Inclusion Complex of β-Cyclodextrin and Quercetin.
Thermodynamic Approach

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The complexation of β-cyclodextrin (βCD) and quercetin (Que) in aqueous alcoholic solutions is investigated by UV-Vis spectroscopy. Three different aqueous media, namely pH 3, unbuffered (pH 5.5) and pH 7, are used to vary the environmental conditions and the interaction between host (βCD) and guest (Que) molecules. The equilibrium constants for the formation of the inclusion βCD-Que complex are estimated from two different absorption bands and the thermodynamics of the complexation is discussed.

Keywords: β-cyclodextrin, quercetin, host-guest complex, equilibrium constant

β-Cyclodextrin is an oligosaccharide, containing seven glucopyranose units linked through the α(1–4) glycosidic bonds into a doughnut shaped ring (fig. 1a). β-Cyclodextrin's internal cavity enables it to act as a molecular host for many molecules [1-28]. Several studies report on the molecular complexation of β-cyclodextrin (βCD) with different guest molecules and evaluate the equilibrium constant for the complexation phenomenon. Among these molecules were: chlorogenic acid [1], rutin [1,18, 26], morin [14], α-tocopherol [4], anilino-naphthalene-sulfonates [6], heptathioether [7], dextromethorphan [8], diazepam [9], adamantyl guests [10], piroxicam [11], imipramine hydrochloride [12], 3-hydroxyflavone[14], flavonols (galangin, kaempferol) [16], myricetin[19], methyl and ethyl salicylates [20], ferrocene [21], 2-naphthyloxyacetic acid and 1-naphthylacetic acid [22], orange G [27], a β-carboline analogue [28], ascorbic acid [23], vitamin B6 [24], vitamin B13 [25], quercetin (fig. 1 b) [1,2,4,5,13-15, 17-19], and quercetin with Zr(IV) [3].

The formation of the host–guest complexes of cyclodextrins with many types of organic, inorganic and organometallic compounds is detected mainly by various spectroscopic methods based on the changes of spectra upon complexation: UV-Vis absorption spectroscopy [4,8,9,11, 12, 14, 20, 22, 23-25, 27, 28], fluorescence spectroscopy [1, 2,3, 6, 22, 26- 28], FTIR [14, 16, 18, 23-25], NMR spectroscopy [14, 15, 16, 23-25], AFM force spectroscopy [7], ROESY (Rotating Frame Overhause Effect Spectroscopy) [10], static and dynamic light scattering (SLS, DLS), but also by phase solubility studies [5, 13-15], liquid chromatography [8, 20], isothermal titration calorimetry [10], differential scanning calorimetry, DSC [14, 16, 18, 23-25, 26], X-ray powder diffractometry [18, 23-25, 26] and by measurements of dielectric properties (relative permittivity) [21] and inhibitory effect investigation [19] .

