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Studies on the interactions between some flavonols and cyclodextrins

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Abstract—The interactions of some natural flavonols with α, β- and γ-Cds have been investigated. Guest molecules were galangin, kaempferol and quercetin. Inclusion complexes were prepared by kneading and freeze-drying. The complexes were characterized using different physico-chemical methods based on differential scanning calorimetry (DSC), infrared spectroscopy (IR) and NMR spectroscopy. In the proton and carbon spectra the effects of complexation on the chemical shifts of the internal and external protons of Cds in the presence of each flavonoid were observed. Moreover, the water-solubility of the flavonols in the presence of Cds was also evaluated. The increased solubility of quercetin and kaempferol in the presence of β-Cd was evidenced. For all three guests, multidimensional NMR experiments in DMSO and water are consistent with dynamic binding processes, dominated by insertion of the B ring into the wider rim of the Cd cavity.

Cyclodextrins (Cds) are cyclic oligosaccharides largely used in pharmaceutical technology.[1,2] They are able to entrap a variety of organic compounds in their cavities, which can influence the dissolution rate, the aqueous solubility of poorly water-soluble substances and their stability in the presence of light, heat and oxidising conditions, as well as modify physico-chemical properties of drugs.[3]

Flavonoids, especially flavonols, occur in most of the vegetables and fruits of the Mediterranean diet but some of them represent also the active constituents or characteristic markers for many herbal drugs[4–6] and they show several biological properties.[5,7] Owing to their phenolic nature, flavonoids are quite polar but poorly water-soluble, and their very scarce absorption is well known.[5,7] These aspects have limited their use in the pharmaceutical field. Thus, we have decided to investigate, both in solution and in the solid state, the supramolecular complexes of some flavonols in the cavity of the natural Cds to improve their solubility. The investigated guests were galangin, kaempferol and quercetin (Fig. 1), in order to analyse the effects of the different hydroxylation pattern of ring B on the ability to form complexes with Cds.

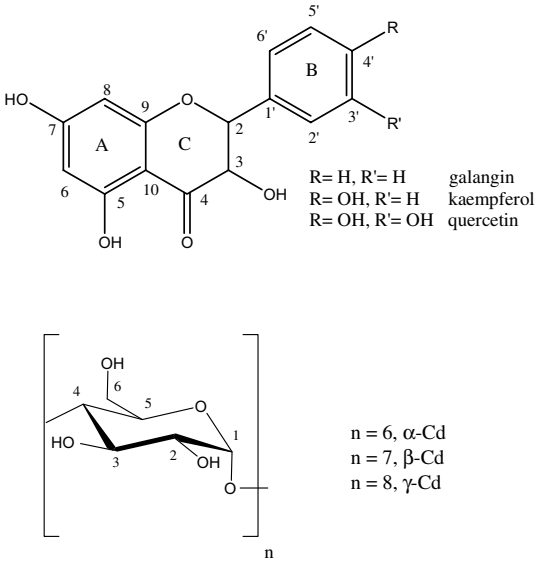

Figure 1. Structure of flavonols and cyclodextrins.

Keywords: α-, β- and γ-cyclodextrins; Flavonols: galangin, kaempferol, quercetin; Phase solubility analysis; ^{1}H NMR, ^{13}C NMR, NOESY, ROESY; IR spectroscopy; DSC.
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Kneading and freeze-drying were evaluated as methods to prepare the complexes.[8]

In the literature a few studies on the interaction between flavonols and cyclodextrins (mainly β-Cd) have been reported,[9–13] but these analyses were performed by thin layer chromatography, UV and fluorescence spectroscopies and were unable to give conclusive details about host-guest interaction modes. Very interesting and exhaustive papers on the investigation of the inclusion complexes of β-Cd and rutin or quercetin have also recently appeared.[14–16]

Thus, FT-IR spectroscopy and DSC were used to characterize the Cd-flavonols systems in the solid state, while NMR spectroscopy was used to obtain information on the inclusion complexes in solution. Solubility studies were performed by HPLC-DAD analysis. The stability constants of the complexes were determined by evaluating drug–cyclodextrin interactions in solution using phase solubility analysis.[17,18]

