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Jorge Carrazana[a]; Belén Reija[a]; Pedro Ramos Cabrer[a]; Wajih Al-Soufi[a]; Mercedes Novo[a]; José Vázquez Tato[a]
[a] Departamento de Química Física, Facultad de Ciencias, Universidad de Santiago de Compostela, Lugo, Spain

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Complexation of Methyl Orange with β-cyclodextrin: Detailed Analysis and Application to Quantification of Polymer-bound Cyclodextrin

JORGE CARRAZANA, BELÉN REIJA, PEDRO RAMOS CABRER, WAJIH AL-SOUFI, MERCEDES NOVO* and JOSÉ VÁZQUEZ TATO

Departamento de Química Física, Facultad de Ciencias, Universidad de Santiago de Compostela, E-27002 Lugo, Spain

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The formation of inclusion complexes between methyl orange (MO) and β-cyclodextrin (β-CD) was studied extensively using UV–visible absorption spectroscopy. In neutral-to-basic media, where MO is an anion, two complexes of stoichiometries 1:1 and 1:2 (guest:host) are formed, as determined by principal components analysis of the absorption data. Global analysis yields precise values of the corresponding association constants and the spectra of each species: free MO, complex MO·CD and complex MO·2CD. In acid media, where MO is a zwitterion, only 1:1 complexes are formed, but the system becomes complicated due to tautomerization of MO. The *microscopic* equilibria among the two tautomers and their corresponding complexes were resolved and estimations of the *microscopic* association constants were obtained. Finally, the application of MO complexation to the quantification of bound β-CD in a polymer sample is presented, with a detailed description of the experimental conditions to be used and a critical discussion of the results obtained.

Keywords: Methyl orange; Cyclodextrins; Association equilibria; Inclusion complex

INTRODUCTION

In the last decades cyclodextrins (CDs) have attracted the attention of numerous researchers due to their widespread industrial use derived from their ability to form inclusion complexes with many organic substrates [1]. Many attempts have been made to develop new derivatives of natural cyclodextrins that show an enhanced binding capacity or can be used as building blocks for supramolecular structures [2–4].

The characterization of such derivatives usually implies quantification of their complexation potential using model guest molecules. Of these, methyl orange (MO) has become very popular since its complexation by cyclodextrins can be studied by visible absorption spectroscopy, a technique which is available in most laboratories. However, the necessary reference values for the association constants of MO with natural CDs are not consistent in the literature. Regarding complexation of MO with β-cyclodextrin (β-CD), which is the most commonly used natural cyclodextrin, there is a large discrepancy between the reported values for the association constant in neutral-to-basic media and no agreement about the complex stoichiometry [5–12]. In acid media, there are also discrepancies related to the association constant [8,13–18] and the system becomes very complex due to tautomerization of MO (see below) [19]. Attempts to resolve the *microscopic* equilibria involved are based on assumptions which must be considered with care [13–16]. Therefore, the complexation of MO with natural cyclodextrins, and in particular with β-CD, must be further investigated in order to clarify the discrepancies found in the literature and to define under which conditions MO can be used as a model guest. This is the purpose of this work, where the use of advanced methods of data analysis allows us to carry out definite determinations of the complexation stoichiometries and solid estimations of the association constants involved. The structure of the complexes formed is also discussed on the basis of their individual absorption spectra.

*Corresponding author. Fax: +34-98-228-5872. E-mail: mnovo@lugo.usc.es

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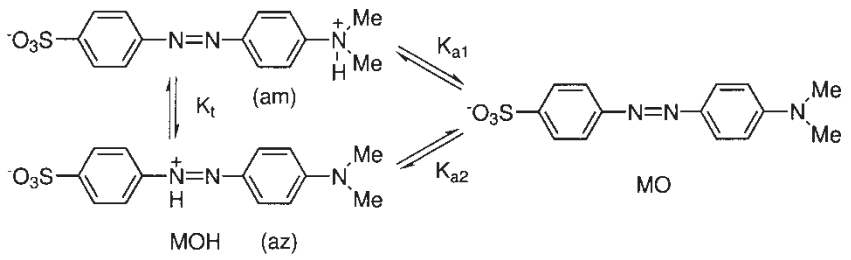


SCHEME 1 Acid–base and tautomerization equilibria of methyl orange in aqueous solution.

Finally, an analytical application of MO complexation is presented where the amount of β-CD bound to a soluble polysaccharide is quantified. The experimental procedure and data analysis are described and a critical discussion of the assumptions involved and the accuracy of the results is given.

MO is a well-known acid–base indicator, with a pK_a of 3.49 at 25°C [19]. In neutral-to-basic media, MO is a sulfonate anion, whereas in acid media (pH less than about 1.5) a zwitterion is formed due to protonation either at the azo group or at the amino group. Thus, two tautomers are present in equilibrium in such media, the azonium and the ammonium (Scheme 1). Further protonation of both tautomers leads to a highly charged molecule, with an absorption maximum at 407 nm, which predominates in very acid media (p$K_a = -6.1$) [19].

EXPERIMENTAL

Materials

Methyl orange (Panreac PA-ACS) was used without further purification. β-Cyclodextrin was kindly supplied by Roquette and recrystallized twice from distilled water and dried in a vacuum oven. Solutions at pH 1 were prepared with HCl from Panreac for analysis. A concentrated phosphate buffer for the experiments at neutral pH was prepared from Na_2HPO_4 and NaH_2PO_4 (Fluka PA), with a total phosphate concentration of 0.10 M and a pH of 6.5.

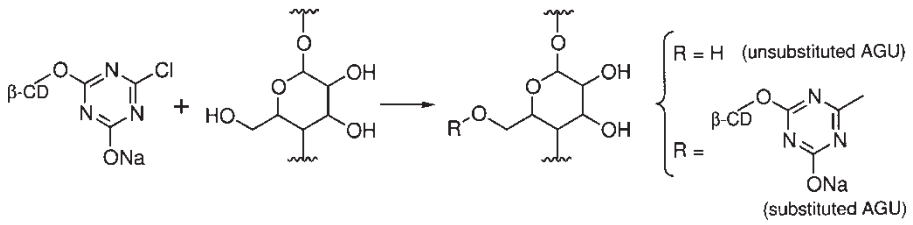
The β-CD-grafted polymer was synthesized using soluble starch purchased from Panreac and monochlorotriazinyl-β-cyclodextrin Na-salt (MCT-β-CD) from Wacker (Scheme 2). Synthesis and purification procedures are described elsewhere [20]. UV and IR absorption spectra confirmed the binding of MCT-β-CD to the polysaccharide.

Sample Preparation

To obtain reliable experimental data in titrations with β-CD it is very important to maintain a highly reproducible concentration of MO in the samples. Therefore, the following procedure was applied: a solution of MO with a suitable concentration for absorption measurements (around 5×10^{-5} mol dm^{-3}) and the desired pH was prepared, and a certain volume of this solution was used to dissolve the necessary amount of β-CD to obtain a β-CD stock with a concentration of about 11×10^{-3} mol dm^{-3}. Samples with different β-CD concentrations were then prepared in 5 ml flasks by adding the necessary amount of the MO + β-CD solution and filling to end volume with the MO solution. The same procedure without MO was followed to prepare the β-CD solutions used as reference in the absorption measurements. The desired pH values were obtained by adding the necessary amounts of 1 M HCl for the solutions at pH 1 or phosphate buffer for the neutral solutions. The final phosphate concentration in the latter solutions was 0.010 M.

