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Review

Determination of equilibrium constants from chromatographic and electrophoretic measurements

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Abstract

Chemical interactions, such as acid–base, complex-forming, ion association and other equilibria, are widely exploited to improve the separation efficiency in liquid chromatography as well as in electrophoresis. On the other hand, these techniques can be advantageously used to study the chemical equilibria affecting the separations. If the equilibium is sufficiently fast in comparison with the separation process, then the retention characteristics in chromatography (retention factors) or the migration characteristics in electrophoresis (effective mobilities) may be expressed as functions of the composition of mobile phase or background electrolyte (BGE), respectively. Using a proper experimental arrangement, the dependencies of retention (migration) characteristics on the mobile phase (background electrolyte) composition can be measured and utilized to calculate the equilibrium constants for equlibria taking place in the mobile phase (background electrolyte). Although principles of these measurements have been known for a long time, only more recent studies utilizing HPLC and capillary electrophoretic techniques are reviewed in this paper.

Keywords: Reviews; Equilibrium constants; Acid–base equilibria; Complexation

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1. Introduction

Nowadays, many routine environmental, industrial and other analyses require determination of an increasing number of substances in a short time, often in complex matrixes and in a wide range of concentrations. Highly effective separation methods—chromatographic and electrophoretic, in the first place—are typically used for those purposes. Using today's advanced separation techniques, a great number of analytes (tens, if not hundreds) may be determined in a single run. Despite the progress in analytical instrumentation accompanied with growing separation efficiency, an in-depth understanding of the chemistry of the separation process is still necessary to solve successfully any analytical task.

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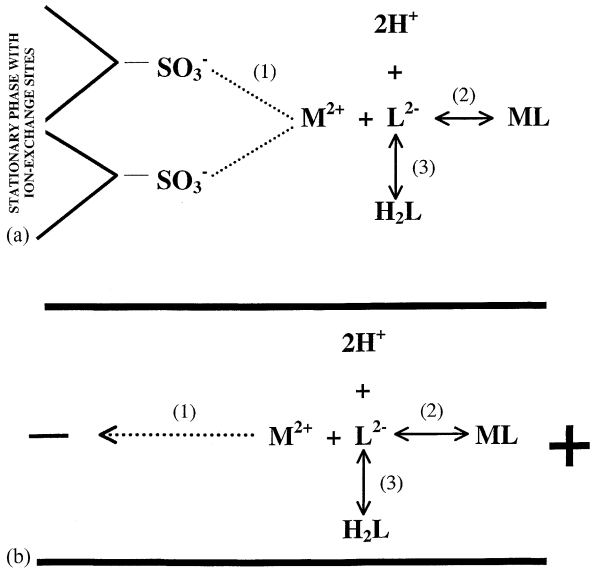

Fig. 1. Equilibria in separations of metal ions with the aid of ion chromatographic (a) and electrophoretic (b) methods. (1) Retention of metal cations M^{2+} on the ion-exchange sites in ion chromatography or attraction of metal cations to the oppositely charged electrode in electrophoresis, (2) complex-forming equilibrium of metal ions with the complexing ligand L^{2-}, (3) protonation equilibrium of the ligand L^{2-}. From [2], with permission.

It is well known that chemical equilibria between the separated compounds and constituents of working solutions (i.e. mobile phase in liquid chromatography or background electrolyte (BGE) in electrophoresis) may be advantageously exploited to govern the separation process and to manipulate the selectivity of the separation [1–3]. Acid–base and complex-forming equilibria belong to those most frequently used in the separation science, although some other interactions have also been employed successfully to improve the separations of certain classes of compounds. As an example, the equilibria effective in the separation of metal cations by ion chromatography (IC) and capillary electrophoresis (CE) are shown schematically in Fig. 1.

The knowledge of the separation mechanisms and model parameters including the respective equilibrium constants allows to predict behavior of analytes during the separation process and to optimize the separation [4,5]. Logically, on the other hand, the equilibrium constants for processes occurring in mobile phases or background electrolytes can be determined from the retention characteristics in liquid chromatography or migration characteristics in electrophoresis.

It should be pointed out that conventional separation methods such as ion-exchange and solvent extraction have been traditionally used for investigations of chemical equilibria and measurements of the equilibrium constants [6–8]. Many other methods and experimental arrangements have been proposed for the determinations of equilibrium constants. Some of them, of course, have lost their importance with time (such as the “frog’s heart method” used to measure the dissociation constants of the alkaline earth metal–citrate complexes [9]), while many others, on the other hand, are still extensively used (potentiometry, polarography, conductimetry, spectrophotometry), and some procedures developed here, e.g. for an evaluation of experimental data can be adopted for an evaluation of data from the chromatographic or electrophoretic measurements. In this work, principles and procedures for the determinations of equilibrium constants with the aid of modern separation methods—high-performance liquid chromatography (HPLC) and capillary electrophoresis, in the first place—are discussed, and selected applications are overviewed.

2. Chemical equilibria and equilibrium constants

The simplest one-to-one interaction of an analyte species, A, with a component of the working solution, C, can be expressed by the following reaction scheme:

$$\mathrm{A} + \mathrm{C} \Leftrightarrow \mathrm{AC} \tag{1}$$

Then a thermodynamic equilibrium constant is defined as:

$$(K)_a = \frac{a_{\mathrm{AC}}}{a_{\mathrm{A}} a_{\mathrm{C}}} \tag{2}$$

where a_{AC}, a_{A} and a_{C} are activities of AC, A and C in the equilibrium, respectively. Replacing the activities with equilibrium concentrations we obtain:

$$(K)_a = \frac{[\mathrm{AC}]}{[\mathrm{A}][\mathrm{C}]} \frac{\gamma_{\mathrm{AC}}}{\gamma_{\mathrm{A}}\gamma_{\mathrm{C}}} = K \frac{\gamma_{\mathrm{AC}}}{\gamma_{\mathrm{A}}\gamma_{\mathrm{C}}} \tag{3}$$

where γ_{AC}, γ_{A} and γ_{C} are activity coefficients of the species AC, A and C, respectively. [AC], [A] and [C] are the equilibrium concentrations in mol/l and K is the stoichiometric equilibrium constant [10,11]. The stoichiometric equilibrium constant is based on equilibrium concentrations of the species involved in the reaction scheme at the used experimental conditions. Its magnitude depends on temperature, pressure and on the ionic strength of the solution. This constant is the easiest to measure. Obviously, the value of K calculated from experimental data holds for the given experimental conditions only. The stoichiometric constants can be converted to the thermodynamic constants using some of the well known relations based on the Debye–Hückel theory, for example a relatively simple Davis equation [11,12]:

$$\log K = \log(K)_a + 0.5\,\Delta z^2 \left(\frac{I^{0.5}}{1 + I^{0.5}} - 0.3I \right) \tag{4}$$

I is the ionic strength and Δz^2 is an algebraic sum of squares of charges of the ions involved in the reaction. However, the stoichiometric equilibrium constants determined from chromatographic and electrophoretic measurements can be often used directly (without further re-calculations) to optimise the chromatographic and electrophoretic separations, because the conditions during the equilibrium constant determinations and analytical separations are usually similar.

