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Correlations between high-performance liquid chromatographic retention, X-ray structural and ^{13}C NMR spectroscopic data of flavonoid compounds

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ABSTRACT

The retention behaviour of selected flavonoid compounds was analysed and correlated with structural properties, as elucidated by X-ray crystallographic and ^{13}C NMR spectroscopic studies. A detailed analysis of group retention contribution ($\Delta\log k'$) makes it possible to establish how the introduction of an OH group in the molecule affects the overall solute polarity and, therefore, its retention, in relation to the position of the OH group and the molecular environment. X-ray structural information and ^{13}C NMR chemical shifts show the formation of strong intramolecular hydrogen bonding (HB) in 5-hydroxy-flavones and a weaker intramolecular HB in 3-hydroxy-substituted compounds. $\Delta\log k'$ values may be explained in terms of these intramolecular HB properties; moreover a quantitative relationship between $\Delta\log k'$ values and chemical shifts was found.

INTRODUCTION

The flavonoids are one of the most diverse and widespread groups of natural constituents; as many as 400 different compounds have been reported to exist and their practical relevance is increasingly described [1,2]. The systematic identification of flavonoid compounds was first well established by coupling the selectivity properties of classical chromatographic separation techniques (paper and column chromatography) with spectroscopic measurements [3,4]. ^{13}C NMR spectroscopy has been successively employed to establish the environment and nature of the carbon and hydrogen atoms and the information gained by this technique is fundamental for flavonoid structure determination. Modern reversed-phase high-performance liquid chromatographic (HPLC) methods are now the most commonly used techniques in the separation and analysis of flavonoid mixtures owing to their greater selectivity, sensitivity and speed [4,5]. The use of HPLC retention values in elucidating flavonoid structures has also been suggested and its potential role as an aid in systematic identification has been demonstrated [6–10].

This topic was further investigated in this work. The specific effects of the introduction of hydroxyl groups on structural features and therefore on HPLC retention were studied in relation to substituent position and number. The relationship

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between HPLC retention values, NMR spectroscopic data and flavonoid molecular structure was investigated. The structural information gained by this comparative approach was examined by comparison with X-ray structural data.

EXPERIMENTAL

Materials

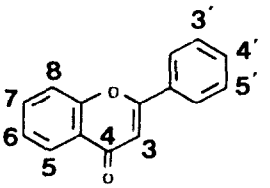
The selected flavonoid standards were obtained from Sarsyntex (Merignac, France) and used as received. Standard solutions in ethanol had a concentration of 10–100 ppm.

HPLC measurements

Capacity factors (k') for various fixed organic solvent compositions were determined for standard compounds with a Waters Model 600 multi-solvent system, equipped with a Rheodyne injection valve (20-μl sample loop) and a Waters Model 990 photodiode-array detector, coupled with an APC III personal computer (NEC, Boxborough, MA, USA). The mobile phases were mixtures of water, purified with a Norganic system (Millipore, Bedford, MA, USA) and HPLC-grade methanol (Rudi-Pont, Hetalab Chemical, USA). The aqueous phase was buffered at pH 2–3 in 80 m*M* acetic acid–8 m*M*

TABLE I

MOLECULAR STRUCTURES OF COMPOUNDS STUDIED



No.	Compound	Substituent position							
		3	5	6	7	8	3'	4'	5'
1	Acacetin		OH		OH			OCH_3	
2	Apigenin		OH		OH			OH	
3	Chrysin		OH		OH				
4	Chrysoeriol		OH		OH		OCH_3	OH	
5	Fisetin	OH			OH	OH	OH		
6	Flavone								
7	3-Hydroxyflavone	OH							
8	5-Hydroxyflavone		OH						
9	6-Hydroxyflavone			OH					
10	7-Hydroxyflavone				OH				
11	6,7-Dihydroxyflavone			OH	OH				
12	7,8-Dihydroxyflavone				OH	OH			
13	Galangin	OH	OH		OH				
14	Isorahmnetin	OH	OH		OH		OCH_3	OH	
15	Kaempferid	OH	OH		OH			OCH_3	
16	Kaempferol	OH	OH		OH		OH		
17	Luteolin		OH		OH		OH	OH	
18	Myricetin	OH	OH		OH		OH	OH	OH
19	Quercetin	OH	OH		OH		OH	OH	
20	Rhamnetin	OH	OH		OCH_3		OH	OH	
21	Robinetin	OH			OH		OH	OH	OH
22	Scutellarein		OH	OH	OH			OH	