Absorption Measurements

Absorption spectra were recorded using a Varian-Cary 300 spectrometer with quartz cells of 10.0 mm path. Baseline was recorded with water in both sample and reference cells. Eventual shifts of the baseline were corrected by subtraction of a constant



SCHEME 2 Synthesis route for the MCT-β-CD-grafted polymer used in this work. AGU: anhydroglucose unit.

absorbance. The important optical effect of β-CD on the absorption spectra was corrected using a β-CD solution as reference with the same concentration as the sample solution. A Haake cryostat was used to maintain a constant temperature of 25°C during the titration measurements and to perform the series with temperature variation.

For quantification of the amount of β-CD bound to the polymer chains, the absorption spectrum of an exactly measured volume ($\approx 3\,cm^3$) of MO solution buffered at pH 7 was recorded. Then, about 10 mg of β-CD-grafted polymer (depending on solubility) was added to the MO solution directly in the measuring cell and dissolved with the aid of magnetic stirring. The spectrum of this mixture was then recorded. To avoid light dispersion it is very important that the entire amount of the solid is dissolved. The total concentration of MO was about 10^{-5}mol dm^{-3} and its precise value was obtained from the absorption spectrum. Also, it was checked that the MCT-β-CD does not absorb in the working wavelength range and that the presence of ungrafted polymer has no influence on the absorption spectrum of MO with or without β-CD.

NMR Measurements

NMR titrations of MO with β-CD were performed only at neutral pH, since the solubility of MO at pH 1 is too low for this kind of experiment. Samples were prepared by mixing different amounts of equimolar stock solutions of MO and β-CD in D_2O, according to the continuous variation method. The pH was adjusted by adding the necessary amounts of KOD to the stock solutions. ^{1}H NMR spectra of those mixtures were recorded using a Bruker AC spectrometer at 300 MHz at 298.1 ± 0.1 K.

Data Analysis

The series of absorption spectra recorded in the titrations with β-CD were analysed using a self-developed procedure based on principal components analysis and global analysis (PCGA) described elsewhere [21]. This method can be applied to any series of spectra which vary with an external parameter such as the concentration of a reagent, pH, time, etc. The first step of PCGA is the principal components analysis (PCA), where the minimal number of spectral components responsible for the observed variations is obtained. This information helps to draw up a theoretical model which is used in the second step as a fit function for a nonlinear least-squares global analysis using the whole spectra as dataset. The results of this global fit are the physicochemical parameters involved in the model and the individual spectra of the components. All other fits presented in this work were performed with commercial programs.

RESULTS AND DISCUSSION

Complexation of Methyl Orange in Neutral Media

Figure 1 shows the absorption spectra of MO at neutral pH in the presence of different concentrations of β-CD. A small but systematic blue shift of the visible absorption band is observed as the concentration of β-CD is increased, leading to the absorbance-concentration profiles shown in the inset of Fig. 1. These features are attributed to the interaction between MO and β-CD to form inclusion complexes. Most of the variations observed could be explained by the formation of a 1:1 complex, but there are some facts which suggest the presence of a complex of higher stoichiometry: there are no clear isosbestic points over the whole range of β-CD concentration, as would be expected for a pure 1:1 complexation, and the absorbance-concentration profiles at wavelengths in the range from 425 to 445 nm show a pronounced increase followed by a slight decrease of absorbance with increasing β-CD concentration, a pattern that cannot correspond to a single complexation equilibrium. Nevertheless, such features can only be observed with very accurate data resulting from a highly constant MO concentration throughout the series and careful correction for the CD baseline. This may explain the discrepancies found in the literature data referred to above.

To deal with this problem an analysis procedure should be performed which allows an objective determination of the complexation stoichiometry

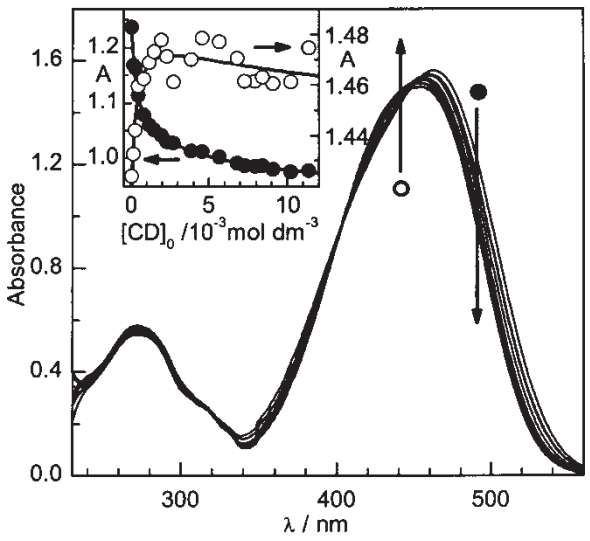


FIGURE 1 Absorption spectra of MO at pH 7 in the presence of different concentrations of β-CD, varying from 0 to 11.3×10^{-3} mol dm^{-3}. Inset: Plot of absorbances at 440 nm (open circles) and at 490 nm (filled circles) versus initial β-CD concentrations. Lines represent the fitted curves. $[MO]_0 = 6.1 \times 10^{-5}$ mol dm^{-3}.

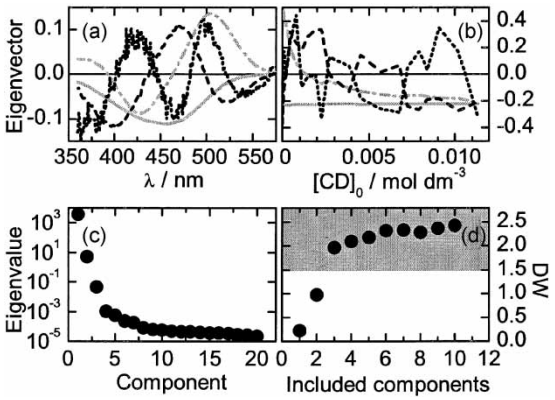


FIGURE 2 Results of PCA for the series of absorption spectra of MO at pH 7 with different β-CD concentrations: (a) the first four spectral eigenvectors in the order grey solid, grey dash-dot, black dash, black dot; (b) the first four eigenvector profiles in the same order as before; (c) logarithmic plot of eigenvalues versus number of components; (d) plot of mean Durbin-Watson test values of residual profiles versus number of included components.

involved and that provides definite values of the association constants, as does PCGA described above. Figure 2 summarizes the results of PCA applied to the series of absorption spectra in Fig. 1. Analysis was limited to the range 360–600 nm, which corresponds to the visible band, since the variations of the UV band are very small and much influenced by the correction of the β-CD baseline. The direct results of PCA are the eigenvalues and the eigenvectors, which provide statistical criteria to determine the number of linearly independent components which reproduce the systematic change in the experimental spectra. The eigenvectors can be calculated in dependence of wavelength, what we call spectral eigenvectors (Fig. 2(a)), or as functions of the external parameter, yielding the eigenvector profiles (Fig. 2(b)). The number of components corresponds to the number of eigenvectors which show systematic variations. Nevertheless, the spectral eigenvectors of absorption data usually show additional spurious components due to instrumental distortions inherent to this type of measurement [21]. Instead, such spurious components do not appear in the eigenvector profiles and hence they provide a better criterion to determine the number of components when treating absorption data. As shown in Fig. 2(b) the first three eigenvector profiles change systematically with β-CD concentration, whereas the fourth oscillates randomly. This implies three components. The logarithmic plot of the eigenvalues versus the number of components (Fig. 2(c)) leads to the same result. The first three eigenvalues are clearly much larger than the rest, which represent the instrumental error. Another powerful statistical criterion is the Durbin-Watson test based on the residual profiles (Fig. 2(d)). In this case, uncorrelated residuals (i.e. DW values above 1.5) are first obtained when three components are included.

