Complexation of morin with three kinds of cyclodextrin A thermodynamic and reactivity study

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Abstract

Properties of inclusion complexes between morin (M) and β-cyclodextrin (βCD), 2-hydroxypropyl-β-cyclodextrin (HPβCD) and Heptakis (2,6-*O*-di methyl) β-cyclodextrin (DMβCD) such as aqueous solubility and the association constants of this complex have been determined. The water solubility of morin was increased by inclusion with cyclodextrins. The phase-solubility diagrams drawn from UV spectral measurements are of the A_L-type. Also $ORAC_{FL}$ studies were done. An increase in the antioxidant reactivity is observed when morin form inclusion complex with the three cyclodextrin studied. Finally, thermodynamics studies of cyclodextrin complexes indicated that for DMβCD the inclusion is primarily enthalpy-driven process meanwhile βCD and HPβCD are entropy-driven processes. This is corroborated by the different inclusion geometries obtained by 2D-NMR.

Keywords: Morin; Cyclodextrin; Reactivity; ROESY

1. Introduction

Flavonoids have recently attracted great interest as potential therapeutic agents against a variety of diseases, most by involving radical damage. These polyphenolic compounds, ubiquitous in higher plants, are commonly major dietary constituents. The biological and medicinal properties of flavonoids have been reviewed extensively, with wealth of data on their activity as reducing agents, hydrogen-donating antioxidants and singlet oxygen quenchers; in some cases metal chelating has been proposed [1–5]. Morin (2′,3,4′,5,7-pentahydroxyflavone) is a flavonoid widely distributed in tea, coffee, cereal grains and a variety of fruits and vegetables [6] (Scheme 1), and has two aromatic rings (A and B) linked by an oxygen-containing heterocyclic (ring C). Abundant in the human diet, morin, with potent antioxidant and metal ion chelating capacities, possesses various biological and biochemical effects including anti-inflammatory, anti-neoplastic, and cardioprotective activities [7,8]. They have aroused considerable interest due to their broad pharmacological activity, but morin is sparingly soluble in water, which limits its absorption in oral administration.

In pharmaceutical product development, β-cyclodextrins (Scheme 1), a category of pharmaceutical excipients, have been widely used to improve solubilities, chemical stabilities and bioavailabilities of a number of poorly soluble compounds.

Cyclodextrins (CDs) are cyclic oligosaccharides composed of glucopyranose units and can be represented as a truncated cone structure with a hydrophobic cavity [9]. The cavity is relatively hydrophobic, while the external faces are hydrophilic [10]. The most extraordinary characteristic of a cyclodextrin is its ability to form inclusion complexes with a variety of compounds, i.e., by trapping foreign molecules (guest) in its cavity (host). Generally, hydrophobic molecules or those with hydrophobic residues have the highest affinity with the CD cavity in aqueous solution, and it is well established that the ability of β-cyclodextrin to enhance drug stability and sol-

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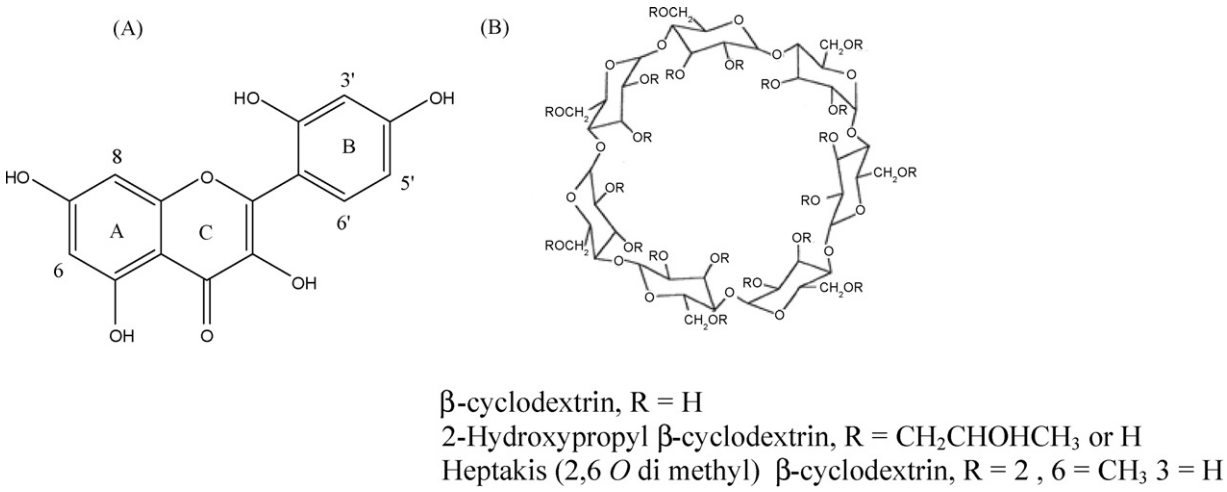

