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Analytical Improvement in Measuring Formation Constants of Inclusion Complexes between β-Cyclodextrin and Phenolic Compounds

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Abstract. Inclusion complex formation between sixteen para-substituted phenols and β-cyclodextrin have been investigated in order to establish Quantitative Structure Affinity Relationships. An analytical methodology is proposed, in order to obtain reliable evaluation of binding affinities. Potentiometry and circular dichroism have been applied to define experimental conditions and to confirm postulated equilibriums. In addition, the use of algorithmic treatments and concentration optimisation to determine formation constants leads to coherent values between ^{1}H NMR, direct UV Spectroscopy and the spectral displacement method. The results emphasise the contribution of van der Waals interactions, provided that no significant difference in the dipole of the molecule arises from the para-substituent.

Key words: β-cyclodextrin complexes, phenols, QSAR, spectral displacement method.

1. Introduction

Among the genuine cyclomaltooligosaccharides, β-cyclodextrin (β-CD) has received considerable attention, especially because of its lower cost [1]. As a result, applications involving β-CD cover many fields [2–5], including pharmaceutics, cosmetology or even the environment. The intervention of β-CD in this latter domain relies in particular on its affinity for aromatic compounds [6–7], such as polycyclic aromatic hydrocarbons (PAH) [8–10] or volatile organic compounds (VOC) [11–13]. As far as increasing degradability or molecular trapping is concerned, a more quantitative complexation represents an interesting way to efficient decontamination. Consequently, the definition of the chemical modifications which lead to stronger affinity constitutes an ultimate aim. The comprehension of the driving forces enables rationalism of such modification, and in this respect QSAR represents a powerful tool. Nevertheless, such studies are quite rare in the cyclodextrin field [14–17], when compared to the numerous papers dedicated to formation constant (K_f) determination [18]. Since reliable affinity constants represent an essential prerequisite to QSAR studies, the divergence often observed in cyclodextrin

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Table I. Characteristics of the sixteen paraphenols studied [substituent, molar refractivity (MR), hydrophobic character (π), Hammett constants (σ)] and experimental results [pKa, ellipticity (θ), formation constants (mean reproducibility: 6%)]

N°	Substituent	MR	π	σ_p	pKa	θ (mDeg)	K_f
1	—F	0.92	0.14	0.06	9.7	−1.38	105
2	—Cl	6.03	0.71	0.23	9.28	−3.28	270
3	—Br	8.88	0.86	0.23	9.21	−4.14	430
4	—I	13.94	1.12	0.18	9.14	−2.71	905
5	—H	1.03	0.00	0.00	9.86	−1.49	105
6	—CH_3	5.65	0.56	−0.17	10.13	−1.09	195
7	—C_2H_5	10.58	1.02	−0.15	10.00	−4.78	520
8	—CN	6.33	−0.57	0.66	7.85	+3.87	200
9	—OH	2.85	−0.67	−0.37	9.97	−2.71	115
10	—OCH_3	7.87	−0.02	−0.27	9.97	−3.21	170
11	—OC_2H_5	12.47	0.38	−0.24	9.96	−4.01	330
12	—OC_3H_7	17.06	1.05	−0.25	9.97	−4.07	550
13	—CHO	6.88	−0.65	0.42	7.53	+4.51	200
14	—CH_2OH	7.48	−1.03	0.00	9.69	−3.13	110
15	—CH_2COOH	11.88	−0.72	−0.07	10.00	−1.86	95
16	—SO_3H	10.38	–	–	8.76	−2.39	70

literature values may be the limiting factor of QSAR expansion. Assuming the crucial importance of the initial data, we were prompted to develop an experimental methodology based on several techniques: potentiometry, circular dichroism, ^{1}H NMR and UV-Visible Spectroscopy. We report in this paper analytical considerations for formation constant determination, applied to sixteen para-substituted phenols (listed in Table I). The interest in such compounds lies in the fact that they represent soluble VOC analogues, which are easier to study.