Therefore, PCA demonstrates that at least three spectral components are necessary to account for the variations of the MO absorption spectrum with β-CD concentration. The simplest assignment of these components to chemical species is based on the formation of two complexes between MO and β-CD of stoichiometries 1:1 and 1:2, which, together with the free MO, contribute to the observed absorption spectra. This interpretation can be expressed by the following chemical equations:

$$\mathrm{MO} + \mathrm{CD} \overset{K_1}{\rightleftharpoons} \mathrm{MO \cdot CD} \tag{1}$$

$$\mathrm{MO \cdot CD} + \mathrm{CD} \overset{K_2}{\rightleftharpoons} \mathrm{MO \cdot 2CD} \tag{2}$$

where K_1 and K_2 are the association equilibrium constants for the formation of the 1:1 complex ($\mathrm{MO \cdot CD}$) and the 1:2 complex ($\mathrm{MO \cdot 2CD}$).

The next step in our analysis is to obtain a mathematical function that relates the absorbance at any wavelength (A^λ) to the total β-CD concentration on the basis of the model proposed. At any wavelength the observed absorbance is the sum of the absorbances of each of the three contributing species, which are proportional to their equilibrium concentrations. These concentrations can be obtained using the mass balances for MO and β-CD and the definitions of the association equilibrium constants. Following this procedure and under conditions of excess β-CD concentration ($[\mathrm{CD}] \simeq [\mathrm{CD}]_0$), the following equation is deduced:

$$A^\lambda = \frac{A^\lambda_{\mathrm{MO}} + A^\lambda_{\mathrm{MO \cdot CD}} K_1 [\mathrm{CD}]_0 + A^\lambda_{\mathrm{MO \cdot 2CD}} K_1 K_2 [\mathrm{CD}]_0^2}{1 + K_1 [\mathrm{CD}]_0 + K_1 K_2 [\mathrm{CD}]_0^2} \tag{3}$$

where

$$A^\lambda_{\mathrm{X}} = \varepsilon^\lambda_{\mathrm{X}} \ell [\mathrm{MOH}]_0 \tag{4}$$

is the absorbance of species X (= MO, $\mathrm{MO \cdot CD}$ and $\mathrm{MO \cdot 2CD}$) when all the MO is in this form, $\varepsilon^\lambda_{\mathrm{X}}$ being the corresponding molar absorptivity.

Using equation (3) as fit function, a nonlinear global analysis is performed using the experimental spectra as the dataset, where K_1 and K_2 and the absorbances A^λ_{MO}, $A^\lambda_{\mathrm{MO \cdot CD}}$ and $A^\lambda_{\mathrm{MO \cdot 2CD}}$ at all the wavelengths are the fit parameters. In our procedure the number of fit parameters is significantly reduced by using the eigenvectors obtained in PCA [21]. The results of the fit for the association equilibrium constants are: $K_1 = (2.97 \pm 0.05) \times 10^3 \mathrm{mol}^{-1}\,\mathrm{dm}^3$ and $K_2 = (0.10 \pm 0.01) \times 10^3 \mathrm{mol}^{-1}\,\mathrm{dm}^3$. The value of K_1 is in good agreement with some of the reported values for this constant [5,6], but it is more precise than all of them. On the contrary, the value obtained for K_2 is about six times smaller than the literature values [6,9]. The main reason for this large discrepancy is probably the assumption made in those works that the two equilibria take place in different concentration ranges

so that they can be treated independently. That is, however, not the case since the two equilibrium constants differ by only one order of magnitude. Therefore, we suggest that our value of K_2, obtained by a simultaneous fit of the two association constants and using the whole range of wavelengths as dataset, is a better estimation of the real equilibrium constant.

The other direct results of the global analysis are the individual absorption spectra of the species MO, MO·CD and MO·2CD, i.e. the absorption spectra of these species when the initial MO amount is free, complexed with one β-CD molecule or complexed with two β-CD molecules (Fig. 3(a)). The spectrum obtained for free MO coincides exactly with the experimental spectrum of MO in the absence of β-CD, and this result validates our method of analysis and the model used. The spectra of the complexes cannot be measured directly and have not yet been published. When compared with the spectrum of free MO (Fig. 3(a), solid line), they differ only in a slight shift of the visible band to higher energies. Nevertheless, a thorough analysis of these spectra makes clear their significant differences and provides information about the microenvironment of MO inside the β-CD cavity: for this, each spectrum is decomposed into two bands using spectroscopic lognormal functions [22], and the contributions of the two bands are compared (Fig. 3(b)–(d)). Reeves and coworkers [23] showed that the visible spectra of MO in different solvents and solvent mixtures can be explained by the combination of two bands centered at about 21000 cm^{-1} and 24000 cm^{-1}. In water the contribution of the low-energy band is very important, whereas the high-energy band predominates in organic solvents. The low-energy band is attributed to a $\pi \rightarrow \pi^*$ transition which is red-shifted due to hydrogen-bond interactions between water and the azo nitrogens, whose basicity is enhanced by the *p*-amino group. Our decomposition of the individual absorption spectra yields two bands analogous to those obtained by Reeves *et al.*, in different solvents. The contribution of the low-energy band is large in the spectrum of free MO (Fig. 3(b)) and decreases significantly in the spectrum of MO·CD (Fig. 3(c)) and still more in the spectrum of MO·2CD (Fig. 3(d)), whereas the contribution of the high-energy band increases. These results show that hydrogen-bond interaction between water and the azo nitrogens is hindered by the inclusion of MO into one β-CD cavity and, to a larger extent, when MO complexes with two β-CD molecules. This suggests that the β-CD cavity partially includes the azo group in the MO·CD complex, but is essentially located around one of the aryl groups. In the case of the MO·2CD complex, the non-negligible contribution of the low-energy band can be explained by incomplete inclusion of the azo group in the β-CD cavities or by hydrogen-bond interactions with water molecules present inside the cavities.

