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ARTICLE *in* JOURNAL OF THE AMERICAN SOCIETY FOR MASS SPECTROMETRY · JULY 2004

Impact Factor: 2.95 · DOI: 10.1016/j.jasms.2004.02.007 · Source: PubMed

Toward the Prediction of the Activity of Antioxidants: Experimental and Theoretical Study of the Gas-Phase Acidities of Flavonoids

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The relative gas-phase acidities were determined for eight flavonoids, applying the kinetic method, by means of electrospray-ion trap mass spectrometry. The experimental acidity order, myricetin > luteolin > quercetin > (±)-taxifolin > kaempferol > apigenin > (+)-catechin > (±)-naringenin shows good agreement with the order obtained by theoretical calculations at the B3LYP/6-311 + G(2d,2p)//HF/6-31G(d) level. Moreover, these calculations provide the gas-phase acidities of the different OH groups for each flavonoid. The calculated acidity values ($\Delta_{ac}H$), corresponding to the most favorable deprotonation, cover a narrow range, 314.8–330.1 kcal/mol, but the experimental method is sensitive enough to differentiate the acidity of the various flavonoids. For all the flavones and the flavanol, catechin, the 4′-hydroxyl group is the most favored deprotonation site whereas for the flavanones studied, taxifolin and naringenin, the most acidic site is the 7-hydroxyl group. On the other hand, the 5-hydroxyl, in flavones and naringenin, and the 3-hydroxyl, in taxifolin and catechin, are always the less acidic positions. The acidity pattern observed for this family of compounds mainly depends on the following structural features: The *ortho*-catechol group, the 2,3 double bond and the 4-keto group. (J Am Soc Mass Spectrom 2004, 15, 848−861) © 2004 American Society for Mass Spectrometry

Flavonoids are polyphenolic compounds, found as natural products, in fruits, in plant extracts and in beverages, which originate from plants, such as teas and wines. They are currently used as therapeutic agents due to their antioxidant capacity, which protects the organisms against oxidative stress. This antioxidant capacity has been related to their ability to function as free radical acceptors [1] as well as to their ability to complex transition metal ions, which inhibits the role of transition metals in Fenton Chemistry [2, 3], where the hydroxyl radical is produced from hydrogen peroxide in presence of a metal by a redox process:

$$H_2O_2 + M^{n+} \rightarrow OH^- + HO^{\cdot} + M^{(n+1)+} \quad (1)$$

Previous studies [4] on flavonoids carried out by means of electrospray ionization mass spectrometry (ESI-MS) proved that the technique has the potential to transfer weakly bound noncovalent complexes from solution and allowed studies at biological concentration levels [5].

In both antioxidant mechanisms the loss of a proton may play an important role, emphasizing the importance of the acidity of these compounds. The antioxidant mechanism, which operates through the chelation of transition metal ions, occurs quite often with at least one deprotonated ligand [6].

On the other hand, in the radical scavenging mechanism, the oxygen reactive species are rendered inactive by accepting a hydrogen atom from a hydroxyl group of the flavonoid. This loss can be visualized as the simultaneous release of a proton and of an electron [7, 8]. The weaker the O–H bond dissociation energy the higher will be the antioxidant activity of the flavonoid. It has been shown [9] for substituted phenols, that O–H bond dissociation enthalpies, in the gas phase and solution are often as close as 1.2 kcal/mol. Bearing this in mind and also that the compounds under study are structurally similar and the acidities to measure are relative acidities, it is hoped that the acidity pattern here obtained might be transferred to solution studies, where antioxidant activity has been measured.

Published online April 9, 2004

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1044-0305/04/$30.00
doi:10.1016/j.jams.2004.02.007

Received November 14, 2003
Revised February 9, 2004
Accepted February 29, 2004

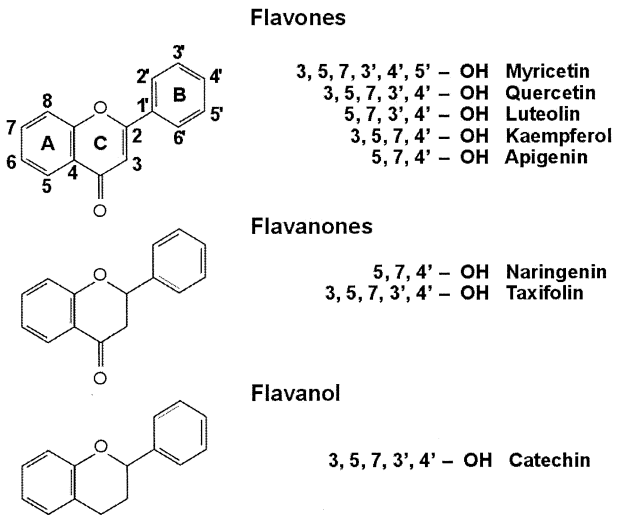


Figure 1. Structures of the flavonoids studied.

The O–H bond dissociation energy can be evaluated in the gas phase through the quantification of the gas-phase acidity of the flavonoid and the electron affinity (E_{ea}) of the respective radical since the ionization energy (E_i) of hydrogen is well established:

$$D(F\text{-}H) = \Delta_{ac}H(F\text{-}H) + E_{ea}(F^{\cdot}) - E_i(H) \qquad (2)$$

The gas-phase acidity (or acidity) of FH, ($\Delta_{ac}G$), is the Gibbs energy change of the reaction and its enthalpy change, $\Delta_{ac}H$, is the proton affinity of the anion F^-. Both are defined at 298 K, and usually referred as acidity of the neutral [10].

$$FH \rightarrow F^- + H^+ \qquad \Delta H_{298} = \Delta_{ac}H \qquad (3)$$

($\Delta_{ac}H$ of reaction is used, in this work, as the measure of acidity).

As far as specific molecular properties are concerned, it is clear that acidity is one of the fundamental factors involved. Therefore, in this study, we decided to investigate the acidity of some common flavonoids with recognized antioxidant capacity [11], in order to correlate this property with their structural/reactivity features. The structures of the flavonoids, whose acidities were measured, are shown in Figure 1.

The experimental method chosen was the well known Cooks Kinetic Method [12]. This is a widely applied method in gas-phase thermochemical measurements. When the samples are impure or non-volatile, which is the case here, this thermokinetic method is a suitable alternative to equilibrium methods for thermochemical determinations. According to this method, the relative acidities of flavonoids (F_1H and F_2H) can be obtained by comparing the dissociation rates of a proton bound heterodimer, of the type $F_1HF_2^-$, into each of the individual monomers F_1^-, F_2^-:

$$F_1HF_2^- \begin{array}{ll} \nearrow^{k_1} & F_2H + F_1^- \\ \searrow_{k_2} & F_1H + F_2^- \end{array} \qquad (4)$$

Here k_1 and k_2 are the rate constants for the competitive dissociations of the cluster anion $F_1HF_2^-$ to produce F_1^- and F_2^-, respectively. The kinetic method is based on the assumptions of negligible reverse activation energies and negligible entropy differences for the competitive channels and also the non-occurrence of isomeric forms of the activated cluster anion. Assuming that these conditions are fulfilled, the ratio in eq 5 lead to the difference in acidities, $\Delta(\Delta_{ac}H)$, of the two acids, where $I(F_1^-)$ and $I(F_2^-)$ are the abundances of both anions and T_{eff} the effective temperature of the proton bound dimer ion.

$$\ln (k_1/k_2) = \ln [I(F_1^-)/I(F_2^-)]$$
$$= [\Delta_{ac}H(F_1H) - \Delta_{ac}H(F_2H)]/RT_{eff} \qquad (5)$$

T_{eff}, the effective temperature of the system, is not known. However, since all compounds are structurally identical, T_{eff} should be similar to all of them. Nevertheless, if the unknown species, F_2H, were combined with a series of F_1H of known acidity, measuring various pairs of dimers, a relative acidity difference can be calculated. Eq 5 can be rewriten as:

$$\ln [I(F_1^-)/I(F_2^-)] = m\Delta_{ac}H(F_1H) - b \qquad (6)$$

where $m = 1/RT_{eff}$, $b = \Delta_{ac}H(F_2H)/RT_{eff}$, from which $\Delta_{ac}H(F_2H)$ can be determined. Thermochemical data obtained by the kinetic method are, in general, in good agreement with values measured by other methods, such as equilibrium and bracketing [13]. However, disagreement has been observed [14], in particular, for heterodimers resulting from structurally different molecules as in those cases assumptions of entropy effects cancelling and/or reverse activation energies being negligible, are unlikely to hold. For this reason the use of reference compounds other than flavonoids was ruled out. Nevertheless, the possibility of entropic contributions in pairs involving a flavone and a flavanol, or a flavone and a flavanone, is addressed.

