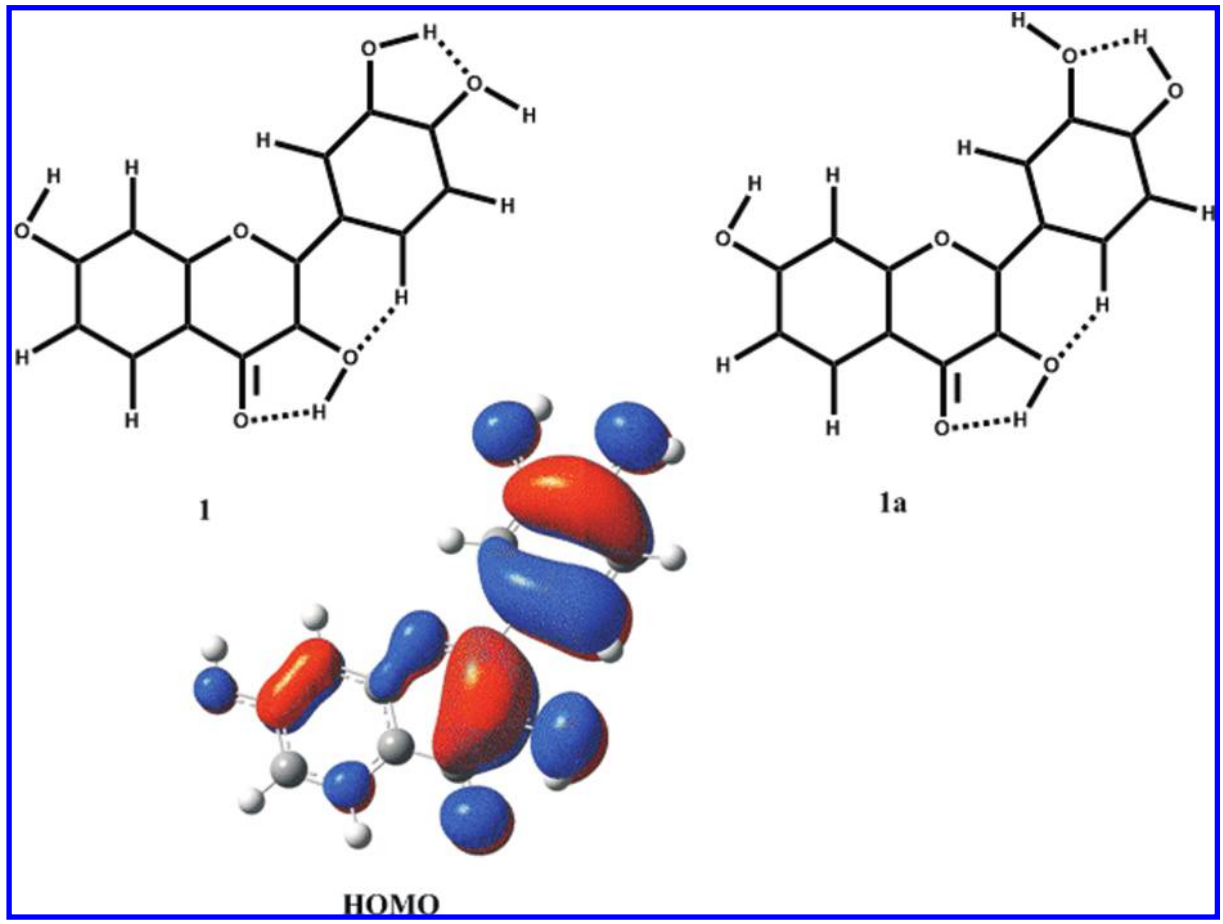


Figure 6. Two most stable B3LYP/6-311+G**-optimized structures of fisetin molecule in the gas phase (1 and 1a). Calculated HOMO orbital of 1.