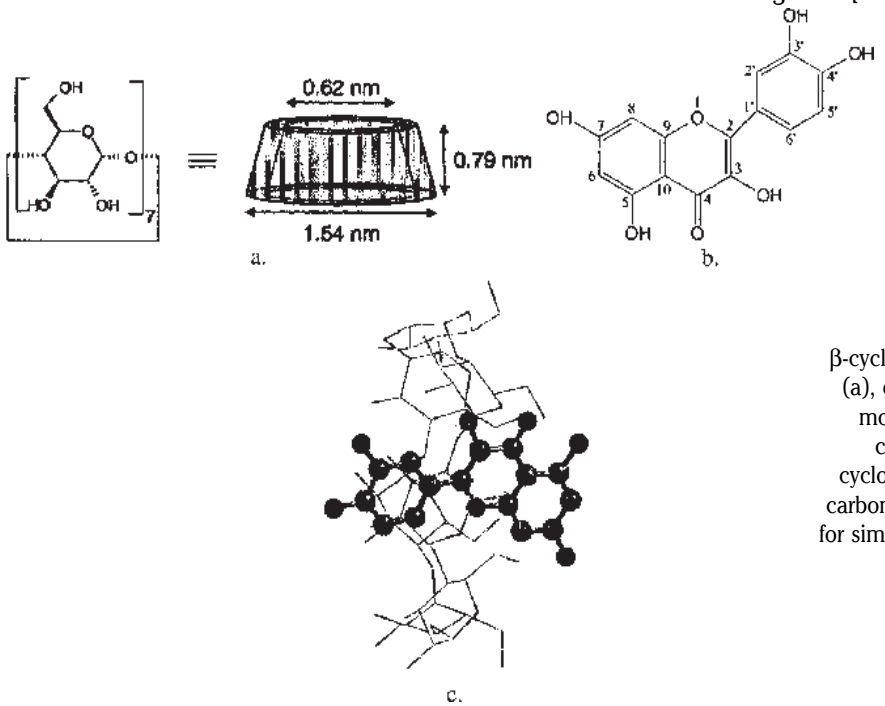

Fig. 1. Chemical formulas of β-cyclodextrin with its geometrical sizes (a), of quercetin (b), and of proposed molecular model for the inclusion compound of quercetin into β-cyclodextrin nanocavity (c; gray shows carbon skeleton; red stands for O atoms; for simplicity, the H atoms are not shown).

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The values of complexation equilibrium constant are strongly dependent on the experimental conditions (*p*H, temperature) and also on the measurement method. Some attempts to correlate the complexation constant values with thermodynamic parameters of molecular recognition have also been reported [7, 10]. However, studies to correlate the complexation constant values with thermodynamic parameters and molecular events in the inclusion process under thermodynamic equilibrium conditions remain scarce.

The present work aims to estimate the equilibrium constant for the formation of 1:1 host-guest complex of βCD with quercetin, Que, 2-(3',4'-dihydroxyphenyl)-3,5,7-trihydroxy-chromen-4-one (fig. 1c):

$$\beta CD + Que \rightleftharpoons \beta CD\text{-}Que$$

The equilibrium constant, K, of a host-guest molecular complexation (known also as the binding constant) can be evaluated from UV/Vis absorption spectra measurements using the mathematical approach of the Benesi-Hildebrand method [29]. For the complex of βCD as host molecule with Que as guest molecule, when the initial concentration of the host, $[\beta CD]_0$, is much larger than the initial concentration of the guest, $[Que]_0$, the usual form of the equation can be written as:

$$\frac{1}{\Delta A} = \frac{1}{l\Delta\varepsilon[\beta CD]_0[Que]_0 K} + \frac{1}{l\Delta\varepsilon[Que]_0} \quad (1)$$

where ΔA is the change in the absorbance of the quercetin solution upon adding βCD, $\Delta\varepsilon$ is the difference of the molar absorptivity for free and complexed quercetin, and l is the path length. The absorbance of βCD is negligible at the absorption maximum of quercetin and of the complex. Maintaining a constant initial quercetin concentration and varying the initial βCD concentration, from the double reciprocal plot, $1/\Delta A = f(1/[\beta CD]_0)$, the equilibrium constant K can be obtained, as the ratio between the intercept and the slope of the linear plot.

A variant is to multiply both members of eq. (1) with $[\beta CD]_0[Que]_0$, thus obtaining:

$$\frac{[\beta CD]_0[Que]_0}{\Delta A} = \frac{1}{l\Delta\varepsilon K} + \frac{[\beta CD]_0}{l\Delta\varepsilon} \quad (2)$$

The linear plot of $\frac{[\beta CD]_0[Que]_0}{\Delta A} = f([\beta CD]_0)$ (known as Scott plot, [30]) presents a more uniform distribution of the experimental points; K is obtained as the ratio between the slope and the intercept.

These methods are not applicable if the intercepts are zero or close to zero, or if they present negative values. A newer approach for the graphical determination of the equilibrium constant [31] uses the following equations:

$$\Delta A = [Que]_0\Delta\varepsilon l - \frac{\Delta A}{K[\beta CD]_0} \quad (3)$$

with the plot of $\Delta A = f(\Delta A/[\beta CD]_0)$, obtaining K from the slope,

and $$([\beta CD]_0 + [Que]_0) = \frac{\Delta\varepsilon l[\beta CD]_0[Que]_0}{\Delta A} - \frac{1}{K} \quad (4)$$

by plotting $([\beta CD]_0 + [Que]_0) = f([\beta CD]_0[Que]_0/\Delta A)$, and finding K from the intercept. An advantage of this approach is that it allows for the determination of K only from the slope (eq. 3) or from the intercept (eq. 4).