Inclusion in the solid-state. The fusion peaks of pure flavonols, those of α-, β-, γ-Cds and all flavonol-Cd systems were evaluated. In the case of galangin, a shift of the melting point (endothermic transition at 221.6 °C) to lower temperatures was evident in all kneaded samples and freeze-dried preparations, except in the case of galangin/α-Cd kneading, indicating the presence of an interaction between the two components of the complex. The DSC data for kaempferol and quercetin were difficult to interpret because of their melting-point values at 284.3 and 316.5 °C, respectively, which were superimposed with those of the degradation of α, β and γ-Cd. However, the melting-point endothermic transition of the guest molecule disappeared in all preparations.

IR spectra of all hosts and guests were recorded first and then those of all the flavonol-Cd systems. All guests showed typical bands from 1656 to 1670 cm^{-1} and from 1598 to 1610 cm^{-1}, attributed to the carbonyl moiety. An examination of some flavonol-Cd systems showed these bands decreased or slightly shifted, likely, as a result of interaction.

A shift from 1177 to 1158 cm^{-1}, attributed to C–C vibration of the aromatic system, was observed in the colyophilized galangin with all three cyclodextrins and in colyophilized kaempferol-γ Cd.

In the spectra of kneading products the characteristic carbonyl stretching band of flavonols was unchanged. A shift (from 1311 to 1321 cm^{-1}) of the vibration of C–O, attributed to aliphatic alcohols, was found in kneading galangin with α and β Cds.

The quercetin spectra did not differ from those of the molecules alone in the areas of the main absorption bands. No shifts were revealed, but only a reduction of intensity was observed, in particular for the complexes prepared by colyophilization.

From the solid state characterization we deduced that the kneading galangin-α and β-Cd showed no evidence of complex formation or even the presence of an interaction between the two components of the Cd-flavonol system.

Structure of complexes in solution. After these preliminary results in the solid state, the investigation of the solubility of the different flavonols in water at 25 °C, in the presence of increasing amounts of Cds, was carried out.

The phase solubility diagrams of quercetin with β-Cd displayed a typical AL type diagram[17] with a stability constant of 396 M^{-1}, according to data reported in the literature.[15] α and γ-Cds did not have any positive influence on solubility of this compound.

In the case of galangin, no CDs were found to enhance water solubility. On the contrary, water solubility of kaempferol increased as a function of the cyclodextrin concentration, evidencing that water-soluble complexes exist in solution. Figure 2 shows the phase solubility diagram of kaempferol with α, β and γ-Cds. The stability constants were 105 M^{-1} for α-Cd, 375 M^{-1} for β-Cd and 160 M^{-1} for γ-Cd.

In order to demonstrate the reasons for the very different behaviour of flavonoids in the phase solubility studies, the complex formation was investigated by means of two distinct NMR procedures. First, the complexes were solubilised in DMSO, in order to obtain the optimum solubility of both species and ^1H NMR, COSY and NOESY spectra were recorded in order to obtain information with regard to the interactions between the Cd and flavonol, as reported in Tables 1–3. Then, NOESY and ROESY experiments were performed using the complexes in D_2O, in order to approach a situation nearer to the phase solubility studies.

The experiments performed in DMSO were carried out both on free host and guest molecules and on complexes. Thus, as a general rule, the penetration of aromatic rings in the cavity of Cd is related to shielding of the inner protons of the glucose units of cyclodextrins, namely H-3 and H-5, because of the ring-current effects of the aromatic units. Consequently, H-3 and H-5 chemical shift displacement to higher fields is the first evidence of inclusion. The shifts experienced by the protons of the host and guest molecules upon com-

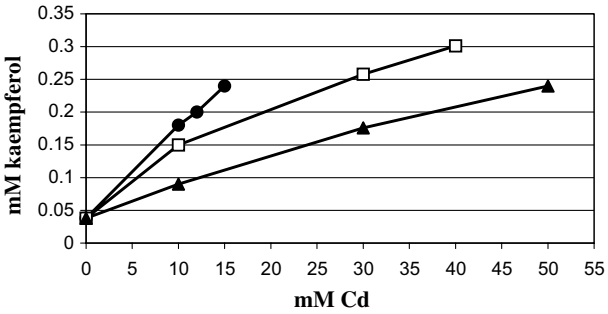

Figure 2. Phase solubility diagram of kaempferol-Cd systems in water at 25 °C. Key: ▲ α-Cd; • β-Cd; □ γ-Cd.