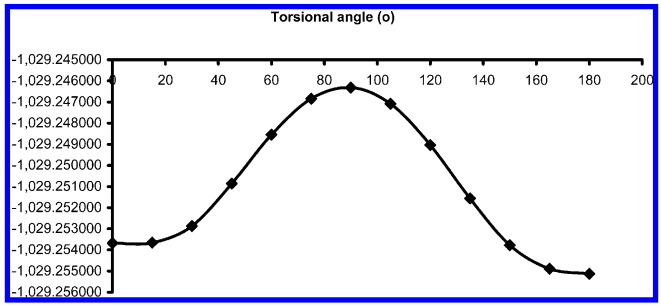


Figure 7. Rotation barrier around the inter bond C2−C1′ calculated with the B3LYP/6-311+G** method for the structure 1.

which biophenols molecules were found to be neither completely planar nor conjugated.[48]

In the molecule structure 1 (Figure 6) three hydrogen bonds are present, between the C3-OH and C4-O carbonyl groups, between ortho hydroxyl groups in the B ring, and between the C3-OH and C6′. Generally all hydrogen bonds have stabilizing effects. Additional stabilization of the C3-OH group is established by the formation of the hydrogen bond between oxygen of the C3-OH group and hydrogen on the C6′. This finding is in agreement with previous results for quercetin.[49] The conformations lacking these bonds (Figure 8) are less stable with respect to the absolute minimum by 9.88, 4.04, and 13.81 kcal mol^{-1}, respectively. The hydrogen bond lacking between the C3-OH and C4-O carbonyl group (1.973 Å) has higher stabilizing effect than that between the C3′-OH and C4′-OH hydroxyl groups (2.166 Å).

In the case of hydrogen bond lacking between the C3-OH and C4-O carbonyl group the torsional angle τ between rings C and B becomes significantly changed (136.7°) in comparison to structure 1 (Figure 6). This significant deviation of the dihedral angle is not only a consequence of lacking hydrogen bond but also the steric hindrance between hydrogen of the OH group and hydrogen bonded to C6′. The similar deviation of the torsion angle between rings C and B (τ = 136.5°) for structure 1e is also present. These geometrical changes make

TABLE 2: DFT Bond Order Values of Fisetin (1) and Its Radical Species (C4, C3′, C3, and C7)

bond order	1	C4′-OH	C3′-OH	C3-OH	C7-OH
O1−C2	0.7876	0.7734	0.7784	0.7971	0.7976
C2−C3	1.2164	1.1734	1.2077	1.0340	1.1794
C3−C4	0.9656	0.9597	0.9620	0.8683	0.9755
C4−C10	0.9610	0.9798	0.9788	0.9546	0.9893
C5−C10	1.0650	1.0676	1.0674	1.0763	1.0807
C5−C6	1.1969	1.1947	1.1948	1.1836	1.1752
C6−C7	1.0912	1.0904	1.0908	1.1005	0.9832
C7−C8	1.1654	1.1595	1.1605	1.1514	0.9969
C8−C9	1.1129	1.1196	1.1220	1.1281	1.1752
C9−C10	1.1144	1.1045	1.1065	1.1191	1.0807
C9−O1	0.8199	0.8096	0.8069	0.7807	0.7838
C2−C1′	0.9545	1.0132	0.9637	1.0194	0.9754
C1′−C2′	1.0961	1.0784	1.1623	1.0693	1.0909
C2′−C3′	1.1565	1.1837	1.0425	1.1682	1.1587
C3′−C4′	1.1017	0.9548	0.9623	1.0898	1.0941
C4′−C5′	1.1579	1.0158	1.1348	1.1472	1.1525
C5′−C6′	1.1504	1.2328	1.1686	1.1604	1.1543
C6′−C1′	1.1021	1.0220	1.0272	1.0706	1.0960
C3−O	0.8596	0.8677	0.8646	1.1760	0.8669
C4−O	1.1935	1.1808	1.1647	1.2108	1.1529
C7−O	0.8388	0.8341	0.8314	0.8287	1.1392
C3′−O	0.8331	0.8734	1.1182	0.8253	0.8301
C4′−O	0.8111	1.1413	0.8936	0.8119	0.8113