Bearing in mind these limitations and taking into account that acidities have not been measured for any flavonoids, this experimental study only yields a qualitative order of relative acidities of some flavonoids. In support of this work, electronic structure calculations were performed. In the past, a number of theoretical studies have focused on the antioxidant properties of flavonoids, most of which have simply been QSAR (Quantitative Structure Activity Relationship) studies that attempt to relate their antioxidant activity with several molecular properties [15]. Many of such studies

have been restricted to molecular mechanics modeling techniques or semiempirical molecular orbital calculations. More recently, however, ab initio and density functional theory level calculations have also been published (see for example references [16–24]). To our knowledge, no systematic theoretical study seems to have yet been reported for the molecular or electronic properties of this set of flavonoids.

Experimental Methods

The flavonoids studied, myricetin, quercetin, luteolin, (±)-taxifolin, kaempferol, (+)-catechin, and (±)-naringenin, were purchased from Sigma (Schnelldorf, Germany), with purities ≥95%. The HPLC grade methanol came from Merck (Madrid, Spain). All chemicals were used without further purification.

The flavonoids were dissolved in HPLC grade methanol. Each flavonoid stock solution was diluted 1:10 with a 1:1 methanol/water mixture. 1:1 solutions of two flavonoids (F_1 and F_2) were prepared from the aforementioned diluted solutions. The concentration of the final mixture solutions in each flavonoid ranged from 17 to 207 μM and the pH was 6, since the flavonoids are slightly acidic.

All the experiments were performed on a Thermoquest LCQ Duo quadrupole ion trap mass spectrometer equipped with an electrospray interface, in negative ion mode. All voltages, such as capillary voltage, lenses and octapole voltages, were optimized for maximum abundance of the dimer under study. No significant differences in the various voltages were necessary in order to optimize the different dimers. The flow rate of the electrospray solutions, with two flavonoids in a ratio 1:1, was 5 μL/min. The capillary temperature was kept at 220 °C. The pressure measured, during the experiments at the skimmer cone with the convectron gauge was typically 0.98 torr. In the ion trap with helium present, the pressure was usually 1.27×10^{-5} torr.

According to Gronet [25], it was assumed that ions in the quadrupole ion trap are slightly above the temperature of the bath gas, typically at 310 ± 20 K.

The full scan spectra were obtained through injection times of 30 ms and an average of at least 10 microscans.

Before collision induced dissociation (CID) experiments, heterodimers under study were isolated. The ion isolation waveform voltage is calculated by the LCQ Duo and automatically applied (for the events sequence, see e.g., [26]), allowing ejection of all but the ion of interest.

All CID spectra were obtained at relatively low values of resonant excitation amplitude. These values were in the range 0.70–0.95 $V_{p\text{-}p}$. For these spectra the minimum ion injection times yielding sufficient dimer abundance, were 200 ms. Each measurement was repeated 9–30 times.

Theoretical Methods

Computational Details

To gain insight into the observed behavior of each phenolic acid, a series of ab initio and density functional theory calculations were performed to find out both the structure and the stability of the neutral phenols and the corresponding anions. The optimal structures of the species were determined by using the ab initio HF/6-31G(d) method. Vibrational frequencies were computed also with this method and then scaled by a factor of 0.9135 [27] to obtain the (scaled) zero-point energies (ZPE) and vibrational contributions. Single-point energy calculations were then carried out at the HF optimal structures, using the B3LYP density functional [28] and the 6-31G(d) basis set. Orbitals from this step were used as input to a final B3LYP/6-311 + G(2d,2p) single-point energy calculation. The total energies of the species at 298 K are then the thermal corrections to the energy (including the translational and rotational corrections) from the HF step plus the B3LYP electronic energy of the final step; these energies can be labeled in standard notation as B3LYP/6-311 + G(2d,2p)//HF/6-31G(d) [or B3LYP/6-31G(d)//HF/6-31G(d)].

For a given phenolic compound FH, the acidity in the gas-phase ($\Delta_{ac}H$) is equated to the enthalpy of the deprotonation reaction (eq 3). This enthalpy, in turn, is estimated according to:

$$\Delta_{ac}H = \Delta E^{0}_{elec} + \Delta ZPE + \Delta E^{298}_{vib} + 5/2RT \qquad (7)$$

where ΔE^{0}_{elec}, ΔZPE, and ΔE^{298}_{vib} stand for the differences between the electronic energies at 0 K, the ZPE and the thermal vibrational corrections, respectively, of FH and F^-. The last term contains the PV work term and the differences between the (classical) translational and rotational energy contributions of FH, F^-, and H^+.

The present methodology was chosen as a compromise between accuracy and computation time as, hopefully, it would provide reliable results for the family of related phenolic compounds considered here. Note that this methodology closely resembles the LLM1 prescription proposed by DiLabio et al. for obtaining gas-phase acidities [29]. All theoretical calculations were performed with the GAUSSIAN 98 program [30].

Results and Discussion

Mass Spectrometry Study

Under the experimental conditions, described above, full mass spectra of the mixtures of two flavonoids were obtained. An example is given in Figure 2, where the spectrum shown, was obtained from a mixture of naringenin (MW = 272) and catechin (MW = 290). In this spectrum there are two groups of relevant peaks. The first group includes peaks at *m/z* 271, 289, 307, and 325. The first two peaks result from the ions derived from deprotonation of naringenin and catechin, the

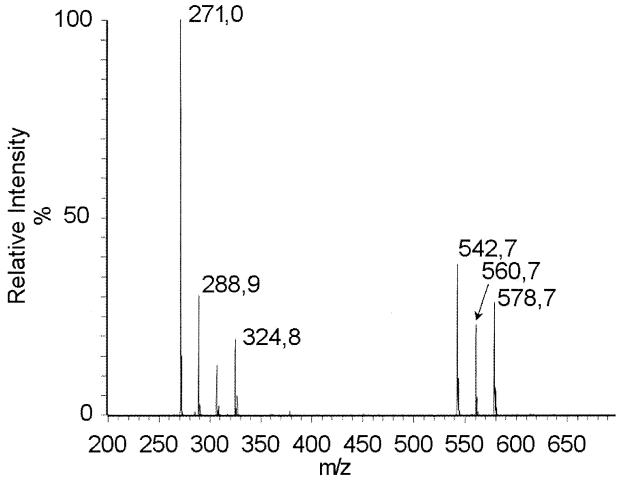


Figure 2. ESI mass spectrum of a mixture of naringenin and catechin.

third and fourth are water adduct ions of the deprotonated catechin (m/z = 289). The second group of peaks is related with the following ions: the proton bound heterodimer anion at m/z 561, the dimer anion of catechin at m/z 579, and the dimer anion of naringenin at m/z 543. The proton bound heterodimer anion abundance is considerable, being ca. 25% of the base peak. The abundances of this heterodimer were always in the range of 23–100% of the base peak.

A doubt may remain about the fact that some m/z 289 might be the water adduct of m/z 271. However, this would not affect the results because all unwanted species (m/z: 271, 289, 307, and 325) were ejected prior to CID study. Moreover, in Figure 2 no water adduct is observed for 579 (highest mass dimer). Thus, all 561 should be the desired heterodimer which was isolated for CID within a mass interval of 561 ± 1 Da and the CID spectra obtained as documented in Figure 3. In this figure, it can be seen that the competitive dissociations of the heterodimer anion, according with both reactions

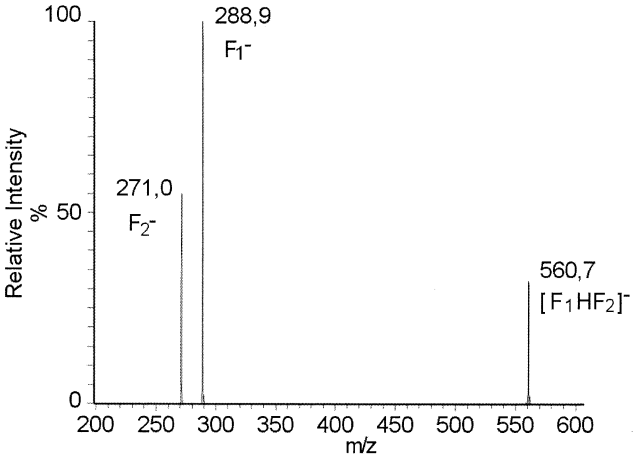


Figure 3. CID spectrum of the proton bound heterodimer $[F_1HF_2]^-$ ion (m/z 561) from a mixture of naringenin and catechin.

