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REVIEW

# Affinity capillary electrophoresis: the theory of electromigration

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**Abstract** We focus on the state-of-the-art theory of electromigration under single and multiple complexation equilibrium. Only 1:1 complexation stoichiometry is discussed because of its unique status in the field of affinity capillary electrophoresis (ACE). First, we summarize the formulas for the effective mobility in various ACE systems as they appeared since the pioneering days in 1992 up to the most recent theories till 2015. Disturbing phenomena that do not alter the mobility of the analyte directly but cause an unexpected peak broadening have been studied only recently and are also discussed in this paper. Second, we turn our attention to the viscosity effects in ACE. Change in the background electrolyte viscosity is unavoidable in ACE but numerous observations scattered throughout the literature have not been reviewed previously. This leads to an uncritical employment of correction factors that may or may not be appropriate in practice. Finally, we consider the ionic strength effects in ACE, too. Limitations of the current theories are also discussed and the tasks identified where open problems still prevail.

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## Introduction

Affinity capillary electrophoresis (ACE) refers to a family of methods. These have in common that a sample reversibly interacts with one (or more) component(s) of the electrophoretic system while it is driven through the capillary or gel by the electric force [1, 2]. These methods are used for studies in proteomics, immunology, drug development, molecular biology, or microbiology, as well as in the separation sciences [1–5]. The interacting component is often referred to as a ligand since the purpose is to study analyte–ligand weak interactions. In separation sciences, however, the interacting component takes the role of a selector that interacts selectively with various components of the sample (analytes) thus affecting their separation. Although gel electrophoresis was first applied in the ACE mode, namely in biophysical studies, the free-solution ACE, where the interacting components are dissolved in the background electrolyte (BGE), has become a highly popular technique especially in the separation sciences. The term electrokinetic chromatography (EKC) became common for these kinds of separations [6, 7], while capillary EKC (cEKC) was suggested by other authors [8]. However, this terminology should not be confused with micellar electrokinetic chromatography (MEKC)—which is based on similar principles, yet described by a conceptually different approach—or even capillary electrochromatography (CEC), which is a completely diverse technique. Since cyclodextrins (CDs) belong to the most common selectors in EKC, CD-mediated EKC (CD-EKC) [9, 10] or CD-modified CZE

(CD-CZE) [6] are idioms that can also be encountered in the literature. Other authors [11] differentiate between ACE and EKC so that "[in EKC] the constituent(s) added to the electrophoresis buffer [BGE] is considered to form a separate phase as is the case for micelle, microemulsion and liposome forming excipients". Then they used the formalism of phase distribution equilibria in the case of the EKC (which applies to MEKC, too), while the weak chemical binding equilibria was considered characteristic for ACE. Specifically, the term mobility-shift ACE [2, 11] is used for the electrophoretic technique that we are going to discuss in this review paper. Although this differentiation between ACE and EKC is natural, it is not generally accepted. Historically, EKC has been used in the sense of what the authors would refer to as the mobility-shift ACE. For example, CD-EKC is a common term in spite of the fact that CDs do not form a separate phase in the BGE. The terminology may also differ upon the authors' perspective. When, for example, the prime interest is in determining the analyte–selector binding constants by means of capillary electrophoresis experiments, the (mobility-shift) ACE designation is sometimes preferred over the EKC, perhaps to put the stress from "chromatography" (resembling separation) back on "affinity" (resembling molecular binding). Since the separation principles in electrophoretic systems with weakly interacting selectors are not directly comparable to chromatography (as we also discuss later in this paper), we suggest to stick to the simple ACE acronym whenever the selectors (ligands) are (i) added to the BGE, (ii) do not form a separate phase, and (iii) affect the migration of the analyte(s) through the affinity interactions.

The detailed theory of the electromigration in ACE is reviewed in this paper. Its application is found namely in chiral separations or when the analyte–ligand weak interactions are to be studied carefully. Numerous review papers exist (we counted over 100 of them since 1993 but here give only those that appeared over the last decade) summarizing applications of ACE [8, 12–21], monitoring usage of various selectors [6, 7, 12, 14, 18, 20], others targeting CDs only [9, 22–27], or focusing on the weak interactions between analytes and selectors (ligands) [28–30]. Only a few out of the numerous review papers cover the fundamentals of the technique as (one of) their prime subject [8, 31–37], whereas some focus more on theoretical aspects of the separation principles [34, 36–40]. As far as we are concerned, the theory of electromigration under the influence of complexation equilibrium has never been summarized to the extent we do in this paper.

## Effective mobility

Although it may seem that the effective mobility of an analyte in ACE is so well understood that it is not worth any special attention, the diversity of various equilibria that may take place in the ACE separation system gave rise to a variety of expressions in the mid-1990s and the research has still not subsided. Simultaneously, these expressions were sometimes derived by several authors in various forms but have apparently never been summarized in one place. This may cause difficulties when getting oriented in the subject. For this reason we summarize the theory in this section, placing especial emphasis on mutual relationships among the equations.