Scheme 1. (A) Structures of morin. (B) Structures of β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin, and Heptakis-2,6-*O*-di methyl-β-cyclodextrin.

ubility depends on formation of inclusion complexes [11]. Unmodified or unsubstituted β-cyclodextrins, i.e., those with no substituent on the glucopyranose unit, have poor water solubility and are parenterally unsafe due to nephrotoxicity. Therefore, several synthetically modified and relatively safe βCD have been made and used in parenteral formulations, such as hydroxypropyl-β-cyclodextrin [12] (HPβCD) and Heptakis-2,6-*O*-di methyl-β-cyclodextrin (DMβCD).

We recently reported a study of quercetin with a number of cyclodextrins and with antioxidant measurements. The results indicated that the complexes formed maintained the quercetin antioxidant activity [13].

Here, we report the preparation of inclusion complexes of morin with three different cyclodextrins, (HPβCD, DMβCD and βCD) in order to improve the aqueous solubility of the drug. Thermodynamic parameters, from van't Hoff plots, were analyzed in order to gain information about the association mechanism. In relating the thermodynamic parameters with the inclusion geometries, we have also examined 2D-ROESY a NMR spectra of the inclusion complexes. We also report the effect of complexation on antioxidant capacity.

2. Experimental

2.1. Apparatus

Spectrophotometric measurements were carried out with a UV_2 UNICAM spectrophotometer, using a 1 cm quartz cell.

A luminescence spectrometer LS 50B (PerkinElmer, Boston, MA, USA), a heating circulator bath DC1–B3 (Haake Fisons, Karlsruhe, Germany) and quartz cuvettes were used for the $ORAC_{FL}$ assay.

NMR spectra were recorded at 300 K on a Bruker Avance DRX spectrometer 300 MHz for ^1H, in unbuffered D_2O.

2.2. Materials

Morin (3,2′,4′,5,7-pentahydroxyflavone), was purchased from Sigma (USA).

βCD (β-cyclodextrin), DMβCD (Heptakis-2,6-*O*-di methyl-β-cyclodextrin), HPβCD (2 Hydroxypropyl-β-cyclodextrin) [M.S. (average molar degree of substitution) = 1.0] AAPH (2,2′-azobis(2-methylpropionamidine) dihydrochloride), FL (Fluorescein disodium salt) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), were from Sigma-Aldrich, Inc., St. Louis, MO. All solvents used in spectrophotometric analyses were of spectroscopic reagent grade, Merck.

2.3. Method

2.3.1. Phase-solubility measurements

Phase-solubility measurements were carried out by the method of Higuchi and Connors [14]. Excess amount of morin (5 mg) was added to 5 mL of deionized water containing increasing amounts of βCD, HPβCD and DMβCD (from 0 to 0.010 M). The resulting mixture was equilibrated in a Julabo thermostatic shaking water bath for 24 h at variable temperature (293, 298, and 303 K) until equilibrium was reached. To minimize photochemical degradation, the flasks were covered with aluminium foil. Suspensions were filtered through 0.45 μm cellulose acetate membrane filters to remove undissolved solid. An aliquot from each vial was diluted and analyzed spectrophometrically at 366 nm. Cyclodextrin did not interfere in the spectrophotometric assay of morin.

