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Do strong intramolecular hydrogen bonds persist in aqueous solution? Variable temperature gradient ^1H, $^1H-^{13}C$ GE-HSQC and GE-HMBC NMR studies of flavonols and flavones in organic and aqueous mixtures

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Abstract—Intramolecular hydrogen bonds in crystals and in apolar media are well documented, however, the degree to which they persist in aqueous solution is controversial. We report here variable temperature gradient ^1H, $^1H-^{13}C$ Gradient Enhanced Heteronuclear Single Quantum Correlation (GE-HSQC) and Gradient Enhanced Heteronuclear Multiple Bond Coherence (GE-HMBC) NMR studies of the flavonols quercetin and kaempferol and the flavone luteolin, in organic solvents and in mixtures of organic–aqueous solutions. It is demonstrated that the strong intramolecular hydrogen bond of the –CO(4) and –OH(5) moieties persists over a wide range of aqueous mixtures and, thus, provide a rare example of non-charged intramolecular hydrogen bonds, which is not overwhelmed by solvation with protic solvents, in particular in aqueous solution.

1. Introduction

Hydrogen bonding is a fundamental aspect of chemical structure and reactivity. It is a key to the structure and properties of water, proteins and DNA, and it is currently of interest for designing systems that exhibit molecular recognition.[1–4] Understanding the electronic nature of the hydrogen bond appears to be more elusive than that for covalent bonds, ionic bonds and van der Waals forces. This is because the term hydrogen bond applies to a wider range of interatomic interactions in relation to those observed in covalent and ionic bonds. Intramolecular hydrogen bonds can be formed between donor and acceptor groups in the same molecule, when molecular configuration and conformation brings them within hydrogen bond geometry. Although intramolecular hydrogen bonds in crystals and in apolar media are well documented, the degree to which these hydrogen bonds persist in protic solvents and especially in aqueous solution is controversial. It is often speculated that intramolecular hydrogen bonds are overwhelmed by solvation of hydroxyl protons with solvent water molecules.[2,5]

Nuclear magnetic resonance (NMR) spectroscopy is among the primary methodologies for investigating hydrogen bonding interactions both in solution and in the solid state. Proton chemical shifts provide evidence of hydrogen bonding and their magnitude is quantitatively proportional to the strength of the hydrogen bond.[4] However, it is difficult to observe directly hydroxyl protons in aqueous solution, because of their rapid exchange with bulk water.[2–7]

An interesting application of the use of ^1H NMR spectroscopy was reported by Leeflang et al.[8] who examined the persistence of an intramolecular hydrogen bond in methyl β-cellobioside dissolved in H_2O-CD_3OD and Me_2SO-d_6. They found that the hydrogen bond persists in non-aqueous solvents, but not in aqueous solvent. Adams and Lerner[9] reported NMR studies of sucrose in water–acetone solutions and showed no distinction between the hydroxyl protons with regard to the temperature coefficients of the chemical shifts, scalar couplings, and exchange rates, which might distinguish the hydroxyl groups involved in intra- and intermolecular hydrogen bonding. Poppe and van Halbeek[5] reported a ^1H NMR study of hydroxyl protons in

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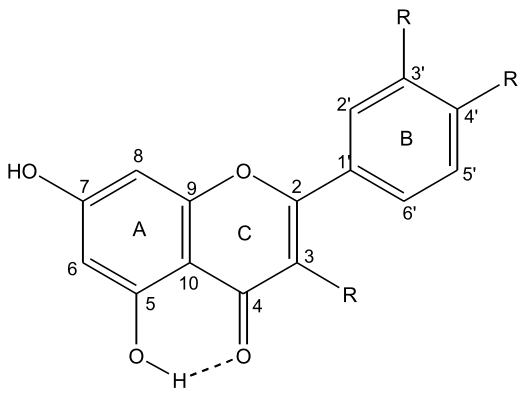

Quercetin: R_3=OH, $R_{3'}$= OH, $R_{4'}$=OH
Kaempferol: R_3=OH, $R_{3'}$= H, $R_{4'}$=OH
Luteolin: R_3=H, $R_{3'}$= OH, $R_{4'}$=OH

Figure 1. Structural formulas of the flavonoids under investigation.

supercooled carbohydrates and showed that although all hydroxyl protons are more or less free rotors, a specific OH appeared to have a smaller vicinal coupling constant, which strongly indicated the formation of inter-residual hydrogen bond.