### Simple systems

The theory of ACE is based on the fundamentals of electrophoresis as postulated by Tiselius in 1930 [41]. If a chemical species exists in various forms among which a chemical equilibrium establishes at fast reaction rate, the individual forms of the species do not migrate apart each other. Instead, they form a single zone whose effective mobility is given by the Tiselius equation:

$$\mu_{\mathrm{A,eff}} = \sum_i \chi_i \mu_{\mathrm{A},i} \tag{1}$$

where

$$\chi_i = \frac{n_i}{\sum_j n_j} \tag{2}$$

is the molar fraction of the $i$-th form of the species $A$, $\mu_{A,i}$ is its individual electrophoretic mobility, and $n_i$ and $\sum_j n_j$ represent the amount of the $i$-th form and the total amount of the species $A$, respectively. (See the Electronic Supplementary Material (ESM) for more detailed explanation of the Tiselius equation.) Equation (1) is applicable to, e.g., the three ionic forms of phosphoric acid as well as the ACE scenario depicted in Fig. 1A. Here, an analyte (A) interacts with a selector (S); the selector is homogenously distributed in the BGE at a total concentration of $c_S$. Thus the analyte exists in two forms, as the free analyte (A0) and as the complex with the selector (AS). The fast equilibrium with 1:1 stoichiometry is assumed between the analyte and the selector. Noticeably, the selector also exists in two forms, as the free selector (S0) and as the complex with the analyte (AS). Nevertheless, it is the analyte that is of prime importance in the separation process; thus, the selector is generally considered as a uniform background. This is only appropriate if the selector is present in sufficient excess of the analyte. Violating this rule has consequences that we will discuss later in this paper. Under these circumstances (fast equilibria, large amount of selector), a constant molar ratio between the two forms of the analyte is kept across its migrating zone during the entire separation process,

$$\frac{n_{AS}}{n_{A0}} = K'_{AS} c_S, \tag{3}$$

where $K'_{AS}$ is the apparent (in contrast to the true thermodynamic) binding constant,

$$K'_{AS} = \frac{c^{rel}_{AS}}{c^{rel}_{A0} c^{rel}_{S0}}. \tag{4}$$

In Eq. (4), $c$ stands for concentrations, while the superscript *rel* reminds of the fact that the relative concentrations should appear in formulas regarding chemical equilibria. Equation (3) results from Eq. (4) after realizing that the relative concentrations are numerically equal to the absolute ones, that $n = cV$ in any finite volume element $V$, and that $c_{S0} \cong c_S$ under the assumption of $c_S \gg c_{AS}$.

The analyte migrates with its own mobility, $\mu_{A0}$. The analyte–selector complex migrates with a mobility of $\mu_{AS}$. In the simplest case of Fig. 1A, $\chi_{AS} = 1 - \chi_{A0}$ and the effective mobility of the analyte results as [34, 42]

$$\begin{aligned}\mu_{A,eff} &= \chi_{A0}\mu_{A0} + (1-\chi_{A0})\mu_{AS} \\ &= (1-\chi_{AS})\mu_{A0} + \chi_{AS}\mu_{AS},\end{aligned} \tag{5}$$

where $\chi_{A0}$ is the fraction of the uncomplexed analyte [42] or (equivalently) $\chi_{AS}$ is the degree of complexation [34]. After expressing the molar ratios by means of Eqs. (2) and (3), the well-known relation for the effective mobility of the analyte in the ACE results,

$$\mu_{A,eff} = \frac{\mu_{A0} + K'_{AS} c_S \mu_{AS}}{1 + K'_{AS} c_S}. \tag{6}$$

The binding constant, $K'_{AS}$, depends on the ionic strength (IS) of the BGE. The same applies to the mobilites of the free analyte and the analyte–selector