Many kinds of interactions can be used to affect behavior of analytes during the separation process. Protonation/dissociation (acid–base equilibria), complex-formation, ion association, ion pairing, host-guest interactions and some others so-called side (or secondary) equilibria [1,2] are frequently used to improve the separability of specific analyte compounds. Various names are used for the corresponding equilibrium constants, e.g. association/dissociation constant, stability constant, complexation constant, binding constant, etc. [13]. Although the nature of chemical binding may be rather different in the above mentioned interactions, the reactions can be described using similar reaction schemes, and nearly identical formulas can be used to express the equilibrium constants. However, the reaction scheme is usually more complex than Eq. (1). A stepwise formation of metal complexes can serve as a good example. It is described by the following set of equations:

$$\begin{aligned} &\mathrm{M}^{x+} + \mathrm{L}^{y-} \Leftrightarrow \mathrm{ML}^{x-y}; \quad K_1 \\ &\mathrm{ML}^{x-y} + \mathrm{L}^{y-} \Leftrightarrow \mathrm{ML}_2^{x-2y}; \quad K_2 \\ &\vdots \\ &ML_{i-1}^{x-(i-1)y} + L^{y-} \Leftrightarrow ML_i^{x-iy}; \quad K_i \\ &\vdots \\ &ML_{n-1}^{x-(n-1)y} + L^{y-} \Leftrightarrow ML_n^{x-ny}; \quad K_n \end{aligned} \tag{5}$$

K_i are the consecutive stability constants. The overall complex-forming reaction for the complex ML_i can be written also:

$$\mathrm{M}^{x+} + i\mathrm{L}^{y-} \Leftrightarrow \mathrm{ML}_i^{x-iy} \tag{6}$$

with the equilibrium constant (called the overall stability constant), β_i:

$$\beta_i = \frac{[\mathrm{ML}_i^{x-iy}]}{[\mathrm{M}^{x+}][\mathrm{L}^{y-}]^i} \tag{7}$$

It holds for the consecutive and overall stability constants [11]:

$$\beta_i = K_1 K_2 \cdots K_i = \prod K_i \tag{8}$$

It is recommended to describe the acid–base equilibria in a similar way as a set of protonation reactions of the base B [11]:

$$\begin{aligned} &\mathrm{B}^{n-} + \mathrm{H}^+ \Leftrightarrow \mathrm{HB}^{1-n}; \quad K_{\mathrm{H1}} \\ &\mathrm{HB}^{1-n} + \mathrm{H}^+ \Leftrightarrow \mathrm{H_2B}^{2-n}; \quad K_{\mathrm{H2}} \\ &\vdots \\ &\mathrm{H}_{n-1}\mathrm{B}^- + \mathrm{H}^+ \Leftrightarrow \mathrm{H}_n\mathrm{B}; \quad K_{\mathrm{H}n} \\ &\mathrm{H}_n\mathrm{B} + \mathrm{H}^+ \Leftrightarrow \mathrm{H}_{n+1}\mathrm{B}^+; \quad K_{\mathrm{H}n+1} \end{aligned} \tag{9}$$

Very often, however, the acid–base equilibrium is considered as a dissociation process. In the simplest case—for monoprotic acid—it can be described by the following equation:

$$\mathrm{HB} \Leftrightarrow \mathrm{H}^+ + \mathrm{B}^- \tag{10}$$

with the dissociation constant K_a:

$$K_a = \frac{[\mathrm{H}^+][\mathrm{B}^-]}{[\mathrm{HB}]} \tag{11}$$

It holds for the protonation and dissociation constants:

$$K_{\mathrm{H}} = K_a^{-1} \tag{12}$$

Great care should be taken when using published values of equilibrium constants, because various concepts and notations can be found in literature.

As can be seen from the above Eqs. (5) and (9), the analytes may be present in mobile phase or background electrolyte in the forms of various species, e.g. in protonated/deprotonated forms or in the form of various complexes. These species differ significantly in their charge, molecular mass and some other properties, and therefore we can expect that their behavior during the separation process will also differ markedly. Taking into account the kinetics of the side equilibria, two basic situations may occur [2]:

(1) The equilibrium is attained slowly in comparison with the duration of the separation experiment (*inert systems*).
(2) The equilibrium is sufficiently fast and the time required for equilibrium is negligible compared with the duration of the separation experiment, i.e. with the separation time (*labile systems*).

In the case of the inert systems, several peaks appear in the chromatogram or electropherogram corresponding to the individual species, i.e. the species may be separated and determined individually. These measurements may be certainly employed for the determinations of equilibrium constants [14–16]. However, this is, at least in principle, a relatively trivial task. Those studies will not be discussed in this review.

In the case of the labile systems, the individual species cannot be determined separately. During the separation, the analyte behaves as one compound exhibiting properties that are a certain "average" of the properties of individual analyte species coexisting in the separation system. As a result, only one peak appears in the chromatogram or electropherogram.

In chromatography, an effect of fast equilibria can be treated using a concept of *limiting retention factors* that has been used in reversed phase [17–22] as well as in ion-exchange [23] separation modes. Let's assume, for example, that fast equilibrium according to Eq. (1) is established in the mobile phase. At any time of the separation process, a part of analyte is present in the form of the "free" (uncomplexed, unbound) species A, whereas the remaining part of the analyte is present in the form of the species AC. The retention behavior of the species A is characterized with the aid of the limiting retention factor k_{A}, whereas the retention behavior of the species AC is characterized with the aid of the limiting retention factor k_{AC}. Only one peak is observed on chromatogram, for which the following

holds true:

$$k = k_A x_A + k_{AC} x_{AC} \quad (13)$$

k is the observed retention factor of the analyte, x_A and x_{AC} are the mole fractions of the species A and AC, respectively. Obviously, the observed retention factor is the weighted average of the limiting retention factors of species, in which the analyte occurs in the mobile phase. The mole fractions in Eq. (13) can be calculated from the equilibrium concentrations of the species A, C and AC. Using the relation for the equilibrium constant Eq. (3), Eq. (13) may be re-written as follows:

$$k = \frac{k_A + k_{AC} K[C]}{1 + K[C]} \quad (14)$$

It is possible to calculate the equilibrium constant K (and also k_A and k_{AC}) when the k values are measured for various concentrations of C. This is a general principle of the determination of equilibrium constants from chromatographic measurements in the case of labile systems.

A similar approach can be used also for more complex sets of side equilibria. The generalized form of Eq. (13) is:

$$k = \sum k_i x_i \quad (15)$$

It is necessary to express the observed retention factor as a function of limiting retention factors, k_i, equilibrium constants, K_i, and a composition of mobile phase, c:

$$k = f(k_i, K_i, c) \quad (16)$$

K_i and k_i are constants that can be determined from the dependence of k on c, e.g. from the changes in the analyte retention with the changes of the mobile phase composition.