Experimental part

Quercetin of about 95% purity was purchased from China β- cyclodextrin, min. 98% purity, was purchased from SIGMA.

Since quercetin is sparingly soluble in water, a 30% (v/v) ethanol and water mixture was used to prepare a 0.25 mM quercetin stock solution. The solution was used shortly after its preparation. Three 0.01 M β-cyclodextrin stock solutions were prepared, by dissolving the necessary amount in (1) water, (2) citrate buffer solution *p*H 3, and (3) phosphate buffer solution, *p*H 7. Deionized water with resistivity of 18 MΩ·cm, obtained from an Elgastat water purification system was used for all solutions. The samples for the spectral investigations had a constant $4\cdot10^{-5}$ M quercetin concentration, and the following βCD concentrations: 0; 0.1; 0.5; 1.5; 2.0; 2.5; 3.0; 4.0; 5.0; 6.0; 7.0; 8.0 mM, each of them at the pH 3; 5.5 (not buffered); 7.

The UV/Vis absorption spectrum of the solutions was recorded using a Jasco UV/Vis V-530 spectrophotometer, with 10 mm path length quartz cuvettes in the 190-500 nm wavelengths range at 2 min after the solution preparation, and after 24 h as well, at a temperature of 25°C. All the measurements were repeated three times, at 5 min intervals.

Results and discussions

The spectra obtained for solutions at *p*H 3 and *p*H 5.5 (unbuffered) presented similar patterns. For example, some of the spectra obtained for *p*H 5.5 are given in figure 2 for the wavelength range from 240 to 500 nm. There are two peaks, with maxima at 372 and 255 nm for all solutions. The maxima presented a general increasing trend with increasing βCD concentrations.

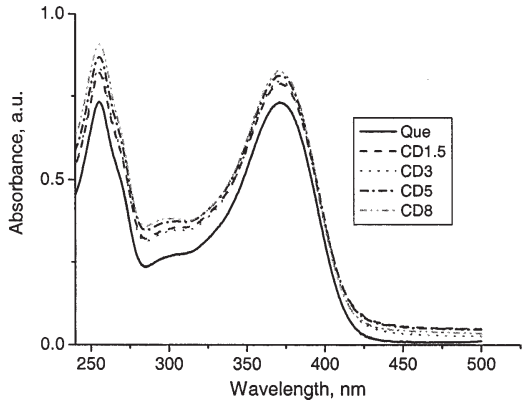

Fig. 2. Optical spectra of 0.04 mM Que solutions with variable βCD contents (mM) at *p*H 5.5

In the buffered solution at *p*H 7, there are important modifications in the spectrum of quercetin, as compared with the spectra at other *p*H values (fig. 3). While for *p*H 3 or 5.5 the spectrum is practical identical between 240 and 500 nm, for *p*H 7 the band at a higher wavelength has its maximum shifted from 372 to 378 nm, and the band at a lower wavelength is split, namely the maximum at 256 nm is lowered and a shoulder appears at 275 nm. The spectra obtained after adding βCD presented no clear trend in the modification of the absorption maxima; some are lower as for quercetin, others are higher. Moreover, these spectra measured at *p*H 7 shortly after preparing the

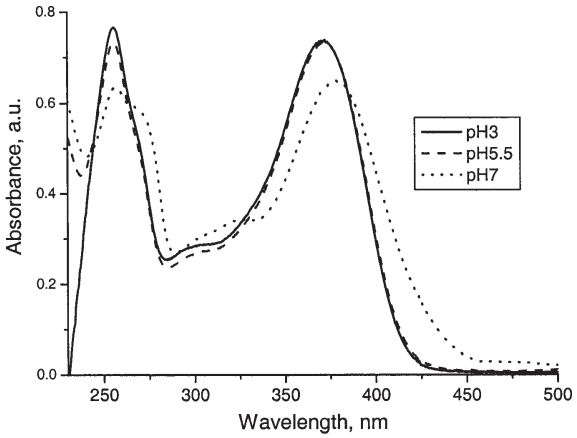

Fig. 3. Optical spectra of quercetin at various *p*H values

solutions were not stable in time and the measurements were not reproducible after an hour.