The apparent stability constants (K_a) of the complexes were calculated from the phase-solubility diagrams according to the following equation:

$$K_a = \frac{\text{slope}}{S_0(1 - \text{slope})} \quad (1)$$

where S_0 is the solubility of morin at 303 K in the absence of cyclodextrin and slope means the corresponding slope of the phase-solubility diagrams, i.e., the slope of the drug molar concentration versus CDs molar concentration graph. The experiment was carried out in triplicate at each temperature.

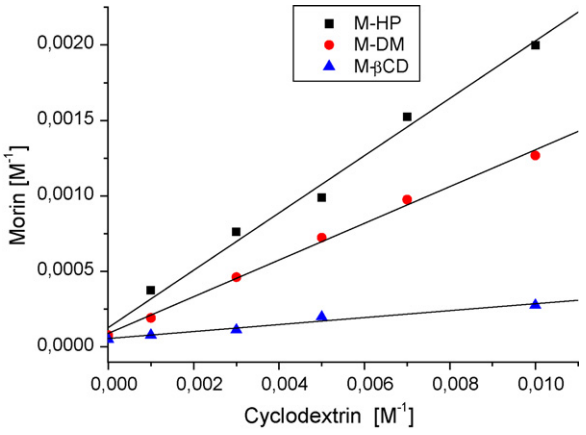

Fig. 1. Phase-solubility diagrams of M-βCD, M-HPβCD and M-DMβCD system in water at 303 K.

2.3.2. NMR

One-dimensional ^{1}H NMR spectra were recorded at 300 K on a Bruker Avance 300 operating at a proton NMR frequency of 300.13 MHz using a 5 mm probe and a simple pulse-acquire sequence. Acquisition parameters were as follows: spectral width 3000 Hz, acquisition time 2.67 s and a relaxation delay 1 s with 128 scans. FIDs were Fourier transformed with LB = 0.3 Hz and GB = 0. The signal at 4.7 ppm of HOD was used as a internal reference. Sample solutions were prepared by dissolving morin and CDs in 1000 μL D_2O in order to obtain the final concentration of 0.5 mM (complexes in a 1:1 molar ratio).

Rotating-frame Overhauser Effect SpectroscopY (ROESY) spectra were acquired in the phase sensitive mode with the same spectrometer and Bruker standard parameters (pulse program roesyph). Each spectrum consisted of a matrix of 16 K (F2) by 8 K (F1) points covering a spectral width of 3000 Hz. Spectra were obtained from sample solutions prepared for the ^{1}H NMR studies, with a spin-lock mixing time of 400 ms, relaxation delay 2 s, and 32 scans were recorded.

2.3.3. $ORAC_{FL}$ assay

The aqueous oxygen radical absorbent capacity (ORAC) assay was based on the original method [15], with modifications by Ou et al. [16] with fluorescein (FL) as the substrate and AAPH as the oxidant generator. Cyclodextrin complexes 15, 30, 45, 60 μL; 0.5–2.0 μM final concentrations and FL solutions were placed in the quartz cuvette. The mixture was preincubated for 30 s at 60 °C. AAPH solution was added rapidly with a single channel pipette. The cuvette was immediately placed in the luminescence spectrometer and the fluorescence recorded every minute for 12 min. As a blank FL + AAPH in phosphate buffer, instead of the studied compounds, were employed and eight calibration solutions with Trolox (1–8 μM, final concentration) as antioxidant positive control were also carried out in each assay. $ORAC_{FL}$ values were expressed as Trolox equivalents by using the standard curve calculated for each assay.