NMR data of MO-β-CD mixtures confirm the results of absorption measurements. Chemical shift displacements (Δδ), i.e. displacements of the observed chemical shift in the mixtures with respect to the pure substance, were determined for three CD protons that face the inner cavity (Fig. 4(a)). All three protons show negative displacements that are larger for the H-5 proton, which is in the middle of the cavity. These displacements reflect the existing interactions between MO and β-CD due to the complexation process. The corresponding Job plots (Fig. 4(b)) confirm the formation of both 1:1 and 1:2 complexes in the mixtures, since their maxima lie at values of the CD molar fraction between 0.5 and 0.66, which are typical for pure 1:1 and 1:2 complexes, respectively. Quantitative analysis of the observed chemical shift displacements, using

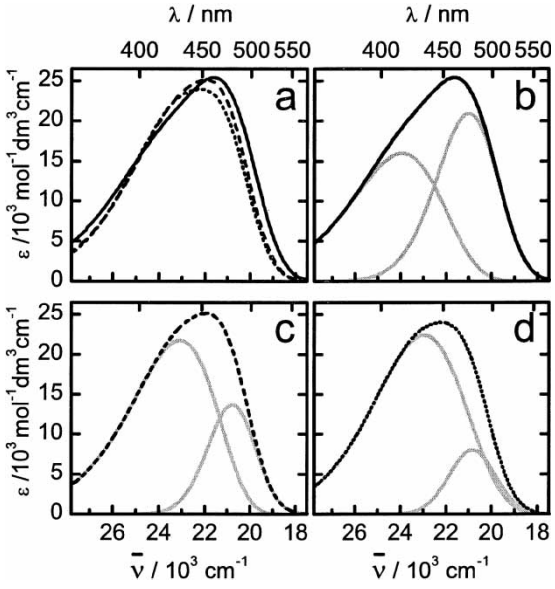


FIGURE 3 (a) Individual absorption spectra of free MO (solid line) and its inclusion complexes MO·CD (dashed line) and MO·2CD (dotted line) as obtained by global analysis of the experimental absorption spectra at pH 7. (b,c,d) Decompositions of the absorption spectra of free MO and the complexes MO·CD and MO·2CD into two contributing bands.

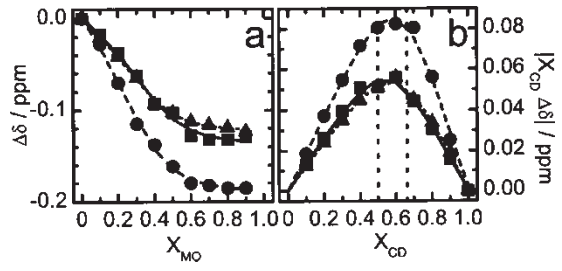


FIGURE 4 (a) Chemical shift displacements (Δδ) of β-CD protons obtained in the ^{1}H NMR titrations of MO with β -CD. Symbols are experimental data for protons H-3 (filled squares), H-5 (filled circles) and H-6 (filled triangles) and lines represent the curves resulting from the fits. (b) Job plots ($X_{CD}\Delta\delta$ versus X_{CD}) corresponding to the data in (a).

global analysis and an analytical solution of the corresponding cubic equation as described elsewhere [24], yields estimations of the association equilibrium constants for the formation of MO·CD and MO·2CD that are in agreement with the much more precise values obtained from absorption spectra: $K_1 = (4.7 \pm 3.3) \times 10^3\,\mathrm{mol^{-1}\,dm^3}$ and $K_2 = (0.37 \pm 0.43) \times 10^3\,\mathrm{mol^{-1}\,dm^3}$. The large errors in these estimations are mainly due to the lower sensitivity of NMR compared with absorption spectroscopy in combination with the strong parameter correlation. Nevertheless, satisfactory fits are also obtained fixing the values of K_1 and K_2 to those determined from absorption data, which indicates good quantitative agreement between the two types of data.

Complexation of Methyl Orange in Acid Media

The ability of MO to form inclusion complexes with β-CD was further investigated at pH 1, at which MO is protonated in aqueous solution (MOH). Figure 5 shows the variation of the MOH absorption spectrum when adding different concentrations of β-CD. The intensity of the visible band decreases strongly when increasing the β-CD concentration, whereas the UV band increases significantly. A clear isosbestic point is observed at about 430 nm, indicating a single association equilibrium between MOH and β-CD. PCA analysis confirms this interpretation, since two components are enough to reproduce the systematic variation of the MOH absorption spectrum with β-CD concentration (Fig. 6). These two components are then assigned to free MOH and a complex between MOH and β-CD

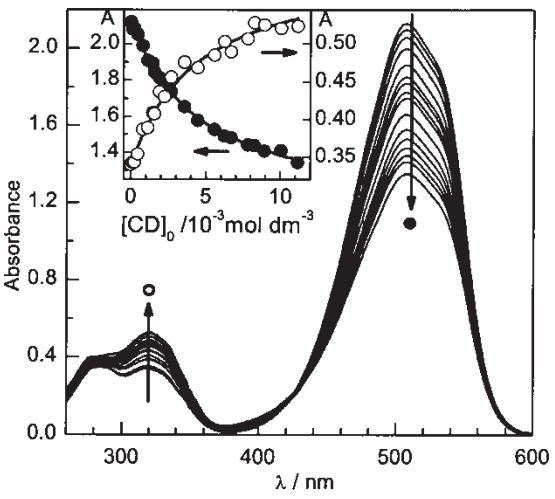


FIGURE 5 Absorption spectra of MO at pH 1 in the presence of different concentrations of β-CD, varying from 0 to $11.3 \times 10^{-3}\,\mathrm{mol\,dm^{-3}}$. Inset: plot of absorbances at 320 nm (open circles) and at 510 nm (filled circles) versus initial β-CD concentrations. Lines represent the curves resulting from the fits to the experimental data. $[\mathrm{MO}]_0 = 5.8 \times 10^{-5}\,\mathrm{mol\,dm^{-3}}$.

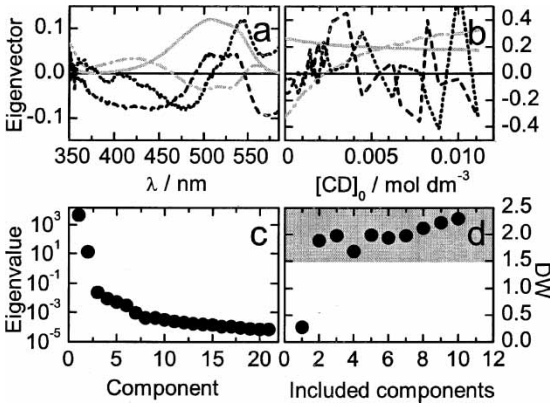


FIGURE 6 Results of PCA for the series of absorption spectra of MO at pH 1 with different β-CD concentrations: (a) the first four spectral eigenvectors in the order grey solid, grey dash-dot, black dash, black dot; (b) the first four eigenvector profiles in the same order as before; (c) logarithmic plot of eigenvalues versus number of components; (d) plot of mean Durbin-Watson test values of residual profiles versus number of included components.

of stoichiometry 1:1 (MOH:CD), which are in equilibrium with constant K:

$$\mathrm{MOH} + \mathrm{CD} \overset{K}{\rightleftharpoons} \mathrm{MOH:CD} \tag{5}$$

For this mechanism and under conditions of excess cyclodextrin, the following mathematical function can be obtained that relates the absorbance at any wavelength (A^{λ}) to the total β-CD concentration:

$$A^{\lambda} = \frac{A^{\lambda}_{\mathrm{MOH}} + A^{\lambda}_{\mathrm{MOH:CD}} K[\mathrm{CD}]_0}{1 + K[\mathrm{CD}]_0} \tag{6}$$

where $A^{\lambda}_{\mathrm{MOH}}$ and $A^{\lambda}_{\mathrm{MOH:CD}}$ are the absorbances at λ when all the MOH present is free and complexed, respectively. This function was fitted in a global analysis using all the experimental spectra in the range from 300 to 600 nm. The fit was very satisfactory at all wavelengths, as exemplarily indicated by the good agreement between the fitted curves and the experimental data (see inset of Fig. 5) and the corresponding random residuals. The value obtained for the association equilibrium constant was $K = 220 \pm 1\,\mathrm{mol^{-1}\,dm^3}$. This value is in agreement with that reported by Gelb and Schwartz [8] ($K = (0.23 \pm 0.04) \times 10^3\,\mathrm{mol^{-1}\,dm^3}$ at 25°C), which was also obtained by nonlinear regression analysis, although our value is much more accurate since it results from global analysis of all spectra. Other published values are significantly larger, but all were obtained by the Benesi-Hildebrand method which involves inadequate weighting of the experimental data with consequent error in the estimation of the equilibrium constant [25].