A quite similar approach can be applied also to electrophoretic measurements, where a concept of *effective mobilities* is even more common than the concept of limiting retention factors in chromatography. The effective (observed) mobility, μ_{eff}, of the analyte may be calculated from the weighted average of the mobilities of the individual analyte species, μ_i, present in the background electrolyte [2,24]:

$$\mu_{eff} = \sum \mu_i x_i \quad (17)$$

Again, the effective mobility has to be expressed as a function of μ_i, K_i, and the composition of the background electrolyte. The equilibrium constants are determined from dependencies of the effective mobility on the composition of the background electrolyte.

It should be pointed out that equations analogical to Eq. (15) or Eq. (17) may be written for any additive property, e.g. for spectrophotometric characteristics [25]. A general expression for an additive property is derived in [25]. However, the discussion in the following chapters will be focused on the chromatographic and electrophoretic measurements only.

3. HPLC determinations of equilibrium constants

3.1. Reversed-phase systems

The retention models in chromatography are usually based on an assumption that equilibrium between mobile and stationary phases is always maintained [26,27], although the non-equilibrium separation theories have also been presented [28]. In reversed-phase liquid chromatography (RP-LC), non-polar stationary phases are used, whereas aqueous solutions containing usually some organic modifier (methanol, acetonitrile) serve as mobile phases. According to the sovophobic theory, the analyte retention is governed by interactions in the mobile phase, whereas contributions from the stationary phase are ignored [29,30]. In this system, non-polar compounds are strongly retained and their retention is affected mainly by the concentration of organic modifier in the mobile phase.

Weak organic acids and bases that represent an important group of compounds determined by RP-LC undergo dissociation/protonation in mobile phase, and their retention depends on the mobile phase pH value. Thus, the analyte retention may be changed by adjusting pH of the mobile phase, and the separation may be easily optimized knowing the dissociation or protonation constants of the separated acids or bases; the appropriate retention models can be found in many papers [31–34]. A general equation relating the observed retention factor to the pH of the mobile phase was derived by Jano and co-workers [35,36] for polyprotic acids and bases using the concept of limiting retention factors:

$$k = \frac{k_0 + \sum_{r=1}^{n} k_r K_a(r) e^{rx}}{1 + \sum_{r=1}^{n} K_a(r) e^{rx}} \quad (18)$$

k_r are the limiting retention factors of the dissociated species, x is related to the pH value ($x = 2.303$pH), and $K_a(r)$ is the product of the first r-dissociation constants:

$$K_a(r) = \prod_{i=1}^{r} K_{a,i} x \quad (19)$$

The derived equation was used to calculate three dissociation constants of leukotriene E_4 from the pH dependence of the retention factor [35]. In [37], RP-HPLC was used to determine the dissociation constants of substituted benzoic acids, phenylacetic acid, 2,4-dinitrophenol, aniline and its derivatives. Sanli et al. [38] determined the K_a values of polyphenolic acids in acetonitrile–water mixtures from retention data measured in the reversed-phase system.

A sound basis for the mentioned studies was laid by Horváth et al. [39], who created the retention model for analytes participating in fast side equilibria in the mobile phase, derived the general equation similar to Eq. (18), and applied RP-HPLC to the determinations of association constants for metal-binding by various nucleotides, crown ethers and nitroso-naphtholsulfonic acids. More recently, the complex-forming reaction between crown ethers and

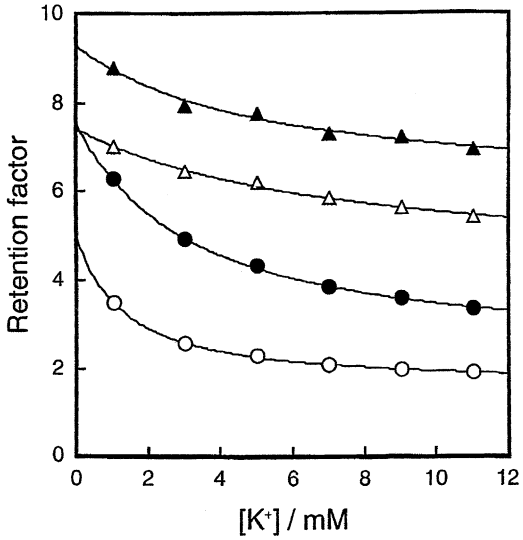

Fig. 2. Changes in retention factor of crown ethers with increasing concentration of K^+ in mobile phase. Column: TSK-GEL ODS-80TM CTR (100 mm × 4.6 mm i.d.). Eluent: 2 mM acetate buffer (pH 4.7) in 55% (v/v) methanol in water + KCl. Temperature 25 °C. Detection: UV, 220 nm. Identification of crown ethers: (○) dibenzo-18-crown-6, (●) dibenzo-21-crown-7, (△) dibenzo-24-crown-8, (▲) dibenzo-30-crown-10. From [40], with permission.

potassium ion was investigated by Takayanagi et al. [40] in water-methanol media. The crown ethers were injected as analytes and potassium ion was present in the mobile phase. Because the interaction of the crown ethers with metal cations may be described by a simple reaction scheme according to Eq. (1), the retention factor can be expressed by the following equation [40]:

$$k = \frac{1}{1 + K_{ML}[K^+]} k_L + \frac{K_{ML}[K^+]}{1 + K_{ML}[K^+]} k_{ML} \quad (20)$$

that is a slightly modified form of Eq. (14). k_L and k_{ML} are the limiting retention factors for the free and metal-bound crown ether, respectively, K_{ML} is the stability constant of the K^+–crown ether complex. The dependencies of the retention factor on the concentration of K^+ ions in the mobile phase are shown in Fig. 2; the stability constants were calculated from these dependencies using a non-linear least-squares analysis. The stability constants of crown ethers with K^+ and Na^+ ions were also measured in [41] in a similar way.

The binding affinity constants of small molecules—enantiometric carboxylic acids—to albumin were measured in the reversed-phase system using various stationary phases and albumin as a constituent of the mobile phase [42]. Apparent association constants of various drugs (psoralen derivatives) with modified β-cyclodextrins were measured by RP-HPLC on an octadecyl-bonded silica column [43]. Determinations of equilibrium constants from chromatographic measurements in the reversed-phase systems were mentioned also in [32,44].

A typical feature of the RP-HPLC systems is the presence of organic modifiers in mobile phases. Changing the organic modifier content in the mobile phase is the most effective way to control the analyte retention. Among other effects, however, the dependence of the magnitudes of equilibrium constants on the concentration of organic modifier should be taken into account. It was shown for weak organic acids that the effect of organic modifier, such as methanol [45,46], acetonitrile [47] or tetrahydrofuran [48], on dissociation constants manifests markedly only at relatively high organic modifier concentrations above ca. 80%. An increase of the pK_a values was found in the range of 1.5–2 pK units with increasing concentration of acetonitrile up to 80 [49], and similar values was found for other solvents [50].

3.2. Ion chromatographic methods

Various HPLC methods serving for the separation of ionic or ionogenic substances are known under the name ion chromatography [51]. Three main mechanisms are employed for the separations, namely ion-exchange chromatography, ion-interaction chromatography, and ion-exclusion chromatography. The following discussion will be focused on the ion-exchange chromatography, although many conclusions may be generalized to the other separation modes.