The first two pK values of quercetin are: $pK_1 = 7.03$, $pK_2 = 9.15$ [32]. The molar fractions of the differently protonated quercetin species in aqueous solutions (QH_2, QH^-, Q^{2-}) were calculated from their protolytic equilibria in a similar way as presented elsewhere [33] for *p*H values from 2 to 14 and are given in the diagram in figure 4.

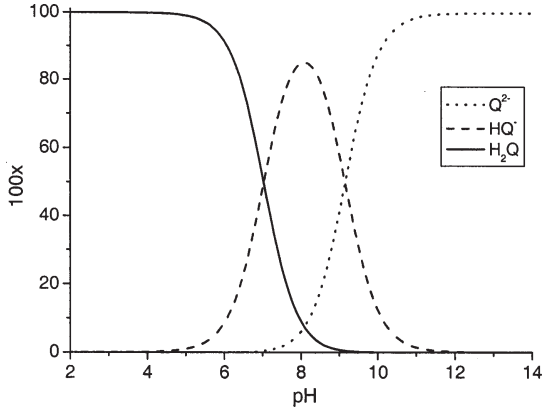

Fig. 4. Mole fractions (x) of quercetin species as a function of *p*H-values

As seen from the diagram (fig. 4), at *p*H 3 non ionized quercetin is the single species present, at *p*H 5.5 only a very small fraction is ionized, whereas at *p*H 7, some 50% of quercetin molecules gave up a proton and are present as mononegative anions. This finding could account for the modified spectrum of quercetin at *p*H 7 and a new peak with the maximum at about 270 nm could be assigned to the quercetin anion.

In order to find the equilibrium constant K of the formation of inclusion complex βCD-Que, equations (1)-(4) can be used. For the spectra measured at pH 3, immediately after mixing the solutions, the points are rather scattered and there is a very poor linear fitting, for instance, using eq. 2:

$y = (2.7\pm1.7)\cdot10^{-6} + (0.79\pm0.39)\cdot10^{-3}\ x$;
$r = 0.558$ (for the 372 nm absorption maximum)

$y = (2.5\pm1.1)\cdot10^{-6} + (0.38\pm0.26)\cdot10^{-3}\ x$;
$r = 0.434$ (for the 256 nm absorption maximum)

From these regression lines, values of about 300 mol^{-1} L and 150 mol^{-1} L, respectively, would be obtained. There is little significance for these values, having in view the scattering of the points, indicating that the process of complex formation is far from equilibrium.

The absorption maxima measured after 24 h, when equilibrium was reached, give the Benesi-Hildebrand plots (eq. 1) represented in figure 5a for the absorption maximum at 372 nm, and in figure 5b for the maximum at 256 nm. The equations of the regressions lines are given in the figure 5 and the K values calculated from them are included in table 1.

The point for [βCD] = 0.1 mmol/L was eliminated here and in the following situations, since the condition that [βCD] >> [Que] is not fulfilled in this case. The correlation is much better for the 372 nm absorption band (fig. 5a) than for 256 nm absorption band (fig. 5b).

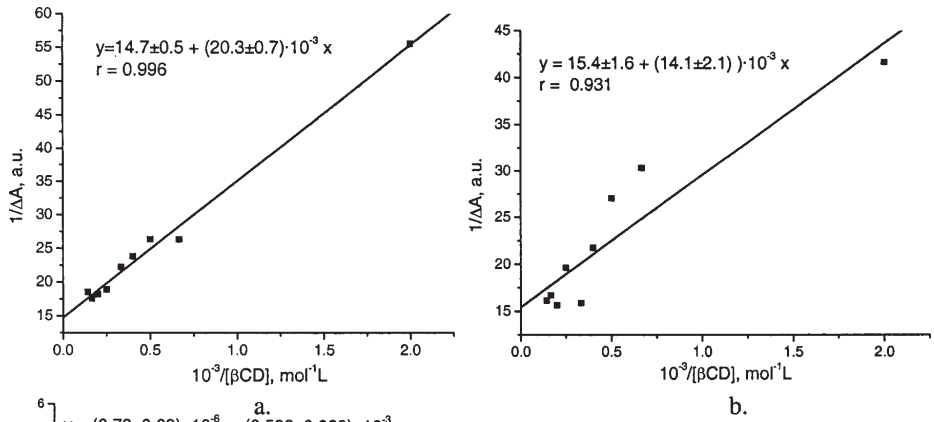

Fig. 5. Benesi-Hildebrand plot for the βCD-Que systems (*p*H 3; 24 h after mixing), for the absorption maxima at 372 nm (a), and 256 nm (b)

