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# Nonlinear analysis of dynamic binding in affinity capillary electrophoresis demonstrated for inclusion complexes of β-cyclodextrin

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**Abstract**

The stability of the molecular host–guest inclusion complexes of β-cyclodextrin with benzoate and four different hydroxybenzoates is investigated. For the measurement of the binding constants an experimental method is devised that is based on affinity capillary electrophoresis (ACE) with indirect UV absorbance detection. We derive an explicit equation for effective mobilities in ACE experiments without violation of rigorous mass balance. This equation is employed in the nonlinear least-squares analyses of the experimental data yielding binding constants of $48\pm2\ M^{-1}$ for benzoate, $299\pm38\ M^{-1}$ for 2-hydroxybenzoate, $37\pm1\ M^{-1}$ for 3-hydroxybenzoate, $228\pm9\ M^{-1}$ for 4-hydroxybenzoate, and $895\pm110\ M^{-1}$ in the case of 2,4-dihydroxybenzoate. 

*Keywords:* Binding constants; Affinity capillary electrophoresis; β-Cyclodextrin; Benzoate; Hydroxybenzoates

## 1. Introduction

The family of native cyclic oligosaccharides called cyclodextrins (CDs) is one of the most intensively studied macromolecular systems [1–5]. Its member β-CD is composed of a ring of seven (1→4)-linked α-D-glucose monomer units which create a three-dimensional structure that is reminiscent of a truncated cone. The cavity of the cone creates a relatively hydrophobic environment, whereas its external surface has a hydrophilic character. This unique conformation is largely responsible for the characteristic physico-chemical properties of β-CD. In particular, its ability to form non-covalent inclusion complexes has created immense interest, possibly because the formation of cyclodextrin-based guest–host complexes is a simple model for the study of molecular recognition processes [6]. Moreover, cyclodextrins have found numerous industrial applications. Examples include use as food additives [7], stabilizers against degradation [8], and carriers in drug delivery systems [9,10]. In analytical chemistry, and specifically in the separation sciences, CDs are commonly used as media modifiers to achieve the separation of isomeric compounds [11,12]. Furthermore, they are highly valued as chiral selectors in capillary electrophoresis (CE) [13–15].

Despite this impressive spectrum of applications, an in-depth understanding of the involved cooperative processes and forces has not been reached.

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Accordingly, one of the immediate challenges in this field is to provide rules that allow for an assessment of the ability of a molecule to form a complex with CD. Such rules should provide useful guidelines for the design of inclusion complexes with desired physico-chemical characteristics by rationalizing the selection of structural changes to a parent compound. This study provides systematic data on the host–guest binding constants for the parent compound benzoate (BA), the structural isomers 2-, 3-, 4-hydroxybenzoate (2-, 3-, 4-hyBA), and 2,4-dihydroxybenzoate (2,4-hyBA). This class of compounds was chosen as a model because its members possess distinct but simple structural features like apolar, polar, and charged regions. In addition, they allow the formation of hydrogen bonds which might influence the stability of the inclusion complex.

In recent years, CE has been proven to be a valuable tool for the measurement of complex formation constants of CD with charged guest molecules [17–19]. The primary interest in many of the earlier studies, however, was the separation of native CDs and the CE methods were optimized accordingly [20,21]. They employed two different flavors of affinity capillary electrophoresis (ACE) which are based on indirect absorbance detection (IAD) or direct absorbance detection (DAD) [17]. ACE utilizes the differences in the mobility of analytes caused by their characteristic affinity to electrolyte molecules [22–25]. In the IAD technique, the background electrolyte (BGE) contains the guest molecules and the mobility of the analyte (CD) is measured as a function of the guest's concentration. On the other hand, in the DAD method the guest is run as analyte and the CD is part of the BGE. Consequently, the mobility of the guest can be determined as a function of the concentration of CD in the BGE. The quantitative analysis of the equilibrium constant for 1:1 guest–host complexes is based on the general form of a binding isotherm and an expression for the effective electrophoretic mobilities derived for the conditions of the experiments. Many earlier CE studies employed a linear plotting method for this analysis, which assumes that the equilibrium concentration of the free guest can be approximated as the total guest concentration [17–19,26]. This simplification, however, severely restricts the range of experimental conditions and is not reasonable if a significant fraction of the guest population is complexed [27]. More recently, this problem was addressed by Guillaume and co-workers, who considered the degree of complexation explicitly [28,29].