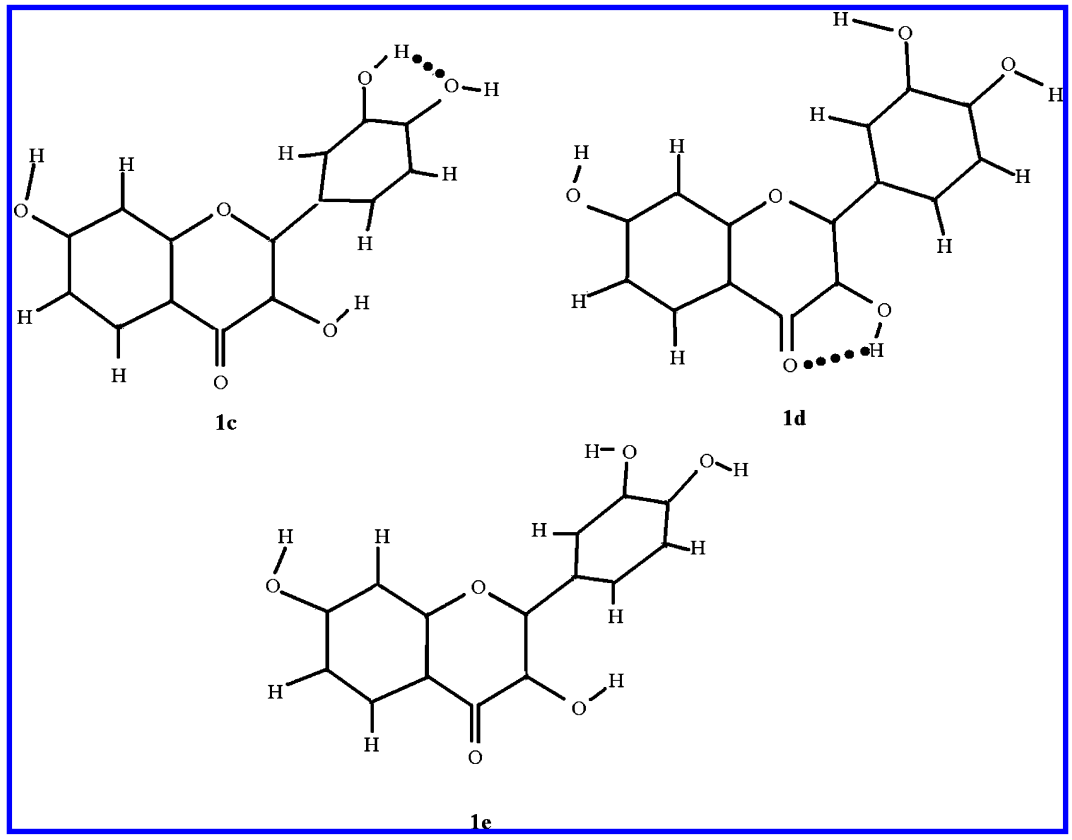


Figure 8. The structures of fisetin in the absence of the C3-OH-C4O (1c), C3′OH-C4′OH (1d) hydrogen bonds, and both of them (1e).

the conjugation stabilizing effect quite impossible for these structures. On the other hand, when hydrogen bond lacks between the C3′-OH and C4′-OH groups, the torsional angle between rings C and B retains equal value (180.0°) as in the structure 1, leaving the molecule completely conjugated.

Radical Species of Fisetin. Geometry optimizations of the radical species are performed starting from the optimized structure of the parent molecule and removing the H-atom from the C3, C7, C3′, or C4′ positions. The radical formed by H-removal from the 3-OH group of fisetin is called the C3-OH fisetin radical, and the same notation is used for the other three radicals. No geometrical parameter constraint is imposed during the optimization. The OH group neighboring the primary radical site in the B-ring is oriented in such way that H bonding is preserved. Indeed, after H abstraction from C4′-OH, the ortho hydroxyl group is titled to form a H-bond with the remaining O-atom.

Starting from the absolute minimum conformation of the structure 1, a hydrogen atom removal from the C4′-OH and C3′-OH groups yields two radical forms of the fisetin molecule (Figure 9), the first one being more stable by 2.63 kcal mol^{-1}. Energy minimum for two other isomers, generated by the loss of the hydrogen atom from the C3-OH and C7-OH groups, are found at 10.28 and 12.61 kcal mol^{-1}, respectively. All these radicals retain planarity and consequently conjugation, similar as parent molecule represented by the structure 1 (Figure 6).

Each of the C4′-OH, C3′-OH, and C7-OH radical structures is characterized by three hydrogen bonds (Figure 9) that contribute to their stabilities. An exception is the C3-OH radical form, which has only two hydrogen bonds. In addition, after H-removal from C4′−OH or C3′-OH positions obtained radical forms are stabilized by relatively strong hydrogen bonds between oxygen and hydrogen from neighboring OH group. On the other hand, there is not additional stabilization via hydrogen bonds for C7-OH and C3-OH radical forms. This occurrence can be attributed to the fact that these structures are less stable than the C4′-OH and C3′-OH radical structures.

