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Diffusion Evidence for a Stepwise Association Between Alkyl *p*-Hydroxybenzoates and β-Cyclodextrin Forming 1 : 2 Complex

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Abstract. The incorporation of a homologous series of alkyl *p*-hydroxybenzoates into β-cyclodextrin was studied by diffusion measurements. In some cases, all the anionic species and the larger neutral solute, butyl *p*-hydroxybenzoate, the occurrence of two species with different diffusion coefficients was observed, as CD concentration increased. Based on their derived hydrodynamic radii, these species were ascribed to 1 : 1 and 1 : 2 (solute:CD) complexes. The interaction of the neutral, mono and bianionic species of *p*-hydroxybenzoic acid with β-CD was also investigated, but, in this case, only 1 : 1 complexes were observed. The strength of solute-CD interaction was estimated from the observed decrease in diffusion coefficients at higher CD concentrations and no significant effect was verified within the homologous series that could be ascribed to hydrophobic effects. A smaller extent of interaction was estimated for the anionic species, possibly due to the contribution from electrostatic repulsion with ionised CD hydroxyl groups. These results prove the potential of diffusion measurements as an invaluable tool in assessing complex stoichiometry and in providing insights in the incorporation process.

Key words: β-CD complex, stoichiometry, diffusion measurement, alkyl *p*-hydroxybenzoates.

1. Introduction

The study of inclusion complexes involving cyclodextrins (CD) has become a major area in both basic and applied research due to their wide range of applications in pharmacy and food science, ranging from controlled drug delivery, solubilisation, or protection against harmful chemical environments [1], besides their fascinating properties connected to molecular recognition [2].

Many different types of molecules are known to be incorporated by cyclodextrins, with different modes of incorporation and complex stoichiometries. For studies dealing with the solution behaviour of such complexes, regardless of the technique employed in such studies, the stoichometry discrimination is performed by fitting the experimental data to previously built models, usually leading to a mixture of different complexes. Some examples of such approach can be found

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in the literature using, for instance, calorimetry [3, 4], NMR techniques [5], potentiometry [6], voltametry [7], electronic spectroscopy [8] and surface tension measurements [9]. Connors has recently presented a thorough review on stability constants [10] for the incorporation of a wide range of molecules by CD, where more details on the incorporation processes are discussed.

Besides the above mentioned experimental techniques, diffusion measurements have recently been used to investigate solute incorporation by cyclodextrins. Vitagliano and coworkers have presented a series of investigations using interferometric techniques to examine the incorporation of amino acids and urea derivatives by α-CD [11], being able to determine a series of stability constants. In addition, the same group has reported measurements of the diffusion coefficients and molar volumes for α and β-CD [12]. Cohen et al. [13] have used pulsed NMR to study γ-CD and macrocycles (crown ethers).

Electrochemical techniques were employed by Manzanares and coworkers [14] to examine the incorporation of vitamin C by β-CD. Raj and Ramaraj [15] also reported studies on the electrochemical behaviour of a phenothiazine dye in β-CD solution. The anomalous behaviour observed for the dye diffusion coefficients was attributed to the occurrence of 1 : 1 and 1 : 2 complexes, although no direct evidence was shown.

When a solute diffuses in a macromolecular solution, its observed diffusion coefficient, D_{obs}, represents an average between those of free, D_w, and bound, D_{complex} , solute, according to

$$D_{\text{obs}} = f D_{\text{complex}} + (1 - f) D_w, \tag{1}$$

where f, is the fraction of incorporated solute. Hence, the measurement of the solute diffusion coefficient in the presence of a macromolecule allows the examination of the extent of interaction. This equation assumes solute independent diffusion and incorporation and, therefore, is valid only when its incorporation is much faster than its diffusion [16]. It has been extensively used to investigate solute interaction with a variety of micelle systems [17–19], providing valuable information on binding constants and other features of the incorporation process.