disodium hydrogenphosphate (Carlo Erba, Milan, Italy). Solvent mixtures were filtered through 0.2-μm Millipore filters. All the data were obtained on a 30 cm × 3.9 mm I.D. 10-μm μBondapak C_{18} column (Waters Assoc., Milford, MA, USA) by following the experimental procedure described previously [8,9].

NMR study

The ^{13}C NMR spectra of compounds **1**, **3**, **8**–**11**, **15** and **22** were all obtained at 300 MHz on a Varian Gemini spectrometer in [$^2H_6$]dimethyl sulphoxide (DMSO-d_6) or $C^2H_3O^2H$ solutions. All the chemical shifts (δ ^{13}C) are reported in ppm with DMSO-d_6 at 35.5 ppm and $C^2H_3O^2H$ at 49 ppm as references.

RESULTS AND DISCUSSION

The selected compounds represent the classes of flavones and flavonols. The structures reported in Table I illustrate the hydroxylation pattern. Standard compounds were chosen on the basis of structure in order to test the effect of substituent introduction into the molecule; some occur naturally and others are synthetic products.

Table II reports the measured log k' values for the extended series of compounds, while other retention data have been reported previously [6–10]. When retention values were correlated with mobile phase composition (as methanol volume fraction, φ) a linear fit was obtained (Table III). The statistical goodness of the fit confirmed the linearity of log k'–φ correlation when methanol is the organic modifier and acetic acid is the acid modifier [11]. For various substituents in the benzopyran ring the group contribution to retention was calculated as $\Delta\log k'$ (*i.e.*, the difference between the retention of one molecule containing a particular substituent and that of a molecule which does not contain that group);

$$\Delta\log k' = \log k'_2 - \log k'_1 \quad (1)$$

where k'_2 is retention of the solute with the added group and k'_1 is that of the molecule without the substituent. The values reported in Table IV were calculated employing only log k' data roughly in the range 0–1 (k' = 1–10) where the experimental accuracy is assumed to be ±5%.

The group retention contributions obtained were largely independent of eluent volume fraction when methanol was the strong solvent, as shown in Fig. 1, where $\Delta\log k'$ values are plotted as a function of mobile phase composition for a 3-OH substituent.

TABLE II

RETENTION VALUES LOG k' MEASURED FOR SELECTED FLAVONOID COMPOUNDS WITH DIFFERENT METHANOL CONCENTRATIONS IN THE MOBILE PHASE

Compound[a]	Methanol concentration (%)							
	30	40	45	50	55	60	70	80
5	1.52	0.96	0.69	0.48	0.19	0.09	−0.39	
6				1.29	0.99	0.85	0.35	0.02
7				1.40	1.10	0.95	0.45	0.07
8				1.58	1.26	1.11	0.60	0.21
9				1.12	0.82	0.67	0.18	−0.16
10				1.07	0.77	0.64	0.14	−0.24
11				0.92	0.60	0.46	−0.01	−0.33
12				0.68	0.40	0.28	−0.15	−0.44
15					1.16	1.01	0.47	0.00
16				0.98	0.65	0.53	0.01	−0.34
18	1.45	0.89	0.63	0.41	0.13	0.02	−0.46	
20				1.16	0.86	0.72	0.19	−0.21
21	1.18	0.64	0.37	0.18	−0.09	−0.18	−0.66	
22				0.51	0.30	0.05	−0.30	

[a] See Table I.