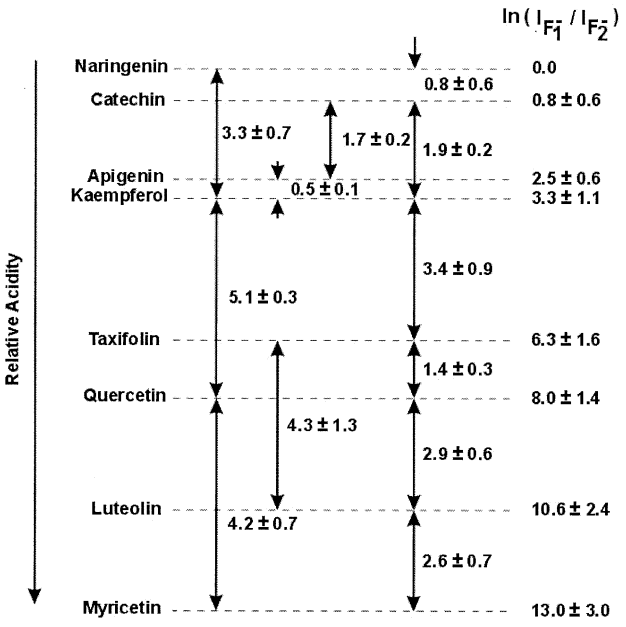


Figure 4. Ladder of values of ln $[I(F_1^-)/I(F_2^-)]$ measured for proton bound heterodimer anions of flavonoids referred to naringenin (under ln $[I(F_1^-)/I\ (F_2^-)]$ heading) and experimental ln $[I(F_1^-)/I(F_2^-)]$ for each pair of flavonoids experimentally equilibrated (near the arrows).

in eq 4, are the only decomposition channels and as no water loss is observed. As a consequence the raised possibility of m/z 289 to be water adduct of m/z 271 was completely disregarded. This observation points to two flavonoid molecular anions weakly bound by a proton, which is one of the assumptions needed for the kinetic method to hold.

In Figure 4 a relative energy level diagram is presented with the measured $[\Delta_{ac}H(F_1H) - \Delta_{ac}H(F_2H)]/RT_{eff}$ difference, for each pair of flavonoids, at 0.75 $V_{p\text{-}p}$. The pairs of flavonoids associated by arrows are those whose acidities were compared through the decomposition of a given heterodimer. Qualitative relative acidity data were derived from the measurements of the ln $[I(F_1^-)/I(F_2^-)]$. Under the heading ln $[I(F_1^-)/I(F_2^-)]$ in Figure 4 is shown an average of these values referenced to naringenin, which, having the lowest relative acidity, was considered to have a zero value for ln $[I(F_1^-)/I(F_2^-)]$.

Some heterodimers were built from different classes of flavonoids; the two flavonoids involved having structural differences. At first it was intended to use only flavones. However, for kaempferol, it was impossible to find suitable pairing among the available flavones. It could not be paired with luteolin because they are isobaric; on the other hand, the ratio involving kaempferol and quercetin is already on the experimental limit to make accurate measurements. Thus catechin, naringenin, and taxifolin were also included. Therefore, for all systems, measurements were made under more than one set of activating conditions in order to test the validity of the method [31]. The variation of the excitation amplitude was in the range 0.70–0.95 $V_{p\text{-}p}$. This

Table 1. Experimental and theoretical relative acidity values for the flavonoids (all values in kcal/mol)

Flavonoid	$\Delta(\Delta_{AC}H)_{exp}$	$\Delta(\Delta_{AC}H)_{theor}$[a]
Naringenin	0.0	0.0
Catechin	0.7 ± 0.6	2.5
Apigenin	2.0 ± 1.1	5.7
Kaempferol	2.6 ± 1.6	3.0
Taxifolin	5.0 ± 2.8	6.0
Quercetin	6.4 ± 3.4	10.7
Luteolin	8.4 ± 4.6	13.0
Myricetin	10.4 ± 5.7	15.3

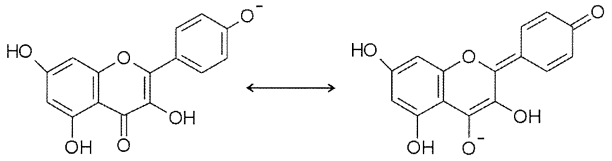
[a] $\Delta(\Delta_{ac}H)_{theor}$ derived from the $\Delta_{ac}H$ values obtained at 298 K using the method B3LYP/6-311 + G(2d,2p)//HF/6-31G(d) (see Table 4).

interval was chosen in such a way that the lower limit corresponds to the minimum ratio of ion intensities accurately measurable and the upper limit to the maximum value of $V_{p\text{-}p}$ only leading to the ions of interest. As a result of these experiments, no variation was observed in the internal consistency of the data; therefore entropic contributions seem to be negligible. However a doubt may still remain about possible entropic contributions that may cancel in certain pairs. This could be the case for taxifolin, a flavanone, when is paired with two flavones, kaempferol, and quercetin. More work will be necessary, in future, with the extended Cooks method [32, 33] in order to clarify whether entropic effects are measurable.

In the absence of calibrants, as in the present case, a T_{eff} has to be chosen to calculate $\Delta(\Delta_{ac}H)$. The effective temperature, T_{eff}, does not correspond to a thermodynamic temperature. It is dependent on the mean internal energy of the fragmenting cluster ion population as well as on the instrumental conditions [34, 35]. In the case of trapping mass spectrometry where the time window of analysis is of the order of milliseconds, the T_{eff} will be lower than in sector instruments where the time window of analysis is of the order of microseconds. In previous experimental studies carried out in ion traps involving different families of compounds, T_{eff} seems to be in a typical range of 300–400 K [36, 37], under CID conditions. The upper limit for T_{eff} in the work of Afonso et al. is 390 K. However our excitation amplitude values were slightly higher and therefore we decided to adopt a value of 400 K. Nevertheless, bearing in mind Armentrout's recommendation [35] to include an error of 50%, precisely in cases where no adequate calibration standards are used, this T_{eff} will be considered as 400 ± 200 K. Using this T_{eff} and the average cumulative values of $\ln [I(F_1^-)/I(F_2^-)]$ listed in Figure 4, the $\Delta(\Delta_{ac}H)$ for each compound relative to naringenin was calculated through eq 5 and is presented in Table 1.

In order to check the validity of the experimental $\Delta(\Delta_{ac}H)$, these are compared in Table 1 with the theoretical $\Delta(\Delta_{ac}H)$ results and, as it can be seen, all of them agree within the experimental error except apigenin.

In the gas phase, since the solvent is absent, the most important factors that determine the acidity of phenolic compounds are the strength of the O–H bond, the electronegativity of the phenoxyl radical and the factors that may stabilize the phenoxide anion relative to phenol. Phenols are well known stronger acids than aliphatic alcohols but considerably weaker than carboxylic acids [38]. In fact, phenols show the possibility of relative stabilization of the anion by delocalization of its negative charge through interaction with the π orbitals of the aromatic ring.



Scheme 1. Resonant structures for phenoxide ion formed by loss of a proton from OH in Position 4′.

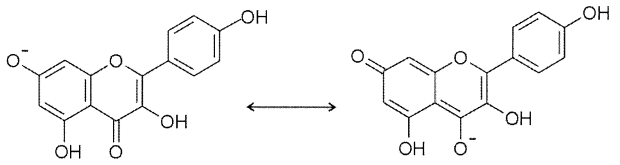
The main differences in acidity found in this experimental study are related with to the structural differences of the three groups studied: Flavones, flavanones, and flavanol.

In Figure 4 it can be seen that flavones are in general, the more acidic flavonoids, ranging from the most acidic myricetin to the less acidic apigenin/kaempferol.