In this work, the experimental data is analyzed in terms of an equation for the effective electrophoretic mobility that rigorously obeys mass conservation. The values of the specific binding constants are obtained by a non-linear least-squares analysis. Moreover, the β-CD inclusion complexes are studied using a modified ACE-IAD technique which is optimized for the measurement of host–guest binding constants. The pH of the BGE is adjusted to a value which assures that the carboxylic acid group of the guest is deprotonated, whereas the hydroxyl group (if present) is protonated. In addition, we refrained from adding commonly used buffer systems or modifiers to the BGE to avoid conceivable competition processes for the binding site of β-CD. Because all of our measurements are performed under similar experimental conditions, this study allows for a direct comparison of the data based solely on the structural differences between the guest molecules.

## 2. Materials and methods

### *2.1. Chemicals*

All solutions were prepared from analytical grade chemicals and used without further purification. The compounds were desolved in deionized water (18.3 MΩ) obtained from a Barnstead Easypure system (Barnstead, Boston, MA, USA). β-Cyclodextrin, 2-, 3-, 4-hydroxybenzoic acid, and 2,4-dihydroxybenzoic acid as well as 1 *M* NaOH were purchased from Fluka (Milwaukee, WI, USA). Benzoic acid was obtained from Acros Organics (Fairlawn, NJ, USA). A 10 m*M* solution of β-cyclodextrin was prepared in deionized water and stored at about 4°C. Prior to each experiment small volumes of the cyclodextrin solution were equilibrated to room temperature and used as analyte. Electrolyte concentrates (0.4 *M*) of the organic acids were prepared by dissolving each of the acids in deionized water. These solutions were then titrated with NaOH to pH 7. Subsequent dilutions in a concentration range of 16–128 m*M* were

Table 1
Experimental conditions for the CE method

| | |
|---|---|
| Capillary: | 43.8 cm ($L_C$)×50 μm (I.D.); $L_E$=39.5 cm |
| Analyte: | 10 m*M* β-cyclodextrin |
| Injection: | pressure injection; 2 p.s.i.×s |
| Detection: | indirect UV at 254 nm |
| Voltage: | 15 kV |
| Temperature: | 25°C |
| BGE: | aqueous solution of the guest compounds in different concentrations as specified in Figs. 2a–e; adjusted to pH 7 with NaOH. |

prepared from the concentrates and the pH was readjusted if necessary. These solutions served as the BGE in each of the experiments.

### *2.2. Instrumentation*

The experiments were performed on a Bio-Rad BioFocus™ 3000 Capillary Electrophoresis system (Bio-Rad, Richmond, CA, USA) using 43.8 cm×50 μm (I.D.) bare fused-silica capillaries purchased from Polymicro Technologies (Phoenix, AZ, USA). Indirect UV on-column detection was carried out at 254 nm. All experiments were run at a constant voltage of 15 kV and the temperature of the sample/buffer compartment as well as the cooling water of the capillary was set to 25°C. Unless otherwise stated, we used the experimental conditions listed in Table 1.

### *2.3. Electrophoretic procedures*

The capillary was conditioned daily with a sequence of high-pressure purges (20 p.s.i.). First, it was rinsed with 1 *M* NaOH for 6.5 min, followed by water and finally the BGE solution, both for 10 min. Before each injection the capillary was filled with BGE using a high pressure purge for 2 min. Whenever the concentration of the BGE was changed the capillary was reconditioned with 1 *M* NaOH for 1 min, water for 3.3 min and the BGE solution for 5 min.

## 3. Results and discussion

The binding constants of the inclusion complexes of β-cyclodextrin with benzoate, 2-, 3-, 4-hydroxybenzoate, and 2,4-dihydroxybenzoate were determined on the basis of ACE-IAD experiments. A typical electropherogram is depicted in Fig. 1. It shows an experiment in which a 10 m*M* β-CD analyte was injected hydrodynamically. In this example, a 32 m*M* benzoate solution was used as the running buffer. The first peak (a) in Fig. 1 represents the water peak which also serves as a marker for the electroosmotic flow (EOF). Peak (b) stems from the elution of the β-CD. The β-CD moves slower than the EOF due to the dynamic process of inclusion complex formation with the negatively charged benzoate molecules in the running buffer. The dependence of the effective mobility of the β-CD on the concentration of the acid in the running buffer is utilized to evaluate the binding constants. The effective electrophoretic mobility, $\mu_{eff}$, can be determined from the measured migration times of the analyte, *t*, and of the water signal, $t_{EOF}$, for each of the initial concentrations of the buffer solution according to the following expression [30]:

$$\mu_{eff} = (L_C/U)(L_E/t - L_E/t_{EOF}) \quad (1)$$

where $L_E$ is the effective length of the capillary, $L_C$ the total length of the capillary, and *U* the applied voltage. The corresponding results are presented in Fig. 2a–e. The squares represent the effective mobilities calculated according to Eq. (1) at different initial concentrations of the acids. Each individual data point is the mean of six separate measurements and the error bar represents their standard deviation.