TABLE III

STATISTICAL PARAMETERS OF LINEAR FIT OF LOG k' *VERSUS* METHANOL CONCENTRATION IN THE MOBILE PHASE

Compound[a]	Intercept	Slope	R^b	S.E.[c]
5	2.93 (±0.13)	−4.89 (±0.26)	0.99	0.06
6	3.56 (±0.19)	−4.57 (±0.32)	1.00	0.05
7	3.69 (±0.18)	−4.63 (±0.30)	1.00	0.04
8	3.93 (±0.20)	−4.76 (±0.33)	1.00	0.05
9	3.39 (±0.17)	−4.58 (±0.29)	1.00	0.04
10	3.31 (±0.20)	−4.51 (±0.34)	0.99	0.05
11	3.11 (±0.20)	−4.46 (±0.33)	0.99	0.05
12	2.66 (±0.18)	−4.02 (±0.31)	0.99	0.05
15	3.79 (±0.37)	−4.71 (±0.59)	0.99	0.06
16	3.29 (±0.25)	−4.68 (±0.42)	0.99	0.06
18	2.86 (±0.12)	−4.87 (±0.25)	0.99	0.06
20	3.51 (±0.20)	−4.73 (±0.34)	0.99	0.05
21	2.52 (±0.13)	−4.65 (±0.28)	0.99	0.07
22	2.82 (±0.13)	−4.60 (±0.23)	1.00	0.02

[a] See Table I.
[b] R = correlation coefficient.
[c] S.E. = standard error of regression.

TABLE IV

EFFECT OF HYDROXY SUBSTITUTION ON RETENTION, EXPRESSED AS GROUP CONTRIBUTION TO RETENTION (ΔLOG k'), AND ON C-4 CHEMICAL SHIFT ($\Delta\delta^{13}$C)

Group	Compounds[a]	$\Delta\log k'$[b]	$\Delta\delta^{13}C$[c]	$\Delta\delta^{13}C$[d]
3-OH	**19–17**	−0.13 ± 0.06	−6.3	−6.5
3-OH	**16–2**	−0.07 ± 0.02	−5.9	
3-OH	**15–1**	0.04 ± 0.01	−5.8	
3-OH	**14–4**	−0.02 ± 0.01	−5.5	
3-OH	**13–3**	0.01 ± 0.01	−5.4	−6.3
3-OH	**7–6**	0.11 ± 0.01	−4.2	−5.3
5-OH	**19–5**	0.20 ± 0.02		
5-OH	**18–21**	0.20 ± 0.02		
5-OH	**3–10**	0.25 ± 0.02	5.9	
5-OH	**8–6**	0.27 ± 0.02	4.5	
6-OH	**9–6**	−0.17 ± 0.01	−0.6	
6-OH	**11–10**	−0.17 ± 0.01	−0.1	
6-OH	**22–2**	−0.34 ± 0.03	1.0	
7-OH	**10–6**	−0.22 ± 0.01		
7-OH	**3–8**	−0.22 ± 0.01		
7-OH	**11–9**	−0.21 ± 0.01		

[a] See Table I.
[b] Average value for three or four different solvent compositions.
[c] Determined in DMSO-d_6 solvent.
[d] Determined in $C^2H_3O^2H$ solvent.

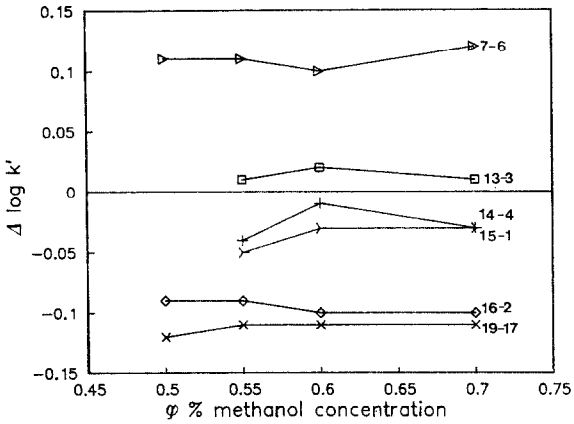


Fig. 1. Dependence of 3-OH retention contribution, $\Delta\log k'$, on methanol concentration in the mobile phase (φ, %). Pairs of numbers represent compounds listed in Table I.