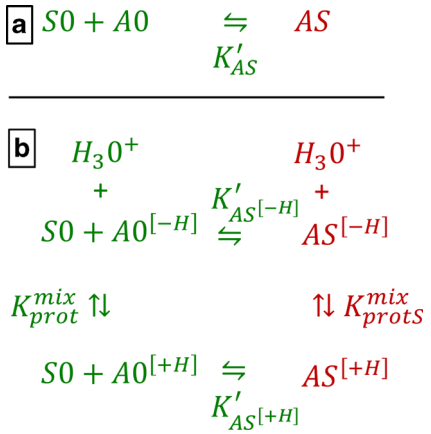


**Fig. 1** Complexation scheme. **A** Simple complexation. **B** Coupled acid–base equilibrium

complex. Thus Eq. (6) is generally valid but the IS-conditional parameters must be entered into it. Typically, the parameters are determined at a certain IS and thus the IS must be kept while changing the selector concentration when applying Eq. (6). This causes difficulties especially when permanently or weakly acidic/basic selectors are used. Alternatively, the IS-conditional parameters can be calculated from the thermodynamic (binding constant) or limiting (mobilities) ones. We discuss the role of the IS of the BGE in more detail in "Ionic strength". Equation (6) is often ascribed to Wren and Row [43] (which has over 600 citations since 1992) but it was already published in this exact form by Guttman et al. in 1988 [42] in the context of the employment of cyclodextrins incorporated within a gel matrix. Explicit forms of Eq. (6) were given for neutral analytes ($\mu_{A0} = 0$ ) [44, 45] or neutral analyte–selector complexes ($\mu_{AS} = 0$ ) [46, 47]. In the former case the selector has to be charged ($\mu_{AS} \neq 0$ ) so as to mobilize the sample, while the latter case is a consequence of analyte and selector bearing the same but opposite charges. An alternative form of Eq. (6) can be encountered in the literature [48], although it does not seem to bring any advantage.

Studies that primarily aim to determine the binding constant, $K'_{AS}$, often start with Eq. (6) expressed as [49]

$$K'_{AS} c_S = \frac{\mu_{A0} - \mu_{A,eff}}{\mu_{A,eff} - \mu_{AS}}. \tag{7}$$

Other rearrangements lead to the so-called *x*-reciprocal, *y*-reciprocal, and double-reciprocal linearized expressions [49], respectively:

$$\frac{\mu_{A,eff} - \mu_{A0}}{c_S} = -K'_{AS}(\mu_{A,eff} - \mu_{A0}) + K'_{AS}(\mu_{AS} - \mu_{A0}), \tag{8a}$$

$$\frac{c_S}{\mu_{A,eff} - \mu_{A0}} = \frac{1}{\mu_{AS} - \mu_{A0}} c_S + \frac{1}{\mu_{AS} - \mu_{A0}} \cdot \frac{1}{K'_{AS}}, \text{ and} \tag{8b}$$

$$\frac{1}{\mu_{A,eff} - \mu_{A0}} = \frac{1}{\mu_{AS} - \mu_{A0}} \cdot \frac{1}{K'_{AS}} \cdot \frac{1}{c_S} + \frac{1}{\mu_{AS} - \mu_{A0}}. \tag{8c}$$

Another approach to the linearization of Eq. (6), which is somewhat similar to the *x*-reciprocal Eq. (8a) and enables specific statistical treatment that we discuss in "Other approaches", can be found in ref. [50]. Results (namely binding constants) obtained by the different linearization approaches, Eq. (8), and the nonlinear regression of Eq. (6) have been compared in several studies showing that the obtained values may or may not differ significantly [51–55]. Nevertheless, Bowser and Chen demonstrated by Monte Carlo simulations in 1998 that the data transformation causes a violation of statistical requirements on that data and thus is not recommended

[56, 57]. Linearization of the fundamental Eq. (6) seems rather obsolete with the contemporary computational power and statistical tools. We recently published freeware CEval (http://echmet.natur.cuni.cz/download) [58] that enables easy evaluation of data from ACE measurements, automatic estimation of the binding parameters by the linearized equations, and subsequent nonlinear regression of Eq. (6). On the other hand, the *x*-reciprocal rearrangement may be advantageous for data visualization since it is sensitive to possible nonlinearities, namely deviations from the 1:1 stoichiometry [59–61].

The existence of the weak interactions between the analyte and the selector makes ACE comparable to chromatography. This may bring about certain confusion reflected both in the nomenclature (as briefly demonstrated in the "Introduction") and the related theory. Bowser et al. [62] opened "a potential pathway to a unified separation science" in 1997. Virtually the same idea was published by Lelièvre et al. [63] that same year. The authors realized the common nature between the molar fraction, Eq. (2), and the chromatographic capacity (retention) factor

$$k' = \frac{n_{SF}}{n_{MF}}, \tag{9}$$

where $n_{SF}$ represents the amount of the analyte adsorbed on the stationary phase and $n_{MF}$ stands for that remaining in the mobile phase. Denoting $n_{MF} \equiv n_0$ (aka the free analyte), it follows that

$$\chi_i = \frac{n_i}{\sum n_j} = \frac{(n_i/n_0)}{\left(\sum n_j/n_0\right)} = \frac{k_i'}{\sum k_j'}. \tag{10}$$

Consequently, Tiselius' Eq. (1) is of general applicability in separation sciences if $k_i'$ is defined as

$$k_i' = \frac{[\text{Amount of analyte of species } i]}{[\text{Amount of free analyte}]}, \tag{11}$$

where the "species *i*" represents various analyte interactions that can appear in the separation system. Comparison between Eqs. (3) and (11) clearly shows that the product of $K'_{AS}c_S$ in ACE gives the counterpart to the retention factor, $k'$, in chromatography. Consequently, Eq. (6) becomes

$$\mu_{A,eff} = \frac{1}{1+k'}\mu_{A0} + \frac{k'}{1+k'}\mu_{AS} \tag{12}$$

as also explicitly derived by Peng et al. in one of their subsequent papers [64]. Although the chromatographic approach to the effective mobility provided its educative purpose in the early days of ACE, it seduces one to think of the ACE systems as chromatographic ones, which is not always appropriate [7, 8, 33, 38]. For this reason we consider it best avoided nowadays. See the ESM for additional information on the chromatographic concept in ACE.