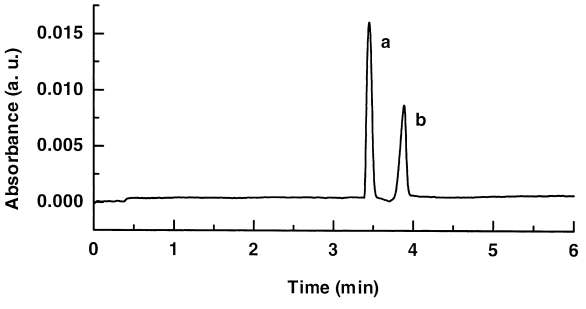


Fig. 1. Typical electropherogram obtained from an experiment using benzoate (32 m*M*) as BGE and β-CD (10 m*M*) as analyte. The experimental conditions are summarized in Table 1. Peak assignments: (a) water (used as EOF marker) and (b) β-CD.

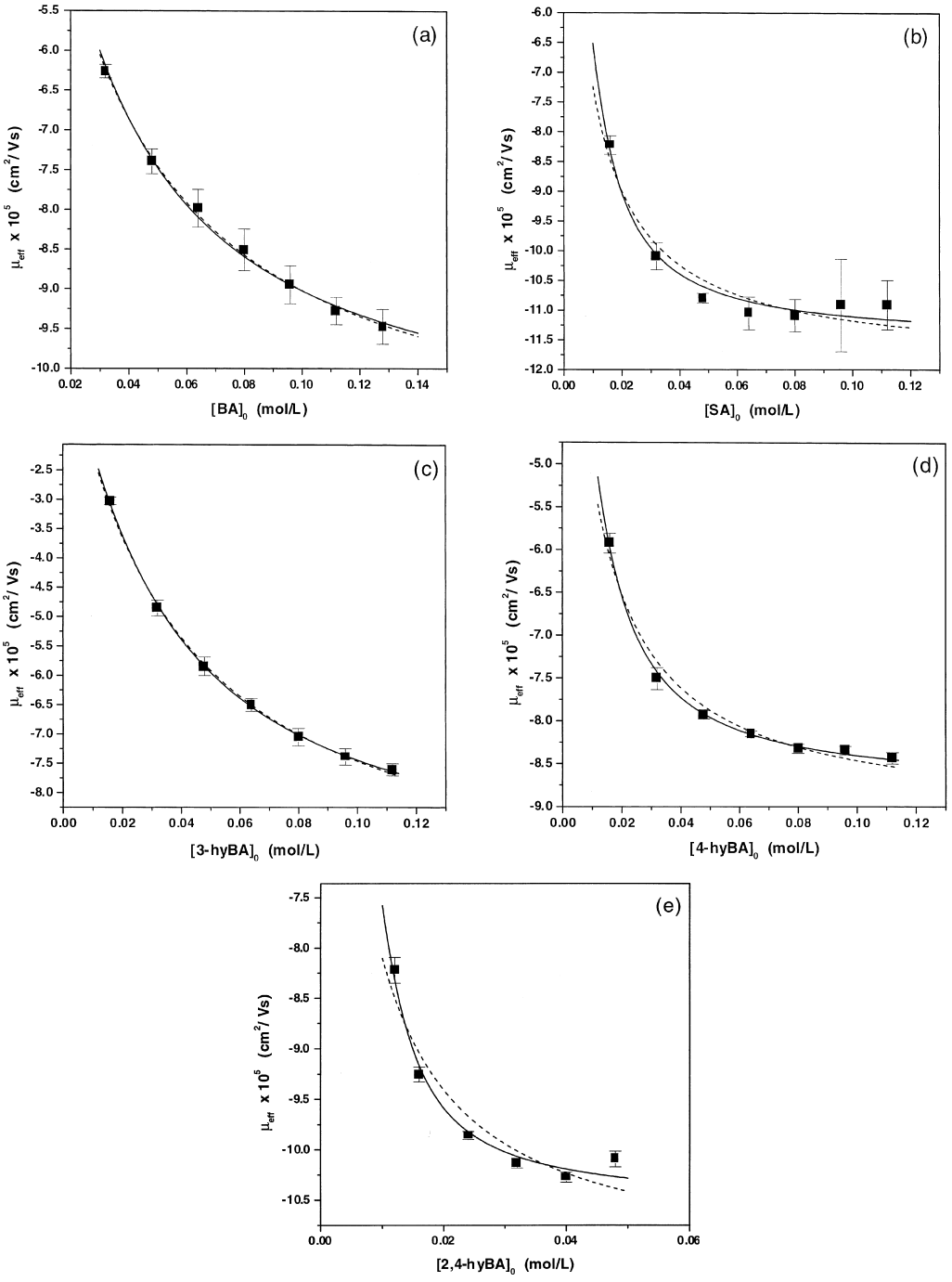


Fig. 2. Effective mobilities, $\mu_{eff}$, as a function of the guest concentration in the BGE. The individual plots are obtained for the guest compounds: (a) benzoate (BA), (b) salicylate (SA), (c) 3-hydroxybenzoate (3-hyBA), (d) 4-hydroxybenzoate (4-hyBA), and (e) 2,4-dihydroxybenzoate (2,4-hyBA). The data points (solid squares) are obtained from the measured retention times according to Eq. (1). The experimental conditions are given in Table 1. The curves show the optimized fits according to the function in Eq. (9). The corresponding binding constants and mobilities of the complexes are summarized in Table 2.

The 1:1 molecular association equilibria between β-CD as host and the different acids as the guest molecules can be described by the following reaction equation:

$$A^- + \beta\text{-CD} + H^+ \rightleftarrows C^- + H^+, \quad (2)$$

where $A^-$ and $C^-$ symbolize the ionized form of the acid and the inclusion complex, respectively. Because the pH of the solution is much higher than the $pK_a$ of the carboxylic acid group ($pH \gg pK_a$), it is reasonable to assume that the acids are present in their ionized form. Therefore, one can neglect the acid–base equilibria as well as the complex formation of β-CD with the protonated form of the acid. Furthermore, the hydroxyl group of the hydroxybenzoates remains predominantly undissociated ($pH \ll pK_a$). In the following we denote the equilibrium concentrations of β-CD, $A^-$, and $C^-$ as a, b, and c, respectively. For Reaction (2) the binding constant, $K$, is therefore given as:

$$K = \frac{c}{ab}. \quad (3)$$

Straightforward mass conservation laws apply to all points within the capillary because the guest–host equilibrium involves reactions that are fast compared to transport-induced perturbations. These laws constrain the sum of $a$ and $c$ as well as the sum of $b$ and $c$ to the constant values $a_0$ and $b_0$:

$$a_0 = a + c, \quad b_0 = b + c, \quad (4a,b)$$

where $a_0$ and $b_0$ equal the initially employed concentrations of the β-CD and organic acid, respectively. The effective electrophoretic mobility can be expressed in terms of the mole fraction of the complex, $x_C$, and the ion mobility of the complex $\mu_C$:

$$\mu_{eff} = x_C\, \mu_C. \quad (5)$$

This expression does not consider corrections for viscosity variations [16]. The mole fraction $x_C$ can be given as:

$$x_C = c/(a + c) = c/a_0. \quad (6)$$

Combining Eqs. (5) and (6) yields:

$$\mu_{eff} = \mu_C c/a_0. \quad (7)$$

Substitution of Eqs. (4a) and (4b) into Eq. (3) yields an expression for c that describes the concentration of the complex as a function of the binding constant $K$ and the initial concentrations of the reactants (i.e., $a_0$ and $b_0$):

$$c = \frac{a_0 + b_0 + (1/K)}{2} - \left(\tfrac{1}{4}(a_0 + b_0 + (1/K))^2 - a_0 b_0\right)^{1/2}. \quad (8)$$

The binding isotherm (8) can be substituted into Eq. (7) resulting in an expression that relates the dependent variable $\mu_{eff}$ to the binding constant and the initial concentrations of the host and the guest:

$$\mu_{eff} = (\mu_C/a_0)\left[\frac{a_0 + b_0 + (1/K)}{2} - \left(\tfrac{1}{4}(a_0 + b_0 + (1/K))^2 - a_0 b_0\right)^{1/2}\right]. \quad (9)$$

This equation should be compared to the approximate result:

$$\mu_{eff} = \mu_C\, [b_0/(b_0 + 1/K)]. \quad (10)$$

that is readily obtained from Eqs. (3), (5), and (6) under the assumption of negligible guest complexation ($b \approx b_0$) (see, e.g., Ref. [18]).