This peculiar behaviour of methanol as organic modifier was previously found with different HPLC and thin-layer chromatographic systems for different flavonoid compounds [9,10]. Owing to this peculiarity, methanol seems to be the solvent of choice for the systematic study of the retention behaviour of these compounds related to their molecular structure. An average of three or four different mobile phase compositions can be taken to obtain the retention contributions (Table IV; see the reported small standard deviations).

The retention of a molecule is determined by various intermolecular solute-mobile-stationary phase interactions, such as dispersive, inductive, orientative and charge transfer, including hydrogen bonding [12]. A detailed analysis of the group retention contributions makes it possible to single out the specific interactions controlling chromatographic behaviour. The data in Table IV show that the position of hydroxylation affects the retention in different ways, depending on the molecular environment. On introducing an OH group in positions 6 and 7 the retention value decreases, whereas substitution in position 5 results in an increase in retention. All these effects, with the exception of the 6-OH substitution, are independent of the presence of other substituents in the molecule. On the other hand, substitution in position 3 results in a very different behaviour: the $\Delta\log k'$ value ranges from positive to negative values and is strongly dependent

on the molecular environment [10] (see Table IV and Fig. 1).

The introduction of a polar, hydrogen-bonding OH group normally increases solvation of the flavonoid molecules with an aqueous solvent and/or decreases hydrophobic interactions with the C_{18} stationary phase. This effect is valid for all 6- and 7-substituted compounds and some of the 3-OH derivatives, whereas for the other flavones introduction of an OH group results in a decrease in solute polarity (see all of the 5-OH and some of the 3-OH substitutions in Table IV). This behaviour shows that substituent retention contributions are not strictly constant and additive, but are a complex result of intramolecular interactions.

In order to obtain further insight into these intermolecular effects, the information on molecular structure obtained by ^{13}C NMR spectroscopic and X-ray crystallographic studies was investigated and correlated with the chromatographic data.

The usefulness of ^{13}C NMR spectroscopy for flavonoid structure analysis is well established and there is good agreement between different workers on the assignment of signals [13–15]. The ^{13}C chemical shift directly reflects the total charge density at the particular carbon nucleus and is therefore a sensitive probe for the structural characterization of the molecule. Many surveys have been published summarizing the results of ^{13}C NMR studies on flavonoids [13]. When not available in the literature, NMR spectra were measured for the flavonoid compounds and the results are reported in Table V. In the flavone derivatives the lowest field carbon resonances are generally those of carbonyl and oxygenated aromatic carbons (due to the lower electron density on ^{13}C), and the highest field resonances are shown by non-oxygenated aliphatic carbons. In particular, the ^{13}C NMR data show the different effects of the introduction of an OH group in position 3 or 5 on the chemical shift of the C-4 carbon according to the different electron density on the carbon (Table V) [13]. The ^{13}C NMR chemical shift of the C-4 nucleus may be selected as a descriptor of electronic density on the carbonyl group, and therefore of the strength of hydrogen bonding (HB) involving this part of the flavonoid molecule. The group contribution to the chemical shift can be calculated in the same way as the group retention contribution:

$$\Delta\delta\ ^{13}C = \delta_2{}^{13}C - \delta_1{}^{13}C \qquad (2)$$

where $\delta\ _2{}^{13}C$ represents the chemical shift of the molecule with the added hydroxyl group and $\delta\ _1{}^{13}C$ that for the compound without the substituent. The values obtained are reported in Table IV.

X-ray crystallographic information on flavonoids is summarized in Table VI, where the available literature data are grouped into four classes: (i) flavones unhydroxylated in positions 3 and 5 [16–20], (ii) flavonols unsubstituted in position 5 [21,22], (iii) 5-hydroxyflavones [22–34] and (iv) quercetin, the only 5-hydroxyflavonol whose crystallographic structure has been determined [35,36]. The reported data are average values calculated from the various measured data available in the literature. The molecular parameters most affected by intramolecular HB with the carbonyl group are shown in Table VI. The summarized structural data demonstrate that the HB between C-4 keto and C-3 hydroxyl groups has no significant effect on carbonyl bond strength, because C-4 = O-4 bond length in the flavonols is not statistically different from that in flavones [*cf.*, $d_{C\text{-}4\text{–}O\text{-}4}$ = 1.235(9) for flanones *vs.* 1.234(2) for flavonols]. Table VI also shows that the HB between the carbonyl oxygen (as a proton acceptor) and the 5-OH group, thus closing a six-membered ring (β-ring), is sterically and energetically more favoured than that of flavonols unsubstituted in position 5, where the HB closes a five-membered ring (α-ring) (compare flavones).