It is interesting to note that the acid used to achieve pH 1 in the above measurements must be carefully selected, since the anions of some usual inorganic acids such as $HClO_4$ and HNO_3 form complexes with β-CD [26,27]. Although the

association equilibrium constants of these anions are relatively low, they have an important effect due to their high concentration in comparison with methyl orange. Thus, if $HClO_4$ is used to prepare the solutions, an apparent association equilibrium constant of $105 \pm 1\,mol^{-1}\,dm^3$ for MOH with β-CD is obtained. This means that the effective β-CD concentration is about a half of $[CD]_0$ due to competitive complexation of the ClO_4^- anions. Such a value can be explained by assuming the value of K obtained above and a stability constant of $10.7 \pm 0.2\,mol^{-1}\,dm^3$ for the ClO_4^- anions, which is in agreement with that reported by Gelb *et al.* [27].

Global analysis also yields the individual absorption spectra of free MOH and of the complex MOH:CD (Fig. 7). The former coincides with the experimental spectrum of MO at pH 1 without β-CD, confirming the validity of the analysis. The spectrum of the complex shows the two typical absorption bands of MOH, but the intensity ratio between the visible band and the UV band decreases significantly, from 6.3 in free MOH to 1.7 in the complex MOH:CD. Nevertheless, the shapes of the two bands remain constant except for a slight broadening of the visible band. To explain this result a more detailed analysis of the *microscopic* equilibria involved in this system must be performed.

Up to now our treatment has not considered the equilibrium between the ammonium (am) and the azonium (az) tautomers of MOH (see Scheme 1). In principle, these tautomers can form two different 1:1 complexes with β-CD (am:CD and az:CD) leading to a system of four chemical species joined by four *microscopic* equilibria, as shown in Scheme 3. This mechanism has already been proposed by other authors [13–16], but has not been satisfactorily resolved. We will now attempt to obtain a proper resolution of the *microscopic* equilibria using as few assumptions as possible. This resolution must be compatible with the analysis performed above and explain questions such as why PCA finds only two components contributing to the absorption spectra of MOH with β-CD, or what is the meaning of the association constant K determined above.

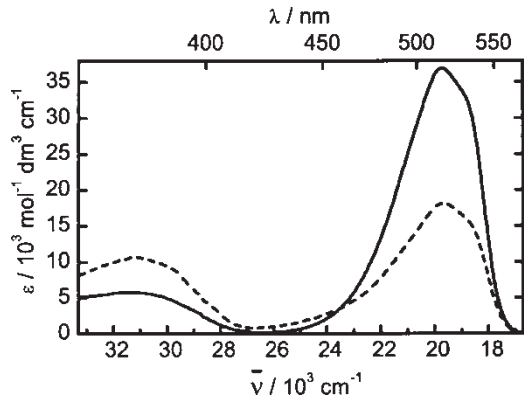
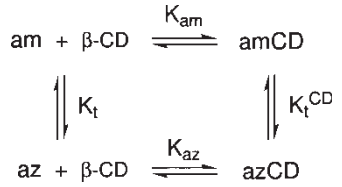


FIGURE 7 (a) Individual absorption spectra of free MO (solid line) and its inclusion complex MO·CD (dashed line) as obtained by global analysis of the experimental absorption spectra at pH 1.



SCHEME 3 *Microscopic* equilibria of the two tautomers of MOH and their corresponding inclusion complexes with β-CD.

From Scheme 3 a relation between the association equilibrium constants of the ammonium and the azonium tautomers, K_{am} and K_{az}, and the tautomerization equilibrium constants of free MOH and complexed MOH, K_t and K_t^{CD}, can easily be derived:

$$\frac{K_{am}}{K_{az}} = \frac{K_t}{K_t^{CD}} \tag{7}$$

This relation shows that at least three of the four equilibrium constants must be determined to resolve the system. We will consider first the tautomerization equilibrium of free MOH, which can be studied in the absence of β-CD.

Previous studies [14,15,19] state that the visible band of the MOH absorption spectrum is due to the azonium tautomer, whereas the UV band with a maximum at about 320 nm is mainly attributed to the ammonium tautomer. The tautomerization constant K_t, defined as the ratio of concentrations,

$$K_t = \frac{[az]}{[am]} \tag{8}$$

was calculated from the observed molar absorptivity of MOH at 316 nm using a molar absorptivity for the ammonium tautomer at 316 nm of $\varepsilon_{am}^{316} = 2.50 \times 10^4\,mol^{-1}\,dm^3\,cm^{-1}$, a value obtained for the model compound 4-phenylazo-*N*,*N*,*N*-trimethyl 4′-sulfonato anilinium ion, which only exists in the ammonium form. Assuming that the azonium tautomer does not absorb at 316 nm, values of the tautomerization constant of 3.2 [19] and 2.65 [15] were calculated. If the azonium tautomer is considered to contribute slightly to the UV band with $\varepsilon_{az}^{316} = 0.2 \times 10^4\,mol^{-1}\,dm^3\,cm^{-1}$, as obtained for a model compound of this tautomer, higher values of K_t result (4.7 [19] and 3.75 [15]). Therefore, the values of K_t found in the literature are not sufficiently accurate for our purposes.

In order to obtain a better estimation of K_t, the temperature dependence of the MOH absorption spectrum was studied. Figure 8 shows the

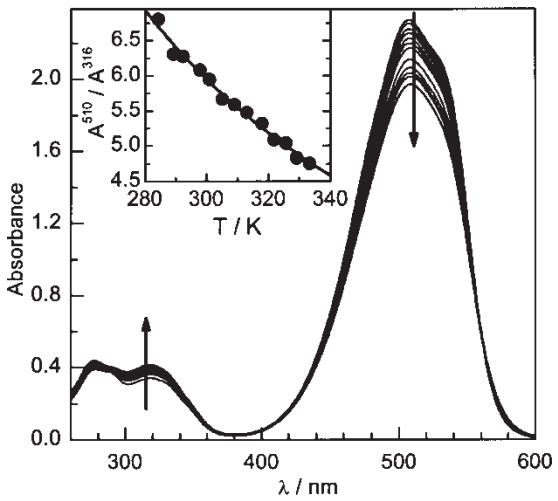


FIGURE 8 Absorption spectra of MO at pH 1 at temperatures varying from 10 to 60°C. Inset: plot of the ratio of absorbances at 510 nm and 320 nm versus temperature. Lines represent the curves resulting from the fits (see text). $[MO]_0 = 4.9 \times 10^{-5}$ mol dm^{-3}.

absorption spectrum of MOH in the temperature range from 10 to 60°C. The intensity of the visible band decreases as temperature increases, whereas the UV band at 320 nm increases in intensity. This indicates a change in the equilibrium concentrations of the two tautomers, decreasing that of the azonium tautomer and increasing that of the ammonium tautomer as temperature is increased. More detailed analysis of the UV region of the spectrum shows that the second UV band with a maximum around 280 nm has a complex variation, first increasing slightly and then decreasing as the temperature is increased. This band must be assigned to the azonium tautomer, so that the observed variation results from the overlap of a decreasing azonium band and an increasing ammonium band. This considerable overlap indicates that the contribution of the azonium tautomer to the observed absorbance at 316 nm cannot be neglected.