In flavones, the phenoxide anions resulting from the loss of a proton either from the hydroxyl groups in Positions 4′ or 7 (present in all the flavones studied) may be the most favored as they lead to two different paraquinoid structures which are very much stabilized by delocalization. These structures are shown in Schemes **1** and **2**.

The first resonant structure, when an *ortho*-catechol group is present in the B ring, may allow the formation of intramolecular hydrogen bonds conferring to the resulting phenoxide ion an extra stabilization. In fact, the flavones possessing an *ortho*-catechol group (myricetin, quercetin, and luteolin) are more acidic than apigenin and kaempferol, which in the B-ring only have a 4′ hydroxyl group. In the most acidic trio, the number of hydroxyl groups in the B-ring ranges from two in luteolin/quercetin to three in myricetin. It can be observed that the larger the number of adjacent hydroxyl groups in the B-ring the higher the acidity. This can be rationalized in terms of the increase in stability obtained through the establishment of one intramolecular hydrogen bond for luteolin/quercetin and two intramolecular hydrogen bonds for myricetin (Scheme **3**).

The flavanones, taxifolin, and naringenin do not possess the 2,3 double bond in the C-ring, differently from the flavones. Consequently, the structure shown in Scheme **2**, where delocalization will occur involving the A- and C-ring, but not including the 2,3 double bond, must be the favored paraquinoid structure for flavanones. Therefore, for this family of flavonoids, their phenoxide ions may assume a structure corresponding to the loss of a proton from the hydroxyl group in Position 7. In the taxifolin anion, the delocal-



Scheme 2. Resonant structures for phenoxide ion formed by loss of a proton from OH in Position 7.

ized structure bearing the negative charge in Position 4, may establish an internal hydrogen bond with the 3-hydroxyl group. This may explain the difference in acidity between taxifolin and naringenin.

For the phenoxide ion of catechin, the studied flavanol, none of the above mentioned paraquinoid structures can occur, due to the lack of both the 2,3 double bond and the 4-keto group. Nevertheless, the anion may be stabilized by intramolecular hydrogen bonding in the *ortho*-catechol group, if the proton is released from any of the hydroxyl groups in the B-ring. The structural differences of catechin relative to the other two families are probably enough to explain its observed relative acidity.

The 2,3 double bond, the *ortho*-catechol group and the 4-keto group seem to be the most important structural features determining the acidity of the flavonoids. A more sound correlation was sought in next the section through molecular orbital calculations.

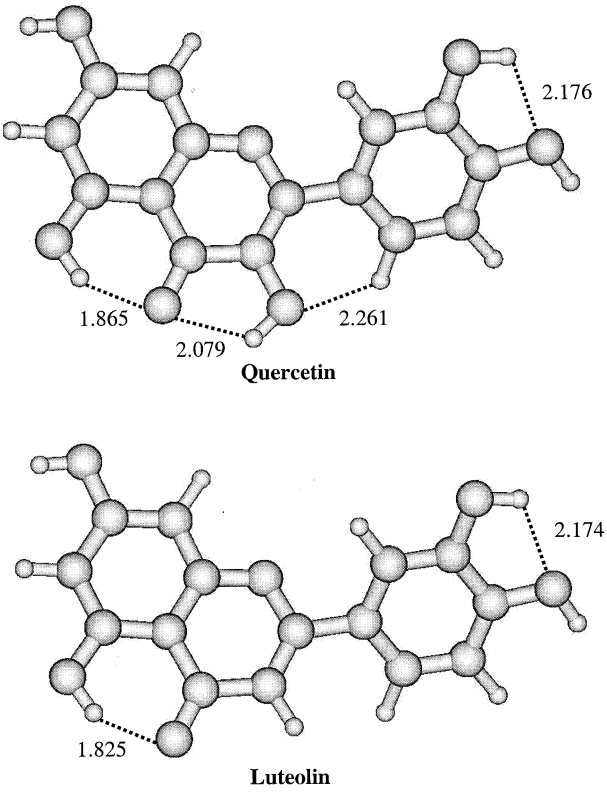
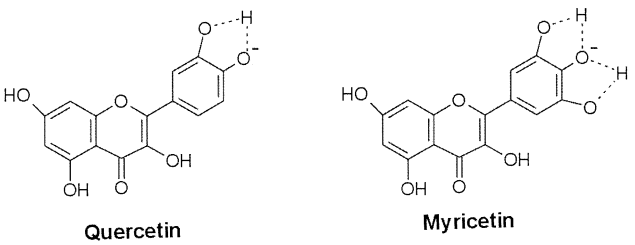


Figure 5. Optimized geometries for the flavones, quercetin, and luteolin.

Electronic Structure Calculations

Structures

Flavones. The question whether the flavones do or do not adopt a planar conformation has been addressed before in a couple of theoretical studies. Van Acker and coworkers [15] studied a variety of flavonoid molecules by the ab initio HF/STO-3G method. They argued that all flavones bearing a 3-OH group on Ring C should be planar, due to the interaction between the oxygen atom of this group and the hydrogen attached to the C2′ or C6′ position which forces Ring B to become coplanar with rings A and C (see Figure 1). However, other theoretical works based on the ab initio HF/6-31G(d) method [19] and on the semiempirical AM1 model [39, 40] found a nonplanar minimum conformation for quercetin (or a complex of myricetin [40]), though with a small barrier for the rotation between rings B and C (AM1: <3 kcal/mol; HF: <4kcal/mol). In addition, Meyer has shown that the simple 3-hydroxyflavone is a nonplanar species in a study using the same HF/6-31G(d) method [17].



Scheme 3. Proposed structures for the phenoxide ions of quercetin and myricetin.

The present HF/6-31G(d) calculations led also to nonplanar minimum energy structures for all neutral flavones that contain the 3-OH group; still, optimal planar conformations are obtained for the respective anions. In the case of the flavones luteolin and apigenin, which lack the 3-OH group, the HF optimizations pointed to a nonplanar structure not only for the neutral species but also for the 5- and 7-anions. Our results for quercetin and myricetin essentially match the previous semiempirical [40, 41] and ab initio findings [20] though with lesser deviations from planarity than the semiempiricals. In addition, the optimized geometric parameters of quercetin are fairly close to the X-ray crystal structure reported by Jin et al. [42]; the main differences are the torsion angle between rings B and C (X-ray: $\tau_{3-2-1'-6} = 5°$; HF: $\tau_{3-2-1'-6} = 19°$) and the orientations of the hydrogens attached to the O3 and O4 atoms (see Figure 5), which are in opposite directions probably due to packing effects.

Since the preferred conformations of such benzenoid-type systems are determined by a balance between non-bonded interactions and electronic delocalization effects, a definitive answer to this question can only be attained by using higher-level quantum chemical meth-

Table 2. Calculated geometrical parameters for the neutral flavones and flavanones

Flavonoid	Method[b]	Parameter[a]			
		$r_{C=O}$	r_{C-C}/ring B[c]	r_{C-OH}[c]	$\tau_{1-2-1'2'}$
quercetin	HF/6-31G(d)	1.222	1.386	1.344	+18.8
	B3LYP/6-31G(d)	1.262	1.399	1.359	−0.12
	HF/STO-3G	1.242	1.500	1.391	−0.29
luteolin	HF/6-31G(d)	1.216	1.386	1.342	+26.5
	B3LYP/6-31G(d)	1.253	1.398	1.359	+18.1
	HF/STO-3G	1.240	1.505	1.390	+16.3
kaempferol	HF/6-31G(d)	1.222	1.388	1.340	−17.9
	B3LYP/6-31G(d)	1.262	1.399	1.356	+0.94
	HF/STO-3G	1.242	1.499	1.392	−0.14
apigenin	HF/6-31G(d)	1.216	1.387	1.336	+24.4
	B3LYP/6-31G(d)	1.253	1.399	1.354	+16.4
	HF/STO-3G	1.240	1.504	1.391	+16.5
(+)-taxifolin	HF/6-31G(d)	1.206	1.386	1.342	−42.8
	HF/STO-3G	1.232	1.545	1.392	−27.6
(−)-naringenin	HF/6-31G(d)	1.209	1.386	1.337	−42.8
	HF/STO-3G	1.232	1.545	1.393	−42.7

[a]Bond distances are in Å and angles in °.
[b]HF/6-31G(d) and B3LYP/6-31G(d): results from this work; HF/STO-3G; results from Ref. [16].
[c]Averaged bond distances.

ods. Additional full-optimizations (or single-point test calculations) were then carried out at the B3LYP/6-31G(d) level for all the flavone species.