In an attempt to investigate whether strong intramolecular hydrogen bonds persist in aqueous solution, we report here detailed variable temperature gradient ^{1}H, ^{1}H–^{13}C Gradient Enhanced Heteronuclear Single Quantum Correlation (GE-HSQC) and Gradient Enhanced Heteronuclear Multiple Bond Coherence (GE-HMBC) NMR studies of the hydroxyl protons of the flavonols quercetin and kaempferol and the flavone luteolin (Fig. 1) in organic solvents and in mixtures of organic–aqueous solutions. Flavonoids are a group of naturally occurring antioxidants, which are widely distributed in plants and have gained tremendous interest over the past years due to their possible biological activity and therapeutic applications.[10–12]

2. Results and discussion

The antioxidant activity of the family of flavonoids is determined by structural arrangements, such as the *ortho* 3′,4′-dihydroxyl moiety in the B ring (e.g. in luteolin and quercetin) and the *meta* 5,7-dihydroxyl moiety in the A ring (e.g. quercetin, kaempferol and luteolin) (Fig. 1). The 2,3 double bond in combination with both the 4-keto group and the 3-hydroxyl group in the C ring (e.g. quercetin), and an *o*-dihydroxyl structure in the B ring, are also significant.[10,11]

In order to investigate the existence of strong intramolecular hydrogen bonds and clarify the solvation and conformational properties of the hydroxyl groups of flavonols and flavones, which are of importance in structural–function relationships, we performed detailed variable temperature ^{1}H NMR studies of quercetin, kaempferol and luteolin in CD_3COCD_3 and CD_3OH solutions (Figs. 2 and 3). Generally, the ^{1}H NMR resonances of the –OH groups appear at room temperature as broad signals especially in protic solvents, due to fast, on the NMR time scale, exchange of the -OH protons with the solvent. By decreasing the temperature, the proton exchange rate is reduced and relatively sharp –OH peaks are revealed. In the case of kaempferol in acetone solution, all the –OH resonances are clearly observed as sharp singlets even at room temperature (Fig. 2). On the contrary, in methanol-d_3 solution only a relatively broad –OH resonance at ~12.3 ppm appears at 280 K, as shown in Fig. 3, whereas the other –OH signals appear at significantly lower temperatures (<240 K). To the best of our knowledge, this is the first time that phenolic OH groups of flavonoids

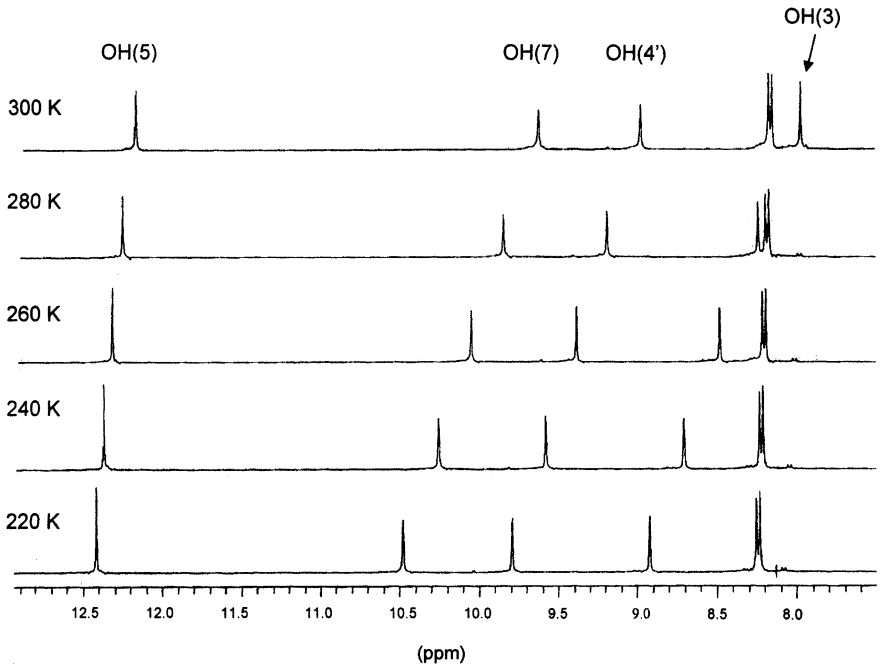

Figure 2. Variable temperature 400 MHz ^{1}H NMR spectra of kaempferol in acetone-d_6, concentration 10 mM.