For a quantitative analysis of the temperature-dependence data the ratio of absorbances at the visible band (A^{510}) and at the first UV band (A^{316}) will be used (see inset of Fig. 8). A mathematical function that relates this ratio to temperature must be derived taking into account that the absorbance at 510 nm is only due to the azonium tautomer, whereas the absorbance at 316 nm is contributed by both tautomers. Since the equilibrium concentrations of the tautomers are related by the equilibrium constant K_t, which has a well-known thermodynamic dependency on temperature, the following equation can easily be obtained:

$$\frac{A^{510}}{A^{316}} = \frac{\left(\varepsilon_{az}^{510}/\varepsilon_{am}^{316}\right) e^{\Delta S/R} e^{-\Delta H/RT}}{1 + \left(\varepsilon_{az}^{316}/\varepsilon_{am}^{316}\right) e^{\Delta S/R} e^{-\Delta H/RT}} \qquad (9)$$

where ΔS and ΔH are the variations of the entropy and the enthalpy in the tautomerization reaction. In this equation the two ratios of molar absorptivities can be calculated using the values of the model compounds and the value of $\varepsilon_{az}^{510} = 6.66 \times 10^4$ mol^{-1} dm^3 cm^{-1} determined from a Lambert-Beer plot using the azonium equilibrium concentrations. Therefore, two parameters, ΔS and ΔH, are left to be estimated in the nonlinear fit to the experimental data. The fit is very satisfactory, with good agreement between the calculated curve and the experimental data (see inset of Fig. 8) and random residuals. The values obtained for the parameters are: $\Delta S = (-13.6 \pm 0.7)$ J mol^{-1} K and $\Delta H = (-6.6 \pm 0.2) \times 10^3$ J mol^{-1}, which yield a value for the tautomerization equilibrium constant at 25°C of $K_t = 2.8 \pm 0.3$. This value is significantly lower than those previously estimated assuming that the azonium tautomer contributes to the UV band. It should be noted that the small absorptivity of the azonium tautomer at 316 nm has a small effect on the goodness of fit, but it affects considerably the fit parameters and, as a consequence, the resulting value of K_t.

Further resolution of the system involves an analysis of the absorption spectra of MOH in the presence of β-CD (Fig. 5) in terms of the *microscopic* equilibria of Scheme 3. The observed absorbance at any wavelength, A^λ, is the sum of contributions of the four species present, which are proportional to their equilibrium concentrations. These concentrations can be derived from the definitions of the *microscopic* equilibrium constants and the mass balance for MOH. Then, and under excess β-CD, the following expression is obtained that relates A^λ to the total β-CD concentration:

$$A^\lambda = \frac{A_{am}^\lambda + A_{az}^\lambda K_t + A_{amCD}^\lambda K_{am}[CD]_0 + A_{azCD}^\lambda K_{az} K_t [CD]_0}{1 + K_t + K_{am}[CD]_0 + K_{az} K_t [CD]_0} \qquad (10)$$

where A_X^λ (X = am, az, amCD or azCD) is the absorbance of species X when all the MOH is in this form, as given by equation (4).

Examining the form of equation (10), one observes that the contributions of the two free tautomers have an identical dependency on $[CD]_0$ and the same is true for the two complexed tautomers. Consequently, it is not possible to distinguish between the contributions of the tautomers by their variation with β-CD concentration. This explains why PCA yields two spectral components even though four species are present. Note that PCA gives the minimum number of components that can explain the observed variation and this may be less than the number of chemical species present in the mixture [21]. In fact, equation (10) can be written in the form of equation (6) with the following relations among

the parameters:

$$A^{\lambda}_{\mathrm{MOH}} = \frac{A^{\lambda}_{\mathrm{am}} + A^{\lambda}_{\mathrm{az}} K_{\mathrm{t}}}{1 + K_{\mathrm{t}}} \tag{11}$$

$$A^{\lambda}_{\mathrm{MOH:CD}} = \frac{A^{\lambda}_{\mathrm{amCD}} K_{\mathrm{am}} + A^{\lambda}_{\mathrm{azCD}} K_{\mathrm{az}} K_{\mathrm{t}}}{K_{\mathrm{am}} + K_{\mathrm{az}} K_{\mathrm{t}}} \tag{12}$$

$$K = \frac{K_{\mathrm{am}} + K_{\mathrm{az}} K_{\mathrm{t}}}{1 + K_{\mathrm{t}}} \tag{13}$$

Therefore, only these *macroscopic* parameters can be obtained from the analysis of the variation of the MOH absorption spectrum with β-CD concentration. The constant K, determined by global analysis of equation (6), is a *macroscopic* or *apparent* equilibrium constant that combines three independent *microscopic* equilibrium constants. The absorbances $A^{\lambda}_{\mathrm{MOH}}$ and $A^{\lambda}_{\mathrm{MOH:CD}}$ constitute the individual spectra of free MOH and complex MOH:CD shown in Fig. 7, each being a linear combination of the spectra of the corresponding tautomers. It should be noted that, even if the analysis is limited to the visible band, where only the free and the complexed azonium tautomer absorb, no further information concerning the *microscopic* equilibrium constants can be obtained from the experimental data.

At this point it is clear that assumptions must be made in order to estimate the *microscopic* association constants. As discussed above, the individual spectra of free and complexed MOH (Fig. 7) differ strongly in the intensity ratio between visible and UV bands without significant variation of the form of these bands. This suggests that the spectrum of each complexed tautomer is very similar to the spectrum of the corresponding free tautomer and that the differences in intensity are only due to a change in the equilibrium concentrations of the two tautomers upon complexation. Since the intensity of the visible band decreases, it is deduced that the concentration of complexed azonium tautomer is lower than that of the free tautomer. On the contrary, the concentration of the ammonium tautomer increases upon complexation. Therefore, it seems reasonable to assume that the molar absorptivities of each complexed tautomer coincide with those of the corresponding free tautomer (for example, $\varepsilon^{510}_{\mathrm{azCD}} = \varepsilon^{510}_{\mathrm{az}} = 6.66 \times 10^4\,\mathrm{mol^{-1}\,dm^3\,cm^{-1}}$). Then the ratio of absorbances at 510 and 316 nm of the MOH:CD spectrum (Fig. 7),

$$\frac{A^{510}_{\mathrm{MOH:CD}}}{A^{316}_{\mathrm{MOH:CD}}} = \frac{(\varepsilon^{510}_{\mathrm{azCD}}/\varepsilon^{316}_{\mathrm{amCD}})(K_{\mathrm{az}}/K_{\mathrm{am}})K_{\mathrm{t}}}{1 + (\varepsilon^{316}_{\mathrm{azCD}}/\varepsilon^{316}_{\mathrm{amCD}})(K_{\mathrm{az}}/K_{\mathrm{am}})K_{\mathrm{t}}} \tag{14}$$