Let us now consider in detail the hydroxylation pattern effects.

TABLE V

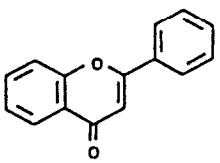
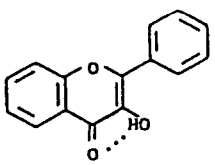
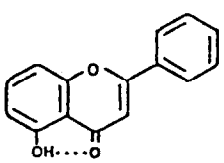
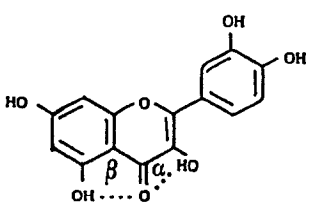
SOME $\delta^{13}C$ NMR CHEMICAL SHIFTS (PPM IN DMSO-d_6) OF FLAVONOID MEASURED SPECTRA

Compound[a]	C-3	C-4	C-5
1	104.2	182.7	161.4
3	105.3	182.0	161.4
8	105.6	182.9	159.8
9	106.3	177.8	129.6
10	107.0	177.2	129.5
11	106.4	177.1	129.6
15	136.7	176.9	161.3
22	104.4	182.8	150.3

[a] See Table I.

TABLE VI

BOND LENGTHS (Å) AND ANGLES (°) FROM X-RAY CRYSTALLOGRAPHIC DATA

Compounds	$d_{C\text{-}4\text{–}O\text{-}4}$	$d_{O\text{-}3\text{–}H}$/ $d_{O\text{-}5\text{–}H}$	$d_{O\text{-}3\cdots O\text{-}4}$/ $d_{O\text{-}4\cdots O\text{-}5}$	$d_{O\text{-}4\cdots H}$	$\alpha_{O\text{-}3\text{–}H\cdots O\text{-}4}$/ $\alpha_{O\text{-}5\text{–}H\cdots O\text{-}4}$
(i) Flavones (n = 6)[a]	1.235(9)				
					
(ii) Flavonols (n = 4)	1.234(2)	0.88(6)	2.72(4)	2.27(9)	122(8)
					
(iii) 5-Hydroxyflavones (n = 5)	1.255(9)	1.00(9)	2.60(3)	1.69(14)	151(11)
					
(iv) Quercetin	1.268(1)				
(n = 2) α-Ring:	0.91[b]	2.795(2)		105[b]	
β-Ring:	0.94[b]	2.581(8)	1.85[b]	133[b]	
					

[a] n = Number of available structures.

[b] The only data reported in the literature [35,36].

C-3 hydroxylation

The introduction of a 3-OH group causes an upfield shift of about 5.5 ppm (on average) in the C-4 signal of differently substituted flavones (Table IV). Taking into account that the *ortho* effect of the OH substituent on the ^{13}C chemical shift of benzene is upfield of 12.7 ppm, the net effect on the C-4 resonance of the introduction of a 3-OH group into the unsubstituted flavone (compound **1**) could corre spond to a net 7.2 ppm downfield shift. This result has been ascribed to the existence of the intramolecular HB interaction between C-4 keto and C-3 hydroxyl groups. The probable decrease in the HB strength between the C-4 keto and 3-OH, when

other hydroxyl groups are also present (see, *e.g.*, the mean distances $d_{O\text{-}3\ldots O\text{-}4}$ in quercetin and in flavonols in Table VI), may be the reason why the net deshielding effect on the C-4 chemical shift decreases in the series going from the pair 3-hydroxyflavone–flavone to the pair quercetin–luteolin. In fact, when hydroxyl groups are also present on C-5 and C-7 atoms, the introduction of a further OH group in position 3 also causes a net downfield effect, but lower than the above, ranging from +7.3 to +6.4 ppm (corresponding to $\Delta\delta^{13}C$ values of −5.4 and −6.3 in Table IV). This behaviour may be considered to be a complex result of the inductive effect of the electron-withdrawing O atoms introduced and of the intramolecular HBs present in the molecule.