A nonlinear least-squares method based on the Levenberg–Marquardt algorithm [31] was employed to fit the functions of Eqs. (9) and (10) to the experimental data. In this fitting procedure, $a_0$ and $b_0$ are the known initial concentrations of the reactants and the values of $\mu_{eff}$ are the results from the CE measurements according to Eq. (1). In both cases, only the values of $K$ and $\mu_C$ are treated as unknown variables. The best fits are given in Figs. 2a–e along with the experimental data. The solid and dashed curves represent the fits obtained from Eqs. (9) and (10), respectively. The results reveal less agreement between experimental and numerical data for the fits obtained from Eq. (10), although both fitting procedures optimize the same number of unknown parameters. The difference in the quality of the fits is most pronounced in the case of 2,4-hydroxybenzoate and marginal for benzoate and 3-hydroxybenzoate.

The values determined for the binding constants, $K$, and the ion mobilities, $\mu_C$, of the complexes are summarized in Table 2 according to the equation used in the fitting procedure. The table is complemented by the $\chi^2$ values of the best fits. This

statistical measure reveals that the fits obtained from Eq. (10) yield poorer agreement with experimental data than those obtained from Eq. (9). This finding is most pronounced in the case of large binding constants. Although the $\chi^2$ values are comparable for equilibria with small binding constants, the two equations still yield significant differences in the values of $K$. These results demonstrate that the rigorous consideration of mass balance is of importance in the analysis of ACE data.

Notice that the systematic error introduced by Eq. (10) increases not only with increasing binding constants but also with increasing values of $a_0$. Hence, the use of Eq. (9) imposes fewer restrictions to the choice of initial concentrations and accordingly these parameters can be adjusted more readily to suit experimental or technical requirements of the ACE experiment. We therefore consider the latter analysis to be a straightforward and a versatile approach. In addition, we note that the mathematical structure of Eq. (9) also allows for fitting procedures that involve the concentration $a_0$ as an additional unknown variable. Although this bonus feature might be valuable for certain applications in analytical chemistry, its applicability requires very large binding constants and low signal-to-noise ratios. We attempted to carry out a three-variable fit of the experimental data obtained for 2,4-dihydroxybenzoate. This analysis yielded a mobility of $\mu_C = -1.04\times10^{-4}$ cm$^2$/Vs and a total β-CD concentration of $a_0 = 0.012$ $M$ which is only 20% larger then the actually employed concentration. The binding constant, however, was optimized at $K = 1590$ $M^{-1}$ which appears to be unreasonably large.

In the following, we focus the discussion of our experimental results on the data obtained from fits with Eq. (9). In the context of this study, the binding constants vary by a factor of about 20 ranging from $37\pm1$ $M^{-1}$ for 3-hydroxybenzoate to $895\pm110$ $M^{-1}$ for 2,4-dihydroxybenzoate. We determined the binding constant of the parent compound benzoate with β-CD to be $48\pm2$ $M^{-1}$. This is in good agreement with earlier CE studies, where binding constants of 23 [19] and 50 $M^{-1}$ [26] have been reported. Furthermore, the binding constant of benzoate with β-CD also compares well to data obtained from techniques such as potentiometry and microcalorimetry. For this particular example, a value of 60 $M^{-1}$ was found by potentiometry [32] and values in the range of 10–20 $M^{-1}$ were determined based on microcalorimetric measurements [33,34]. In the case of 2-hydroxybenzoate (salicylate), earlier CE studies report binding constants in the range of 50–82 $M^{-1}$ [17,19]. A dynamic dialysis technique yielded a value of 51 $M^{-1}$ [35] and based on conductivity measurements the binding constant was determined to be $105\pm15$ $M^{-1}$ [36]. In comparison, we determined the binding constant for the salicylate-β-CD inclusion complex to be $299\pm39$ $M^{-1}$. This value is higher than the data reported in the literature and deviates significantly from the ones obtained in earlier CE experiments. We attribute a major part of this discrepancy to the method of data analysis used in these prior studies which assumed that the equilibrium concentration of the free guest equals its initial concentration. For example, our experimental data yielded binding constants of $38\pm1$ and $155\pm25$ $M^{-1}$ in the cases of benzoate and salicylate, respectively, if the nonlinear least-squares fitting was carried on the basis of Eq. (10) (compare Table 2). In regard to the other guest compounds studied, we are not aware of any data in the literature.