In analytical practice, the IC methods are used for the determinations of inorganic anions in the first place. Selectivity coefficients of common inorganic anions on strong basic anion exchangers are sufficiently different and, therefore, there is a relatively little need to improve separations with the aid of side equilibria in mobile phase. The analyte retention is governed mainly by an eluent concentration. The degrees of dissociation of eluting (driving) species (and sometimes also the separated ions and even the ion-exchange groups on the stationary phase [52]) play an important role in separation, and thus the acid–base equilibria are incorporated in the retention models [1]. Only exceptionally, however, the modern IC in its anion-exchange mode has been used for measurements of equilibrium constants.

A quite different situation is in ion chromatographic analysis of metal cations. Typically, the IC separations of metal cations are carried out on strong, low-capacity cation exchangers. While cations of alkaline and alkaline earth metals can be readily determined, e.g. by “standard cation IC” [53] using diluted solutions of strong inorganic acids as eluent, separations of other divalent and polyvalent cations (heavy and transition metals, lanthanides) are almost impossible without the presence of complexing agents in mobile phase.

The ion-exchange equilibria on conventional resin-based exchangers were studied extensively dozen years ago. A great boom in almost every branch of separation science started during the World War II in relation to a project of nuclear fission. There was an urgent need to separate a number of fission products having very close atomic numbers and properties. As a result of extensive research, an “elution method” utilizing synthetic ion-exchangers (ion-exchange chromatography, in principle) was developed, among others. It was helpful in an early stage of the project to work

with non-radioactive mixtures, which closely approximated the radioactive fission products. Thus, the separation methods were tested on a group of rare earth elements, exploiting a strong analogy between lanthanides and actinides [54]. One of the most important findings of this research was discovering the key role of complexing agents for the separations of metal cations with similar properties. Citric, lactic, ethylenediamine tetraacetic, oxalic and other organic acids were used to improve the separation of metal cations on synthetic ion exchangers [55–59]. The ion-exchange technique was used to study the properties of metal ions (determination of a charge of transition metal cations with organic ligands [60]) and to measure the stability constants. In addition to a batch experimental arrangement [7,61–63], also chromatographic methods were used, such as a conventional ion-exchange column chromatography [64,65], or, probably even more often, paper chromatography with ion-exchange papers [66,67].

Weak organic acids are widely employed also in modern IC; tartaric acid is a typical agent for the separation of transition metals [68], whereas α-hydroxyisobutyric acid (HIBA) is the most effective agent in the chemistry of lanthanides [69]. However, many other agents, such as glycolic, lactic, oxalic, malic, citric and succinic acids, have been successfully applied, too [70].

After an injection of an analyte (metal cation) into the mobile phase containing complexing ligand the equilibrium according to Eq. (5) is established; hence the species carrying positive charge ("free" cations, cationic complexes), neutral complexes as well as negatively charged anionic complexes can coexist in the mobile phase. The general Eq. (15) can be used to express the retention factors, and it may be modified using the overall stability constants as follows [1]:

$$k = \sum_{i=0}^{n} k_{\mathrm{ML}_i} x_{\mathrm{ML}_i} = \frac{\sum_{i=0}^{n} k_{\mathrm{ML}_i} \beta_i [\mathrm{L}]^i}{\sum_{i=0}^{n} \beta_i [\mathrm{L}]^i} \tag{21}$$

Retention of positively charged species (cationic complexes as well as free metal cations) on a cation-exchange column can be described as follows:

$$z\mathrm{ML}_i^{x-iy} + (x-iy)(\mathrm{E}^{z+})_\mathrm{s} \Leftrightarrow z(ML_i^{x-iy})_\mathrm{s} + (x-iy)\mathrm{E}^{z+} \tag{22}$$

where E^{z+} is the eluting cation and the subscript s refers to the stationary phase. The equilibrium constant for Eq. (22) (selectivity coefficient) is:

$$K^{\mathrm{E}}_{\mathrm{ML}_i} = \frac{[\mathrm{ML}_i^{x-iy}]_\mathrm{s}^z [\mathrm{E}^{z+}]^{x-iy}}{[\mathrm{ML}_i^{x-iy}]^z [\mathrm{E}^{z+}]_\mathrm{s}^{x-iy}} \tag{23}$$

Ion-exchange sites on the stationary phase are occupied with the eluting cations and the analyte species. As the amount of the analyte injected on the column is small, the column capacity can be expressed by the simple relation:

$$Q = z[\mathrm{E}^{z+}]_\mathrm{s} \tag{24}$$

The limiting retention factor for the species ML_i is defined as the ratio of its amount in the stationary phase to that in the mobile phase [1]:

$$k_{\mathrm{ML}_i} = \frac{w}{V_\mathrm{m}} \frac{[\mathrm{ML}_i^{x-iy}]_\mathrm{s}}{[\mathrm{ML}_i^{x-iy}]} \tag{25}$$

where w is the weight of stationary phase and V_m is the volume of mobile phase in the column. When combining Eqs. (21) and (23)–(25) we obtain after rearrangement:

$$k = \frac{w}{V_\mathrm{m}} \frac{\sum_{i=0}^{n} (K^{\mathrm{E}}_{\mathrm{ML}_i})^{1/z} (Q/z)^{(x-iy)/z} [\mathrm{E}^{z+}]^{(iy-x)/z} \beta_i [\mathrm{L}^{y-}]^i}{\sum_{i=0}^{n} \beta_i [\mathrm{L}^{y-}]^i} \tag{26}$$

The terms w, V_m, Q and $K^{\mathrm{E}}_{\mathrm{ML}_i}$ are constant for the given chromatographic system. Eq. (26) expresses both the "pushing effect" of the eluting cation E^{z+} as well as "pulling effect" of the complexing ligand L^{y-} [68,71]. If the concentration of the eluting cation is kept constant in a proper experimental arrangement, then the retention factor is a function of the ligand concentration and the stability constants β_i only, which is suitable for measurements of stability constants.

There are, in principle, two approaches to a mathematical treatment of experimental data from ion-exchange experiments, in which complex metal ions take place. According to the classical Fronaeus approach [7], it is assumed that the metals are retained not only in the form of the free metal cations (e.g. M^{2+}) on the cation-exchange sites of the sorbent, but also in the form of the positively charged complex ions (e.g. ML^{+}, etc.). In this case, Eq. (21) or Eq. (26) have to be used in their complete forms. This approach may be applied, after a slight modification, also to anion-exchange measurements.

More often is assumed that the only form retained on cation exchangers is the free metal cation; complex metal ions are excluded from the sorbent regardless of their charge, e.g. because of a steric hindrance (classical Schubert's approach [7,8]). It means that all the limiting retention factors k_{ML_i} in Eq. (21) (and similarly the selectivity coefficients $K^{\mathrm{E}}_{\mathrm{ML}_i}$ in Eq. (26)) are equal to zero except of the limiting retention factor for the free metal cation. Then Eq. (21) can be rearranged as follows (Broul's equation [72]) (charges are omitted):

$$\frac{1}{k} = A(1 + \beta_1[\mathrm{L}] + \beta_2[\mathrm{L}]^2 + \cdots + \beta_n[\mathrm{L}]^n) \tag{27}$$

The constant A combines the column parameters (w, V_m, Q), selectivity coefficient $K^{\mathrm{E}}_\mathrm{M}$, and also a constant concentration of the eluting cation. A similar equation was derived also for the ion interaction chromatographic system [73]. A more general equation expressing effects of more than one kind of complexing agents was derived in [72].