Inspection of Table 2 allows further comment on the electronic structure of the four possible radical isomers of fisetin. In the C4′-OH radical species the complete delocalization involves only ring A, while ring C is characterized by three double bonds strongly localized on the carbonyl group, C2−C3, and C9−C10 bonds. Significant difference in comparison to the structure 1 of the parent molecule is the existence of strong localized double bonds on the carbonyl group at C4′ as well as between the C2′-C3′ and C5′-C6′ atoms. Slightly higher bond order for C2-C1′ than the corresponding bond in parent molecule offers electron delocalization over the whole C ring.

In the B ring of the 3′-OH radical species there are also three double bonds, localized on the carbonyl group at C3′ as well as between the C1′-C2′ and C5′-C6′ atoms. The bond order between C2 and C1′ is close to 1 meaning that the conjugation between the C and B rings is hindered.

In the C3-OH radical species the complete delocalization involves only rings A and B, while the ring C is characterized by two double bonds strongly localized on the carbonyl groups. However, due to the planarity around the torsional angle τ, the electronic flow between the ortho-diphenolic moiety and the ring C is still possible. This radical species appears to be a cross-conjugated system as parent molecule fisetin.

The C7-OH radical gives rise to good electron delocalization between rings B and C and poor conjugation between the rings C and A. The C7−O carbonyl group, derived from the hydrogen removal, determines two separated double bonds in the ring A.

The DFT calculated BDE values (Table 3) for the four radical forms, formed by the H-abstraction from the parent molecule, give the following BDE sequence for the present OH groups: C7-OH > C3-OH > C3′-OH > C4′-OH. This sequence clearly