Table 1
Apparent stability constants (K_a) of morin inclusion complexes with the different cyclodextrins and temperatures

Temperature	K_a (M^{-1})		
	M-βCD	M-HPβCD	M-DMβCD
293 K	200	1180	2090
298 K	396	1480	1700
303 K	500	1830	1400

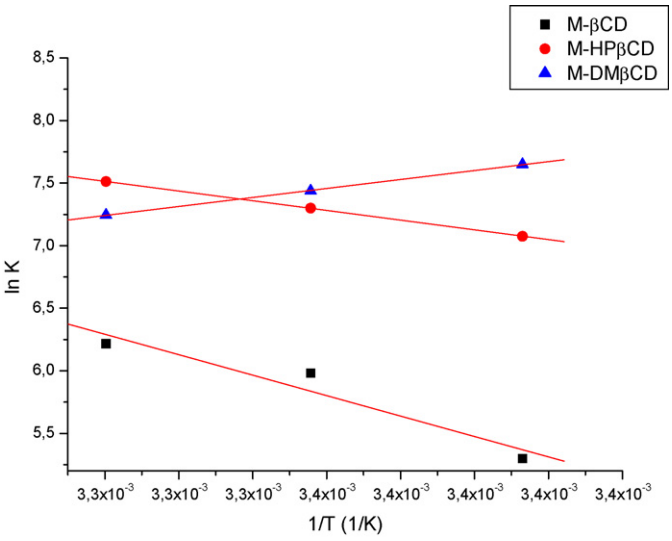

Fig. 2. van't Hoff plots (ln K vs $1/T$) for morin cyclodextrin association, determined by solubility diagrams experiments.

Table 2
Thermodynamic values for complexation of morin with cyclodextrins

	M-βCD	M-HPβCD	M-DMβCD
ΔH (kJ mol^{-1})	67.96	32.20	−29.82
ΔS (kJ mol^{-1} K^{-1})	0.28	0.17	−0.038
ΔG (kJ mol^{-1})	−14.46	−18.08	−18.48

3. Results and discussion

Stoichiometric ratios and stability constants describing the extent of formation of the complexes were obtained by mon-

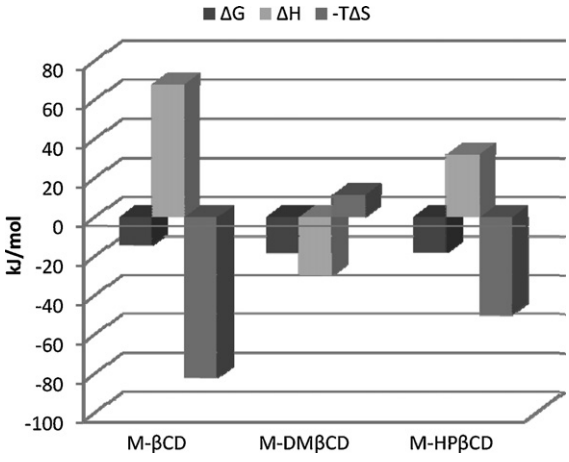

Fig. 3. Changes in free energy (ΔG°), enthalpy (ΔH°) and entropy ($-T\Delta S^\circ$) for inclusion complexation of morin with βCD, DMβCD and HPβCD at 298 K.

itoring the UV–vis absorbance of morin, in the presence of increasing concentrations of cyclodextrins. The phase-solubility diagram is a widely accepted method for evaluation of the effect of CD complexation on drug solubility [17]. The 1:1 drug/cyclodextrin complex is typical of association where a single drug molecule is in the cavity of a cyclodextrin, with a stability constant K_a for the equilibrium between the free and associated species. Fig. 1 presents the phase-solubility diagrams of morin with βCD, HPβCD and DMβCD at 293 K. The solubility of morin increased linearly as a function of CD concentration, a feature of A_L-type complexes, showing formation of a water-soluble complex, although slopes lower than unity can be indicative of 1:1 stoichiometry. These studies were carried out at three temperatures to calculate stability constants, K_a, and thermodynamic values for the complexes. The stability constants K_a, of the complexes at 293, 298, 303 K were calculated from the slopes of the linear phase-solubility diagram, and results are summarized in Table 1 are in agreement with those obtained by Calabrò et al. [18]. They formed inclusion complexes of some hydroxyflavones, including morin, with βCD, and the association constant obtained at 25 °C by using phase solubility was 330.95 M^{-1}, in total agreement with our value of 396 M^{-1}.