We report here on the application of this approach, measuring diffusion coefficients for the study of the incorporation of a series of alkyl p-hydroxybenzoates (from methyl to butyl) into β-CD. These solutes, commonly known as parabens, are widely used as preservatives in food and cosmetics products. Their association with other macromolecular systems, including polymer aggregates [18] and micelles [20], have been already studied, revealing a strong hydrophobic character for these interactions, attributed to the removal of the solute alkyl chain from water to a less polar environment. Therefore, the present study aims at the complementary investigation of alkyl p-hydroxybenzoate association with cyclodextrin, another important class of macromolecular hosts.

2. Experimental

The solutes, methyl (Aldrich), ethyl (Knoch-Light), propyl (BDH) and butyl *p*-hydroxybenzoates (Apin) were of the best analytical grade available and were used as received. A reverse-phase HPLC analysis confirmed their purity. *p*-Hydroxybenzoic acid, p.a., was obtained from Aldrich. β-cyclodextrin was purchased from Sigma and their solution concentrations were corrected by assuming a hexahydrate compound [4]. All the solutions were prepared with MilliQ-Plus grade water .

The experiments dealing with the anionic species of the alkyl *p*-hydroxybenzoates were performed in 0.025 mol L^{-1} borate buffer solutions, at pH = 11, as previously [20, 21]. *p*-Hydroxybenzoic acid was investigated at three different pH: in its neutral form, with a HCl/KCl buffer, at pH 2.2; as the mono-anionic species with phosphate buffer at pH 6.7 and as the bi-anionic species, with borate buffer at pH 11. The presence of the different species was confirmed by shifts in the UV absorption spectra, in agreement with their literature pKa values [22]. At pH 11, a fraction of β-CD hydroxyl groups is ionised, estimated as 6%, from its literature pKa = 12.2 [10].

Solute diffusion coefficients were determined by using the Taylor dispersion technique. It is based on the analysis of the gaussian-like profile caused by the dispersion of a solute pulse in a solvent flow along a long and narrow tubing [21, 23]. The diffusion coefficient is calculated from the parameters of the recorded solute dispersion curve, according to

$$D = \frac{r_i^2 t_R}{24\sigma^2}, \tag{2}$$

where r_i is the tubing internal radius, t_R, is the solute retention time and σ is the variance of the dispersion curve.

The equipment used was similar to the one previously described [21], consisting of a peristaltic pump operating in a 1.5 mL/h flow, a HPLC injector with a 20 μL loop, a 10 m stainless steel tubing, encased in a temperature controlled environment, at 298 K, and a UV/VIS detector (CECIL spectrophotometer 1020S) interfaced to a computer for data acquisition and analysis. The solute concentration profile was detected at 256 nm, where no contribution from CD absorption is verified. Diffusion coefficients of α and γ-cyclodextrins were determined using the UV detector at 210 nm.

The solute sample dissolved in CD aqueous solution was injected into a CD solution of the same concentration, so that the only concentration gradient was due to the solute. Solute injected concentrations varied from 1 to 0.05 mmol L^{-1}, respectively, for solutions of methyl and butyl *p*-hydroxybenzoates, and CD concentrations were in the range of 10^{-5} to 10^{-3} mol L^{-1}. During the experiment, the solute band is diluted as it disperses through the tubing. At the detector, this solution band bears a Gaussian profile, with solute concentrations ranging from a

maximum, in the middle of the solute band, to zero, at its borders. It is possible to estimate a minimum dilution factor by using the ratio of maximum absorbance of the solute dispersion curve at the detector to the absorbance of the injected solution. According to this procedure, in our experimental setup, this minimum dilution factor is approximately 65 times. Therefore, the solute/CD concentration ratio expressed throughout refers to the injected solution but, in order to reinforce that the concentration ratio changes during the experiment, estimates of minimum concentration ratios at the detector were also added to the figures.