## Acid–base equilibria

Only rare analytes are permanently charged or neutral molecules. Usually they bear one or more ionizable (acidic or basic) groups. Monovalent analytes are common. Larger molecules can often be treated as "stepwise ionizable" in the entire pH range as long as the p$K_a$ values of all individual ionizable groups are sufficiently different. Thus the natural extension of Eq. (6) is to account for monovalent weakly acidic and basic analytes. By "monovalent" we mean that only one ionizable group is considered at a time in the sense of the "stepwise ionization". Thus the protonated form of an acidic analyte does not necessarily need to be the neutral one and the same applies to the deprotonated form of a basic analyte. This situation is depicted in Fig. 1B. The protonated/deprotonated forms of the analyte are denoted by subscripts $[+H]$ / $[-H]$ respectively, so as not to impose a perception of any charge state.

The complete solution was published by Williams and Vigh in 1997 [65]. After expressing the molar fractions, $x_i$, for the individual forms of the weak acid or base in Fig. 1B, its effective mobility results as

$$\mu_{A,eff} = \frac{\mu_{A^{[-H]}} + \mu_{AS^{[-H]}}K'_{AS^{[-H]}}c_S + K^{mix}_{prot}a_{H_3O^+}\cdot\left(\mu_{A^{[+H]}} + \mu_{AS^{[+H]}}K'_{AS^{[+H]}}c_S\right)}{1 + K'_{AS^{[-H]}}c_S + K^{mix}_{prot}a_{H_3O^+}\cdot\left(1 + K'_{AS^{[+H]}}c_S\right)}. \tag{13}$$

In Eq. (13), subscripts *A* and *AS* refer to the free analyte (acid or base) and the analyte–selector complex. $\mu$ and $K'$ stand for electrophoretic mobilities and apparent equilibrium constants (cf. Fig. 1B), respectively. $K^{mix}_{prot}$ is the so-called

mixed protonation constant, either of the acid or the base. For acids, $K_{prot}^{mix} = 1/K_A^{mix}$, for bases, $K_{prot}^{mix} = K_B^{mix}/K_W$, where $K_A^{mix}$ is a mixed acidity constant, $K_B^{mix}$ is a mixed basicity constant and $K_W = a_{H_3O^+} \cdot a_{OH^-}$ is the ionic product of water. Finally, $a_{H_3O^+}$ and $a_{OH^-}$ are activities of hydroxonium and hydroxide ions in a BGE, respectively. Equation (13) does not originally contain the $K_{prot}^{mix} a_{H_3O^+}$ product, but it was expressed in terms of $K'_{prot} c_{H_3O^+}$, i.e., an apparent protonation constant and a concentration of hydroxonium ions. Using the product of $K_{prot}^{mix} a_{H_3O^+}$ is more appropriate though [66], and thus we will use it throughout this paper (cf. the ESM). Notice that the mixed constant applies only in the context of the acid–base equilibrium. The complexation equilibria are described by apparent binding constants. The activity of the hydroxonium ions can be obtained directly from a measurement of $pH = -\log a_{H_3O^+}$. The only precaution is needed regarding IS of the BGE. As a matter of fact, $K_{prot}^{mix}$ actually results when the protonation constant is determined by means of the CE (a dependence of the effective mobility of a weakly acidic/basic analyte on *pH* at a constant IS) [66] and thus can be directly used for the purpose of Eq. (13). Another approach was adopted in one of our very recent studies [67]. From Eq. (15a) given below it follows that

$$K_{prot}^{mix} a_{H_3O^+} = \frac{\mu_{A0^{[-H]}} - \mu_{A0}^{pH}}{\mu_{A0}^{pH} - \mu_{A0^{[+H]}}}, \tag{14}$$

where $\mu_{A0}^{pH}$ is the mobility of the analyte at the given *pH* (and IS) without presence of any selector. All mobilities in Eq. (14) are easily accessible experimentally and the resulting product of Eq. (14) can be input into Eq. (13) when the analysis is performed at defined *pH* and IS with changing $c_S$ only. Derivation of Eq. (13) is a relatively simple task. For example Rizzi summarized the derivation process in a highly instructive way in his review paper in 2001 [34]. On the other hand, this enabled various authors to make this derivation from scratch; this resulted in numerous different-looking expressions, additionally depending on whether acids or basis were of interest [26, 65–70]. We explore these expressions and their mutual relations in the ESM.