X-ray structural data provide another important insight into flavone structure [35,36]: it was observed that, in the crystal, quercetin exists in the dihydrated form. The HB formed by the 3-OH group is so weak that the hydroxyl proton is able to form a second bifurcated bond with an acceptor water molecule. The existence of this hydrated form may further justify the increased affinity of 3,5-OH derivatives to water and therefore their decreased retention values in reversed-phase chromatography ($\Delta\log k'$ = −0.13 in Table IV for the pair quercetin–luteolin).

A quantitative description of this behaviour was attempted by linearly relating retention with molecular structure descriptors, expressed as group contribution to retention ($\Delta\log k'$) or with chemical shift ($\Delta\delta^{13}C$). The calculated linear relationship is expressed by the following equation:

$$\Delta\log k' = 0.58\ (+0.059) + 0.11\ (+0.011)\ \Delta\delta^{13}C$$

$$n = 6;\ R = 0.981;\ \text{S.E.} = 0.017 \qquad (3)$$

where R is the correlation coefficient and S.E. is the standard error of the regression (Fig. 2). This result shows that both ^{13}C NMR spectroscopy and reversed-phase HPLC retention values are equally affected by the same electronic properties: the increase in retention follows the increase in deshielding of the C-4 atom as a result of complex electronic rearrangements due to intramolecular HBs. The correlation between two independent measures of solute electronic interactions may be very promising for the structural characterization of molecules and solute retention prediction purposes.

The $\Delta\log k'$ *vs.* $\Delta\delta\ ^{13}C$ correlation may contain

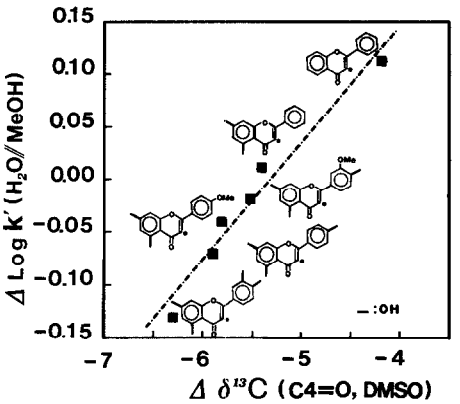


Fig. 2. Linear relationship between retention ($\Delta\log k'$) and chemical shift ($\Delta\delta\ ^{13}C$ in DMSO). Me = methyl.

some uncertainty due to the solvent effect, as the retention data were measured in methanol–water mixtures whereas the NMR spectra were determined in DMSO. In order to estimate solvent effects on the ^{13}C chemical shifts, the NMR spectra of some flavonoid compounds were determined using $C^2H_3O^2H$ as solvent. A downfield shift of the ^{13}C resonance for the solvent was observed; the solvent effect reflects the major ability of methanol to form a HB complex with the solute, particularly with the basic carbonyl group [37–39]. Moreover, the trend between $\Delta\delta^{13}C$ and $\Delta\log k'$ values is close to that observed in DMSO. Because of solubility problems, only a few spectra could be determined in $C^2H_3O^2H$ (Table IV), and the lack of experimental data prevents a better elucidation of specific solvent effects.