According to the B3LYP calculations, the neutral and all anionic species of the flavones myricetin, quercetin, and kaempferol are predicted to be planar. On the other hand, in close agreement with the HF predictions, the neutral luteolin and the neutral apigenin as well as their 5- and 7-anions are found to be nonplanar. Overall, B3LYP and HF structures closely resemble each other concerning the trends in bond lengths, valence angles and the orientations of the OH groups. It should be noted, however, that the B3LYP computed bond lengths are in general larger than the HF ones (except for the C2–C1′ bond which is slightly smaller). But the largest deviations between the geometries predicted by the two methods are the torsional angles related to the rotation about Rings C and B. Nevertheless these geometric differences do not affect the following B3LYP single-point energy calculations much. (As checked, differences between the HF and B·LYP absolute energies are constant and ~4–5 kcal/mol for such compounds; see also Table 2).

Above all, the B3LYP results corroborate the hypothesis of van Acker et al. [16] regarding the planarity of the flavones and the key role of the 3-OH moiety. They also suggest that the HF/6-31G(d) method underestimates the stabilizing π-delocalization contributions, at least for the 3-hydroxy flavones. They suggest too that the semiempirical methods underestimate far greater π-delocalization contributions (especially the PM3 model), since we have confirmed that the AM1 (or PM3) geometry optimizations always give the B-ring non coplanar with the chromane moiety (C- and A-rings). Here, it is worth noting that Toth and coworkers [43] have shown before the particular unreliability of the PM3 model to describe the torsional angles and barrier heights of simple flavone. In addition, Meyer [17], who performed a detailed conformational study of simple flavone by comparing the results from methods such as HF/6-31G(d), MP2/6-311G(d,p)//HF/6-31G(d), or B3LYP/6-311G(d,p), concluded that the B3LYP DFT method tends to overestimate the stability of the planar-π flavonoid systems.

Taking into account the above considerations and the computational difficulties in performing, for the large number of complex systems under study, the optimization of the geometries plus the calculation of vibrational frequencies at a higher ab initio level, this study solely relies on the HF geometry optimizations.

Figure 5 shows, as an example, the optimized structures of the neutral flavones quercetin and luteolin obtained at the HF/6-31G(d) level. The most remarkable feature of the flavone's structures is the pattern of intramolecular hydrogen bonds. Hydrogen bonds are always found between the hydroxyl groups on the catechol moiety (B-ring) and between the 5-OH and the 4-keto groups, as well as between the 3-OH and 4-keto groups when applicable. The flavones bearing a 3-OH group present also a hydrogen bond-like interaction involving donation of the hydrogen at Position 6′ to the 3-hydroxyl oxygen. In addition, among all H-bonds, presumably the strongest one (i.e., the shortest one that has also the higher donor-hydrogen-acceptor angle) is the bond formed with the 5-hydroxyl group, which might explain why Site 5 is the less acidic for all these flavones.

Our results for the neutral flavones can be compared with the ones obtained in the former theoretical study by van Acker et al. [16] (Table 2). Such comparison shows that the HF/6-31G(d) method predicts shorter bond distances and larger deviations from planarity

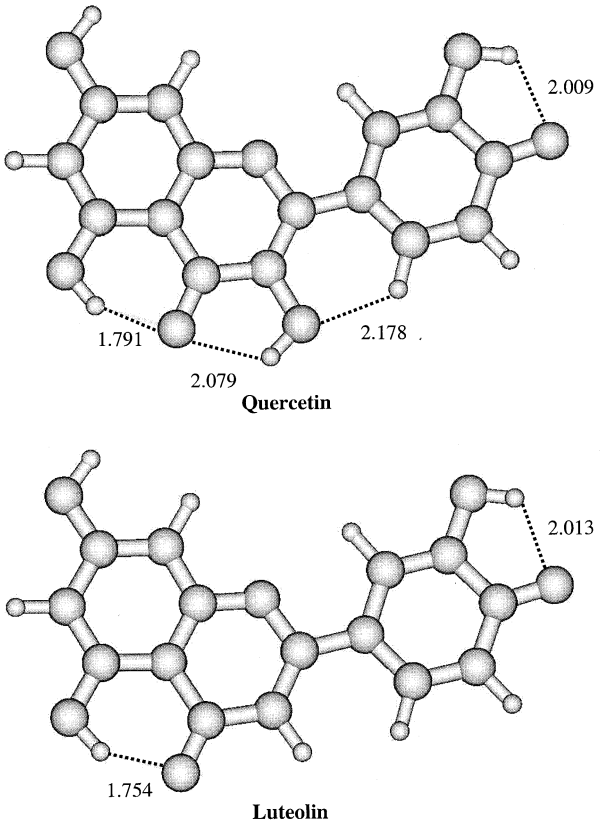


Figure 6. Structures of the 4′-phenoxide anions of the flavones, quercetin, and luteolin.

than the HF/STO-3G method. Nevertheless, when the HF/STO-3G geometrical parameters are compared with those from the B3LYP/6-31G(d) optimizations, one finds out that the former method tends to predict too large bond distances.

The optimal structures of the anionic species of the flavones are very similar to those of the parent neutral compounds. As an example, Figure 6 displays the optimized geometries of the 4′-phenoxide anions, which are the most stable anions for all the flavones. As can be seen, the pattern of hydrogen bonding is retained except for the hydrogen bonds of Ring B, which now rotate to the more stable conformation shown. Notice that in each case we have assumed that regardless of which proton is being lost in the parent, the anion is allowed to rearrange to the most stable conformer. In fact, we have checked that these OH rotations in the anions (e.g., rotations of the OH groups from Positions 5′ and 4′ for the 3′-anion of myricetin) can occur at room temperature as their barrier heights are low (B3LYP//HF calculations: <4 kcal/mol). Note also that the structures of the 4′-anions of luteolin and apigenin are completely planar, thereby suggesting that for these ions the gain in delocalization overcomes the steric hindrance of the hydrogens. It is also clear (see Figures 5 and 6) that the hydrogen bonds of the catechol moiety and that of the 5-OH group are shorter and presumably stronger in the 4′-anions than in the neutral flavones (or other anions), which therefore stabilize particularly these anions.

As expected, the 5-anions are the less stable species upon all the series of compounds, further indicating that the loss of the 5-OH hydrogen bond causes the highest energy destabilization to the parent flavones.

Flavanones. The flavanones used in the experiments correspond to a mixture of the two (+) and (−) isomeric forms, and for that reason both forms have been considered here. Figure 7 depicts the optimized geometries calculated at the HF/6-31G(d) level for the two isomers of taxifolin and naringenin. It should be pointed out that the (−) isomer of taxifolin is more stable than its (+) isomer, being indeed 6.0 kcal/mol lower in energy (B3LYP/6-311 + G(2d,2p) thermal corrected values). On the other hand, the two isomers of naringenin are almost isoenergetic, the (+) isomer being more stable by 0.13 kcal/mol.

In (+)-taxifolin, the B-ring adopts an equatorial position with respect to the chromane moiety, whereas in (−)-taxifolin it adopts an axial position. However, both isomers present an identical pattern of hydrogen bonding, which in turn is similar to that observed for the flavones, except for the 3-OH group in (+)-taxifolin that has an H-bond interaction with the hetero oxygen in Ring C. Figure 7 also shows that, excluding the orientation of the B ring, the structures of the two isomers of naringenin are not all that different and contain the usual hydrogen bond between the 5-OH and 4-keto groups. It should be referred that the structural aspects of the neutral (+)-taxifolin and (−)-naringenin have already been investigated in two previous theoretical studies [16, 44]. Although the authors did not provide enough geometric parameters to enable a full comparison, one can see that the reported optimized geometries look similar to the present ones (see also Table 2).