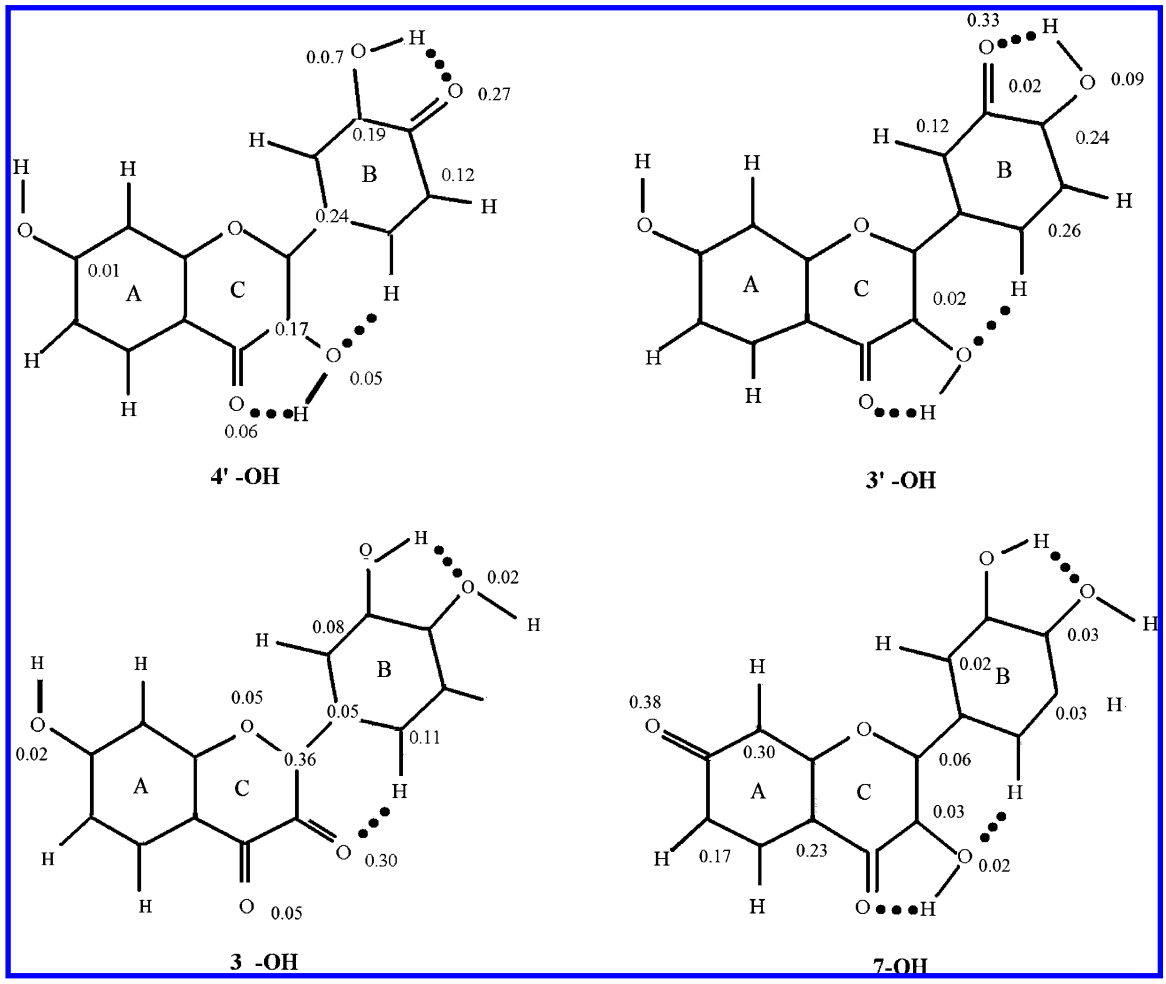


Figure 9. The optimized structures of fisetin radicals in the gas phase. Values of corresponding spin densities are presented by numbers.

TABLE 3: Some of the Calculated Energies of the Fisetin Molecule (1 and 1a) and Its Radical Isomers (C4′-OH, C3′-OH, C3-OH, and C7-OH) in Their Ground States[a]

	C4′-OH	C3′-OH	C3-OH	C7-OH	1	1a
E+ZPE	−1028.413205	−1028.409016	−1028.396828	−1028.393109	−1029.030757	−1029.030566
BDE	72.41	75.04	82.69	85.02		
HOMO	−0262	−0.252	−0.252	−0.256	−0.217	−0.216
LUMO	−0.097	−0.099	−0.090	−0.096	−0.079	−0.081
SOMO	−0.221	−0.227	−0.213	−0.231		

[a] BDE, bond dissociation energy; E+ZPE, electronic and zero-point energy; HOMO, highest-occupied molecular orbital; LUMO, lowest-unoccupied molecular orbital. E+ZPE is in Hartree; BDE is in kcal/mol; the rest of the energies are in eV.

confirms that H transfer from the B-ring (C4′-OH and C3′-OH groups) is easier than that from the ring A (C7-OH group), which is consistent with the literature data concerning structure−activity relationship of antioxidant flavonoids.[50]

According to the performed calculations, the BDE value for the C7-OH site on the A-ring is higher than those for C4′-OH and C3′-OH sites on the B ring by 12.61 and 9.98 kcal mol^{-1}, respectively. Whatever the kind of oxidative system is involved these results clearly implicate higher reactivity of the B ring in comparison to the ring A. This hierarchy may only be overcome if the oxidation of the molecules takes place via an enzymatic action for which the binding configuration with the protein receptor governs the location of the redox reactions. It is worth noting that the BDE for the C4′-OH species is lower only by 2.6 kcal mol^{-1} than for C3′-OH, whereas the BDE for C4′-OH is lower by 2.4 kcal mol^{-1} than for C3′-OH of quercetin.[31]

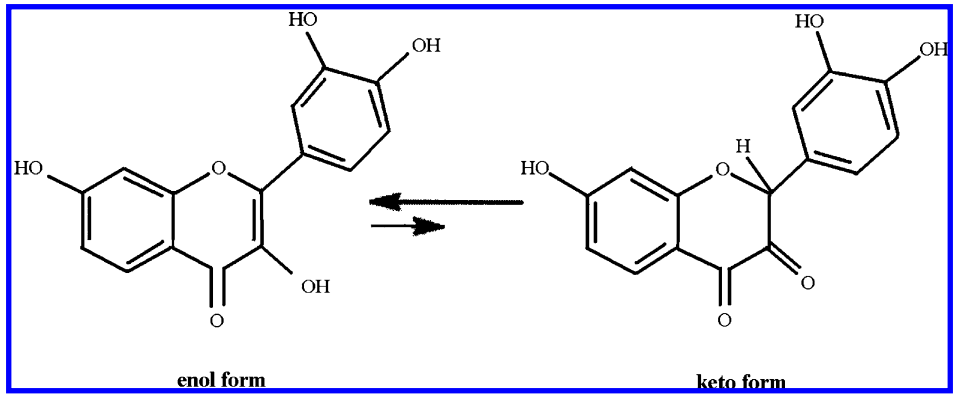
The BDE value for the C3-OH radical form of fisetin (82.69 kcal mol^{-1}) lies between the values of the C4′-OH and C3′-OH forms on one hand and C7-OH form on the other. It is by 10.3 and 7.6 kcal mol^{-1} higher than the corresponding values for C4′-OH and C3′-OH forms and only by 2.3 kcal mol^{-1} lower than the value for C7-OH specie. Obviously, the BDE values have similar trend as for quercetin molecule.[31]