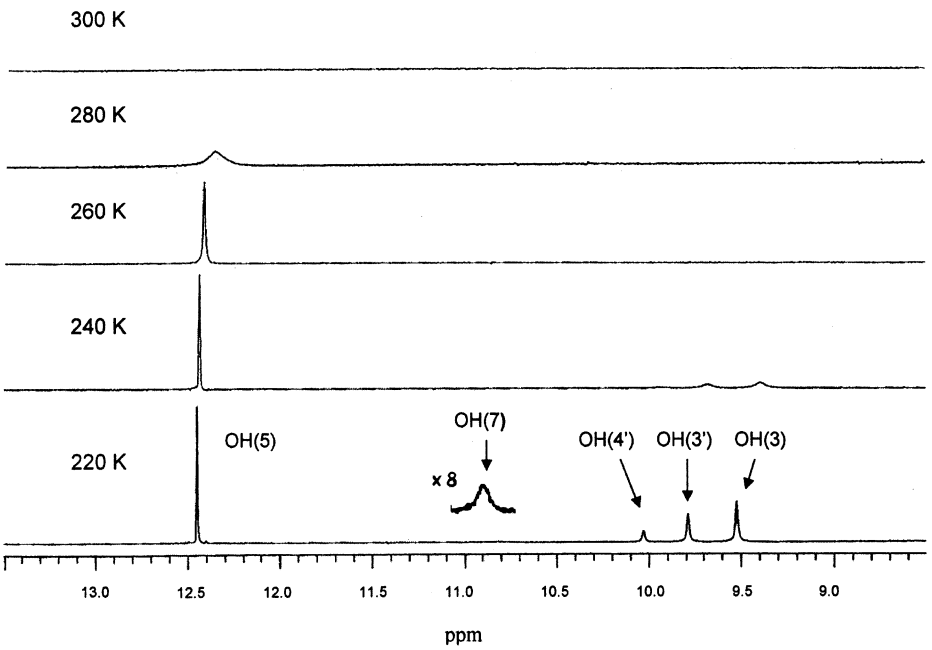

Figure 3. Variable temperature gradient 400 MHz ^{1}H NMR spectra of quercetin in CD_3OH, concentration 10 mM.

have been observed in a protic solvent like methanol-d_3. This is due to the utility of the WATERGATE pulse sequence for solvent suppression,[13,14] which does not eliminate fast exchanging OH resonances with the solvent.

Heteronuclear $^{1}H-^{13}C$ experiments can be used in order to reveal long range coupling of hydroxyl protons, and thus to provide unequivocal assignment of the –OH signals of the flavonoids under investigation. The $^{1}H-^{13}C$ GE-HSQC and GE-HMBC NMR experiments provide assignment information for protons and carbons that are connected via one and multiple (up to four) bonds, respectively. Gradient-enhanced experiments have particularly beneficial effects in eliminating the t_1-noise, improving the suppression of solvent signals, removing spectral artifacts and facilitating the observation of very weak interactions across several bonds.[15,16] Fig. 4 shows the $^{1}H-^{13}C$ HMBC spectrum of kaempferol in acetone at 243 K. The OH(5), which resonates at 12.3 ppm, shows cross-peaks with C(6), C(10), and C(9), the OH(7), at 10 ppm, shows cross-peaks with C(8), C(6), and C(7) and the OH(3), at 8.3 ppm, a cross-peak with C(2). Cross-peaks between OH(4′), at 9.3 ppm, and C(3′5′), C(4′) are also observed.

As mentioned before, the sensitivity of ^{1}H chemical shifts to changes in the electronic environment makes it a useful probe for detecting hydrogen bonded protons. Thus, the OH(5) group results in the most deshielded signal at 12.3–12.4 ppm and its proton is the least mobile and accessible to the solvent, due to its participation in a strong intramolecular hydrogen bond of the C(5)OH···OC(4) moiety (Fig. 1).