As shown in Table 1, the stability constants K_a, for the M-DMβCD complex decrease with increasing temperature, as expected for an exothermic process. Similar temperature effects on the stability constants were found by Tommasini et al. [19] and by Rajewski et al. [20]. However, for the other complexes, βCD and HPβCD, stability constants increase as temperature rises.

The integrated form of the van't Hoff equation (Eq. (2)) permits calculation of enthalpy and entropy changes, from variations of the stability constants with temperature [21].

$$\ln K_a = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (2)$$

The van't Hoff plots for the complexes are linear (Fig. 2). Standard formation enthalpies (ΔH) of the host–guest inclusion compounds are in Table 2. These values are positive for M-βCD and M-HPβCD indicating that formation of host–guest inclusion complexes is endothermic, while for M-DMβCD the negative standard formation enthalpies indicate that for this complex the process is exothermic. The negative values of standard Gibbs energy change (ΔG), given by enthalpy changes and entropy changes, indicate the spontaneous formation of host–guest inclusion complexes in aqueous solution.

The formation of an inclusion complex with cyclodextrin is classically caused by interactions such as hydrogen bonding with the OH groups at the periphery of the cavity, van der Waals interactions and hydrophobic effects [22]. Generally, solute inclusion in the cyclodextrin cavity is associated with large negative values of ΔH and ΔS values are either negative or slightly positive, indicating inclusion complexation of the guest without extensive desolvation, indicating that these inclusion reactions are

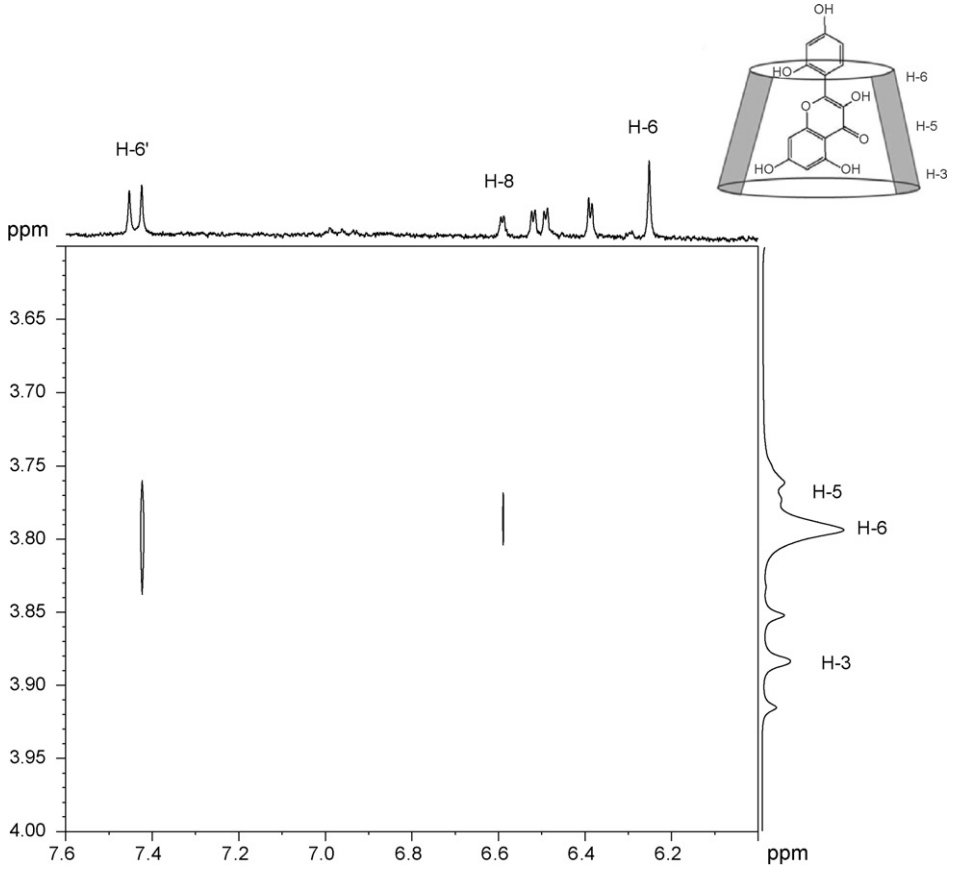

Fig. 4. Partial contour plot of the two-dimensional ROESY spectrum of morin in the presence of βCD in D_2O.