Diffusion measurements in solutions of interacting species should consider that flows of components 1 and 2 are interconnected due to cross-term diffusion coefficients. A thorough description of these terms and their implications on diffusion/binding processes can be found in the literature (see, for instance, references [24] and [25]), with examples that allow the evaluation of binding constants through a more comprehensive fitting procedure. However, as in our experiments there is no gradient of CD concentration, the host/solute concentration ratio is large and we are not dealing with CD diffusion, since our detector is unable to follow the cyclodextrin, we found it reasonable to neglect contributions of the cross coefficients and assume the determined diffusion coefficient to be the true solute diffusion coefficient.

This apparatus was assembled in accordance with the theoretical requirements for the application of this technique [23]. The internal radius of the dispersion tubing was calibrated as $r = 0.605$ mm, by accurately weighing a piece of tubing filled with water. This value was confirmed by measuring the diffusion coefficients of caffeine [26], toluene [27] and benzoic acid [28] in water. The reproducibility of the experimental data was better than 3% and no sign of solute adsorption onto the tubing walls [21] was detected. All the data shown represent the average of, at least, three measurements.

3. Results and Discussion

The measured diffusion coefficients, for neutral and anionic species of the methyl to butyl *p*-hydroxybenzoates in aqueous β-CD solutions, at 298 K, are shown in Figures 1 and 2, respectively. The decreases of the observed solute diffusion coefficients as CD concentration increases confirm its incorporation into the large macromolecule, as expected due to the reduced average diffusivity of the incorporated solute, as described by Equation (1). Moreover, some curves present an inflexion point allowing the discrimination of two plateau regions. Constant diffusion coefficients are expected to appear in such experiments when all the solute is incorporated by the host, leading to the determination of the complex diffusion coefficient. However, in some cases, this inflexion point and the observation of a second plateau region suggest the appearance of another complex species, in agreement with a stepwise association to form 1 : 2 complexes.

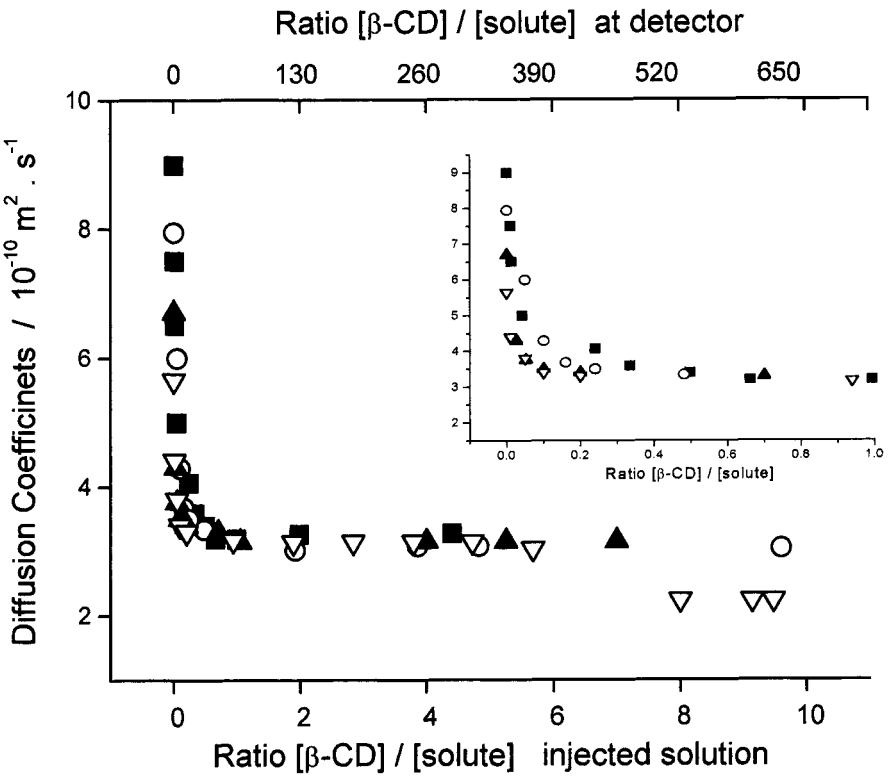

Figure 1. Diffusion coefficients of alkyl *p*-hydroxybenzoates at increasing β-cyclodextrin concentration: (■) methyl, (○) ethyl, (▲) propyl and (▽) butyl. The concentration ratios indicate the injected samples and the minimum ratio at the detector (see text).