The temperature coefficients of the chemical shift of hydroxyl protons can also be utilized in order to identify intramolecular hydrogen bonding. This method has been extensively used to identify backbone peptide proton intramolecular hydrogen bonding in peptides and proteins[17–21] and intramolecularly hydrogen bonded hydroxyl groups of carbohydrates. Temperature coefficients below −4 ppb/K suggest strong solvent shielding and probably the formation of an intramolecular hydrogen bond. Thus, small $\Delta\delta/\Delta T$ values have been observed for hydroxyl protons of amylose,[7] cellulose,[22] and methyl β-chitobiose[23] in aprotic solvents and were interpreted by their participation in intramolecular hydrogen bonds. Since no literature data of $\Delta\delta/\Delta T$ values of flavonoid hydroxyl protons have so far been reported, the above $\Delta\delta/\Delta T$ values of the carbohydrate hydroxyl protons could possibly be used as a criterion for the identification of intramolecular hydrogen bonds of the –OH protons.

Fig. 5 shows the temperature dependence of the hydroxyl protons chemical shifts of kaempferol in acetone solution. Table 1 lists $\Delta\delta/\Delta T$ values of the hydroxyl proton of kaempferol, quercetin and luetolin in acetone and methanol solutions. It is obvious that the temperature coefficient $\Delta\delta/\Delta T$ of OH(5) is an order of magnitude smaller than those of other hydroxyl protons.[24] This indicates that the OH(5) is the only proton involved in an intramolecular hydrogen bond in the molecules and solvents under study.

A certain number of crystal structures of flavonoids and their derivatives have been resolved.[25–27] An X-ray structure determination of quercetin, showed that the benzopyran and phenyl rings are both essentially planar; the dihedral angle between the two rings is 9° and that between the annellated rings is 6°.[27] The bond lengths of C(4)–C(10) (1.423 Å) and C(4)–O(4) (1.269 Å) are shorter and longer respectively, than the average,[27] which is indicative of the formation of the hydrogen bond between –CO(4) and –OH(5) in a six-membered ring (Fig. 1). A second intramolecular hydrogen bond was also suggested between OH(3) and CO(4), resulting in the formation of a five-membered ring. The formation of a strong intramolecular hydrogen bond between CO(4) and OH(5) in