Lelièvre, Gareil, and Jardy [63] expressed the dependence of the effective mobility of an analyte on its ionization state and the concentration of the selector in terms of *pH*-conditional parameters. The conditional constants were referred to as apparent ones in the original work. In one of our recent papers [67] we used a term "overall". However, the adjective "conditional" is the more appropriate from the physicochemical point of view [71]. In the frame of the last paragraph, the parameters were actually $c_{H_3O^+}$-conditional, i.e., requiring constant concentration of the hydroxonium ions. Nevertheless, we can adopt the concept of the mixed protonation constants, which will make the model truly *pH*-conditional. The resulting formula takes the same form as that for the effective mobility in simple systems, Eq. (6):

$$\mu_{A,eff} \frac{\mu_{A0}^{pH} + \mu_{AS}^{pH} K_{AS}^{pH} c_S}{1 + K_{AS}^{pH} c_S}, \tag{15}$$

with

$$\mu_{A0}^{pH} = \frac{\mu_{A^{[-H]}} + \mu_{A^{[+H]}} K_{prot}^{mix} a_{H_3O^+}}{1 + K_{prot}^{mix} a_{H_3O^+}}, \tag{15a}$$

$$\mu_{AS}^{pH} = \frac{\mu_{AS^{[-H]}} K'_{AS^{[-H]}} + \mu_{AS^{[+H]}} K'_{AS^{[+H]}} K_{prot}^{mix} a_{H_3O^+}}{K'_{AS^{[-H]}} + K'_{AS^{[+H]}} K_{prot}^{mix} a_{H_3O^+}}, \text{ and} \tag{15b}$$

$$K_{AS}^{pH} = \frac{K'_{AS^{[-H]}} + K'_{AS^{[+H]}} K_{prot}^{mix} a_{H_3O^+}}{1 + K_{prot}^{mix} a_{H_3O^+}}. \tag{15c}$$

Notice that not only *pH* but also IS of the BGE must be kept constant to ensure the validity of Eq. (15). The IS-conditional equilibrium constants, $K'_{AS}$ and $K_{prot}^{mix}$, make the *pH*-conditional parameters, Eqs. (15a–15c), IS-conditional, too. Equations (15a–15c) appear in a different form from that in the original paper [63]. We deliberately applied some rearrangements to give them the shape resembling Eq. (13). See the ESM for details. Mofaddel et al. [54] further extended the *pH*-conditional model by considering two simultaneously dissociating acidic groups of binols. The prime message is that even such complicated systems can be treated from the *pH*-conditional perspective and the protonation equilibria can be omitted as long as the *pH* (and IS) stays constant.

The *pH*-conditional mobilities and binding constants can be obtained in yet another way, which is of general applicability for systems with multiple equilibria. For this reason we briefly introduce the rearrangement strategy in the ESM, too. On the other hand, when the *pH* dependency is taken into account but the concentration of the selector, $c_S$, is kept constant, the same rearrangement (ESM) can be applied to Eq. (13) with respect to the $a_{H_3O^+}$, rather than $c_S$, yielding

$$\mu_{A,eff} = \frac{\mu_{A^{[-H]}}^{c_S} + \mu_{A^{[+H]}}^{c_S} K_{prot}^{mix,c_S} a_{H_3O^+}}{1 + K_{prot}^{mix,c_S} a_{H_3O^+}}, \tag{16}$$

where

$$\mu_{A^{[-H]}}^{c_S} = \frac{\mu_{A^{[-H]}} + \mu_{AS^{[-H]}} K'_{AS^{[-H]}} c_S}{1 + K'_{AS^{[-H]}} c_S}, \tag{16a}$$

$$\mu_{A^{[+H]}}^{c_S} = \frac{\mu_{A^{[+H]}} + \mu_{AS^{[+H]}} K'_{AS^{[+H]}} c_S}{1 + K'_{AS^{[+H]}} c_S}, \text{ and} \tag{16b}$$

$$K_{prot}^{mix,c_S} = \frac{1 + K'_{AS^{[+H]}} c_S}{1 + K'_{AS^{[-H]}} c_S} \cdot K_{prot}^{mix} = Z_{prot} K_{prot}^{mix} \tag{16c}$$

are the $c_S$ -conditional parameters. Specifically, $K_{prot}^{mix,c_S}$ is the $c_S$ -conditional (mixed) protonation constant. Equation (16b) implies $pK_{prot}^{mix,c_S} = pK_{prot}^{mix} + pZ_{prot}$. This means that the system behaves as if no selector was present but the $pK_{prot}^{mix}$ value was shifted by a factor of $pZ_{prot}$. This effect is known as the complexation-induced $pK_A$ shift. We recapitulate the key papers [72–77] in the ESM. More studies are summarized in the review paper [7]. A list of related studies (not limited to electrophoresis) is also provided in the introduction of ref. [78]. Yet again, the $c_S$ -conditional constants, Eqs. (16a–16c) are simultaneously IS-conditional.