2. Experimental

2.1. MATERIALS

D_2O (>99.9% isotopic purity) was obtained from SDS. β-CD, from Roquette, was used without further purification; methyl orange (MO), and each phenol derivative (>99% purity) were purchased from Acros. All samples were prepared in phosphate buffer solutions in order to keep the pH and the ionic strength constant.

2.2. EQUIPMENT AND EXPERIMENTAL CONDITIONS

Potentiometric studies. pKa values were calculated from the pH titration data obtained at 20 °C with a MOLSPIN automatic titration system. Changes in pH were followed by using a glass-calomel electrode (Mettler). All solutions were prepared in 0.1 M KCl. Titrations were performed for each system over the pH range 3–11 using volumes of 2 ml. pKa values were calculated with the aid of the SUPERQUAD computer program.

Circular dichroism studies. Circular dichroism experiments were carried out using a Jobin Yvon CD6 spectrometer, between 200–350 nm, at 20 °C. Guest solutions were prepared in such a way that the optical density was equal to 0.8, in order to optimise the signal to noise ratio. Complexes were prepared from these stock solutions by adding a given amount of β-CD (concentration fixed to 10 mM).

UV-Visible spectroscopic studies. Spectra were recorded with a Perkin Elmer Lambda 2S spectrometer. The 10 mm cell used was placed in a cuvette holder, the temperature of which was kept constant at 20.0 $\pm$ 0.1 °C by means of a thermostated bath.

The quantitative determination of the formation constant is based on the absorbance variation of the guest in the presence of β-CD for several concentrations. A critical point when measuring these variations lies in the constancy of the guest concentration. Indeed, the spectral change upon complexation is very low if compared to the incidence of a difference in guest concentration. This assertion is true even if the difference represents only 1% of the overall signal. Thus, if the variation of the β-CD concentration is obtained by mixing two stock solutions where each species is alone, a significant part of the spectral variations is not due to complexation. In order to avoid this kind of error, the host guest stock solution has to be prepared by dissolution of β-CD powder directly from guest solution. Variation of the β-CD concentration is then obtained by mixing these two stock solutions, in which guest concentrations are strictly equal.

For the special case of the spectral displacement method, β-CD was dissolved in MO solution, and each phenol derivative was dissolved in the resulting solution.

NMR spectroscopic studies. ^{1}H NMR spectra were recorded at 20 °C on a Bruker ASPECT 3000 spectrometer operating at 250 MHz. The inner protons of β-CD were considered rather than the aromatic ones, since the observed variation of their chemical shifts are generally higher. As explained for UV-Visible spectroscopic studies, the concentration of the species responsible for the signal has to remain rigorously constant. Thus, host–guest solutions were obtained by adding a given quantity of guest to a β-CD solution (D_2O used as solvent) whose concentration was equal to 1 mM. TMS was used as an external reference.

2.3. METHODS

Direct measurement of formation constants. This could be performed both by NMR and UV-Visible spectroscopy, and is based on the spectral variation observed for one compound upon complexation, that is to say upon the addition of the other component. Data treatment is accomplished by a numerical method. Assuming a 1 : 1 equilibrium, complex concentration could be described as follows:

$$[\beta\text{-CD}/G] = -\frac{1}{2}\sqrt{\left[\left(\frac{1}{K_f} + [\beta\text{-CD}]_A + [G]_T\right)^2 - 4[\beta\text{-CD}]_A[G]_T\right]} + \frac{1}{2}\left(\frac{1}{K_f} + [\beta\text{-CD}]_A + [G]_T\right), \quad (1)$$

where G stands for guest, β-CD/G for complex, T for total and A for available. Since only one guest is considered, the cyclodextrin concentration available to this guest is $[\beta\text{-CD}]_T$.