The BDE values clearly demonstrate that H-transfer from the B-ring is energetically more favorable than H-transfer from the rings A and C. Depending on the oxidative system the H-transfer from the 3-OH group is also possible in fisetin molecule. This finding is confirmed with several in vitro studies that have shown the participation of the 3-OH group in redox reactions.[51,52]

For explaining the reactivity of the C3-OH group and the C2−C3 double bond in the fisetin molecule another possible mechanism, keto−enol tautomerism is also considered (Scheme 2).

Obtained BDE value of H transfer from the C2 atom in the keto form is 61.1 kcal mol^{-1}. This very low BDE value indicates high capacity of H-transfer from the C2 site in the keto form. However, it should be noted that the keto form of fisetin is less

SCHEME 2: Structures of Keto and Enol Forms of the Fisetin Molecule



stable than the enol form, the latter being stabilized by π-conjugation from the B-ring to the C4 carbonyl group through the C2−C3 double bond. The difference in stability is close to 19.6 kcal mol^{-1} and indicates that the contribution of the keto form can be considered negligible for fisetin as a free molecule. On the other hand, it is possible to presume that, in some enzymatic processes, the difference in stabilities between two tautomeric forms is reduced. In that case, the keto form becomes relevant enabling the H-abstraction from the C2 atom and thereby contributing to the antioxidant properties.

The difference in antioxidant activity of different hydroxyl groups of fisetin, reflected in the corresponding BDE values, is often attributed to π-electron delocalization, which leads to the stabilization of the radical forms obtained after the H abstraction. This conclusion is made on the basis of an assumption that if π-electron delocalization exists in the parent molecule it also exists in the corresponding radical. To better understand the relationship between the electron delocalization and the reactivity of the radical species, the electron distribution in the singly occupied molecular orbital (SOMO) should be examined. The SOMOs of the C3-OH, C3′-OH, and C4′-OH radical forms (corresponding to H-removal from one of the three OH groups) are mainly delocalized over the rings C and B (Figure 10). Their shapes are mutually very similar exhibiting no sufficient variations for explaining the differences in activities between these OH groups. On the other hand there is a slightly different picture concerning SOMO of the C7-OH radical form in which the delocalization of SOMO exists over the whole molecule of fisetin. As SOMO is the α-highest occupied molecular orbital it does not describe the global electronic behavior of the radical form, and its shape is not a reliable indicator of the reactivity of fisetin radicals.

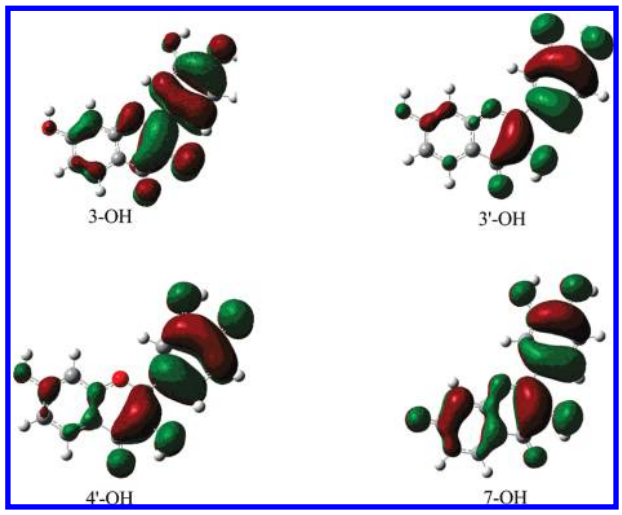


Figure 10. SOMO of the C3-OH, C3′-OH, C4′-OH, and C7-OH radicals formed by H removal from fisetin.