### Multiple equilibria

Separation systems with two or more selectors, i.e., dual- or multi-selector systems, respectively, are often encountered in ACE. The former occurs in an attempt to increase the separation efficiency (cf., e.g., [35]), whereas the latter out of necessity since commercial derivatives of selectors (namely functionalized cyclodextrins) are usually produced as mixtures of isomers [40, 79–82]. We reviewed the theoretical aspects of ACE separations in such systems recently [31].

When an analyte interacts with a mixture of selectors and the requirements of the fast equilibria, the 1:1 stoichiometry, and the large amount of the selector are fulfilled for every analyte–selector interaction, the effective mobility of the analyte results as a straightforward extension of Eq. (6),

$$\mu_A^{eff} = \frac{\mu_{A0} + \mu_{AS^{[1]}} K'_{AS^{[1]}} c_{S^{[1]}} + \cdots + \mu_{AS^{[N]}} K'_{AS^{[N]}} c_{S^{[N]}}}{1 + K'_{AS^{[1]}} c_{S^{[1]}} + \cdots K'_{AS^{[N]}} c_{S^{[N]}}}. \quad (17)$$

Equation (16c) was expressed in terms of the capacity factors, Eq. (11), from the "unified separation science" approach by Bowser et al. [64] and derived by Lurie et al. earlier in 1994 [83] for dual-selector systems ($N = 2$ ). The indexes $[1]\ldots[N]$ refer to the first up to the $N$-th selector in the mixture. When all concentrations of the selectors in the mixture are kept constant except one, e.g., the first one ($c_{S^{[1]}}$ ), the rearrangement introduced in the ESM provides the $c_{S^{[1]}}$ -conditional parameters. Similarly to the previous cases, such parameters are IS-conditional, too. They were originally published for dual-selector systems [84] but we provide universal formulas here:

$$\mu_{A0}^{c_{S^{[1]}}} = \frac{\mu_{A0} + \sum_{q=2}^{N} \mu_{AS^{[q]}} K'_{AS^{[q]}} c_{S^{[q]}}}{1 + \sum_{q=2}^{N} K'_{AS^{[q]}} c_{S^{[q]}}}, \quad (18a)$$

$$\mu_{AS}^{c_{S^{[1]}}} = \mu_{AS^{[1]}}, \text{ and} \quad (18b)$$

$$K'^{c_{S^{[1]}}}_{AS} = \frac{K'_{AS^{[1]}}}{1 + \sum_{q=2}^{N} K'_{AS^{[q]}} c_{S^{[q]}}}. \quad (18c)$$

Alternatively, the mixture composition may be held constant while changing its total (analytical) concentration. So every selector concentration can be expressed as $c_{S^{[q]}} = \chi_q c_S$, where $\chi_q$ is the (constant) molar fraction of the $q$-th selector in the mixture and $c_S$ now represents the total concentration of the mixture. Then, the same rearrangement yields the $\chi$-conditional (and simultaneously IS-conditional) parameters [85, 86],

$$\mu_{A0}^{\chi} = \mu_{A0}, \quad (19a)$$

$$\mu_{AS}^{\chi} = \frac{\sum_{q=1}^{N} \mu_{AS^{[q]}} K'_{AS^{[q]}} \chi_q}{K'^{\chi}_{AS}}, \text{ and} \quad (19b)$$

$$K'^{\chi}_{AS} = \sum_{q=1}^{N} K'_{AS^{[q]}} \chi_q. \quad (19c)$$

We give preference to the $\chi$-conditional perspective for two reasons. First, the $\chi$ parameter is well constrained between 0 and 1, which makes the inspection of the parametric space of the effective mobility easier in the case of dual-selector systems. Second, the $\chi$-conditional perspective is natural to the multi-selector systems composed of a mixture of unknown but constant composition. The model can explain the extraordinary selectivity of randomly charged CDs as often encountered in practice [87]. Recently we showed that the $\chi$-conditional perspective enables determination of relative enantiomer migration order of enantiomers using a racemic sample [88]. If an enantiomer A migrates first with one single selector, it is possible to decide whether the first migrating enantiomer with another selector is also the enantiomer A or it is the opposite one. Such a decision can be made without the need of having the pure enantiomers available if the two selectors are combined into a dual-selector mixture in a defined way. This allows for assigning the complexation parameters obtained in single-selector systems to the correct enantiomeric forms as necessary for computation of the $\chi$-conditional parameters, Eq. (19), e.g., for subsequent method optimization.