No change in the solution viscosity was verified within the studied range of solute and cyclodextrin concentrations, which may cause such changes in diffusion coefficients. Also, the possibility of solute aggregation may be ruled out since the injection of solutions with different concentrations, for all the homologues, produces the same diffusion coefficients. In addition, these values conform to the expected behaviour of an homologous series according to the Stokes–Einstein Equation [21]. Moreover, a similar behaviour, with inflexion points and multiple plateau regions, was verified in diffusion studies on the incorporation of a drug, violacein, by β-CD [29]. In that case, stepwise association was simultaneously confirmed by circular dichroism measurements, supporting the connection between the shape of these curves and stepwise association processes.

One striking point about the curves shown in Figures 1 and 2 is that a consecutive association process produces, in some cases (as, for instance, for the neutral butyl *p*-hydroxybenzoate), two discrete behaviours, ascribed to the two plateaux. It would be possible fitting these experimental data according to derivations from Equation (1), accounting for the stepwise association. Such an approach, however,

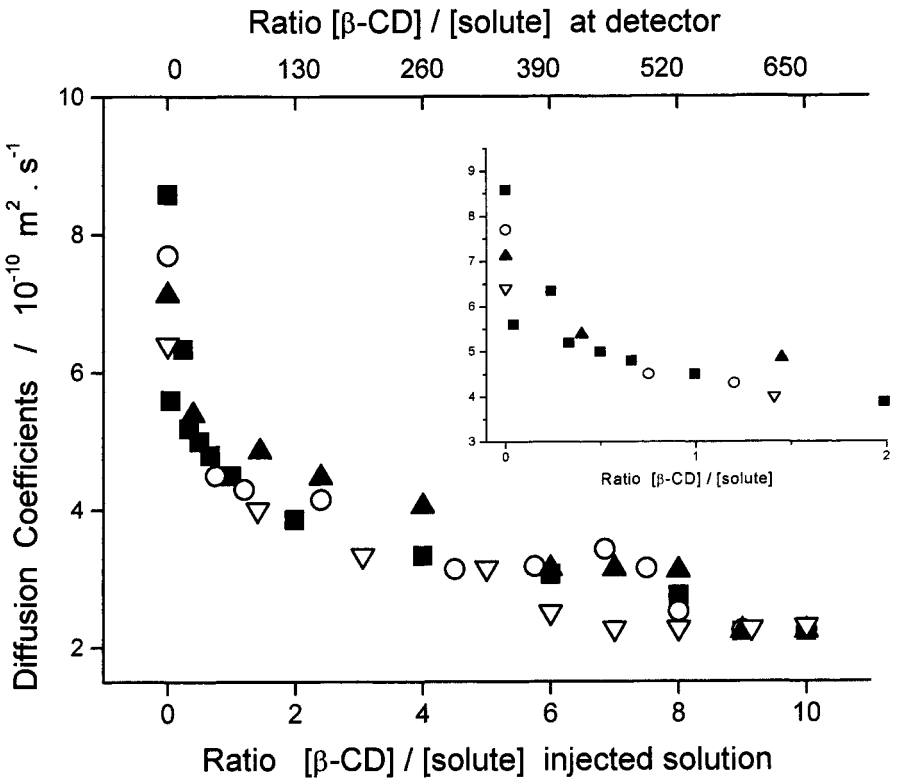

Figure 2. Diffusion coefficients of anionic alkyl *p*-hydroxybenzoates at increasing β-cyclodextrin concentration: (■) methyl, (○) ethyl, (▲) propyl and (▽) butyl. The concentration ratios indicate the injected samples and the minimum ratio at the detector (see text).