Table 1. ^{1}H NMR shifts ($\times 10^{-2}$) of the complexes galangin-β and γ-Cd; sign +: up-field, sign −: down-field (Kd, kneading; Col, colyophilized)

	Galangin								Cd						
	OH-3	OH-5	OH-7	H-6	H-8	H-2′ H-6′	H-4′	H-5′ H-3′	H-1	H-2	H-4	H-5	OH-2	OH-3	OH-6
Kd +β	+4	−1.13	0	−0.63	−0.41	−0.81	n.d.	−0.15	+0.43	+0.61	+0.33	+0.42	+0.56	+0.82	+1.30
Kd +γ	+2	−0.83	+1.90	−0.37	−0.13	−0.13	+0.13	+0.07	+0.63	+2.18	+0.21	+0.45	+0.61	0	+0.63
Col +β	+8	−1.19	−5	−0.78	−0.6	−0.87	−0.17	−0.24	+0.44	+0.66	+0.33	+0.43	+0.61	+0.88	+1.37
Col +γ	+9	−1.26	n.d.	−0.93	−0.7	−0.87	−0.17	−0.23	+0.20	+2.00	+1.88	+0.20	+0.56	+0.71	+1.25

Table 2. ^{1}H NMR Shifts ($\times 10^{-2}$) of the complexes of Kaempferol-β and γ-Cd; sign +: up-field, sign −: down-field (Kd, kneading; Col, colyophilized)

	Kaempferol								Cd						
	OH-3	OH-5	OH-7	H-6	H-8	H-2′ H-6′	OH-4′	H-5′ H-3′	H-1	H-2	H-4	H-5	OH-2	OH-3	OH-6
Kd +β[a]	+1.75	−0.97	n.d.	−0.76	−0.67	+9.47	−0.7	−0.46	+0.46	+1.30	−1.2	+0.48	+0.33	+0.90	+0.69
Kd +γ	+0.85	−0.87	+0.7	−0.19	−0.12	−0.55	−0.55	−0.3	+0.48	+2.30	+2.0	+0.37	+1.00	+0.76	+1.0
Col +β[a]	+1.00	−0.88	−3.00	−0.50	−0.63	+1.0	−0.88	−0.48	+0.43	+0.48	+0.38	+0.41	+0.19	+0.39	+0.20
Col +γ[a]	+0.27	−1.20	−3.90	−0.68	−0.54	−0.43	−0.87	−0.60	+0.20	+2.10	+1.80	+0.21	+0.60	+0.40	+1.22

[a] Kd +β, H-5 shifted to $-4\ 10^{-4}$; Col+β, H-4 shifted to $-1.9\ 10^{-3}$; Col+γ, H-2 shifted to +0.023.

Table 3. ^{1}H NMR Shifts ($\times 10^{-2}$) of the complexes of quercetin-Cds; sign +: up-field, sign −: down-field (Kd, kneading; Col, colyophilized)