yields the following relation among the *microscopic* constants, after substituting the molar absorptivities by their values in the free tautomers:

$$\frac{K_{\mathrm{az}}}{K_{\mathrm{am}}} K_{\mathrm{t}} = 0.69 \tag{15}$$

Combining this equation and equation (13) and using the values of the *macroscopic* association constant $K = 220\,\mathrm{mol^{-1}\,dm^3}$ and of the tautomerization constant $K_t = 2.8$, the following values for the *microscopic* equilibrium constants are obtained: $K_{\mathrm{am}} = 4.9 \times 10^2\,\mathrm{mol^{-1}\,dm^3}$ and $K_{\mathrm{az}} = 1.2 \times 10^2\,\mathrm{mol^{-1}\,dm^3}$. Moreover, equation (7) allows determination of the tautomerization constant between the complexed tautomers, $K^{\mathrm{CD}}_{\mathrm{t}} = 0.69$, which is much lower than that of the free tautomers, as expected.

Quantification of Polymer-bound β-CD

Complexation with a dye has been proposed as an analytical method for quantifying CDs [28,29]. MO would be a good dye for this purpose since its formation of inclusion complexes can be followed by UV–Visible spectroscopy. Nevertheless, we have shown that MO exhibits complex behaviour when interacting with β-CD, so that suitable experimental conditions must be chosen to simplify the system. First, the working pH must be selected. If there are no particular chemical features of the sample to be considered, the larger binding capacity of MO compared with MOH together with its greater solubility suggest working with neutral-to-basic media, in spite of the smaller spectral change on complexation in this pH range. Also, tautomerization of MOH complicates its use as a guest. But, in order to simplify the data analysis, the formation of a 1:2 complex between MO and β-CD must be avoided. The equilibrium concentration of MO·2CD is only important when β-CD is in large excess, since K_2 is much smaller than K_1. Therefore, for a typical $[\mathrm{MO}]_0$ value of $1 \times 10^{-5}\,\mathrm{mol\,dm^{-3}}$, there is a wide $[\mathrm{CD}]_0$ interval up to $1 \times 10^{-3}\,\mathrm{mol\,dm^{-3}}$ where less than 1% of the total MO forms MO·2CD.

The quantification procedure involves registration of the absorption spectrum of MO in the presence of a certain amount of the CD derivative (in our case, a β-CD-grafted polymer) and analysis of the data on the basis of a 1:1 complexation between MO and the β-CD units. This procedure implies two assumptions. The first is that the polymer backbone does not itself affect the absorption behaviour of MO in the wavelength range used. This must be verified experimentally by registering the MO absorption spectrum in the presence of ungrafted polymer. The second assumption is that the binding of β-CD to the polymer does not change the nature of its complex formation with MO, so that the association equilibrium constant and the spectrum of the MO·CD complex determined with free β-CD can be applied to bound β-CD. There are reports [9,30,31] concerning the effects of linking cyclodextrins on its complexation ability, mainly with substrates that can interact with more than one CD at a time.

In the case of the polymers synthesized here, this is a hypothesis that should be tested.

Under experimental conditions where formation of the 1:2 complex can be neglected, the absorbance, at a given wavelength (A^λ), of MO in the presence of β-CD-grafted polymer depends on the equilibrium concentrations of free MO and MO·CD as follows:

$$A^\lambda = \varepsilon^\lambda_{MO}\ell[MO] + \varepsilon^\lambda_{MO\cdot CD}\ell[MO\cdot CD] \quad (16)$$

where ε^λ_{MO} and $\varepsilon^\lambda_{MO\cdot CD}$ are the molar absorptivities of the two species at wavelength λ. The equilibrium concentrations are related to the total concentrations of MO and β-CD as given by the definition of the association equilibrium constant K_1:

$$K_1 = \frac{[MO\cdot CD]}{([MO]_0 - [MO\cdot CD])([CD]_0 - [MO\cdot CD])} \quad (17)$$

Note that, for generality, no approximation of an excess of β-CD has been made, so that a quadratic equation in [MO·CD] results. Solving this equation for [MO·CD] and substituting the meaningful root in equation (16), a solution for the total concentration of cyclodextrin in the sample is obtained:

$$[CD]_0 = \frac{\varepsilon^\lambda_{MO} - \varepsilon^\lambda}{\varepsilon^\lambda - \varepsilon^\lambda_{MO\cdot CD}}\frac{1}{K_1} + \frac{\varepsilon^\lambda_{MO} - \varepsilon^\lambda}{\varepsilon^\lambda_{MO} - \varepsilon^\lambda_{MO\cdot CD}}[MO]_0 \quad (18)$$

where ε^λ is the observed molar absorptivity of the sample calculated as follows:

$$\varepsilon^\lambda = \frac{A^\lambda}{\ell[MO]_0} \quad (19)$$

Thus, using the spectrum of MO in the presence of β-CD-grafted polymer and the spectra of free MO and MO·CD (Fig. 3), with the value determined for K_1 in the titration with β-CD ($K_1 = (2.97 \pm 0.05) \times 10^3\,mol^{-1}\,dm^3$) and the initial concentration of MO, the total concentration of β-CD in the sample can be determined. Although the calculation can be performed with the data at a certain wavelength, a more accurate value of the total β-CD concentration is obtained using a selected wavelength range. The best range is that where the difference between the absorption spectra of free MO and MO·CD is largest and the influence of the MO·2CD complex is small. Observing the absorption spectra of the three species in Fig. 3(a), the wavelength range from 500 to 540 nm is the most suitable for analysis. Regarding the approximation of an excess of β-CD with respect to MO, it leads to equation (18) without the second term and simplifies the calculation only slightly.

Once the total concentration of β-CD in the sample is known, other quantities can be obtained that allow characterization and comparison of different derivatives. The weight percentage of bound β-CD, defined as the weight of the β-CD residues grafted to the polymer (w_{CD}) related to the total weight of polymer in solution (w), can be calculated as follows:

$$\%w_{CD}/w = 100[CD]_0 V M_{CD}/w \quad (20)$$

where V is the volume of the solution and M_{CD} is the molar mass of the β-CD residue grafted to the polymer (R of substituted AGU in Scheme 2). Another interesting quantity is the fraction of β-CD residues per monomeric unit of the polymer chain, f, which measures the degree of grafting:

$$f = \frac{w_{CD}}{w - w_{CD}}\frac{M_{mon}}{M_{CD}} \quad (21)$$

with M_{mon} being the molar mass of a monomeric unit.