was not able to fit our experimental data within the studied range of concentration ratios, producing only monotonic curves. Nevertheless, respecting the restrictions that $K_{11} > K_{12}$, this equation does show an inflexion point for some K_{11} and K_{12} values, although not for the studied concentration ratios. This inflexion being more pronounced as the K_{12}/K_{11} ratio decreases, as expected due to the competition between the two equilibria. Similar behaviour is observed when comparing Figures 1 and 2, where the plateau regions are more pronounced for the neutral species than for the anionic ones. This observation suggests that the K_{12}/K_{11} ratios are smaller for the anions.

This apparent disagreement between experimental diffusion data and the approach described by Equation (1) arises from peculiarities from the Taylor dispersion technique. As previously discussed, this technique is based on following the solute dispersion over a long time interval. In this experiment, the solute band within the dispersion tubing presents a time-dependent concentration profile, which resembles a Gaussian one at the detector. Therefore, any concentration ratio would

not only change in time during the experiment but also depend on the position within the dispersion tube. For this reason, the values expressed in Figures 1 and 2 should be only taken as estimates of limit values for concentration ratios, and not as true descriptions of the concentration profiles. Moreover, the observed diffusion coefficients and the effect of binding upon them accounts for an average of all the processes occurring from injection until reaching the detector.

The complexity of the dispersion process and of its effect on the binding processes are not accounted for by the assumptions leading to Equation (1) and hence, although its use has been proposed to analyse diffusion data determined by this dispersion technique [17, 19], we do not believe it is appropriate to quantitatively describe these binding equilibria [18, 31], as the impossibility of fitting this more complex data confirms. Therefore, the analysis of Equation (1) will only be restricted to derive qualitative information on the binding equilibria.

However, more evidence supports the assumption that 1 : 1 and 1 : 2 complex species are represented by these two plateau regions. These diffusion coefficients may be related to the complex sizes, through the Stokes–Einstein Equation

$$D = \frac{kT}{6\pi\eta r_h}, \tag{3}$$

where k, T and η are, respectively, the Boltzmann constant, the absolute temperature and the solution viscosity, and r_h is the hydrodynamic radius of the diffusing entity.

Care should be taken when applying the Stokes-Einstein equation in cases where the solute and the solvent possess similar sizes [32], as in the case of parabens diffusion in water. In such cases, the calculated hydrodynamic radii can only be regarded as estimates [21] and used as relative values, for the purpose of comparison. This limitation, however, is not found when describing the diffusion of larger species as cyclodextrin complexes.

The calculated hydrodynamic radii for the 1 : 1 and 1 : 2 complexes are shown in Table I. For comparison, Table II shows literature diffusion coefficients and derived hydrodynamic radii for α-, β- and γ-CD. By using our apparatus with a UV detector, we were not able to study the diffusion of β-CD, due to its lower aqueous solubility. Nevertheless, it was possible to determine the diffusion coefficients for the more soluble α- and γ-CD, also shown in Table II, which are in good agreement with the literature data. Comparing these Tables, one verifies that the diffusion coefficients for the 1 : 1 complexes, both of neutral and anionic species, are all very close and slightly smaller than the one of the macromolecule alone. For the 1 : 2 complexes, there is a decrease in diffusion coefficient of *ca*. 50% and, again, there is no significant change of hydrodynamic radii for the 1 : 2 complex within the homologous series of anions. The average hydrodynamic radii for the 1 : 1 complexes are very close to estimates of CD dimensions, allowing for molecule rotation during diffusion, and the same is verified for the 1 : 2 complex radii when compared to estimates taking into account the geometry of the β-CD molecule.