Within an unrestricted scheme, the spin density is often considered to be a more realistic parameter providing a better representation of the reactivity.[53] The importance of the spin density for the description of flavonoids has been pointed out by the recent literature data.[24,25] Therefore, the spin density on the various fisetin radical forms is also analyzed in this paper in order to be able to understand the differences in reactivity of the various OH groups in fisetin and consequently the differences in BDE values. It is important to point out that the more delocalized the spin density in the radical form is, the easier the radical is formed, and thus the lower the BDE value is.[54]

The spin density appears to be slightly more delocalized for radicals formed on the B ring (at C4′-OH and C3′-OH) than for that formed on the A ring (at C7-OH) (Figure 9). This is most probably the consequence of the presence of a C2−C3 double bond, which allows spin presence on the C3 atom (0.17 for C4′-OH, Figure 9). The spin distribution indicates the oxygen O atom bonded to C4′ as the most probable radical center (Figure 9), followed by the carbon C1′ atom. This effect can also be explained by using the classical resonance effects occurring in the phenoxy radical. Such a scheme explains the presence of the radical (high spin density) on the C1′ atom in para position for the C4′−OH radical, and the subsequent possible delocalization effect due to the presence of the C2−C3 double bond. It is to be noted that such delocalization cannot happen for the C3′-OH radical form since the high spin density is located both on the C6′ and C4′ sites.

It is clear that the 3-OH radical can be formed either from keto or enol tautomeric forms. It is nonetheless important to note that whatever is the mechanism of H-transfer the radical formed is the same. Present calculations show that the spin density on the O3 atom is 0.30, confirming the high capacity for H removal from the enol tautomeric form of fisetin. On the other hand, the presence of a high spin density (0.39) localized on the C2 atom implies a high reactivity for this site. This result indicates the carbon C2 as the most probable radical center (Figure 9) followed by the oxygen atom bonded to carbon C3.

Nevertheless, the differences in the BDE values cannot be explained only on the basis of the spin density values, on the O atom where H abstraction occurs, and delocalization of the spin densities. The BDE value of C3-OH form is by about 10 and 7.5 kcal mol^{-1} higher than those of the C4′-OH and C3′-OH

forms, respectively, whereas the spin density on the O-atom of C3-OH is similar as on the other two. This difference is related to the fact that the H bond exists between the C3-OH group and the C4 carbonyl group. As a consequence, the BDE on this site is higher because the H removal also implies the cleavage of the H-bond.

Conclusion

The oxidation of fisetin is a pH-dependent process that proceeds by eHHe mechanism characteristic to other flavon-3-ols. First to oxidize is the C4′-C3′ catecholic group which according to the theoretical calculations lies at lower energetic values than other OH groups. The second oxidation process includes the C3-OH group, after an intramolecular rearrangement in which C4′-C3′ catecholic group becomes recovered. Oxidation potential values indicate good antioxidant capacity of fisetin in the whole range of the pH investigated.

On the basis of the obtained theoretical results, fisetin appears to be a planar molecule exerting a cross-conjugation effect. The most stable radical species in the gas phase is the C4′-OH one. The antioxidant action is generated by two most stable radicals showing a planar conformation. It means that radical structures allow extended electronic delocalization between adjacent rings. This theoretical approach confirms the important role of the B ring and sheds light on the role of the C3-OH group in the antioxidant properties. The reactivity of this site is dependent on the presence of the C2−C3 double bond.

Results indicate that H-abstraction from the C3-OH group can be induced by two possible chemical pathways. The first one is H abstraction from the C3-OH group of the enol form of fisetin, which induces spin delocalization to the C2 atom. The second one is H abstraction from the C2 atom of the keto form of fisetin, which can be formed as a consequence of keto−enol tautomerism via the C2−C3 double bond. While the enol form is the most stable tautomer in the free molecule, the keto form, which possesses a much lower BDE value for H-abstraction on the C2 atom, might play a significant role in the real system, depending on the molecular environment of fisetin and on the oxidative system acting on the molecule. Results also indicate that the C7-OH radical form lies at higher energetic values with respect to the absolute minimum, so, its presence in the antioxidant mechanism is rather improbable. In spite of fact that there is no TEAC value for fisetin, all calculated values (BDE, dipole moment and IP) indicate that fisetin has high antioxidant capacity.

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