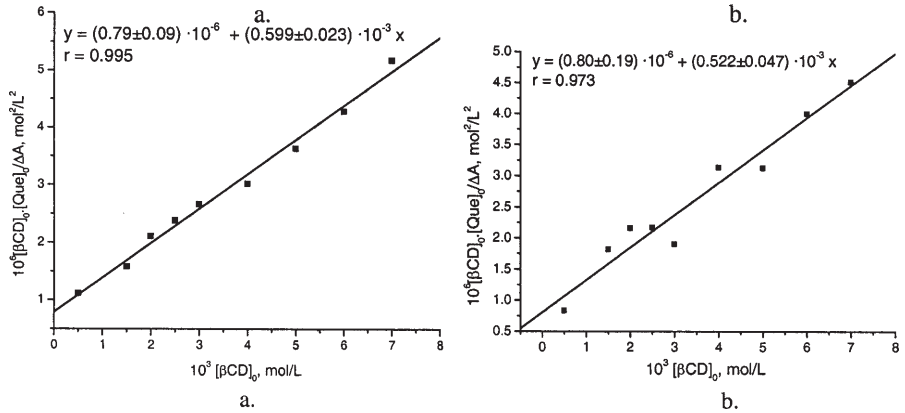

Fig. 6. Scott plots for the βCD-Que systems (*p*H 3; 24 h after mixing), for the absorption maxima at 372 nm (a), and 256 nm (b)

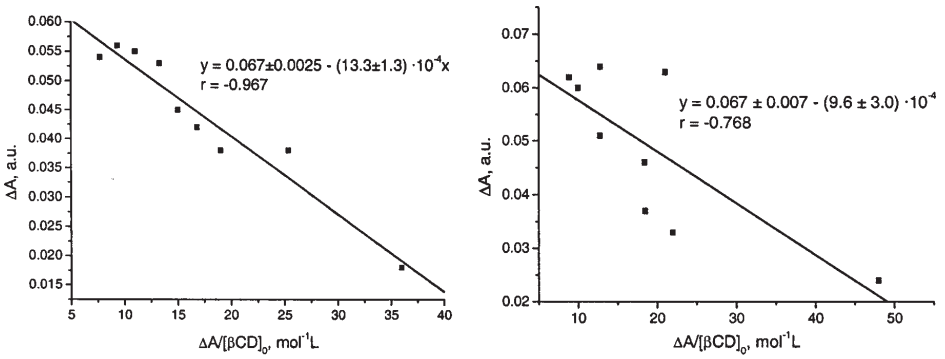

Fig. 7. Plots from eq. (3) for the βCD-Que systems (*p*H 3; 24 h after mixing), for the absorption maxima at 372 nm (a), and 256 nm (b)

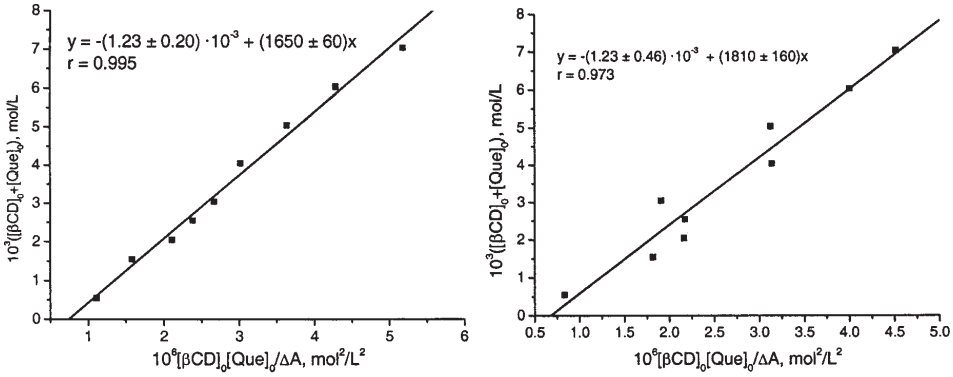

Fig.8. Plots from eq. (4) for the βCD-Que systems (pH 3; 24 h after mixing), for the absorption maxima at 372 nm (a), and 256 nm (b)

For the same absorption maxima, the Scott plots (eq. 2) are given in figure 6, the representations obtained from eq. (3) in figure 7, and finally those from eq. (4) in figure 8.