primarily enthalpy-driven processes [23]. As shown in Fig. 3, only the M-DMβCD complex has this behavior. The enthalpic term for morin complexation by DMβCD indicates that the binding forces include strong van der Waals-London dispersion interactions, associated with a negative value of ΔS, related to the apparent low degrees of freedom of the solute in the rigid cyclodextrin cavity. However, for a morin guest in βCD and HPβCD, results are different. Apparently, when morin is free in solution, it seems to have a strong interaction with the solvent shell. Upon binding, this solvent shell is broken up, leading to the partly unfavourable enthalpic change. Furthermore, the inclusion complexation involves desolvation of both morin and cyclodextrin, which takes place when morin penetrate totally inside the CD cavity [24]. On the other hand, the inclusion complexation of morin in βCD and HPβCD gave positive enthalpic changes, indicating that these inclusion reactions of morin are mainly entropically driven.

^{1}H NMR spectroscopy is an effective method for studying spatial conformations of cyclodextrin inclusions. Two-dimensional (2D) NMR is a powerful tool for investigating inter- and intra-molecular interaction. The presence of NOE cross-peaks between protons from two species indicates spatial contacts within 0.4 nm. To gain more conformational information, we used 2D ROESY to study the inclusion complexes.

Fig. 4 shows a partial contour plot of 2D-ROESY spectra of the inclusion complex of morin and βCD. There are two intermolecular cross-peaks, the first one between H-6′ of morin with H-6 of βCD and the second one between H-8 of the A-ring with H-5 of βCD indicating that morin is inserted in the cyclodextrin cavity with the A-ring oriented towards the secondary hydroxyl group and the B-ring oriented towards the primary hydroxyl group. Fig. 5 shows a partial 2D-ROESY contour plot of the M-HPβCD complex. In order to assign unambiguously H-3, H-5 and H-6 of the 2-hydroxypropyl-β-cyclodextrin region, an HSQC spectrum of the M-HPβCD system was obtained in the conditions used for the ROESY spectrum (data not shown). The ROESY spectrum of the M-HPβCD complex shows correlation between H-8 of the A-ring of morin with H-5 and H-6 of the cyclodextrin, indicating that the entire chromene is included in the HPβCD cavity and the B-ring protrudes towards the primary hydroxyl group. Fig. 6 shows the partial contour plot of the ROESY spectra of the complex M-DMβCD. We observe dipolar interaction between H-8 of the A-ring with H-3 of DMβCD clearly indicating a different form of inclusion. The chromene is inserted in the CD cavity and the B-ring is oriented towards the secondary rim. This observed different form of inclusion could be related to the driving forces of the complexes indicating that DMβCD offers a different microenvironment for guest addition.

The $ORAC_{FL}$ assay expresses antioxidant activity relative to a standard (Trolox) while measuring the oxidation of the fluorescent substrate by peroxy radicals generated during the reaction. This method follows a hydrogen atom transfer path-