	Quercetin									Cd						
	OH-3	OH-5	OH-7	OH-3′	OH-4′	H-6	H-8	H-2′	H-5′	H-6′	H-1	H-2	H-4	H-5	OH-2	OH-3	OH-6
Kd +α	−0.64	−1.00	+1.20	+1.10	+0.91	−0.74	−0.66	−1.40	−0.78	−1.05	+0.21	+0.25	+0.77	+0.28	+0.40	+0.51	+0.56
Kd +β	−1.00	−1.50	+3.10	+2.43	0	−0.55	−0.32	−1.30	−0.57	−0.90	+0.24	+0.51	+0.10	+0.14	+0.96	+1.09	+2.26
Kd +γ	−1.00	n.d.	0	+1.00	+1.00	−0.30	−0.63	−0.95	−0.53	−0.60	+0.29	+1.20	+0.02	+0.31	+0.32	+0.29	+0.20
Col +α	−2.00	−1.60	+1.0	−1.00	+3.80	−0.90	−0.70	−0.15	−0.86	−1.20	+0.15	+0.31	+0.08	+0.06	+0.02	+0.72	n.d.
Col +β	−0.56	−0.74	+3.70	+0.63	+0.90	−0.43	−0.41	−0.72	−0.20	−0.01	+0.35	+0.33	+0.40	+0.37	+0.51	+0.19	+0.19
Col +γ	−2.00	n.d.	0	0	+2.00	−0.50	−0.50	−0.84	−0.56	−1.43	+0.21	+1.10	+1.90	+0.21	+0.22	+0.35	+0.38

plex formation showed slightly different chemical shift values between complexed and uncomplexed form. All the data, with the exception of the complexes galangin/α-Cd due to their lack of significant shifts, are reported in Tables 1–3. Unfortunately, because of the overlapping of the shifts of H-3 and H-6a,b, proton signals were not considered in the evaluation of the proton shift in the complex formation.

In the proton spectra of complexes, Cd signals showed differential shifts greater than 10^{-3} ppm, and among them resonances of H-2 and H-4 of γ-Cd were displaced by more than 10^{-2} ppm for all the samples. Concerning the shift values of the proton resonances of the flavonol moieties, all phenol OH protons were shifted more than 10^{-2} ppm and the phenol protons of ring B and ring A showed, in general, differential shifts from 5×10^{-3} to 10^{-2} ppm. The greatest shifts of both guest and host protons are related to hydroxyls, probably due to a formation of hydrogen bonds, assisted by the non-protic solvent DMSO.

NOESY experiments were performed as a general strategy to have complete identification of the geometry of the complex in solution.

In these experiments, all the signals corresponding to the protons of phenolic OH showed a very strong interaction with the Cd proton signals.

In addition, benzopyran proton signals of galangin and kaempferol-Cd systems showed more intense cross-peaks with Cd secondary OH groups at C-2 and C-3 than those observed for phenyl protons of ring B. These data indicate a greater proximity between benzopyran and secondary OH at the wider end of the Cd cavity. Furthermore, H-6 and H-8 were involved in an interaction with the primary hydroxyl OH-6 of Cds and also with internal H-5 protons of Cds.

Concerning the cross-peaks of aromatic ring B proton signals and those of Cds, H-2′ and H-6′ of kaempferol and galangin, among others, showed an interaction, with H-5 of Cds. The same interaction was revealed by the signals of H-3′, H-4′ and H-5′ of galangin and also by the signals H-3′ and H-5′ of kaempferol. Similar results were obtained with quercetin-Cd systems, with regard to the interactions of protons of phenolic OH and those of aromatic ring A with H-3, H-5, H-1, OH-2 and OH-3 of Cds. No interactions between OH-2 and OH-3 of Cds and H-5′ and H-6′ of quercetin were present. From these data it was concluded that in the presence of non-protic solvents such as DMSO, and probably in the solid state, there is the possibility for all the flavonols to insert ring A in the Cd, as confirmed from the cross-peaks between the resonances of H-6 and 8 of flavonols and the Cd resonance attributable to H-5. The cross-peaks of benzopyran protons with the Cd secondary OH groups at C-2 and C-3 suggested that the

interaction was throughout the greater rim of the Cds. However, in principle, also the aromatic ring B, besides the different substitution patterns of the three flavonols, due to the same type of cross-peaks, can penetrate into the internal cavity of the Cds.