Then, for a given value of K_f, $[\beta\text{-CD}/G]$ is known and the spectral characteristic of the complex can be calculated. This includes the molar absorptivity ($\epsilon_{\beta-\text{CD}/G}$) for UV-Visible spectroscopy, or the intrinsic chemical shift ($\delta_{\beta-\text{CD}/G}$) for NMR. These characteristics are respectively based on the following relations:

$$A = 1 * (\epsilon_G * [G] + \epsilon_{\beta\text{-CD}/G} * [\beta\text{-CD}/G]) \quad (2)$$

$$\delta = \delta_{\beta-\text{CD}/G} * [\beta\text{-CD}]/[\beta\text{-CD}]_T + \delta_{\beta-\text{CD}/G} * [\beta\text{-CD}/G]/[\beta\text{-CD}]_T, \quad (3)$$

where A represents the absorbance, and δ the chemical shift.

The estimation of the spectral characteristic ($\epsilon_{\beta-\text{CD}/G}$ or $\delta_{\beta-\text{CD}/G}$) is achieved for each concentration of β-CD for UV-Visible spectroscopy, or of G for NMR. The difference over the parameter of concern has then to be minimised relative to K_f. All calculations were performed in Excel (minimisation algorithm: Newton–Raphson).

Spectral displacement method. The competing equilibria involved in this method may be described as follows:

$$\beta\text{-CD} + \text{MO} + G \Leftrightarrow \beta\text{-CD/MO} + G \Leftrightarrow \beta\text{-CD}/G + \text{MO}.$$

The absorbance variation induced by the addition of G to a solution which contains MO and β-CD is directly related to the concentration of β-CD which is involved in complexation with G.

Spectra were recorded between 520–530 nm for [MO] = 0.1 mM. Data treatment was realised by means of an algorithmic method. Its principle consists in

the calculation of the concentrations of the complexes, by considering the two equilibriums successively in an iterative way:

Step 1: a value is postulated for K_f (estimation for β-CD/G)

Step 2: $[\beta\text{-CD/MO}]_1$ is calculated from relation (1) with $[\beta\text{-CD}]_A = [\beta\text{-CD}]_T$

Step 3: $[\beta\text{-CD}/G]$ is calculated from relation (1) with $[\beta\text{-CD}]_A = [\beta\text{-CD}]_T - [\beta\text{-CD/MO}]_1$

Step 4: $[\beta\text{-CD/MO}]_2$ is calculated from relation (1) with $[\beta\text{-CD}]_A = [\beta\text{-CD}]_T - [\beta\text{-CD}/G]$

Step 5: if $[\beta\text{-CD/MO}]_2 \neq [\beta\text{-CD/MO}]_1$
then go to *Step 3* with $[\beta\text{-CD/MO}]_1 = [\beta\text{-CD/MO}]_2$
if $[\beta\text{-CD/MO}]_2 = [\beta\text{-CD/MO}]_1$ then go to *Step 6*

Step 6: Calculation of theoretical absorbance

$$A_{\text{theoretical}} = 1 * (\epsilon_{\text{MO}} * [\text{MO}] + \epsilon_{\beta\text{-CD/MO}} * [\beta\text{-CD/MO}])$$

Step 7: if $A_{\text{observed}} \neq A_{\text{theoretical}}$ then go to *Step 1* with a new value for K_f
(determined by the algorithm as a function of $A_{\text{observed}} - A_{\text{theoretical}}$)
if $A_{\text{observed}} = A_{\text{theoretical}}$ then the formation constant is the postulated K_f value

In this way, all concentrations are known, without approximation. The Excel spreadsheet affords a convenient basis to perform such customised numerical treatment.

3. Results and Discussion

3.1. POTENTIOMETRY

pKa determination has been investigated in order to define the pH for which little interference is expected to arise from the phenolate form of our compounds.

The pKa corresponding to hydroxyl groups are listed in Table I. Among the sixteen phenols studied, two present a pKa relative to the para-substituent. The pKa resulting from the primary hydroxyl of hydroxybenzyl alcohol is higher than its secondary analog, so that no interference is expected for neutral pH. This is not the case for hydroxyphenyl acetic acid which exhibits a second pKa close to 4.4. It implies that acetate represents the predominant species for neutral pH, and that the inclusion formation constant has to be measured in acidic medium.