For the "total concentration" of the mixture it applies

$$c_S = \sum_{q=1}^{N} c_{S^{[q]}} = \frac{\sum_{q=1}^{N} n_{S^{[q]}}}{V}. \quad (20)$$

Equation (19a) may come in useful especially when dealing with deliberately prepared mixtures, typically the dual-selector ones, for which the individual values of $n_{S^{[q]}}$ or $c_{S^{[q]}}$ are known. When, on the other hand, dealing with mixtures of constant, yet unknown, composition, it is best to express the total concertation, $c_S$, and the $\chi$-contitional binding constants in terms of masses. Additionally, one may be unsure whether or not the $\chi$-contitional parameters can be further combined into yet other $\chi$-contitional parameters. To illustrate such a need, imagine that two

commercial mixtures of CD derivatives are to be combined into a final "dual-selector"-like mixture. Although intuitively accepted, this problem has apparently never been addressed in the literature explicitly. We provide a proof that it is indeed so in the ESM.

Another example of a multiple analyte–selector interaction is represented by chiral separations of *cis*-diols (and related compounds, such as saccharides) in borate buffers. The borate–diol interaction provides achiral resolution of analytes or mobilizes the neutral ones, while addition of a chiral selector, typically cyclodextrin, allows for the chiral recognition. We again provide detailed discussion on the key papers [89–92] in the ESM. The theoretical treatment of such coupled equilibria shows that the $\chi$-conditional perspective has stronger limits than the $c_{S^{[i]}}$ -conditional (or any equivalent) one. The 1:1 stoichiometry is the only prerequisite for the latter, while no mixed complexes are an additional constrain put on the former. Apart from multiple interactions among one analyte and a mixture of selectors, one can imagine multiple interactions between one analyte and one selector, yet occurring among various binding sites. This issue was addressed by Asnin and Nikitina only recently in 2014 [93]. Their model aimed exclusively at the 1:1 complexation stoichiometry, which means that a certain portion of the molecules of the analyte interacts with the selector through an interaction "A", another portion through an interaction "B", etc., but these interaction modes are independent of each other and there is no space for a mixed mode. The model is also summarized in the ESM. In brief, the theory reveals that what manifests itself as a single analyte–selector interaction can actually be made up of more elementary equilibria (between one analyte and one selector) that, however, remain hidden and inaccessible.

Despite the long-term existence of the above equations, we identified in our recent review [31] that a model that would adequately describe both complexation equilibria with multiple selectors and acid–base equilibria was still missing. This led us to formulation of the so-called $M_AM_S$ -conditional parameters in our recent work in 2015 [94]. The $M_AM_S$ nomenclature shortens the "multi-analyte multi-selector" model and the parameters are also IS-conditional, as usual. The model is designed for an analyte that exists in $L$ forms among which fast equilibria are established. The $L$ forms are not specified but the protonated/deprotonated forms of a weakly acidic/basic/amphoteric compound are definitely of prime interest. Such compounds are no longer limited to monovalent or "stepwise ionizable" ones. These $L$ forms of the analyte are allowed to interact with $N$ selectors under the exclusive 1:1 stoichiometry and no mixed interactions. Noticeably, $R$ out of the $N$ selectors ($R \leq N$ ) may alternatively represent $R$ (de)protonated forms of weakly acidic/basic selector(s). The conditional parameters read

$$\mu_{A0}^{M_AM_S} = \sum\nolimits_{i=1}^{L} \chi_{i0}\mu_{A0^{[i]}}, \tag{21a}$$

$$\mu_{AS}^{M_AM_S} = \frac{\sum_{i=1}^{L}\chi_{i0}\sum_{q=1}^{N}\mu_{AS^{[i][q]}}K'_{AS^{[i][q]}}\chi_q}{K'^{\chi}_{AS}}, \text{ and} \tag{21b}$$

$$K'^{M_AM_S}_{AS} = \sum_{i=1}^{L}\chi_{i0}\sum_{q=1}^{N}K'_{AS^{[i][q]}}\chi_q, \tag{21c}$$

where $\chi_{i0}$ is the molar fraction of the $i$-th form of the free (not complexed) analyte, $\mu_{A0^{[i]}}$ are their (the $i$-th forms') electrophoretic mobilities, $K_{AS^{[i]}}\,[q]'$ are apparent binding constants between the $i$-th form of the analyte and the $q$-th (form of the) selector, $\mu_{AS^{[i]}}\,[j]$ are the electrophoretic mobilities of such complexes, and $\chi_q$ are the molar fractions of each $q$-th ($r$-th form of the) selector.