Therefore, in order to better understand the different solubilities in water of the different flavonols in the presence of Cds, NOESY and ROESY experiments in D_2O were also performed. Only data with the kaempferol-Cd complexes were recorded because the very low water solubility of the other complexes, as resulted also by the solubility studies. Thus, when D_2O was used as the solvent, an initial striking phenomenon takes place: upon dissolution of however small a quantity of the powder of complexes (as long as easily handled), the solution becomes slightly turbid and in no case was a 1:1 integral ratio between host and guest resonances observed (obviously scaled to the number of equivalent Cd protons). This is in remarkable contrast with the data collected in DMSO, which confirmed the equimolar composition of the sample. We must conclude that in water the drug is in equilibrium between a free (insoluble) and a bound (soluble) form. As described above, specific interactions can be sought with magnetisation transfer experiments: ROESY yields weak but detectable intermolecular cross peaks. In particular, ortho and meta protons on phenyl ring B seem in proximity of the H-3 and H-5 of γ-Cd, respectively, as depicted in Figure 3. This is in agreement with the findings with DMSO solution and demonstrates that also in water the adducts take place by including ring B into the Cd cavity, with the rest of the flavonol protruding from the larger rim.

The dynamic picture was filled up by measuring a NOESY spectrum. Remarkably, in this case, *all* diagonal *and cross* peaks are negative, including those of kaempferol (intramolecular). This behaviour is common to macromolecular systems, characterized by slow tumbling motion (it verifies that the rotational correlation time of the flavanol is apparently larger than 600 ps). We can easily justify it by assuming that it reorients together with the cyclodextrin host.

In conclusion, the solid state analyses show an interaction between Cds and flavonols in almost all the preparations and the strength of the interaction appears to be related to both steric factors of host molecules and preparation method of the supramolecular system. The freeze-drying method is the best to prepare the inclusion complexes, although in some cases, also kneading can be used. β-Cd is in general the Cd with the best characteristics to obtain inclusion complexes.

Through complexation with the β-Cd the aqueous solubility of quercetin and kaempferol has been improved in neutral aqueous solution. Thus, β-Cd may be useful in improving the dissolution and the bioavailability of these flavonols in pharmaceutical formulations.

The NMR analysis in DMSO showed that flavonols possess several moieties capable of interacting with Cds, namely the benzopyran-4-one unit and the phenol group (ring B). From our results the inclusion of the phenyl group inside the Cd cavity fits with the observed data for all the guests investigated and furthermore, these experiments have revealed that flavonol exists with the phenolic end inside the cavity through the larger rim. In addition, some smaller interactions exist with protons of benzopyran (ring A) and the protons inside the Cd cavity, probably according to a dynamic binding be-