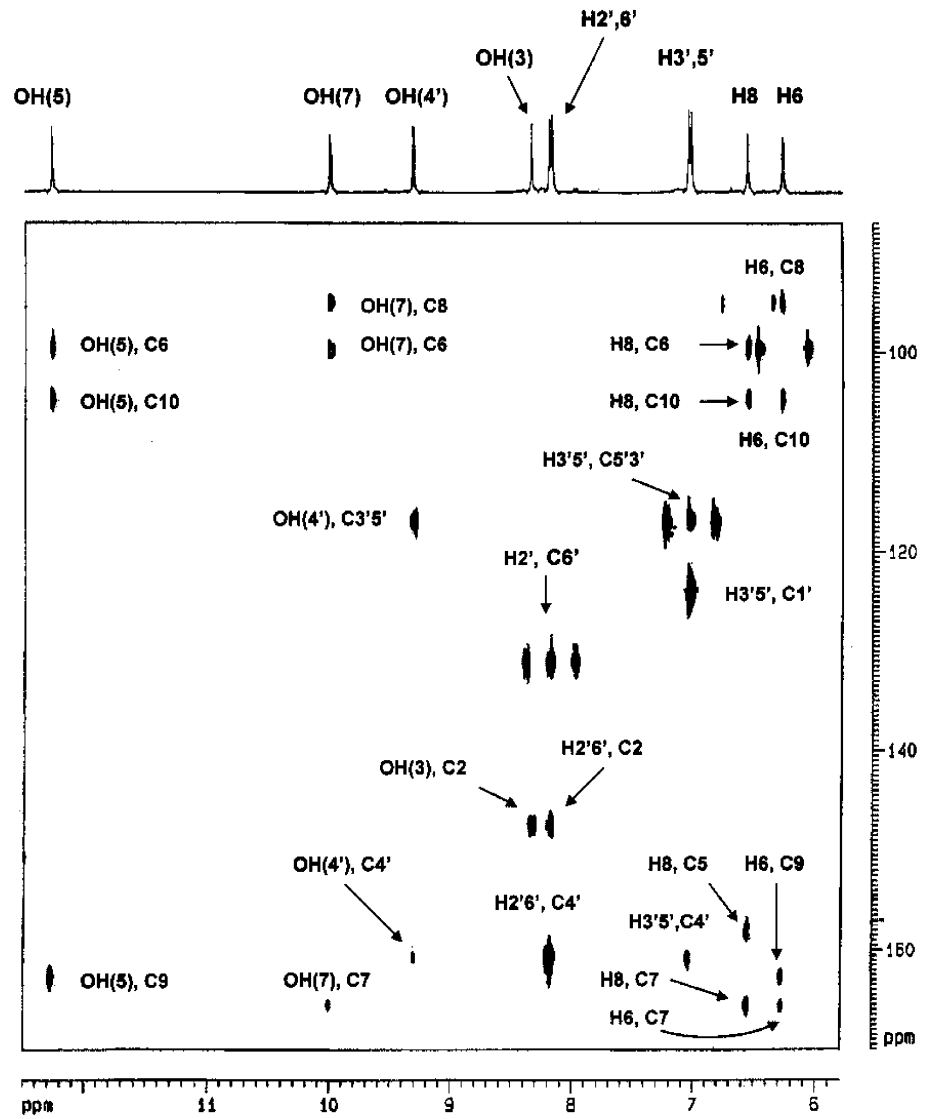

Figure 4. 400 MHz $^{1}H-^{13}C$ GE-HMBC spectrum of kaempferol in acetone-d_6 at 243 K, concentration 10 mM, illustrating connectivities between OH protons and carbons that are connected via multiple bonds. The $^{1}J_{CH}$ filter was set to 3 ms and the delay for the evolution of the long-range coupling was set to 60 ms.

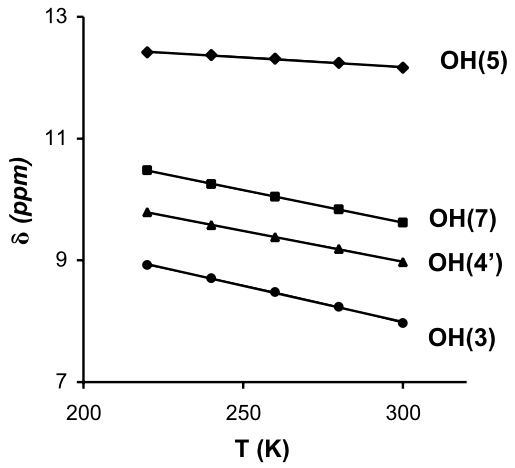

Figure 5. Temperature dependencies of the OH protons of kaempferol in acetone-d_6, concentration 10 mM.

the X-ray structure determination, is in agreement with our NMR results, that indicate: (i) very slow exchange rate; (ii) very small $\Delta\delta/\Delta T$ values and (iii) highly deshielded OH(5) proton in comparison with the other –OH protons. Contrary to the X-ray results, the OH(3) proton appears to be solvent accessible both in acetone-d_6 and methanol-d_3 solution.

Table 1. Chemical shift temperature coefficients ($\Delta\delta/\Delta T$) of hydroxyl protons of kaempferol, quercetin and luetolin in acetone-d_6 and methanol-d_3 solution

	$\Delta\delta/\Delta T$[a]					
	Kaempferol		Quercetin		Luteolin	
	Acetone	Methanol	Acetone	Methanol	Acetone	Methanol
OH(5)	−3.1	−0.5	−2.9	−0.5	−2.3	0.5
OH(7)	−10.7	−4.3	−11.1	−4.5	−17.2	−4.4
OH(4′)	−10.1	−3.8	−11.2	−5.5	−15.0	−5.0
OH(3′)	–	–	−8.4	−5.8	−14.9	−4.2
OH(3)	−11.8	−6.3	−11.6	−6.4	–	–

[a] Expressed in parts per 10^9 (ppb) per K.

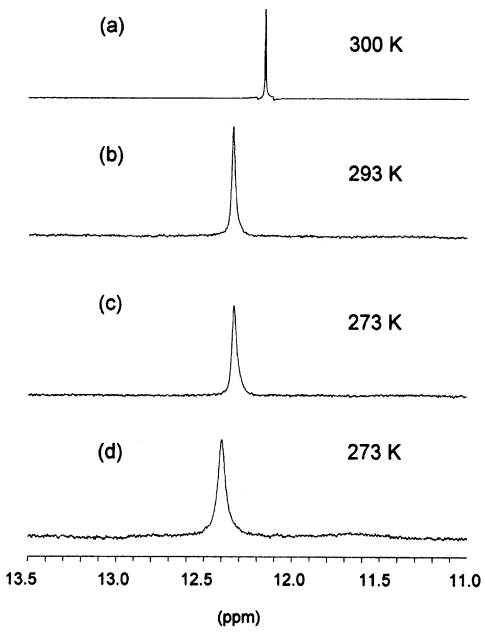

Figure 6. 400 MHz ^{1}H NMR gradient spectra of quercetin in mixtures of water–CD_3COCD_3, in molar ratios: (a) 0, (b) 82, (c) 88, and (d) 91% in water. Number of scans 32, 128, 128 and 320 for (a), (b), (c), and (d), respectively.