Most of the other compounds have pKa values greater than 9. It implies that the phenolate fraction is less than 0.1% for pH = 5.8, thus leading to very slight interference. The effect of such values on the calculation of inclusion formation constants will remain negligible. This leads to the conclusion that the stability

constants may be calculated for pH = 5.8 (for each methods) without important deviation due to the phenolate fraction.

3.2. CIRCULAR DICHROISM

Circular dichroism affords a convenient way to prove the existence of real inclusion complexes [20]. Indeed, observed changes in the absorption spectra may result from the formation of association complexes: considering that no optical activity can be induced by these flexible supramolecular systems, only inclusion complexes may be responsible for the dichroic signal.

Optical activity has been observed for each of the sixteen phenols, even when the affinity for β-CD was presumed to be weak. Induced circular dichroism (ICD) intensities corresponding to $S_0 \rightarrow S_1$ are reported in Table I. It has to be stressed that the formation constant may be evaluated on the basis of ICD intensity, but the signal to noise ratio which is exhibited by phenol complexes are too weak to lead to accurate determination of the stability constant.

The observed variation of ICD as a function of $[\beta\text{-CD}]_T$ may be helpful for the detection of multi equilibrium complexes, since molecular ellipticity may change significantly from one stoichiometry to another. No abnormal behaviour was noticed for any of the sixteen compounds, thus confirming that a single 1 : 1 equilibrium process is a reasonable assumption for quantitative determination by NMR and UV-Visible spectroscopy.

The ICD signs of the adducts can also be extracted from Table I. It has to be emphasised that each halogenophenol complex presents a negative signal. This suggests that inclusion modes are similar, whatever halogenophenol is used. The same conclusion is to be applied for alkylphenols, as well as for alkoxyphenols. The inclusion mode is of crucial importance when dealing with QSAR, since no relationship may be found if the inclusion geometry differs significantly from one compound to another.

3.3. COMPARISON OF THE FORMATION CONSTANTS OBTAINED BY ^{1}H NMR, UV SPECTROSCOPY AND THE SPECTRAL DISPLACEMENT STUDY

In order to cross validate quantitative methods, we have submitted compounds 1, 5, 8 and 9 to UV, NMR and spectral displacement studies, at pH 5.8. Agreement is generally difficult to obtain for such compounds due to the relatively low values of their formation constants.

UV spectroscopic studies. UV absorption has been extensively applied for the determination of formation constants [18, 25–26]. Nevertheless, it has to be stressed that the evaluated K_f often differ from one author to another, illustrating the lack of reproducibility of the results and the existence of bias. The main analytical difficulty when studying cyclodextrins inclusion complexes lies in the relatively

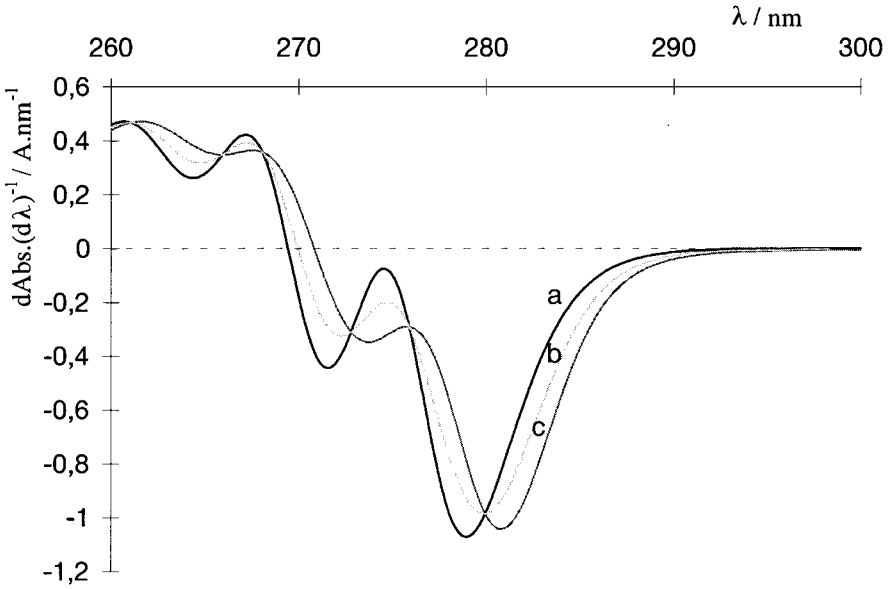

Figure 1. Derivatives of the absorption spectra for phenol solutions in the absence (a) and in the presence of 3.5 mM β-CD (b), 10 mM β-CD (c).