When only one kind of complex, e.g. of ML type, predominates in the mobile phase, Eq. (27) can be further

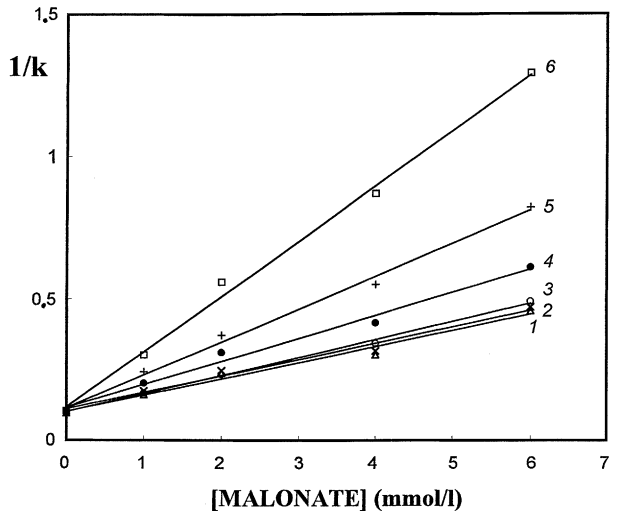

Fig. 3. Dependence of reciprocal value of retention factors on malonate concentration in mobile phase. Column: Separon SGX C_{18}, 150 mm × 3 mm, 7 μm, permanently coated with dodecylsulfate. Mobile phase: 0.1 M Na^+ + malonate, pH 6.5 ± 0.05 adjusted with perchloric acid. 1: Cd^{2+}, 2: Mn^{2+}, 3: Zn^{2+}, 4: Fe^{2+}, 5: Co^{2+}, 6: Ni^{2+}. From [1], with permission.

simplified:

$$\frac{1}{k} = A(1 + \beta_1[\mathrm{L}]) \quad (28)$$

In most systems investigated the dependencies of $1/k$ versus ligand concentration were straight lines—see Fig. 3 as an example. Then the stability constants can be simply calculated as a quotient of the line slope and the *y*-axis intercept.

Eq. (27) and similar ones were derived under certain assumptions that can be summarized as follows (see also [4]):

(i) The column capacity remains constant during the series of experiments, especially it does not change with pH, which is satisfied for the strong cation-exchangers.
(ii) The concentration of eluting cations is kept constant. An eluting power of H^+ or OH^- ions is neglected.
(iii) The metal cations are injected in "trace amounts" to simplify mass balance equations in the stationary and mobile phases, and to be able to work in a linear part of the sorption isotherm.
(iv) The pH value of mobile phase is kept constant to assure a constant degree of dissociation of complexing agents.
(v) The formation of polynuclear and mixed-ligand complexes is not presupposed (although this may be relatively easily incorporated into the retention model).
(vi) Ionic strength remains nearly constant.

It is worth noticing that the specified conditions are almost identical with those assumed by Schubert [8] in his pioneer's work.

An experimental arrangement for determining the stability constants from ion chromatographic measurements was described in [72,74]. The mobile phases were prepared by mixing known and constant amounts of the LiOH or NaOH solutions in a sufficient excess with varying amounts of complexing agent in the form of the respective acid. Then a required constant pH value was adjusted with diluted perchloric acid. It is assumed that perchlorate ions do not participate in complex-forming equilibria. In this way, the concentrations of eluting cations (Li^+ or Na^+) as well as the mobile phase pH were maintained constant. The ionic strength governed mainly by the concentration of lithium or sodium perchlorate was also nearly constant.

The IC methods have been used to determine the stability constants of metal ions with tartrate, citrate, oxalate, malonate, pyridine-2,6-dicarboxylate, HIBA and several inorganic anions. The stability constants measured by IC were in a good agreement with those published in literature and measured by other well established methods, such as potentiometry or conventional ion-exchange, as demonstrated e.g. in [75] for tartrate complexes with divalent metal ions in the temperature range of 20–40 °C. Comparisons of the IC-determined stability constants with reference values for other complexing agents can be found in the original papers [72,74,76]. Some examples of stability constants measurements for metal complexes are listed in Table 1. As can be seen, the cation-exchange systems are used almost exclusively for the stability constants determinations. Anion-exchange systems have been seldom utilized for investigations of complex-forming equilibria [80], although

Table 1
IC determinations of stability constants of metal complexes

Metal cations	Complexing agents(s)	Stationary phase	Medium	Reference
Zn^{2+}, Mg^{2+}, Ca^{2+}, Cd^{+2}, Co^{2+}, Mn^{2+}, Fe^{2+}	Tartrate	Strong cation-exchanger	I = 1–13 mmol/l	[75]
Cu^{2+}, Ni^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, Pb^{2+}	Tartrate	C_{18} coated with SDS	0.04 mol/l $LiClO_4$, pH 3.6	[72]
Cd^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, Pb^{2+}	Tartrate, oxalate, citrate, malonate, PDCA	C_{18} coated with SDS	0.1 mol/l $NaClO_4$, pH 6.0	[74]
Cd^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Fe^{2+}	Thiocyanate, sulphate	C_{18} coated with SDS	0.1 mol/l $NaClO_4$, pH 6.0	[76]
Pb^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+}	Citrate, tartrate	Strong cation exchanger	I = 3.84 mmol/l, pH 3.8	[77]
Cd^{2+}	Chloride, nitrate	Strong cation exchanger	I = 0.05–0.5 (perchlorate)	[78]
Cd^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}	HIBA	Strong cation exchanger	0.15 mol/l $NaClO_4$, pH 6.0	[79]
Zn^{2+}, Ni^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+}, Fe^{2+}, Pb^{2+}	Oxalate	C_{18}[a]	0.1 mol/l $NaClO_4$, pH 6.0	[73]

SDS: sodium dodecylsulfate, PDCA: Pyridine-2,6-dicarboxylic acid, HIBA: α-hydroxyisobutyric acid.
[a] Reversed-phase ion interaction system, sodium octane sulfonate used as ion interaction reagent.

they have been successfully applied to the separations of some metals in the form of their anionic complexes. Hirayama et al. [81] used various anion-exchange columns for ion chromatographic determinations of dissociation constants of several aromatic carboxylic acids.