A comparison of the binding constants with respect to the position of the hydroxyl group yields no simple trend. However, the substitution of a hydroxyl group in 2- and 4-position results in a significantly increased stability of the inclusion complex. The binding constants in these cases are approximately five times higher than the binding constant of benzoate with β-CD. The effect of increased stability is further amplified in the 2,4-dihydroxybenzoate complex. On the other hand, substitution of a hydroxyl group in 3-position leads to a decreased binding constant even compared to the relatively weakly bound β-CD–benzoate complex.

In conclusion, this work provides quantitative data on the binding constants for β-CD-inclusion complexes involving a set of structurally related organic acids. These data should prove to be valuable for future attempts to formulate phenomenological rules regarding the stability of this important type of inclusion complex. In addition, our study employed optimized conditions in the IAD-ACE experiments and demonstrated the successful application of an expression for $\mu_{eff}$ that accounts for the concen-

Table 2
Binding constants for the solutes to β-cyclodextrin and values of the electrophoretic mobility of the respective complexes

| Compound | Analysis based on Eq. (9) | | | Analysis based on Eq. (10) | | |
|---|---|---|---|---|---|---|
| | Binding constant $K$ ($M^{-1}$) | Mobility of the complex $\mu_C$ ($cm^2/Vs$) | Minimum of $\chi^2/10^{-13}$ | Binding constant $K$ ($M^{-1}$) | Mobility of the complex $\mu_C$ ($cm^2/Vs$) | Minimum of $\chi^2/10^{-13}$ |
| Benzoate | 48±2 | $-1.11\times10^{-4}\pm1\times10^{-6}$ | 6.46 | 38±1 | $-1.14\times10^{-4}\pm1\times10^{-6}$ | 3.34 |
| Salicylate | 299±38 | $-1.15\times10^{-4}\pm1\times10^{-6}$ | 37.5 | 155±25 | $-1.19\times10^{-4}\pm2\times10^{-6}$ | 86.9 |
| 3-hyBA | 37±1 | $-9.64\times10^{-5}\pm8\times10^{-7}$ | 1.86 | 28±1 | $-1.01\times10^{-4}\pm1\times10^{-6}$ | 4.79 |
| 4-hyBA | 228±9 | $-8.81\times10^{-5}\pm4\times10^{-7}$ | 2.25 | 124±11 | $-9.14\times10^{-5}\pm1.2\times10^{-6}$ | 18.8 |
| 2,4-hyBA | 895±110 | $-1.06\times10^{-4}\pm1\times10^{-6}$ | 14.8 | 260±49 | $-1.12\times10^{-4}\pm3\times10^{-6}$ | 64.2 |

The data listed in columns 2–4 and 5–7 show the results of nonlinear least-squares fits carried out on the bases of Eqs. (9) and (10), respectively. The errors estimated for the binding constants and the mobilities are the values of $\sigma_i = (C_{ii}\chi^2)^{1/2}$, where $C_{ii}$ is the diagonal element of the variance–covariance matrix.

tration difference between free and complexed guest ions. The presented analysis is a significant improvement compared to earlier investigations and should be applied if the concentration of the sample and/or the involved binding constants are high. However, our analysis also reveals a general problem of the measurement of equilibrium constants by ACE methods. The effective mobility and hence the results obtained depend not only on the concentration of the reactant in the BGE but also on the shape and the amplitude of the sample peak. The latter dependence gives rise to non-constant drift speeds within the moving sample (see Eq. (9)), which in extreme cases could induce asymmetric peak shapes and erroneous results. This problem is nontrivial in the sense that the peak shape is stabilized by diffusion only [37,38], thus, requiring a numerical analysis of the underlying reaction–diffusion–electromigration equations. Also in benign cases, the question as to the most appropriate value of $a_0$ is difficult to answer. For the analyses presented in this study, all peaks were assumed to be rectangular with plateau concentration equaling the initially employed concentration of β-CD. This assumption appears to be justified, because the binding constants of interest were not extremely high and the employed concentration of the host were relatively low. Notice that also the shapes of the observed sample peaks were typically symmetric (see Fig. 1). However, a more detailed analysis of this complex problem is needed in order to provide a general method of ACE-data analysis, which is also applicable to equilibria with very large binding constants.

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