C-5 hydroxylation

The presence of a C-5 hydroxyl group causes, on average, a downfield shift of the C-4 resonance of about 5.2 ppm (see Table IV). A comparison with the *meta*-OH substituent effect on the benzene molecule [2] (+1.4 ppm) shows that an intramolecular HB between C-4 keto and C-5 hydroxyl groups overrides the substituent effect of the 5-OH group to produce a greater downfield shift. The internuclear distance lengths reported in Table VI for 5-hydroxyflavone and quercetin show the intramolecular HB between C-4 keto and C-5 hydroxy groups, forming a six-membered ring (β-ring), has a strong effect on the molecular structure. This intramolecular HB is

surprisingly strong owing to a synergistic mechanism similar to that found in β-diketones and called resonance-assisted hydrogen bonding (RAHB) through which π-bond delocalization strengthens and shortens the intramolecular HB [40–42]. This specific type of HB is characterized by a strong correlation between the bond strength (measured by the $d_{O\cdots O}$ contact distance) and the delocalization of the π-system of the conjugated double bonds. In quercetin, for example, the C-3 hydroxyl group, which forms an additional intramolecular HB, further strengthens the 5-OH hydrogen bond with consequent slight shortening of $d_{O\text{-}4\cdots O\text{-}5}$ and more pronounced lengthening of $d_{C\text{-}4\text{–}O\text{-}4}$ in the β-ring (Table VI). Accordingly, the chemical shifts of the C-4 carbon nucleus are strongly downfield. The group retention increments (Table IV) may be explained in terms of the above-reported structural information: the positive Δ log k' values may be considered as the net effect of intramolecular HB in masking polar groups in solute molecules and therefore in making the whole molecule less polar.

The degree of resonance through the HB fragment affects the ^{13}C NMR chemical shifts: as the degree of π-bond delocalization increases, C-5 should become less shielded whereas C-4 becomes more shielded. Therefore, the greater the degree of resonance, the smaller is the $\Delta\delta_{C\text{-}4\text{–}C\text{-}5} = \delta_{C\text{-}4} - \delta_{C\text{-}5}$ difference is. The chemical shift differences for 5-hydroxy-substituted flavones and flavonols were calculated from the data available in the literature and reported in Table VII. The $\Delta\delta_{C\text{-}4\text{–}C\text{-}5}$ values obtained, ranging from 14.2 to 23.2 ppm, agree with the previously reported data for β-diketones where the formation of RAHB was verified [41,42]. Large $\Delta\delta_{C\text{-}4\text{–}C\text{-}5}$ values (20.1–23.2) are found for 5-hydroxyflavones and smaller values (14.2–15.3) for 5-hydroxyflavonols; this behaviour indicates that the presence of a 3-OH substituent further increases the delocalization of the π-system. This may be one reason for the lower 5-OH contribution to retention in the flavonoid molecules whenever a 3-OH group is present (see the pairs quercetin–fisetin and myricetin–robinetin in Table IV).

TABLE VII

$\Delta\delta_{C\text{-}4\text{–}C\text{-}5}$ CHEMICAL SHIFT VALUES FOR VARIOUSLY SUBSTITUTED FLAVONES AND FLAVONOLS

Compound[a]	$\Delta\delta_{C\text{-}4\text{–}C\text{-}5}$ (ppm)	Compound[a]	$\Delta\delta_{C\text{-}4\text{–}C\text{-}5}$ (ppm)
1	20.1	**14**	15.1
2	20.7	**15**	15.3
3	20.3	**16**	15.2
4	23.2	**17**	20.1
8	23.1	**19**	15.1
13	14.2		

[a] See Table I.

C-6 hydroxylation

The introduction of a hydroxyl group in position 6 always leads to a negative Δ log k' increment. The same values were obtained when 6-OH was added to flavone or to 7-hydroxyflavone (Table IV). A study of NMR spectra shows that the introduction of a 6-OH group produces a slight upfield shift of *ortho*-related C-5 and C-7 nuclei and a slight shielding effect on the C-4 keto group (Table V). A major perturbation of the electron distribution in the ^{13}C NMR spectra is evident when the scutellarein–apigenin pair is considered, where a 5-OH group is present: the signal of the C-4 keto nucleus shifts downfield of 1 ppm. The reason for this effect may be ascribed to the strong interaction of the three vicinal hydroxyl groups with consequent weakening of the HB in the β-ring. The same reasoning may apply to the greater increase in molecule polarity and therefore may explain the more negative Δlog k' value.

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