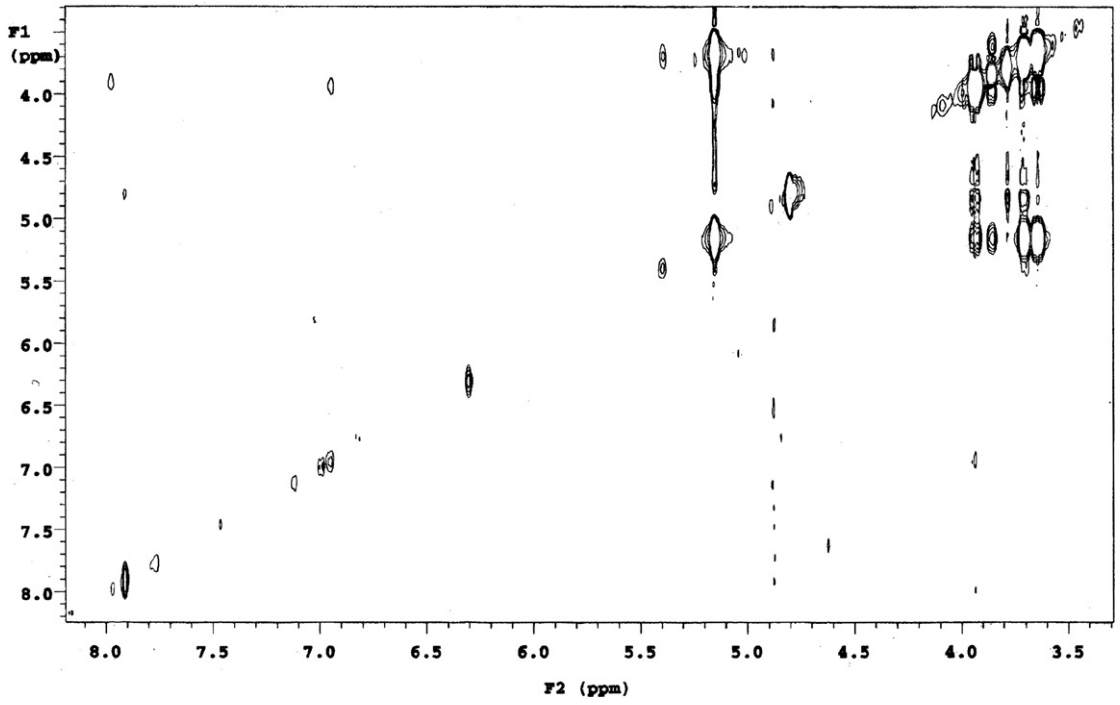

Figure 3. Portion of a ^{1}H ROESY spectrum of the coliophilized kaempferol-β Cd in D_2O, with the addition of 5% DMSO-d_6. Continuous-wave irradiation (B_1 = 2800 Hz) was used during a mixing time of 0.6 s.

tween flavonol and Cd systems, even if in the NMR studies of kaempferol complexes, carried out in deuterated water, these interactions were not present.

Recently, Banerjee and Sengupta,[19] reported a study on the interaction between β-Cd and two flavonols, 3-hydroxyflavone, a synthetic molecule (no hydroxyls in rings A and B), and fisetin (3,7,3′,4′-OH flavone), using molecular mechanics calculations in vacuo. The authors suggested that the interaction of A ring with the β-Cd was the most favoured but reported the need of further NMR experiments to confirm such data. These observations are not in contrast with our results because the greater affinity of ring A for the Cd cavity can presumably be related to the greater hydrophobicity with respect to the flavonols we have investigated. In addition, in the case of fisetin which is the flavonol more similar to quercetin, the insertion of ring B in the Cd cavity is also proposed with a similar energy of formation value.

Our results are in agreement with the six studies on flavanones, flavonols and dihydrochalcones previously reported in the literature.[13,14,20–23] However, if we compare our results with those reported for the dihydrochalcone and flavanones, a deeper inclusion of these molecules in the β-Cd's cavity should be justified by the higher flexibility of ring C in flavanone and by the absence of ring C in the dihydrochalcone as compared to the flavonol skeleton. The presence of interactions between the outer surface of Cds and also Cd cavity was found. Moreover, the OH groups in ring B affects the solubility, such as in the case of kaempferol and quercetin.

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18. *Characterization of the complexes:* Temperature and enthalpy values were measured with a Mettler TA4000 apparatus equipped with a DSC 25 cell. The heating rate was 10 °C min^{-1} over the 30–300 °C range.
    Infrared spectra were measured using a Perkin-Elmer 983 spectrophotometer. The samples were suspended in nujol and used on KBr disks. Thirty scans were obtained at a resolution of 2 cm^{-1}, from 4500 to 400 cm^{-1}.
    ^1H spectra in DMSO-d_6 were recorded at 300 K on a Bruker Avance-600 spectrometer. Data processing was achieved with a SGI/02 computer using XWin-NMR software version 2.6. The spectra in D_2O as the solvent were recorded on a Varian INOVA 600. The chemical shifts due to the solvent at 2.49 ppm and 39.0 ppm were used as internal references, in the proton and carbon spectra, respectively. For the water solutions, 5–10 mg of the freeze-dried complex/0.4 ml was used; a small fraction (less than 5%) of DMSO-d_6 was added to obtain a clear solution. The residual water signal at 4.80 ppm was used for referencing. NOESY and ROESY (cw-irradiation during mixing at $B_1 = 2600$ Hz.) spectra with variable mixing times were recorded.
    Phase solubility studies were carried out in water, according to the method previously reported by Higuchi and Connors[17] and the flavonol concentration was determined by HPLC-DAD analysis by flow-injection.
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