4. Capillary electrophoresis

Electrophoretic methods together with ion-exchange techniques belong to those that have played traditionally a crucial role in investigations of chemical equilibria both of inorganic species (metal ions) as well as complex organic substances and macromolecules of biological interest [67,82,83]. For a very long time, the techniques employing various stabilizing media, such as paper electrophoresis or gel electrohoresis, predominated in the research as well as in analytical practice. Paper electrophoresis, for example, is still utilized successfully to study intricate ternary and mixed-ligand complexes of metals with chelating agents, aminoacids or drugs, and to determine their stability constants [84–87], or to study the speciation of actinide elements [88,89]. Electrophoretic methods utilizing more complex arrangements of discontinuous buffers, especially capillary isotachophoresis (ITP), have gained a great attention in a certain time period, and they have been used extensively also for physicochemical measurements [82]. In the 1980s, Hirokawa and co-workers [90–100] published a series of fundamental papers on complex-forming and acid–base equilibria in ITP, in which sophisticated migration models were developed, and stability and dissociation constants as well as other parameters (ionic mobilities of complex ions) were calculated using a computer simulation.

Extremely rapid development of the electrophoretic techniques began more than a decade ago, after that instrumentation for new progressive separation modes, such as capillary electrophoresis (CE), became commercially available. In CE, the separations are accomplished usually in narrow bore silica capillaries without other stabilizing media (free zone electrophoresis), although several other separation modes are available and increasingly employed [101,102]. Short separation times, high separation efficiency together with a relative simplicity to change experimental conditions characteristic for the modern CE methods have opened new perspectives in investigations of complex systems, e.g. in the anti-cancer metallodrug research [103,104] or in the metal speciation [105]. The discussion in this chapter will be restricted to the labile systems again (similarly to the previous chapter), i.e. the studies will be reviewed, in which the equilibrium constants were estimated from the changes in migration behavior with the changes of the composition of background electrolyte. In this way, acid–base, complex-forming, ion association, and some other equilibria (interactions with cyclodextrins) have been studied in BGE, in the first place. However, as was shown recently, similar approaches can be used also to study the interactions of analyte species with modifiers (diazacrowns, in this case) coated on an inner wall of the separation capillary [106].

General equations derived for additive properties [25] or for retention factors in chromatography, such as Eq. (18), may be readily adopted to express the dependence of the effective mobility on pH for analytes undergoing dissociation/protonation in BGE. A simple form of this equation was derived by Cai et al. [107] for monoprotic acids:

$$\mu_{\text{eff}} = \frac{K_a/[\text{H}^+]}{1 + K_a/[\text{H}^+]}\mu_{0a} \tag{29}$$

μ_{0a} is the electrophoretic mobility of the fully deprotonated species. Eq. (29) represents a sigmoidal dependence between the effective mobility and pH. Two modifications were suggested for the determination of K_a from the μ_{eff}–pH dependencies:

$$\frac{1}{\mu_{\text{eff}}} = \frac{1}{K_a\mu_{0a}}[\text{H}^+] + \frac{1}{\mu_{0a}} \tag{30}$$

and

$$\text{pH} = \text{p}K_a + \log\frac{\mu_{\text{eff}}}{\mu_{0a} - \mu_{\text{eff}}} \tag{31}$$

Eq. (30) shows that a plot of $1/\mu_{\text{eff}}$ against $[\text{H}^+]$ should be a straight line with a slope equal to $1/K_a\mu_{0a}$ and an intercept equal to $1/\mu_{0a}$. K_a can be easily determined from this plot. According to Eq. (31), a plot of pH against $\log[\mu_{\text{eff}}/(\mu_{0a} - \mu_{\text{eff}})]$ will result in a straight line with a slope equal to -1 and an intercept equal to $\text{p}K_\text{a}$. This plot, however, requires that μ_{0a} is known. Similar equations were derived also for bases and ampholytes [107]. The suggested methods were used to measure the $\text{p}K_\text{a}$ values of aniline, *p*-anisidine and *p*-aminobenzoic acid [107]. Some examples of the determinations of K_a with the aid of CE methods are listed in Table 2. A general equation applicable to polyprotic acids and bases can be found in [111]. It was shown that the relationship for the effective mobility is a particular case of the Bolzman sigmoid; this mathematical formula was used in a computer evaluation of experimental data by non-linear curve fitting [114]. In [119], an electrophoretic model allowing simultaneous determinations of K_1 and K_2 was applied to the determination of dissociation constants of zwitterionic drugs (quinolones) in water–acetonitrile mixtures. In this work, two analogical approaches to the dissociation constant measurements were compared—namely, the electrophoretic approach based on the concept of effective mobilities, and the liquid chromatographic approach based on the concept of limiting retention factors. The electrophoretic and chromatographic measurements were also compared with the results obtained by other independent methods, such as potentiometry and UV–Vis spectrophotometry.

The separation of metal ions by CE has been a major research focus. In particular, the formation of metal complexes to aid the separation and detection of metal ions has been thoroughly investigated and the separation models have been elaborated [2,120–122]. The stability constants

Table 2
CE determinations of equilibrium constants

Equilibrium constant/interaction	Compounds(s)	Technique/conditions	Reference
K_a	Aniline, *p*-anisinine, *p*-aminobenzoic acid	CZE/phosphate and acetate buffers	[107]
	Pyridine, aniline, cinnamic acid, benzoicacid, *p*-bromoaniline, salicylic acid, *o*-bromoaniline	CZE/citrate and acetate buffers	[108]
	p-Nitrophenol	CZE/series of buffers	[109]
	o-Bromoaniline, salicalic acid, *p*-bromo-aniline, 2-ethylaniline, aniline, pyridine, 4-nitrophenol, quinine, methylbenzylamines, phenetylamine, phenol	CZE/series of buffers	[110]
	2,4-Dinitrobenzoic acid, 2,6-dimethoxy-benzoate, 3,6-dichloro-2-anisic acid, benzenesulfinic acid, 3,5-dinitrobenzoic acid, terephthalic acid, isophthalic acid, phthalic acid	CZE/acetate, phosphate and formate buffers	[111]
	Galactose, allose, mannose, altrose, idose, talose	CZE/0.1 M NaOH	[112]
	Quinolones	CZE/phosphate buffers (0.025 and 0.05 M)	[113]
	Cytokinins	CZE/phosphate, acetate and MES buffers	[114]
	Nortriptyline, diphenhydramine, quinine, codeine, procainamide, benzylamine	CZE/Tris CAN–aqueous buffer	[115]
	Peptides (bacitracin A_1)	CZE/series of buffers	[116]
	Sulfonated azodyes	CZE/phosphate buffers, $I \sim 0.1$	[117]
	Pharmaceuticals	CZE/phosphate and acetate buffers, $I = 0.05$	[118]
Stability constants	Metal–sulfate complexes (Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+})	CZE/perchlorate medium	[123]
	Metal–HIBA complexes (Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+})	CZE/pH of BGE 4.5	[126]
	Metal–18-crown-6 complexes (K^+, Sr^{2+}, Ba^{2+})	CZE/pH of BGE 4.5	[126]
	Cu^{2+}–Alizarin complexone+ amines	CZE/borate buffer, pH 9.1	[127]
	Metal–18-crown-6 complexes (K^+, NH_4^+, Ba^{2+}, Sr^{2+}, Ca^{2+}, Na^+)	CZE/pH of BGE 4.5	[128]
	K^+, NH_4^+, Na^+ complexes with 18-crown-6	ITP/10 mM HCl as leading electrolyte	[128]
	Metal–PEG complexes (Li^+, Na^+, Cs^+, Mg^{2+}, Sr^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Pb^{2+}, Lu^{3+}, Y^{3+})	ITP/acidic non-buffered system	[129]
	Zn^{2+}–peptides (bacitracin A_1)	CZE/series of buffers	[116]
Stability constants/ interactions with CD	Inclusion complexes of 3,3-dihydro-2-H-1-benzopyran enantiomers	CZE/50 mM phosphate buffer, pH 7.0	[140]
	Inclusion complexes of salicylic acid and benzylamine with various CDs	CZE	[141]
	Inclusion complexes of procaine hydrochloride	CZE	[142]
Ion-association constants	Aromatic sulfonate and carboxylate anions—quaternary ammonium cations	CZE/10 mM borate buffer, pH 9.2	[146]
	Divalent aromatic anions—quaternary ammonium cations	CZE/10 mM phosphate buffer, pH 7.0	[147]
	Anionic azo dyes—quaternary ammonium cations	CZE/10 mM borate buffer, pH 9.2	[148]
	Aromatic anions—quaternary ammonium cations	CZE/$I = 0.025$ (NaCl)	[145]