The anionic species of the flavanones, unlike the flavone anions, can adopt conformations rather different from those of the parent neutral compounds owing to their greater conformational flexibility. In fact, although the basic structural features of the rings are preserved, the torsional angle of Ring B varies significantly in some anions. Also, in contrast to the flavones, the most stable anion of the flavanones is that corresponding to the loss of the proton from Position 7. Here the higher stability of the 7-anion might result from a reinforcement of the hydrogen bonds with respect to the parent, which is not so strong in the 4′-anion. This can be confirmed by comparing, for instance, the structures of the 7- and 4′-anions of the more stable isomers of taxifolin and naringenin shown in Figure 8 with those of the corresponding parent compounds (Figure 7). Regarding the less stable anions, for naringenin they correspond naturally to the 5-deprotonated species but for taxifolin they result from deprotonation at Position 3. The lower stability of the 3-phenoxide anions of taxifolin is probably determined by an increase of the

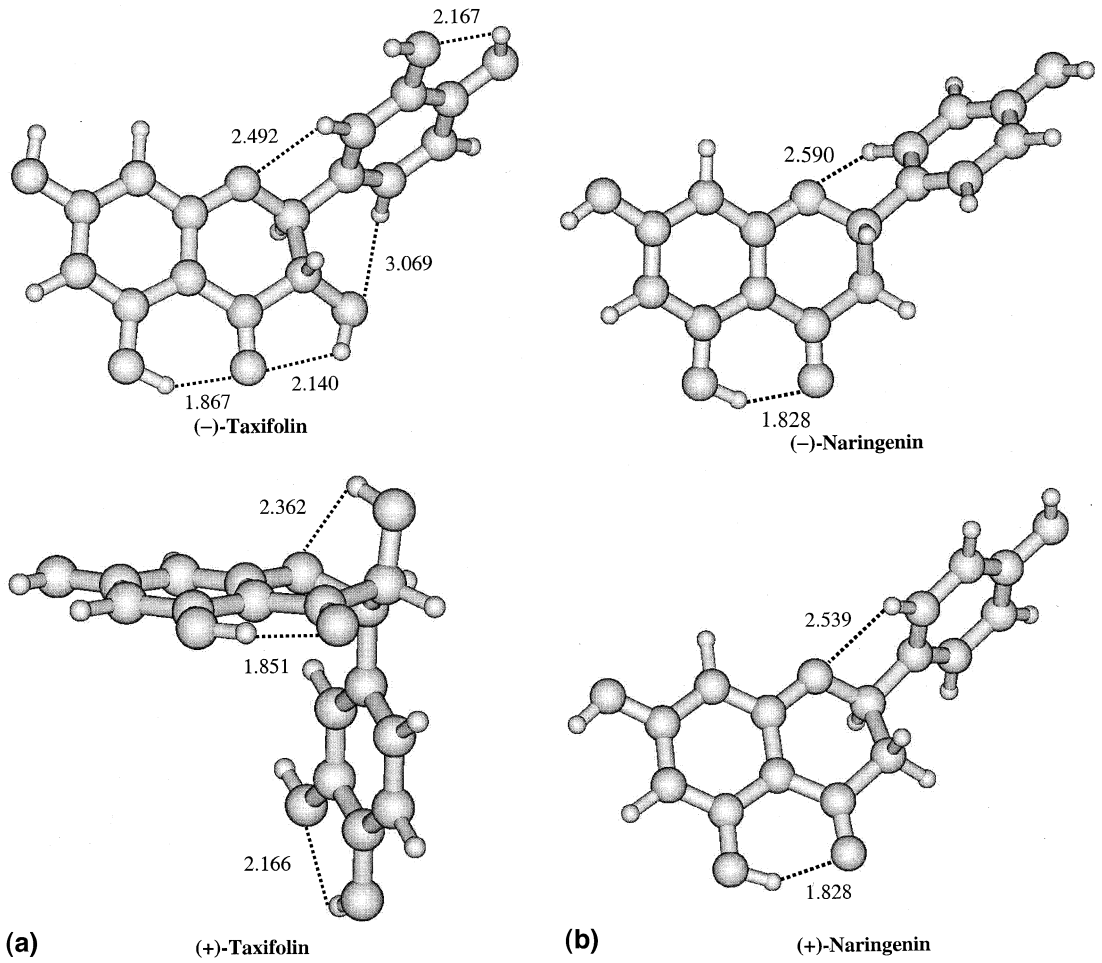


Figure 7. Optimized geometries for the (+) and (−) isomers of taxifolin and naringenin.

non-bonding repulsive interactions in relation to the parent.

Flavanol. Very recently Cren-Olivé et al. [22] determined the gas-phase geometries and corresponding electronic energies of (+)-catechin and its 3′, 4′, 5, and 7-anions by performing full-optimizations at the B3LYP/6-31G(d) level. The authors found that the most stable conformation of both the neutral and anionic species was a staggered conformation in which the B-ring adopts an equatorial position in relation to the chromane moiety. However, apart from this equatorial conformation, our HF geometry search led to another possible minimum for (+)-catechin and its anions, i.e., a conformation in which the B-ring assumes instead an axial position. It is noteworthy that all optimizations that started from one particular conformation never ended up in the other conformation. Even so, conversion between both conformations in the gas-phase may well take place at room temperature, as it requires little energy (<3 kcal/mol)[45]. The authors performed a thorough conformational study of (+)-catechin using the semiempirical AM1 model.

For all the flavanol species, the axial conformations were found to have lower energies than their equatorial counterparts at the B3LYP/6-311 + G(2d,2p)//HF/6-31G(d) level (or at the B3LYP/6-31G(d)//HF/6-31G(d) level), and were therefore employed in the following calculations. Nevertheless, the energies obtained here for the equatorial conformations are compared in Table 3 with those determined by Cren-Olivé et al. [22]. As can be seen, the single-point B3LYP calculations slightly overestimate the absolute energies with respect to the full-B3LYP method but provide the same order of stability. The relative energies show however much lesser deviations, which lends further support to the present B3LYP//HF approach.

Figure 9 shows the HF optimized axial conformations of catechin and its 4′- and 3-anions which are, respectively, the more and less stable anions of this flavanol. Worth mentioning is the great conformational flexibility of these compounds and the geometry changes induced by the break of the O–H bonds in the parent. The stability of the flavanol anions seems to be determined by the interplay between non-bonding repulsions and H-bonding, similarly to the flavanones.