small spectral differences, which increases the influence of experimental errors. Moreover, such errors remain of relative importance when spectra are used as usually recorded. This leads to specific conditions such as reduced scan speed, or integration of values over a wavelength range as wide as possible (under the condition that the absorbance difference between free and complexed guest is high enough). Another origin of error arises from the optical presence of β-CD, which results in very slight but not reproducible values of absorbance, even below solubility limits. Thus, taking a solution with β-CD alone as reference leads to an imprecise spectrum of the complex. To avoid this difficulty, we have used the derivatives of the spectra, since the absorbance of a β-CD solution is nearly constant over a short wavelength range. This assertion is verified by isosbestic points (Figure 1), excellent definition of which could not be reached with absorption curves. Moreover, the calculated constants for different wavelength ranges leads to identical values and confirms the efficiency of the derivatives of the spectra compared to the absorption curves. Excellent reproducibility is obtained, and the remaining variations may be mainly due to irreducible concentration imprecision.

NMR spectroscopic studies. If NMR is a powerful tool for qualitative characterisation of β-CD complexes, it has many drawbacks for quantitative determination. First of all, formation constants are slightly different in D_2O than in water. Secondly, deuterated solutions are generally prepared in small amount, leading to concentration miscalculation if compared to water solutions. Thirdly, if the integ-

ration of values over a wavelength range results in a considerable accuracy gain for UV-Visible studies, no similar tool may be applied for NMR. This has to be compared to the low difference in chemical shifts between free and complexed β-CD. The ratio between reproducibility and spectral variation (between free and complexed species) observed for our compounds greatly differ for the two spectroscopic techniques: 5 to 10% for NMR while only 1% for UV-Visible. We have simulated the maximum error on the K_f value which could arise from deviations of this order, for a titration involving four different concentrations. The resulting inaccuracy varies from 18 to 300% for a ratio equal to 1% and 10% respectively. These values represent extreme upper limits but clearly show that K_f determination is less accurate by means of NMR than UV-Visible spectroscopy for this kind of complex.

Spectral displacement method. The principle of competition with MO has already been used to determine formation constants, especially in the case of α-cyclodextrin and in acidic condition, for which important spectral variations are observed [22–24]. The data treatment generally utilised for this kind of method lies in the assumption that guest and cyclodextrin are employed in great excess with respect to MO [24]. This hypothesis allows the neglect of [β-CD/MO] if compared to [β-CD/G], and can be considered as valid since a 0.01 mM concentration is required for [MO].

Nevertheless, if measurements involve the basic form of MO, and this is the case in our experiments, differences in absorbance between free and complexed dye are not so large, in such a way that specific conditions are required. In order to optimise spectral variation, spectra were recorded between 520–530 nm for [MO] = 0.1 mM. Under these conditions, no assumption could be used, so that we developed the algorithmic treatment described in the experimental section. This numerical method not only allows the calculation of the formation constant but also the simulation of absorbance variation for hypothetical data of concentrations and formation constants. Simulations are of particular interest for concentration optimisation. Indeed, using $[\beta\text{-CD}]_T$ as high as possible in order to maximise the absorbance variation between MO alone and MO + β-CD does not result in an optimised absorbance variation between MO + β-CD and MO + β-CD + G. Figure 2 describes the optimal β-CD concentration as a function of K_f, for $[G]_T$ fixed to 5 mM. For the range corresponding to the formation constants of phenols, it appears that $[\beta\text{-CD}]_T$ has to be closed to 0.5 mM (especially in the case of a low K_f value for which such optimisation is crucial) in order to obtain a maximum absorbance variation.