As an example of the above-described procedure we report the results obtained for a sample of soluble starch grafted with MCT-β-CD, but the method has also been satisfactorily applied to other β-CD-grafted polymers [20]. The absorption spectrum of MO in the presence of 0.0073 g of the grafted polymer shows variations typical for MO complexation with β-CD when compared with the spectrum of free MO. In the range from 500 to 540 nm the absorbance of the β-CD-containing sample decreases by 20–40% with respect to absorbance in the absence of β-CD, so that the effect is quite significant. Data analysis with equation (18) yields reasonably uniform values of the total β-CD concentration over the whole range of wavelengths, with a mean value of $[CD]_0 = (8.36 \pm 0.07) \times 10^{-4}\,mol\,dm^{-3}$. This value corresponds to a $[CD]_0/[MO]_0$ ratio where formation of the [MO·2CD] complex can be neglected, indicating suitable experimental conditions. For routine work the calculation can be performed with the data at a certain wavelength representative of the useful interval. Nevertheless, it is convenient to register the whole absorption spectrum of the sample and to compare it with the absorption spectra of free MO and the MO·CD complex to detect possible artifacts that could interfere in the analysis and to validate the assumption that bound β-CD has the same complexation behaviour as free β-CD. Regarding the error in the estimation of $[CD]_0$, it depends on the accuracy of the absorption measurements, especially determined by the baseline correction, and on the suitability of the experimental conditions to guarantee the validity of the assumptions made.

Transforming the total β-CD concentration into the weight percentage of bound β-CD by means of equation (20), a value of (44.3 ± 0.7)% is obtained for this sample. The error in this result is predominantly influenced by the error in w, so that the purity and moisture content of the solid sample are critical

variables to control. Also, the volume of the MO solution should be measured precisely.

Finally, the fraction of β-CD residues per monomeric unit of the polymer chain can be calculated, resulting in a value of $f = 0.1$. This result indicates that, on average, there is one β-CD molecule per 10 anhydroglucose units of the polymer chain. Taking into account the helical conformation of starch (amylose) molecules, this suggests that there is no close interaction between the bound β-CD molecules during the formation of inclusion complexes with MO. Therefore, the hypothesis of no effect on the complexation ability of linked cyclodextrins is probably valid for the kind of polymers studied.

CONCLUSION

In neutral-to-basic media MO forms inclusion complexes with β-CD of stoichiometries 1:1 and 1:2 (guest:host). The association equilibrium constants, determined by absorption studies, are $K_1 = (2.97 \pm 0.05) \times 10^3\,\mathrm{mol^{-1}\,dm^3}$ and $K_2 = (0.10 \pm 0.01) \times 10^3\,\mathrm{mol^{-1}\,dm^3}$. In acid media MO exists as a mixture of two tautomers (ammonium and azonium) and both form 1:1 inclusion complexes with β-CD. The *macroscopic* association constant is $K = 220 \pm 1\,\mathrm{mol^{-1}\,dm^3}$ and the tautomerization equilibrium constant is $K_t = 2.8 \pm 0.3$. With these values, estimations of the *microscopic* association constants of the two tautomers can be made. The study shows that the MO/β-CD system exhibits complex behaviour that must be fully understood in order to use it as a model system. Thus, any application of MO complexation for quantification or characterization purposes of β-CD derivatives implies a good selection of experimental conditions, as shown in the example presented. This statement is also valid for many other guest models that, due to their bifunctional structure or their tautomerization ability, show complex behaviour in their association with CDs that often is not taken into account in analytical applications.

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References

[1] Szejtli, J. *Cyclodextrin Technology*; Kluwer Academic: Dordrecht, 1988.
[2] Wenz, G. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 803.
[3] Leung, D. K.; Atkins, J. H.; Breslow, R. *Tetrahedron Lett.* **2001**, *42*, 6255.
[4] Álvarez Parrilla, E.; Ramos Cabrer, P.; Al-Soufi, W.; Meijide, F.; Rodríguez Núñez, E.; Vázquez Tato, J. *Angew. Chem., Int. Ed. Engl.* **2000**, *39*, 2856.
[5] Tabushi, I.; Kuroda, Y.; Mizutani, T. *Tetrahedron* **1984**, *40*, 545.
[6] Fujita, K.; Ejima, S.; Imoto, T. *J. Chem. Soc., Chem. Commun.* **1984**, 1227.
[7] Hirai, H.; Toshima, N.; Uenoyama, S. *Bull. Chem. Soc. Jpn* **1985**, *58*, 1156.
[8] Gelb, R. I.; Schwartz, L. M. *J. Incl. Phenom.* **1989**, *7*, 537.
[9] Schneider, H.-J.; Xiao, F. *J. Chem. Soc., Perkin Trans. 2*, **1992**, 387.
[10] Tawarah, K. M.; Wazwaz, A. A. *Ber. Bunsenges. Phys. Chem.* **1993**, *97*, 727.
[11] Hamai, S.; Handa, M. *Anal. Lett.* **1999**, *32*, 1037.
[12] Sueishi, Y.; Kasahara, M.; Inoue, M.; Matsueda, K. *J. Incl. Phenom. Macrocyclic Chem.* **2003**, *46*, 71.
[13] Matsui, Y.; Mochida, K. *Bull. Chem. Soc. Jpn* **1978**, *51*, 673.
[14] Buvári, Á.; Barcza, L. *J. Incl. Phenom.* **1989**, *7*, 313.
[15] Tawarah, K. M.; Abu-Shamleh, H. M. *J. Incl. Phenom.* **1991**, *11*, 29.
[16] Tawarah, K. M. *J. Incl. Fen. Mol. Recogn.* **1992**, *14*, 195.
[17] Matsui, Y.; Ogawa, K.; Mikami, S.; Yoshimoto, M.; Mochida, K. *Bull. Chem. Soc. Jpn* **1987**, *60*, 1219.
[18] Wang, A. S.; Matsui, Y. *Bull. Chem. Soc. Jpn* **1994**, *67*, 2917.
[19] Reeves, R. L. *J. Am. Chem. Soc.* **1966**, *88*, 2240.
[20] Carrazana García, J., Villamarin, S.F. and Vázquez Tato, J. (2000) In *Cyclodextrin: From Basic Research to Market, International Cyclodextrin Symposium, 10th*, Ann Arbor, MI, May 21–24, pp. 318–323.
[21] Al-Soufi, W.; Novo, M.; Mosquera, M. *Appl. Spectrosc.* **2001**, *55*, 630.
[22] Siano, D. B.; Metzler, D. E. *J. Chem. Phys.* **1969**, *51*, 1856.
[23] Reeves, R. L.; Kaiser, R. S.; Edward, A.; Sylvestre, M. S. M.; Lawton, W. H.; *Can. J. Chem.* **1973**, *51*, 628.
[24] Al-Soufi, W.; Ramos Cabrer, P.; Jover, A.; Budal, R. M.; Vázquez Tato, J. *Steroids* **2003**, *68*, 43.
[25] Álvarez Parrilla, E.; Al-Soufi, W.; Ramos Cabrer, P.; Novo, M.; Vázquez Tato, J. *J. Phys. Chem. B* **2001**, *105*, 5994.
[26] Rohrbach, R. P.; Rodríguez, L. J.; Eyring, E. M. *J. Phys. Chem.* **1977**, *81*, 944.
[27] Gelb, R. I.; Schwartz, L. M.; Radeos, M.; Laufer, D. A. *J. Phys. Chem.* **1983**, *87*, 3349.
[28] Li, W.; Zhou, J.; Zhang, L.; Corke, H.; Yang, L. *Anal. Lett.* **1996**, *29*, 1201.
[29] Basappa, C.; Rao, P.; Narashima, D.; Divakar, S. *Int. J. Food Sci. Technol.* **1998**, *33*, 517.
[30] Harada, A.; Furue, M.; Nozakura, S. *Macromolecules* **1976**, *9*, 705.
[31] Fujita, K.; Ejima, S.; Imoto, T. *Chem. Lett.* **1985**, 11.