Table I. Diffusion coefficients (10^{-10} m^2. s^{-1}) and hydrodynamic radii (10^{-10} m) for the 1 : 1 and 1 : 2 complexes

Solute	$D_{1:1}$	$D_{1:2}$	$R_{h\,1:1}$	$R_{h\,1:2}$
Methylparaben	3.27	–	7.5	–
Ethylparaben	3.06	–	8.0	–
Propylparaben	3.15	–	7.8	–
Butylparaben	3.14	2.23	7.8	11.0
Methyl, anion	3.14	2.30	7.8	10.6
Ethyl, anion	3.15	2.30	7.8	10.6
Propyl, anion	3.15	2.27	7.8	10.8
Butyl, anion	3.14	2.30	7.8	10.6
p-HBZ	3.30	–	7.4	–
p-HBZ $^-$	3.25	–	7.5	–
p-HBZ^{2-}	3.30	–	7.4	–

p-HBZ = *p*-hydroxybenzoic acid, in its different ionisation states.

Table II. Diffusion coefficients for α-, β- and γ-cyclodextrins in water at 25 °C

	Diffusion coefficient/10^{-10} m^2 s^{-1}
α-CD	3.4[a], 3.57[b], 3,52[c]
β-CD	3.21[c]
γ-CD	3.09[b], 3.2[d]

[a] Data from Reference [11].
[b] This work.
[c] Data from Reference [12].
[d] Data from Reference [13].

This agreement supports the attribution of the two plateaux regions to 1 : 1 and 1 : 2 complex species.

In this sense, diffusion measurements provide a valuable tool for assessing stoichiometries of these inclusion compounds, as well as for obtaining insights on the association process. As both species have significantly different sizes, their diffusion coefficients would also differ, leading to direct evidence for their occurrence and with advantages over the traditional fitting procedures described in the Introduction.

It is interesting to notice that for methyl, ethyl and propyl *p*-hydroxybenzoates, in their neutral forms, only 1 : 1 complexes are formed within the studied CD concentration range. For these neutral species, only the larger homologue, butylparaben, is able to interact with a second CD molecule. For the anions, however, the