In these conditions, the spectral displacement method proved to be an accurate and easy to run tool for quantitative studies. Indeed, few variations were observed between each run, and a single guest concentration permits the formation constant calculation, thus allowing multiple attempts. Formation constants thus obtained are compared to UV and NMR results in Table II.

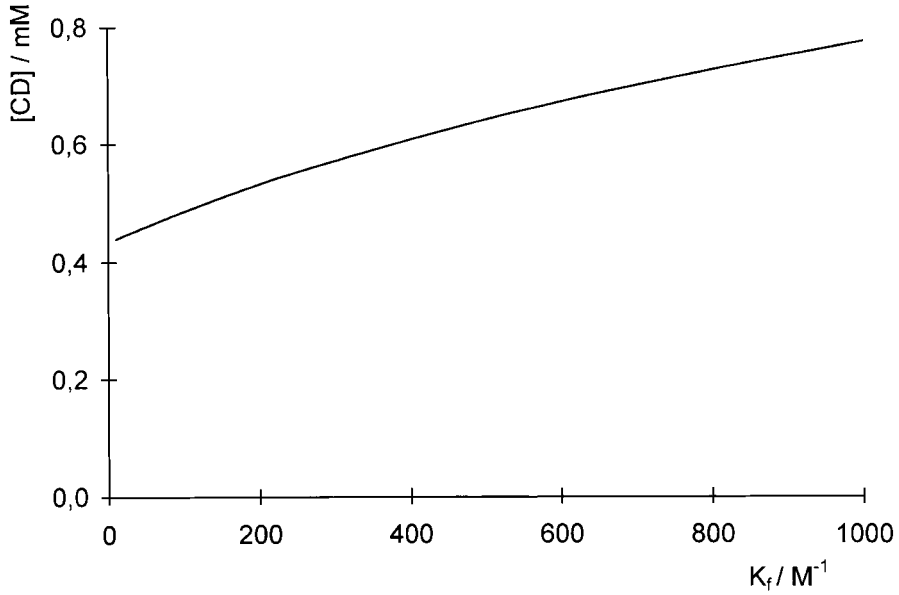

Figure 2. Plot of the optimal CD concentration to be employed for the competitive method as a function of formation constant, for $[G]_T$ fixed to 5 mM.

Table II. Comparison of formation constants (M^{-1}) obtained for inclusion complexes between β-CD and four phenol derivatives (**1**, **5**, **8** and **9**).

No.	Substituent	^{1}H NMR	Direct UV absorption	Displacement method
1	—F	105 ± 25	107 ± 7	105 ± 7
5	—H	120 ± 15	95 ± 6	105 ± 5
8	—CN	180 ± 30	195 ± 8	200 ± 10
9	—OH	100 ± 10	115 ± 6	115 ± 6

Excellent agreement is obtained, but, as already mentioned, the resulting precision differs greatly from one technique to another. NMR shows important deviations between each run and does not constitute the most suitable technique for such complexes. In contrast, no significant difference may be extracted from values obtained by the two spectrophotometric methods. Thus, in the following, the spectral displacement method is performed five times for each para-substituted phenol, and direct measurement is used within a single run to confirm previous data. Such methodology associates the speed involved in the spectral displacement method, and the reliability of cross-validation.