Several factors are known to influence the strength of intramolecular hydrogen bonds: the electronegativity of the proton-acceptor group, the charge densities on the participating groups, steric effects and the resonance stabilization of the ring formed by the internal hydrogen bond.[28–30] The O···O separation of a hydrogen bond is a function of three parameters, the O–H covalent bond length, the H···O hydrogen bond length and the O–H···O angle.[31] The planar configuration and the formation of a strong CO(4) and –OH(5) hydrogen bond in flavonoids can be explained by a greater resonance stabilization of the molecule, due to the formation of a six-membered ring that stabilizes the negative charge density in the carbonyl O atom more effectively.

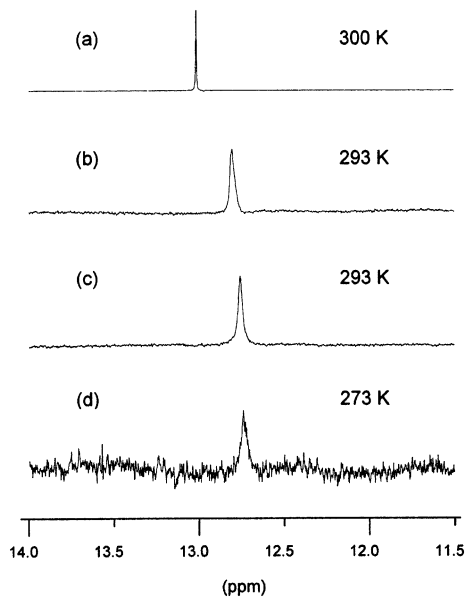

Figure 7. 400 MHz ^{1}H NMR gradient spectra of luteolin in mixtures of water–CD_3COCD_3, in molar ratios: (a) 0, (b) 75, (c) 88, and (d) 91% in water. Number of scans 32, 128, 128 and 1024 for (a), (b), (c), and (d), respectively.

Fig. 6 shows the ^{1}H NMR spectra of quercetin in mixtures of water and acetone-d_6 in molar ratios of: (a) 0, (b) 82, (c) 88 and (d) 91% in water. With a progressive increase in the water content, the highly deshielded signal at 12.3 ppm commences to broaden at room temperature. For molar ratio 88% in water, the ^{1}H NMR spectra were recorded at 273 K due to extensive broadening at room temperature. Similar results were obtained at a molar ratio 91% in water that clearly demonstrates the persistence of this intramolecular hydrogen bond (Fig. 6(d)).

At a molar ratio of 95% in water the peak is beyond recognition due to extensive broadening and precipitation of the compound. Connaturally, similar results were observed for luteolin (Fig. 7) and kaempferol (spectra not shown) in mixtures of water and acetone-d_6. Interestingly, the OH(5) resonance of quercetin is slightly deshielded, by about ~0.2 ppm, upon the addition of water. This is probably due to the extensive hydration of the OH(3) group, which reduces its polar effect on the carbonyl group CO(4) and, therefore, increases the strength of the OH(5)···OC(4) intramolecular hydrogen bond. The OH(5) resonance of luteolin, contrary to the case of quercetin, shows a slight