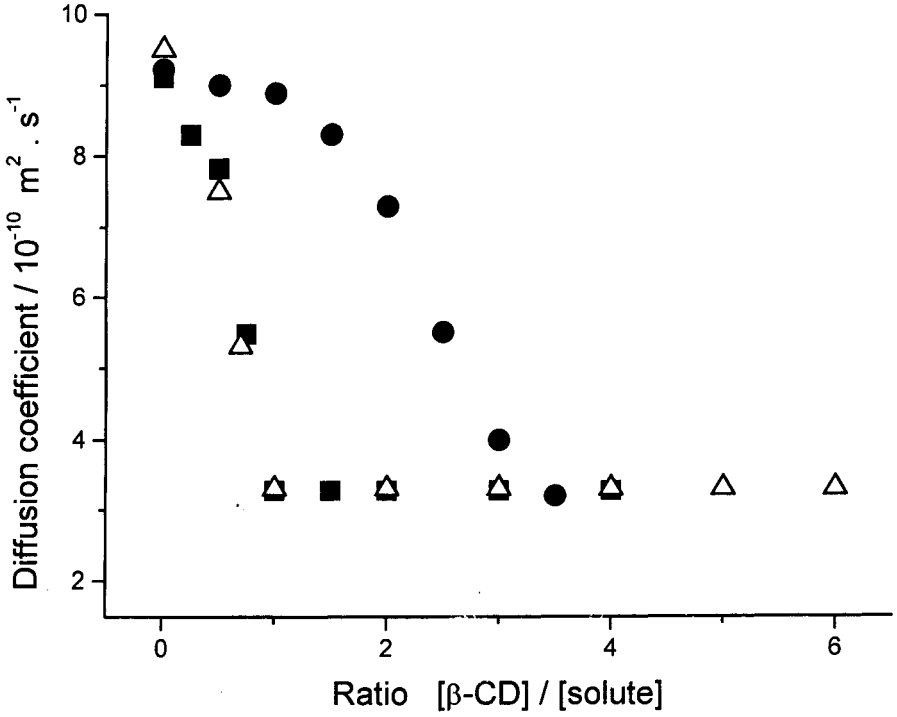

Figure 3. Diffusion coefficients of: (■) neutral (pH 2.2), (△) mono-anionic (pH = 6.7) and (■) bi-anionic (pH = 11) species of *p*-hydroxybenzoic acid, at different β-cyclodextrin concentrations. The concentration ratio indicates the injected samples, changing during the solute dispersion (see text).

presence of a negative charge seems to allow the accommodation of another CD molecule and all the anionic homologues form 1 : 2 complexes. Indeed, there is a critical size dependence, as shown by the results of the diffusion coefficients for the neutral, mono-anionic and bi-anionic species of *p*-hydroxybenzoic acid, presented in Figure 3.

For all these three species, only one plateau is observed, with a diffusion coefficient close to that previously ascribed to 1 : 1 complexes. In a similar investigation, Lin and Connors [33] verified that, for a series of 4-substituted phenols, some compounds were able to interact with a second CD molecule, although in that study this discrimination was ascribed to electronic effects, rather than to solute size.

For the case of *p*-hydroxybenzoic acid, , it is possible to notice that the extent of solute incorporation, inferred by the decrease of diffusion coefficients, varies upon the acid ionisation. The neutral and monoanionic species display similar behaviour, but there is a large decrease in the solute incorporation as the second electric charge appears due to the ionisation of the phenolic group. This behaviour is also verified when relating data of Figures 1 and 2 for the same homologue,

which differ regarding the ionisation of the phenolic group, the anions interacting less strongly with CD than the neutral esters. Changes in the solute interaction and orientation within cyclodextrin were also observed to occur with benzoic acid and benzoate, as revealed by a NMR spectroscopy study [30]. The presence of electric charges on the solute would also increase its aqueous solubility. In addition, it is important to consider that in the present experiments, at pH 11, *ca.* 6% of CD hydroxyl groups are ionised, leading to a degree of electrostatic repulsion upon the anion incorporation.

This may explain an apparent disagreement with earlier findings that suggested a stronger incorporation of related anionic compounds, when compared to their neutral species [33]. These earlier investigations interpreted the stronger anion incorporation as a reflection of increased dipole interactions with the host due to charge delocalisation in the solute molecule.

When these data are analysed within the neutral or anionic homologous series, as in Figures 1 and 2, no significant difference is observed, suggesting no major hydrophobic contribution, possibly due to the solute incorporation with its aromatic moiety inside the CD ring. From estimates based on bond lengths and angles for the parabens and for the β-CD [1], one can infer that a perfect fit of the paraben molecule inside the cavity of β-CD would leave the alkyl chain exposed to the aqueous environment. This hydrophobic moiety could interact with another β-CD molecule to form a 1 : 2 complex.

Considering the whole set of experimental evidence, one may propose that the incorporation of alkyl *p*-hydroxybenzoates and of *p*-hydroxybenzoic acid into β-CD occurs with the inclusion of their aromatic moieties inside the cavity, with the alkyl chains remaining in solution. Also, there seems to be a significant interaction of the phenolic group with the hydroxyl groups of the CD ring, which is affected upon its ionisation, possibly due to contribution from electrostatic repulsion leading to solute displacement inside the cavity, allowing its interaction with a second host molecule.

In summary, this work has proven the potential of diffusion measurements in assessing the occurrence of complexes with different stoichiometries, as well as in providing valuable information about the strength of this interaction and the mode of solute incorporation. The only restriction of the Taylor dispersion technique seems to be its incapacity of providing more quantitative data on the incorporation, in the form of binding constants. This obstacle may be overcome with the use of other techniques to determine diffusion coefficients, as, for instance, pulsed NMR techniques.

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