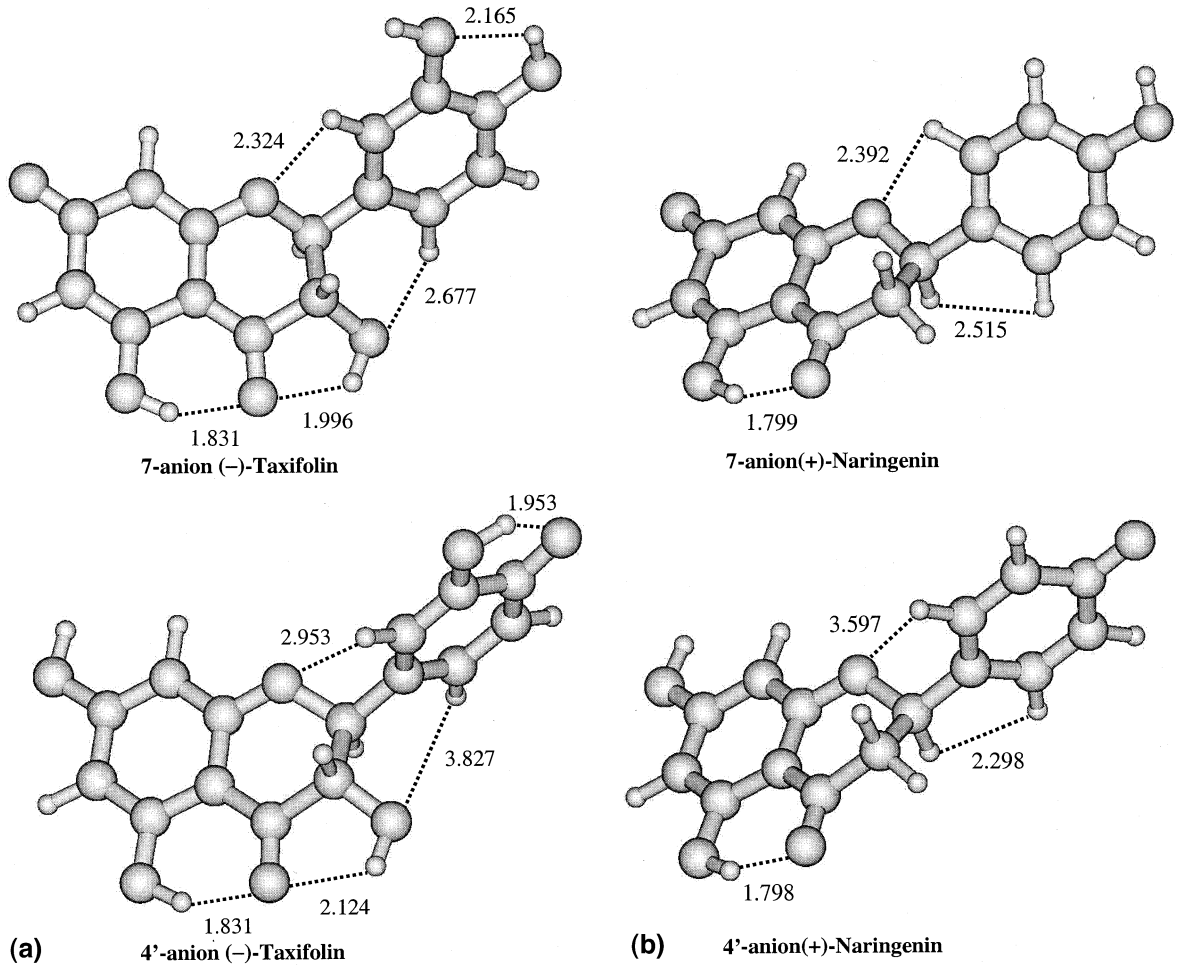


Figure 8. Comparison between the structures of the 4′- and 7-phenoxide anions of (−)-naringenin and (−)-taxifolin.

Gas-Phase Acidities

Table 4 gives the gas-phase absolute and relative acidities for the studied flavonoids, derived from the quantum chemical calculations at the B3LYP/6-311 + G(2d,2p)//HF/6-31G(d) and B3LYP/6-31G(d)//HF/6-31G(d) levels. As can be seen, the calculated acidities are in the range of ca. 315–364 kcal/mol. The $\Delta_{ac}H$ values obtained using the 6-31G(d) basis set are slightly larger than the 6-311 + G(2d,2p) results, yet both methods predict similar relative acidities. In fact, inspection of Table 4 shows that the 6-31G(d) calculation of $\Delta(\Delta_{ac}H)$ generally agrees with the extended-basis set

Table 3. Energies calculated for the equatorial conformation of catechin and its anions

Compound	Method[a]	E^{elec} [b]	ΔE [c]
catechin	B3LYP/6-31G(d)//HF/6031G(d)	−1031.322824	0.0
	B3LYP/6-31G(d)	−1031.328852	0.0
4′-anion	B3LYP/6-31G(d)//HF/6031G(d)	−1030.769910	347.0
	B3LYP/6-31G(d)	−1030.776211	346.8
3′-anion	B3LYP/6-31G(d)//HF/6-31G(d)	−1030.769975	346.9
	B3LYP/6-31G(d)	−1030.776255	346.8
5-anion	B3LYP/6-31G(d)//HF/6-31G(d)	−1030.759569	353.4
	B3LYP/6-31G(d)	−1030.767532	352.2
7-anion	B3LYP/6-31G(d)//HF/6-31G(d)	−1030.754684	356.5
	B3LYP/6-31G(d)	−1030.762459	355.4

[a]B3LYP/6-31G(d)//HF/6-31G(d):B3LYP energies calculated on top of HF optimised geometries; results from this work. B3LYP/6-31G(d): full-B3LYP results from Ref. [23].
[b]Absolute electronic energies in hartrees.
[c]Energies (in kcal/mol) are relative to neutral catechin.

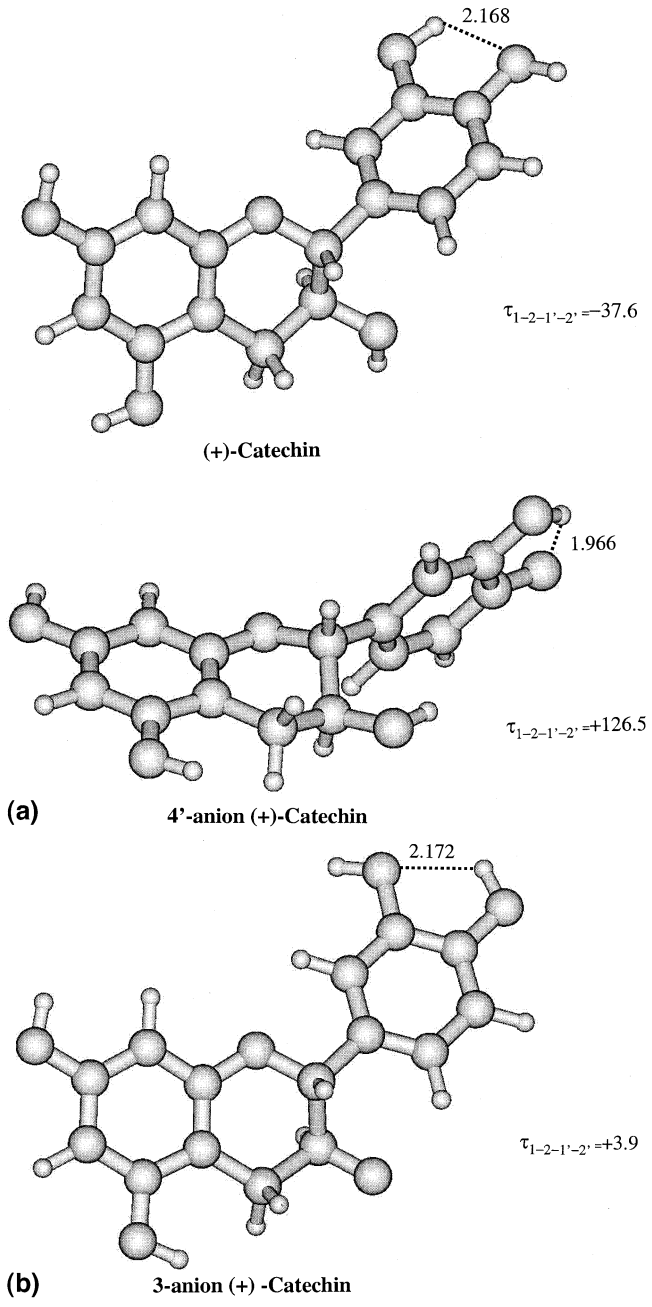


Figure 9. Optimized geometries for (+)-catechin and its 4′- and 3-phenoxide anions.

result within 4.6 kcal/mol or better, with a mean squared deviation of 1.1 kcal/mol.

The gas-phase acidities listed in Table 4 have been determined according to eq 7. An analysis of the different contributions to the calculated $\Delta_{ac}H$ demonstrates the importance of especially the ZPE corrections. These decrease the $\Delta_{ac}H$ values by about 8–10 kcal/mol and, in general, are higher for the species deprotonated at Positions 3 and 5. They are therefore essential for a correct prediction of the gas-phase acidities of these flavonoids.

Examination of Table 4 indicates that the 4′-hydroxyl group on Ring B is the most favored deprotonation site for all the flavones. The 4′-OH group is also the most acidic site of catechin, but the $\Delta_{ac}H$ values of this group and those of the 3′-OH differ by only a small amount (ca. 0.4–0.5 kcal/mol). In taxifolin and naringenin, however, the preferred deprotonation site is the 7-OH group on Ring A. On the other hand, the 5-OH (flavones and naringenin) and the 3-OH (taxifolin and catechin) are always the less acidic positions. Accordingly, one may establish the following order of acidity for the several sites. Flavones: 4′-OH (> 3′-OH ≈ 5′-OH) > 7-OH (> 3-OH) > 5-OH; flavanones: 7-OH > 4′-OH (≈ 3′-OH) > 5-OH (> 3-OH); flavanol: 4′-OH ≈ 3′-OH > 5-OH > 7-OH > 3-OH, where the parentheses depict those OH groups wich are not present in all flavonoids. Naturally, the more (less) acidic positions of the flavonoids are those that generate the most (least) stable anionic species. As noted before, the overall stability of the flavonoid species can be understood when their pattern of intramolecular interactions are considered, i.e., they tend to be H-bonded as much as possible and, for *ortho* functional groups, H-bonding occurs preferentially to a carbonyl group in the anions as opposed to a hydroxyl group in the parent neutrals. Non-bonding repulsions, π-delocalization effects, and conformational flexibility play also a key role in the stability of these species.