CZE: capillary zone electrophoresis, MES: 2-(*N*-morpholino)ethanesulfonic acid, ACN: acetonitrile, HIBA: α-hydroxyisobutyric acid, ITP: isotachophoresis, PEG: poly(ethylene glycol), CD: cyclodextrin.

of labile metal complexes can be calculated from the relationship between ligand concentration and a measured electrophoretic mobility:

$$\mu_{\text{eff}} = \sum_{i=0}^{n} \mu_{\text{ML}_i} x_{\text{ML}_i} = \frac{\sum_{i=0}^{n} \mu_{\text{ML}_i} \beta_i [\text{L}]^i}{\sum_{i=0}^{n} \beta_i [\text{L}]^i} \quad (32)$$

This equation in its general form is rather complicated and a great number of experimental data is needed for reliable calculations. Some possible simplifications are discussed in [2]. In the simplest case when only one kind of complex with zero charge is formed (e.g. by interaction of divalent metal cation with divalent ligand at low ligand concentrations), Eq. (32) can be simplified to [123]:

$$\frac{1}{\mu_{\text{eff}}} = \frac{1}{\mu_{\text{M}}}(1 + \beta_1[\text{L}]) \quad (33)$$

From the dependencies of reciprocal effective mobilities on the ligand concentration, the stability constant can be easily determined using a graphical method [123]. Unfortunately, this is not generally applicable to a majority of real electrophoretic systems, where common complexing ligands, typically anions of weak organic acids are employed, and a series of complexes ($\text{ML}, \text{ML}_2, \ldots, \text{ML}_i, \ldots, \text{ML}_n$)

are formed. Relatively simple equations were derived for "non-conventional" complexing agents, such as crown ethers, where the complex-forming reaction may be described by a rather simple reaction scheme [124].

Because several complexes with unknown ionic mobilities are formed in real metal–ligand systems, a simple graphical approach is seldom utilizable and more sophisticated numerical methods are recommended. A general least-squares CELET program was developed for an evaluation of the CE data [125], which is able to treat the data of effective mobilities as a function of pH, ligand or metal concentration up to quaternary systems.

Some examples of measurements of stability constants with the aid of the CE methods are listed in Table 2 again. Various complexing agents (crown ethers, sulfated β-cyclodextrin, zwitterionic buffers) and their interactions with alkaline and alkaline earth metal cations were studied by CE [130] and the respective stability constants were calculated using the CELET program. Rasmussen and Bjerrum [131] have developed a more sophisticated experimental arrangement for measurements of Ca^{2+} and Na^{+} binding to calcium-containing proteins. The association constants were calculated for proteinase K and α-lactalbumin from the changes in electrophoretic mobility of the protein, whereas EDTA was utilized to control the free Ca^{2+} concentration.

The separation of optical isomers is an important subject of research especially in the pharmaceutical field, since it became evident that the biological or pharmacological activity of compounds is often restricted to one of the enantiomers. CE has been found to be a powerful alternative to chromatographic techniques. Many original papers and several reviews on this topic can be found in literature [132–134]. A comprehensive collection of applications to drugs, pesticides, aminoacids and other compounds of interest is given in tables in [134].

The different interactions of enantiomers, forming a racemate, with a chiral selector are the only way to discriminate between them. Cyclodextrins (cyclic oligosaccharides consisting of 6, 7, or 8 glucose units) belong to the most commonly used chiral selectors. The strength of the interaction of enantiomers with a proper selector in BGE governs their electrophoretic behavior. If the corresponding stability constants are known, the optimal concentration of the chiral selector in BGE can be calculated. Basic models describing the separation of enantiomers in the presence of chiral selectors are relatively simple [5,135–139], especially when a simple one-to-one interaction is considered without further side (e.g. acid–base) equilibria [13]. When the compound A interacts with the chiral selector C according to Eq. (1), and A consists of enantiomers *R* and *S*, then the effective mobilities of the enantiomers can be described as follows [13]:

$$\mu_R = \frac{1}{1 + K_R[\mathrm{C}]}\mu_\mathrm{A} + \frac{K_R[\mathrm{C}]}{1 + K_R[\mathrm{C}]}\mu_\mathrm{AC} \quad (34)$$

$$\mu_S = \frac{1}{1 + K_S[\mathrm{C}]}\mu_\mathrm{A} + \frac{K_S[\mathrm{C}]}{1 + K_S[\mathrm{C}]}\mu_\mathrm{AC} \quad (35)$$

μ_A and μ_AC are the ionic mobilities of the free and complexed compound, respectively. Eqs. (34) and (35) differ only in stability constants K_R and K_S for the enantiomers *R* and *S*, respectively. This simplification is founded on an assumption that the ionic mobilities of enantiomers are identical [13]. The stability constants K_R and K_S may be calculated from the effective mobilities measured at various concentrations of the chiral selector. Three effective mobilities of each enantiomer are sufficient for calculations. However, the experimental data always suffer from random errors. Therefore, from six to ten pairs of raw data $\mu_\mathrm{eff} = f([C])$ are usually necessary to reach reasonable precision by computer fitting. The ionic mobility of the non-complexed compound, μ_A, is accessible experimentally, in principle, whereas μ_AC is treated as an adjustable parameter. The CE methods were used to determine stability constants of various inclusion complexes; some examples are given in Table 2. Possible mechanisms for the chiral recognition of enantiomer with γ-cyclodextrin were investigated using various techniques including CE [143]. The measurements provided clear evidence of the formation of diastereometric complexes between the enantiomers and γ-cyclodextrin with a 1:1 stoichiometric ratio. The binding constants of the complexes obtained by the CE, reversed-phase HPLC and circular dichroism techniques were compared. CE has been used also to study the interactions of some borane cluster anions with β-cyclodextrin, and to estimate the respective stability constants [144].