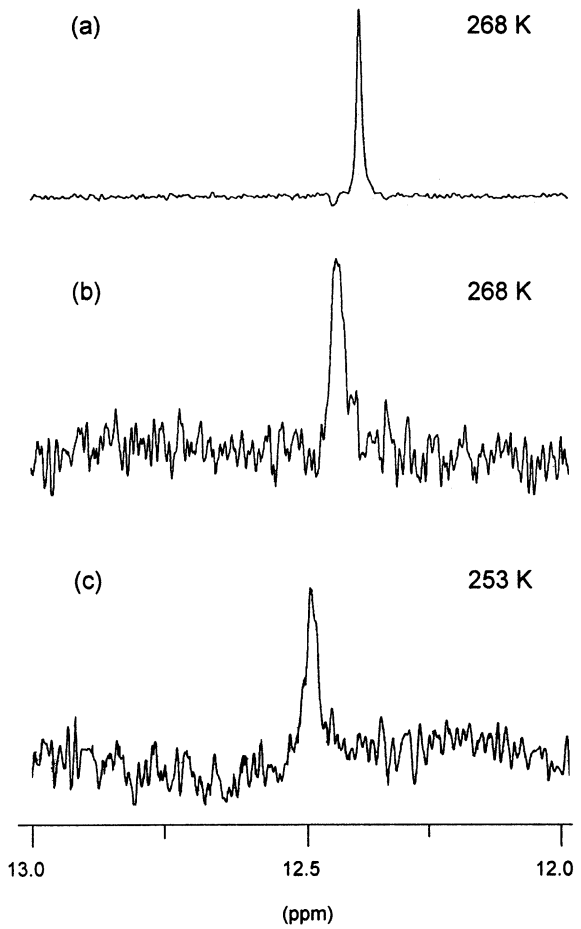

Figure 8. 400 MHz ^{1}H NMR gradient spectra of quercetin in mixtures of water–CD_3OH, in molar ratios: (a) 0, (b) 62, and (c) 71% in water. Number of scans 128, 728, and 1344 for (a), (b), and (c), respectively.

shielding upon the addition of water. Presumably, this is due to the absence of the OH(3) group and, thus, the partial hydration of the OH(5) group results in a slight weakening of the OH(5)···OC(4) intramolecular hydrogen bond.

The persistence of the –CO(4) and –OH(5) intramolecular hydrogen bond in solution was also investigated in water–CD_3OH mixtures. Fig. 8 shows the ^1H NMR spectra of quercetin in mixtures of water and CD_3OH in molar ratios of: (a) 0, (b) 62, and (c) 71% in water. The spectra were recorded at temperatures ≤268 K, because of the extensive broadening of the peaks at greater temperatures. The intramolecular hydrogen bond clearly persists up to a molar ratio of 71% in water, as shown in Fig. 8(c). Increasing the molar ratio of water, results to an extensive broadening of OH(5) resonance and precipitation of the compound, which greatly deteriorates the quality of the resulting spectra. Similar results were obtained for luteolin and kaempferol (spectra not shown) in mixtures of water and CD_3OH.

From the above, it is evident that the strong intramolecular hydrogen bond in the CO(4) and OH(5) moiety of flavonoids occurs in organic non-protic and protic solvents and in a wide range of water–acetone-d_6 and water–CD_3OH solutions. As emphasized previously, the strength of this intramolecular hydrogen bond may be attributed to the greater resonance stabilization, due to the formation of a six-membered ring. A further stabilizing factor may be due to entropy reasons. For an intramolecular hydrogen bond, the process of H-bond formation involves no significant restriction in the number of translational and rotational degrees of freedom, which give rise to inappreciable entropy changes. On the contrary, the entropy change accompanying the formation of an intermolecular hydrogen bond is significantly unfavorable because the independent translational and rotational motions of solute and solvents have to be 'frozen out' in order to create the hydrogen bond.[32]

Although there are several examples in the literature of charged intramolecular hydrogen bonds that persist in DMSO-d_6 and non-protic solvents,[33] the OH(5)···OC(4) hydrogen bond of flavonoids is a rare example of non-charged intramolecular hydrogen bond which is not overwhelmed by solvation, in particular in aqueous solution.

3. Experimental

Quercetin, kaempferol and luteolin were commercial products and used without further purification. All NMR experiments were performed on a Brüker AMX-400 spectrometer equipped with a *z*-gradient unit.[34] The suppression of the water resonance was achieved with the use of the WATERGATE pulse sequence for gradient.[13–14] Data were processed using UXNMR (Brüker) software. Chemical shifts were measured with reference to internal d_4-TMSP in H_2O–CD_3OH solutions and TMS in H_2O–CD_3COCD_3 solutions. CD_3OH was used instead of CD_3OD in order to remove the exchange of D with the mobile OH of the flavonoid molecules. ^1H–^{13}C GE-HSQC and GE-HMBC experiments were acquired with a spectral width of 6024 Hz in the F_2 (^1H) dimension and 20867 in the F_1 (^{13}C) dimension and with an acquisition time of 0.34 s and 16 scans per increment and 0.25 s and 56 scans per increment respectively. The relaxation delay was 1 s, the data collection matrix was 2k×512, the t_1 dimension was zero-filled to 1k real data points and a π/2 square sine bell window was applied in both dimensions.

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