As far as available solution studies are concerned the acidity order of several hydroxyl groups is not dramatically different from the one in the gas-phase. The sequence set up in this study for the flavanones and flavanol agrees well with that measured in solution (naringenin: 7-OH > 4′-OH > 5-OH [44]; catechin: 3′-OH ≈ 4′-OH > 5-OH > 7-OH [22]). The former series, 7-OH > 4′-OH > 5-OH, applies also to the flavones in solution, that is to say, an inversion is found for the two first hydroxyls relatively to the gas-phase data. However, in condensed phase, only two flavones with a hydroxyl group in Position 4′ were studied (morin and apigenin) and the difference in acidity between the 7-OH and the 4′-OH was not quantified. Moreover naringenin was the only flavonoid whose solutions, either from NMR studies or electrospray ionization, were prepared in the same solvent mixture. One might also expect different conformations or/and stability for the neutral and anionic species in solution from those in the gas-phase, especially if the solvent(s) may establish hydrogen bonds.

By comparing the lowest $\Delta(\Delta_{ac}H)$ values in Table 4 (B3LYP/6-311 + G(2d,2p) results), the flavonoids can be ordered regarding to their acidity behavior, i.e.: myricetin > luteolin > quercetin > (−)-taxifolin ≅ (+)-taxifolin > apigenin > kaempferol > (+)-catechin > (+)-naringenin ≈ (−)-naringenin. This acidity order agrees remarkably well with the observed experimental order, except for the particular case of apigenin. Note that the calculated $\Delta_{ac}H$ values for these compounds span a narrow range (314.8–330.1 kcal/mol), especially those for the set taxifolin, kaempferol, apigenin, and

Table 4. Gas-phase absolute acidities ($\Delta_{ac}H$) and relative acidities [$\Delta(\Delta_{ac}H)$] calculated for the flavonoids (all values in kcal/mol)

		$\Delta_{ac}H$[a]		$\Delta(\Delta_{ac}H)$[b]	
Flavonoid	O^- position	6-31G(d)	6-311+G(2d,2p)	6-31G(d)	6-311 + G(2d, 2p)
Myricetin	3	340.8	334.8	19.4	20.0
	5	347.9	340.8	26.5	26.0
	7	333.8	327.3	12.4	12.5
	3′	334.3	326.5	12.8	11.7
	4′	*321.4*	*314.8*	0.0	0.0
	5′	335.0	326.9	13.6	12.1
Quercetin	3	342.3	337.0	17.3	17.6
	5	348.7	343.5	23.7	24.1
	7	332.8	328.2	7.8	8.8
	3′	334.2	326.6	9.2	7.2
	4′	*325.0*	*319.4*	0.0	0.0
Luteolin	5	354.8	347.4	32.5	30.4
	7	329.4	330.2	7.1	6.1
	3′	330.9	323.2	8.6	13.1
	4′	*322.3*	*317.1*	0.0	0.0
Kaempferol	3	343.3	338.0	9.8	10.9
	5	348.4	342.2	14.8	15.1
	7	333.7	328.1	0.2	1.0
	4′	*333.5*	*327.1*	0.0	0.0
Apigenin	5	357.0	349.6	26.5	25.1
	7	338.5	328.8	8.0	4.3
	4′	*330.5*	324.4	0.0	0.0

Taxifolin		(+)-form	(−)-form	(+)-form	(−)-form	(+)-form	(−)-form	(+)-form	(−)-form
	3	357.3	363.5	348.3	355.6	26.3	33.5	24.2	31.4
	5	349.7	346.5	341.6	340.0	18.7	16.5	17.5	15.8
	7	*331.0*	*330.0*	*324.1*	*324.1*	0.0	0.0	0.0	
	3′	332.7	338.4	329.7	331.4	1.7	8.4	5.6	7.3
	4′	334.4	336.4	328.0	330.0	3.4	6.4	3.8	5.9

Naringenin		(+)-form	(−)-form	(+)-form	(−)-form	(+)-form	(−)-form	(+)-form	(−)-form
	5	354.5	354.7	347.1	347.2	17.8	17.8	17.1	17.1
	7	*336.7*	*336.9*	*330.0*	*330.1*	0.0	0.0	0.0	0.0
	4′	343.8	344.5	336.8	337.6	7.1	7.6	6.9	7.5

(+)-Catechin				
3	363.1	354.5	29.6	26.9
5	345.8	338.6	12.3	11.0
7	348.6	341.5	15.1	13.9
3′	323.9	328.1	0.4	0.5
4′	*333.5*	*327.6*	0.0	0.0

[a] $\Delta_{ac}H$ estimated according to eq. (7) using the B3LYP method; ZPE and thermal corrections determined from HF/6-31G(d) frequency calculations and scaled by a factor of 0.9531. For each flavonoid, the lowest $\Delta_{ac}H$ is shown in italic.
[b] Difference between the $\Delta_{ac}H$ values relative in each case to the lowest $\Delta_{ac}H$.

catechin (3.5 kcal/mol), thus suggesting the importance of accurate predictions. Possible sources of errors come from the thermal vibrational corrections since they were determined within the harmonic oscillator approximation, which can be problematic due to the presence of low torsional modes in the vibrational spectra of these compounds. However, the contributions of the vibrational corrections to $\Delta_{ac}H$ are rather small (<1 kcal/mol) and the errors introduced in $\Delta(\Delta_{ac}H)$ can be expected to largely cancel out. Nevertheless, a more careful treatment of the vibrational contributions to the enthalpies $\Delta_{ac}H$ should not be disregarded without further investigations and might improve the agreement with experiments.

Conclusions

The compounds investigated vary in the number and type of acidic sites, number and orientation of the hydroxyl groups and degree of conformational flexibility. Semiquantitatively the acidity of flavonoids was evaluated experimentally, applying carefully the underlying assumptions of the kinetic method. An experimental order of relative gas-phase acidities was established. The electronic structure calculations provided an absolute gas-phase acidity order as well as acidities of the various hydroxyl groups in each flavonoid. Both orders agree pretty well. Nevertheless, the great conformational flexibility expected on the basis of the elec-

tronic structure calculations for the flavones, flavanones, and flavanol strongly recommend us to revisit experimentally these flavonoids by applying the extended kinetic method [32, 33], in order to evaluate the entropic contributions and quantify experimentally their acidities. In addition, the theoretical calculations, besides supporting the experimental trends, supply a sound correlation between the structure and acidity for the three groups of flavonoids studied. Should the 2,3 double bond and the 4-keto group be present, the proton is released from the 4′hydroxyl group. In the absence of the 2,3 double bond, two situations may occur: Either the proton is freed from 7 hydroxyl group if the 4-keto group is present, or it is freed from the 4′hydroxyl group in the absence of the 4-keto group. That is to say, for the flavones and the flavanol, the most acidic site is the 4′-hydroxyl group, whereas for the flavanones, the most favored deprotonation site is the 7-hydroxyl group. On the other hand, the *ortho*-catechol group was found to increase the stability of the flavonoid anions through the establishment of intramolecular hydrogen bonds. Structural features recognized as crucial in determining the antioxidant potential of flavonoids [47], such as the 2,3 double bond, the *ortho*-catechol group, and the 3-OH group, have been shown here to play also an important role in determining the acidity of flavonoids. Furthermore, the experimental and theoretical acidity orders reached for this group of flavonoids are, in general terms, comparable to the antioxidant activity order measured for the very same compounds on a DPPH assay [11] which measures the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging capacities of the flavonoids. Myricetin has the highest antioxidant ability, naringenin the lowest, and the others lie in between; though for the latter some inversions are found in relation to the measured acidity order. However, this is not surprising as the antioxidant capacity depends not only on the acidity of flavonoids but also on the electron affinity of the respective radicals. Most important, our theoretical results clarify the intrinsic reactivity of each ring for every case, thus providing new insights into the chemical processes that involve a proton transfer from the flavonoid to either an enzyme or another substrate.[46]

Acknowledgments

This work was supported in part by POCTI/1999/QUI/ 35406. A BIC grant from POCTI/1999/QUI/ 35406 is gratefully acknowledged by HFPM. MNDSC thanks also Fundação para a Ciência e Tecnologia (Lisbon) for a sabbatical grant (SHFR/BSAB/274/2002). MTF thanks Professor Carlos Borges for kindly allocating experimental time on the mass spectrometer. The authors are also grateful to Professor Christopher Maycock for helpful discussions and to Professor M. Lurdes Mira and Professor M. Helena Florêncio to introduce one of us (MTF) to the knowledge and importance of flavonoids.

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