Ion-pairing or ion-interaction reactions belong to the equilibria that may be examined with the aid of electrophoretic methods, too. It is assumed that bulky (usually organic) anions or complexes are capable to form stable associates with oppositely charged pairing ions, typically with quaternary cations. The stability of ion associates may be expressed with the aid of ion-association constants. It was observed that the electrophoretic mobility of large anions decreased with increasing concentration of the ion pairing agent in BGE (see Fig. 4) [145]. The ion-association constants can be estimated by analyzing these dependencies (Table 2). For example, interactions of basic dyes (Methylene Blue) with aromatic sulfonates were studied by CE and the equilibrium constants were determined from the shift of the Methylene Blue peak after an addition of the sulfonate into BGE [149]. Affinity capillary electrophoresis using mobility-shift analysis was utilized to characterize the binding of peptide ligands to cyclophilins, which are members of the enzyme family of peptidyl-prolyl *cis*/*trans*-isomerases [150]. A similar approach has been used to study the interactions between neutropsin and double-stranded DNA [151]; methods of CZE and affinity capillary electrophoresis were compared here for the determination of the binding constants. The capillary affinity electrophoresis was used to study the interactions of lecitin with sugar [152]. The complex formation constants were calculated from the change in mobility of lecitin at different concentrations of fucose derivative.

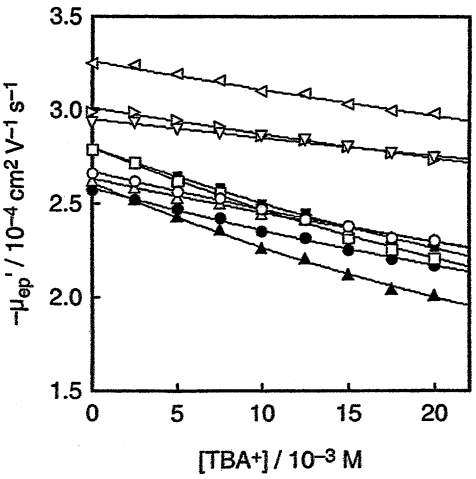

Fig. 4. Change in electrophoretic mobility of aromatic anions with an increase in concentration of tetrabutylammonium (TBA^+) cation in BGE. Capillary: polymer-coated capillary (CElect-N, Supelco), 195 nm. Migrating buffer: 5×10^{-3} M NaOH + NaCl + (0 + 20). 10^{-3} M TBA-Cl, ionic strength was adjusted to 0.025 with NaCl. Applied voltage 15 kV, temperature 25 °C. Detection: UV, 210 nm. Analyte anions: (▽) phenol, (▷) benzoic acid, (◁) sodium benzenesulfonate, (○) 1-naphthol, (△) 1-naphthoic acid, (□) 1-naphthalenesulfonate, (●) 2-naphthol, (▲) 2-naphthoic acid, (■) 2-naphthalenesulfonate. From [145], with permission.

The CE methods have been utilized to estimate the binding constants between carbonic anhydrase and benzenesulfonamides [153], or to study the interactions between DNA and cationic surfactants [154]. A brief review of recent applications of CE for studying molecular interactions in biotechnology has been published by Galbusera and Chen [155].

Electrophoretic techniques may be successfully utilized in investigations of solute interactions with surfactants, for determinations of critical micelle concentrations [156,157], as well as for determinations of solute–micelle association constants [158,159], which is very useful, because surfactants and their interactions play an important role in some CE separation modes.

5. Some practical considerations, data evaluation

As was shown in previous chapters, a proper experimental arrangement allows to use the chromatographic column or separation capillary in CE as a "physical chemistry laboratory" for measuring physicochemical characteristics [30].

Reliable determinations of equilibrium constants require precise measurements of retention or migration characteristics, namely retention factors in HPLC or effective mobilities in CE. The retention factor is calculated from the retention time (volume) of analyte and the void volume of the column. For most chromatographic system, these parameters can be measured with sufficient precision. The column void volume can be determined using a suitable non-retained marker, or from disturbances on chromatograms caused by the injection of sample or pure solvent. Migration times in CE exhibit usually somewhat lower repeatability. To obtain reliable values of effective mobilities, an utilization of two internal mobility standards is recommendable [13,160].

Chromatographic and electrophoretic measurements of equilibrium constants should be carried out at, as much as possible, a constant composition of mobile phase or BGE (at constant ionic strength, concentration of organic modifier, etc.). At the same time, however, it is necessary to vary some parameters, such as a ligand concentration or pH. Although this seems like a contradiction, it is possible to find a reasonable compromise. A suitable experimental arrangement was described in Section 3.2 for IC measurements [72,74], and it may be used after a proper modification also in other modes [125].

Investigations of acid–base equilibria require an exact measurement of pH. This is certainly not a big problem in aqueous solutions. However, the mobile phases as well as BGEs contain very often a certain portion of organic modifiers. Thus, the pH meter should be calibrated with proper buffers of the known pH values for the given solvent composition [48,115,161–163]. It should be pointed out that, according to the new IUPAC recommendation [164–166], various "pH scales" are not longer utilized, and the traceability of pH measurements should be accomplished with the aid of certified reference materials. As was mentioned in the chapter 3.1, organic modifiers affect the values of equilibrium constants, to some extent.

Experimental dependencies of the retention or migration characteristics on the composition of mobile phase or BGE may be rather complex, as can be seen from the above presented equations. It is highly desirable to simplify them by a proper selection of experimental conditions, if possible. In some cases, the experimental dependencies can be transformed into the form of the straight line (linearized)—see, e.g. Eqs. (28), (30), (31) and (33). The equation for the simple one-to-one interaction, Eq. (14), sometimes called a "general binding isotherm" [167], may be linearized in several different ways; some examples are listed in Table 3.

Table 3
Linearized forms of general binding isotherm (Eq. (14)) for graphical evaluation of equilibrium constants

Plotting form	Calculation of equilibrium constant, K
$\frac{[C]}{k-k_A} = \frac{1}{k_{AC}}\frac{k[C]}{k-k_A} + \frac{1}{k_{AC}K}$	Slope/intercept
$\frac{k-k_A}{[C]} = -Kk + k_{AC}K$	−Slope
$\frac{1}{k-k_A} = \frac{1}{(k_{AC}-k_A)K}\frac{1}{[C]} + \frac{1}{k_{AC}-k_A}$	Intercept/slope
$\frac{[C]}{k-k_A} = \frac{1}{k_{AC}-k_A}[C] + \frac{1}{(k_{AC}-k_A)K}$	Slope/intercept
$k-k_A = \frac{1}{K}\frac{k-k_A}{[C]} + (k_{AC}-k_A)$	−1/slope

Slightly adapted from [39].

Various linearized forms of Eq. (14) or similar ones can be found also in [114,167,168]. The way of linearization may affect the uncertainty of the equilibrium constant estimation when a graphical method is used, as discussed in [39]. Although today's curve-fitting computer programs allow to treat virtually any set of experimental data, a graphical presentation and visual inspection of the experimental dependencies are still very useful. Conditions for correct estimations of equilibrium constants by CE, including the data treatment, are discussed in detail in [13]. A new mathematical treatment method was proposed for the CE measurements of binding constants of inclusion complexes [142].

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