The data shown in table 1 indicate that the most methods give here similar values for the equilibrium constant, K. The average value obtained from the 372 nm absorption band is 750 ± 150 mol^{-1} L, while from the 256 nm absorption band an average K value of 900 ± 400 mol^{-1} L is determined. Clearly, the data obtained from the first absorption band are more reliable.

Without adding buffer solutions, the β-cyclodextrin and quercetin mixtures have a *p*H value of 5.5. For the spectra of these solutions, the same four variants of linearization were applied and the correlations found were similar in what concerns their precision to those determined for *p*H 3. The equations of the regression lines are given in table 2.

Using the parameters *a* and *b* of the regression lines (table 2), the equilibrium constants are also calculated for *p*H 5.5 and shown in table 1.

In table 1 again the values calculated from the absorption maximum at 372 nm correlate much better than those obtained at 256 nm. The average value, obtained from the 372 nm absorption band, is 680 ± 150 mol^{-1} L, and 620 ± 230 mol^{-1} L from the 256 nm absorption band. The values of the binding equilibrium constant are somewhat smaller for the *p*H 5.5 than for the *p*H 3. An average K value, obtained from both absorption maxima, can be estimated to be 825 mol^{-1} L at *p*H 3, and 650 mol^{-1} L at *p*H 5.5 (unbuffered solutions).

Method	K, mol^{-1}L, 372 nm absorption band	K, mol^{-1}L, 256 nm absorption band
pH 3, after 2 min		
from eq. (2)	300 (r = 0.558)	150 (r = 0.434)
pH 3, after 24 h		
from eq. (1)	725 ± 50 (0.996)	1100 ± 250 (0.931)
from eq. (2)	760 ± 100 (0.995)	650 ±170 (0.973)
from eq. (3)	750 ± 70 (-0.967)	1040± 250 (-0.768)
from eq. (4)	810 ± 100 (0.995)	810 ± 220 (0.973)
Average	750 ± 150	900 ± 400
Average of the two maxima	825	
Reference [15] 24° C	1028	
pH 5.5		
from eq. (1)	710 ± 70 (0.990)	570 ± 170 (0.858)
from eq. (2)	610 ± 100 (0.990)	530 ± 160 (0.959)
from eq. (3)	740 ± 90 (-0.928)	670 ± 190 (-0.711)
from eq. (4)	680 ± 110 (0.990)	710 ± 210 (0.959)
Average	680 ± 150	620 ± 230
Average of the two maxima	650	
Reference [17] 37° C	709	
Reference [5] 30° C	602	
Reference [2] 25° C	402	
Reference [16] 25° C	396	
Reference [18] 28° C	251	
Reference [14] 25° C	129	
Reference [13] 37 C°	230	

Table 1
ESTIMATED VALUES FOR THE BINDING EQUILIBRIUM CONSTANT, K, FOR THE βCD-Que (HOST-GUEST) INCLUSION COMPLEX

Table 2
PARAMETERS OF THE REGRESSION LINES ($y = a + bx$) FROM EQUATIONS (1)-(4) APPLIED TO UV-Vis MEASUREMENTS AT pH 5.5; a (eq. 1 AND Eq. 3, in a.u.); a (eq. 2, mol^2 L^{-2}); a (eq. 4, mol L^{-1}); b (eqs. 1-3, mol L^{-1}); b (eq. 4, mol^{-1} L)

Eq.	372 nm absorption band			256 nm absorption band		
	a	b	r	a	b	r
(1)	9.6±0.5	$(13.6\pm0.7)\cdot10^{-3}$	0.990	5.4 ± 0.8	$(9.5 \pm 2.1)\cdot10^{-3}$	0.858
(2)	$(6.0\pm0.8)\cdot10^{-7}$	$(3.68\pm0.18)\cdot10^{-4}$	0.990	$(3.8\pm1.0)\cdot10^{-7}$	$(2.03\pm0.21)\cdot10^{-4}$	0.959
(3)	0.103±0.005	$(13.5 \pm 1.9)\cdot10^{-4}$	-0.928	0.179 ± 0.021	$(15 \pm 6)\cdot10^{-4}$	-0.711
(4)	$-(1.48 \pm 0.29)\cdot10^{-3}$	2670 ± 130	0.990	$-(1.4 \pm 0.6)\cdot10^{-3}$	4520± 470	0.959

As mentioned above, at pH 7 the spectra are modified and no usable Benesi-Hildebrand type plots could be drawn in order to estimate the equilibrium constant. As seen on figure 4, about half of the quercetin molecules are ionized at pH 7, as anions. Quercetin in its anionic form is unstable [15], so the ionization and uncontrolled degradation could explain the failure of determining K values at this pH.