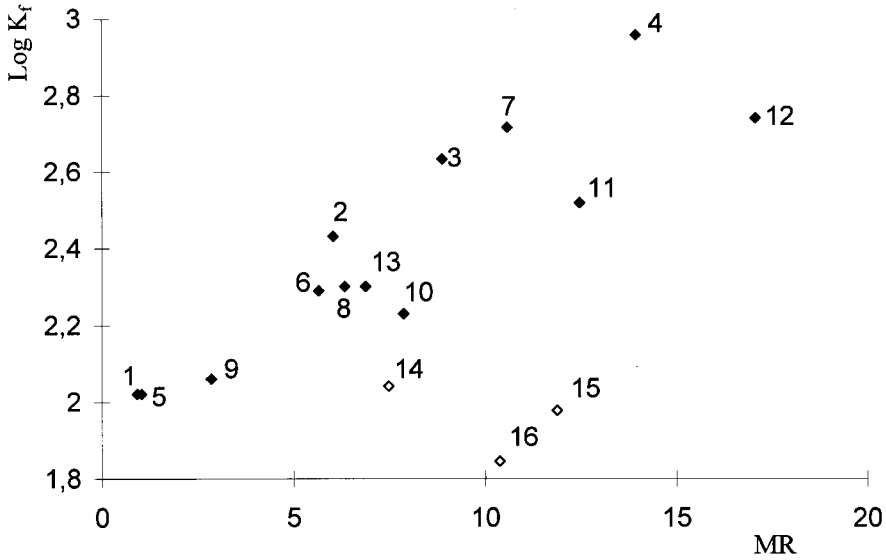

Figure 3. Correlation between log K_f and molecular refractivity (MR), for the sixteen phenols considered. Open squares corresponds to outliners discarded for quantitative evaluations.

3.4. QSAR

Formation constants for the sixteen phenols considered are listed in Table I (mean reproducibility close to 6%), as well as a set of indicator variables for the various substituents (taken from reference [27]).

Linear regressions between inclusion constants, taken in the logarithmic form, and each variable have been achieved and show the essential contribution of van der Waals interactions. Indeed, as described in Figure 3, log K_f tends to increase as the MR values become higher. The major outlines observed in Figure 3 correspond to compounds which exhibit a significant dipole not coplanar to the phenyl ring: phenol sulfonate, phenol acetate and hydroxybenzyl alcohol. Thus, if these aromatics are discarded, better insight into the influence of van der Waals interactions is obtained. Considering compounds **1–13**, 89.7% of the formation constant variation is explained by MR variation. From the resulting graph, two major trends can be distinguished, which separate alkoxy derivatives and the other *p*-substituted phenols. Indeed, the formation constants relative to the alkoxy series are significantly lower, and this could be attributed to dipolar influences but also to the increased flexibility of such substituents. The correlation coefficient increases to 0.986 for phenols **9–13**, and to 0.987 for aromatics **1–8**, if considered separately. Thus, separate regressions obtained for compounds **1–8** and **9–13** are assumed to illustrate the contribution of van der Waals interactions to complexation phenomena.

While no reliable relation could be obtained with σ_p (R = 0.055), a correlation coefficient equal to 0.774 is obtained between log K_f and π values. This latter point was predictable, according to the dependence between π and MR. If the

three descriptors MR, π and σ_p are used simultaneously to explain the formation constants, the best fit is obtained for the relation:

$$\text{Calculated } \log K_f = 1.978 + 0.042 * \text{MR} + 0.216 * \pi + 0.273 * \sigma_p. \quad (4)$$

The correlation coefficient between the logarithm of experimental and theoretical formation constants is then equal to 0.965. Relation (4) shows that hydrophobic forces also positively contribute to binding, which is in agreement with the assumption that the substituent interacting with the cavity is less exposed to the solvent. Moreover, this relation demonstrates the influence of dipolar interaction, since a withdrawing substituent favours the complex formation. Indeed, if such a substituent penetrates into the cavity, it implies that the dipoles of the host and guest are antiparallel: the positive end of the guest dipole faces the primary rim of the β-cyclodextrin (which corresponds to the negative end of the host dipole), thus leading to a stabilisation of the inclusion compound.

4. Conclusion

The combined use of potentiometry, circular dichroism, ^{1}H NMR and UV spectroscopy allows a reliable description of inclusion compounds, even with low affinity. The use of algorithmic treatments and specific conditions has been discussed and leads to coherent values of formation constants. Such a correlation represents an essential requirement for the study of structure affinity relationships. Applied to a phenolic series, our methodology has permitted us to evaluate the influence of van der Waals, hydrophobic and dipolar interactions. Nevertheless, the molar refractivity only represents an approximation for host/guest complementarity, so that a molecular modelling study is under progress to correlate formation constants obtained in this work with computed energetic differences between free and complexed species.

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