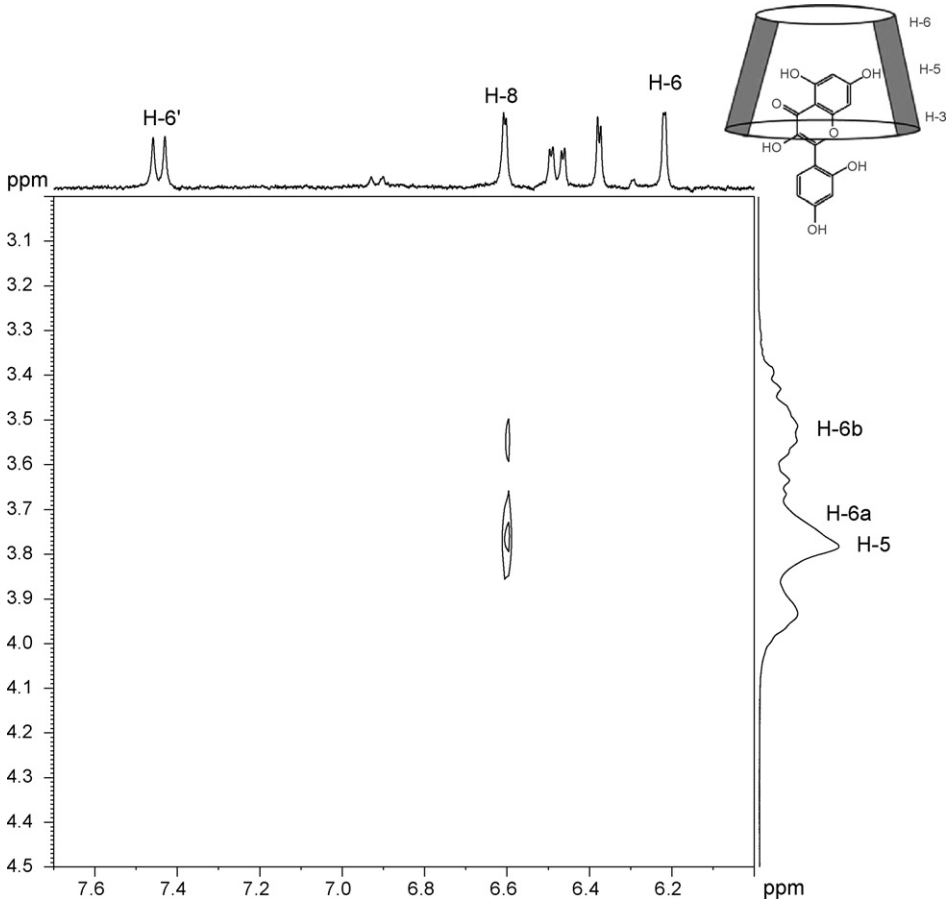

Fig. 5. Partial contour plot of the two-dimensional ROESY spectrum of morin in the presence of HPβCD in D_2O.