In table 1, the values obtained by us for the equilibrium constant, K, from different graphical treatments are summarized and compared with values from literature. Our value for pH 3 is somewhat smaller than that reported by [15] from phase solubility studies. The values given in literature for unbuffered solutions are rather different, depending on the method used for their determination. Our K values are close to the upper limit of the values given in literature, mainly close to the values obtained from phase solubility studies [5].

From estimated values of the binding constant, K, the Gibbs free energy of formation, ΔG^0, for the host-guest molecular complex, can be calculated:

$$\Delta G^0 = -RT \ln K \quad (5)$$

The ΔG^0 values obtained from this relation (5) are -16.6 kJ/mol (pH 3) and -16.0 kJ/mol (pH 5.5). They are comparable with the value determined at pH 3 and 297 K, by Zheng and al., of about -17.2 kJ/mol [15]. The authors found for the entropy of complex formation, ΔS^0, a value of -32.2 J/(mol.K), but with a standard deviation as high as 11.2 J/(mol.K). Assuming this ΔS^0 value, we found for the binding enthalpy, ΔH^0,

$$\Delta H^0 = \Delta G^0 + T\Delta S^0 \quad (6)$$

the values of about -26.2 kJ/mol (pH 3), and -25.6 kJ/mol (pH 5.5).

Therefore, the negative binding Gibbs free energy, ΔG^0, makes possible the complex formation and it is due to the important negative enthalpy variation associated with this process. The negative entropy change, due to the formation of the host-guest association is not essential. The enthalpy change can be assigned to the intermolecular interactions by hydrogen bonding and van der Waals forces. The main driving force for the complex formation could be related to the replacement of water molecules in the β-CD hydrophobic cavity by the guest molecules [34].

A theoretical study on the complexation of β-cyclodextrin with quercetin [35], by the PM3 quantum-mechanical semi-empirical method, calculated for the enthalpy variation, ΔH^0, values of about -48.8 and -46.46 kJ/mol for different orientations of the guest molecule (head up and head down, respectively), but failed completely in estimating the entropy variation. More specific, they calculated ΔS^0 = -255.61 J mol^{-1} K^{-1}, thus leading to a positive ΔG^0 value. The authors attributed this discrepancy mainly to the neglect of the hydrophobic effect involving a gain in entropy due to the assimilation of the solvation water molecules by the medium after the inclusion takes place. Similarly, AM1 and PM3 calculations on methyl and ethyl salicylates as guest molecules in βCD gave exceeding negative ΔS^0 values (from -190 to -260 J mol^{-1} K^{-1}) and therefore, positive ΔG^0 values are estimated [20], the cause being the same neglect in the entropy estimation.

On the other hand a thermodynamic study on β-CD complexation with anilinonaphthalene-sulfonates [6] found similar values for the enthalpy variation and small entropy variations, as in our situation for β-CD complexation with quercetin.

Conclusions

Spectroscopic investigations on the 1:1 host-guest molecular complex of β-cyclodextrin and quercetin allow for the estimation of the binding equilibrium constant, K, in aqueous solutions, in acidic medium (pH 3) and in unbuffered medium (pH 5.5). At pH 7, the partial ionization of quercetin and the low stability of the quercetin anionic species avoid the estimation of reliable K values. Four different approaches to the linearization of the Benesi-Hildebrand equation were tested, and applied for two different absorption peaks of the mixed β-cyclodextrin and quercetin solutions. The obtained results are mostly in fairly good agreement with one another and with literature data, especially for the 372 nm absorption band. From the K values, the thermodynamic ΔG^0 and ΔH^0 values of the complex formation between β-cyclodextrin and quercetin are estimated and discussed on the basis of intermolecular interactions within of inclusion complex.

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