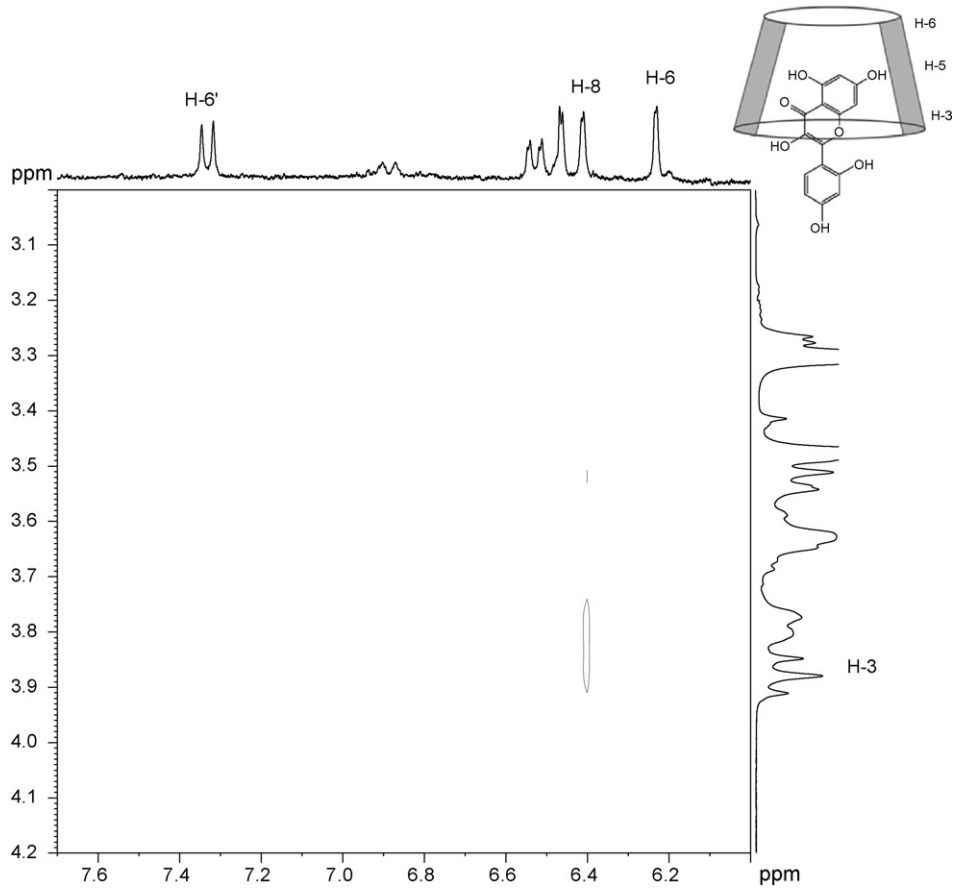

Fig. 6. Partial contour plot of the two-dimensional ROESY spectrum of morin in the presence of DMβCD in D_2O.

way, where the antioxidant and a peroxy radical form a stable antioxidant radical that breaks the radical chain oxidation. Fig. 7 shows that for the three morin complexes the Trolox equivalents are higher than for free morin. The complexes behave as better antioxidants than morin alone. This increment in the antioxidant activity is more prominent in DMβCD than in the other complexes. Alvarez-Parrilla et al. [25] reported a slight increment, less than 10%, when quercetin (an isomer of morin) was included in βCD. They attributed the increment to a modification in the redox behavior of the polyphenols. Our result showed an increase of 100% for M-βCD and M-HPβCD, and about 130% for M-DMβCD. These increases could be related to the different inclusion geometries for these complexes. These strong enhancements of the antioxidant activity may be due to effective stabilization of radical species in the cyclodextrin cavity.

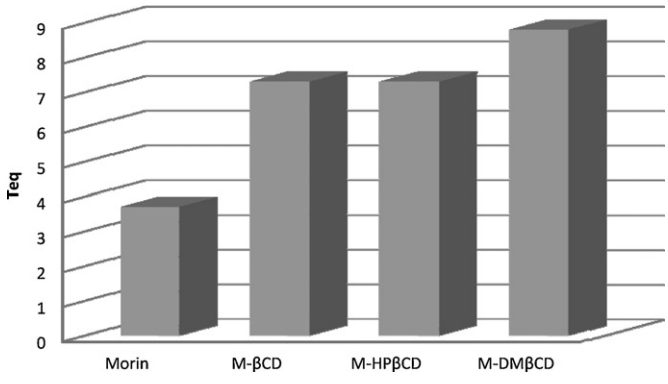

Fig. 7. Trolox equivalent for free morin and in the presence of the cyclodextrins.

4. Conclusions

The effect of β-cyclodextrin, 2-hydroxypropyl-β-cyclodextrin and 2,6-*O*-di methyl-β-cyclodextrin on the UV–vis absorption spectrum of morin was studied in water. There was cyclodextrin complexation with an increase in the solubility. Concentrations of the complexed substrates were obtained from UV–vis intensity. Plotting these intensities as a function of total CD concentration, gave the stability constants from slopes of the linear plots. The phase solubility studies demonstrated the formation of 1:1 stoichiometric complexes of morin with the CD, at each temperature. Stability constants K_a were evaluated and showed an increasing complex stability with the increasing temperature for M-βCD and M-HPβCD, indicating an endothermic and spontaneous process of association. However, behavior was opposite for M-DMβCD, indicating that here complexation is primarily enthalpically driven while βCD and HPβCD provide an entropically driven processes. This result may be related to a best fit of morin in the CD cavity, in agreement with different modes of inclusion obtained by NMR spectroscopy.

The complexation of morin with different cyclodextrins is of great interest, because complexation increases solubility and antioxidant capacity. This result is of special practical interest in the pharmaceutical field, because formulations with higher drug concentrations in solution should provide improved therapeutic options.

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