The most important outcomes of the model—as discussed in more detail in the ESM—are the following. First, the molar fractions of the free analyte, $\chi_{i0}$, can be evaluated, e.g., from the given pH without the need to take the complexation into account if the $K_{prot}^{mix}$ constants are known for every ionization state (cf., e.g., [95, 96]). The same possibly holds true for the fractions of, $\chi_q$ (aka $\chi_r$), if a weakly acidic/basic selector is present alone or in a mixture. The latter must be applied with caution that the selector—being present in a large amount—may participate in the buffering properties of the BGE and also significantly contribute to the IS of the BGE, which both would invalidate this approach. Having the values of $\chi_{i0}$ and $\chi_q$ in hand, one can deduce the $M_AM_S$-conditional parameters directly from Eq. (21). Similarly, pH-conditional parameters can be determined at defined pH (and IS) for several selectors (or several mixtures of selectors) and simply combined into the $\chi$-conditional parameters, Eq. (19). Likewise, the $\chi$-conditional parameters can be determined with a mixture of selectors and inserted into any of the pH-dependent models; this latter case is only applicable to mixtures of neutral or permanently charged selectors. Such a method was demonstrated in the experimental part of one study [67]. It was observed that the $\chi$-conditional parameters can be evaluated from the pH-conditional ones (a partially dissociated weakly acidic analyte) with an excellent precision. The precision was not so perfect in the opposite case of inputting the $\chi$-conditional parameters (of a mixture of selectors) into the pH-conditional model. This was attributed to the difficulties with which the complexation constant of the neutral form of the weakly acidic analyte are determined (regardless of whether the selector is a single compound or a mixture). Nevertheless the pH-conditional parameters were still satisfactory for practical use.

## Violating the basic assumptions

Apart from the 1:1 complexation stoichiometry (which is taken as granted here), fast equilibrium rate, sufficient excess of the selector, and the requirement that the selector does not

interact with any component of the BGE are the prime prerequisites of the aforementioned models. We briefly summarize consequences of violating these assumptions in this section.

If the complexation equilibrium is not accomplished with fast enough rates, the peak (zone) of the analyte broadens, while it eventually splits into two peaks interconnected with an intermediate plateau at medium reaction rates, until the peaks become completely separated at slow reaction rates [97]. The process is characterized by two characteristic times,

$$t_{\mathrm{eq}} = \frac{1}{k_{+} \cdot c_{S0} + k_{-}} \tag{22}$$

and

$$t_{\mathrm{sep}} = \frac{w}{|v_{\mathrm{A0}} - v_{\mathrm{AS}}|}, \tag{23}$$

where $k_{+}$ is the rate constant of the complex formation, $k_{-}$ is that of the complex dissociation, $w$ is the width of the initial zone, and $v$ stands for velocities. (Other authors define $t_{\mathrm{sep}}$ as half of the value of Eq. (23) [98].) It is possible to determine the individual rate constants, $k_{+}$ and $k_{-}$, from the specific peak shape distribution caused by the slow kinetics. The most relevant outcome for our purposes, however, is that the peak shape distribution was indistinguishable from the one that would result if no complexation equilibrium took place by computer simulation with $t_{\mathrm{sep}}/t_{\mathrm{eq}} > 10$. This relation along with Eqs. (22) and (23) somewhat quantifies the adjective "fast" used to describe the equilibrium rates.

If the selector is present in small amounts, the effective mobility of the analyte is not affected but nonlinear peak dispersion occurs. Some authors tried to calculate the free amount of the selector, $c_{S0}$, in the sample zone in an attempt to compensate for the selector consumption in Eq. (6) [99–101] (see ESM). However, such a procedure is not adequate. Le Saux, Varenne, and Gareil [102] reported highly convincing experimental evidence that the analyte peak becomes HVL-distorted with decreasing selector/analyte concentration ratio, where HVL stands for the Haarhoff–Van der Linde function (see the ESM). The HVL function was proven to be an exact solution of the linearized governing equations of capillary zone electrophoresis (CZE) with a first-order nonlinear term (yet not concerning the complexation equilibrium) [103, 104]. We made the same conclusion as Le Saux et al. on the basis of a series of papers combining experiments, computer simulations, and computations by means of the linearized theory of CZE (newly partially accounting for the complexation equilibrium, too) [105–109]. The complexation-induced distortion in ACE can be understood when realizing that there is always low enough concentration of the analyte at the diffusive edges of its migrating zone (peak). Consumption of the selector can be severe only inside the zone. Thus the peak edges migrate with the expected mobility, Eq. (6), while the possible consumption of the selector pushes the peak apex out of its central position. More about this topic can be found in the ESM. These findings provide a strong reasoning for using the HVL function for peak evaluations in ACE. The $a_1$ parameter of the fitted HVL function is the proper migration time that should be used for the effective mobility calculations. To the contrary, the migration time should not be read from the peak apex since it can be shifted out of its expected position. The CEval software introduced above [58] performs automated estimates of the HVL parameters followed by a HVL fitting procedure and reading the effective mobility from the $a_1$ parameter.

Presence of any selector, even a neutral one, can alter the BGE properties if any of the BGE components interacts with it [78, 110]. The interacting component is possibly subject to the complexation-induced p$K_{\mathrm{A}}$ (generally p$K_{\mathrm{prot}}$) shift (cf. "Acid–base equilibria" and the ESM). Consequently, the pH of the buffer may be compromised. pH shifts as high as 0.5 pH units were recently reported with some common buffer constituents (benzoic acid, CHES) and neutral CDs at a concentration as low as 10 mM. When complexation parameters are determined in such systems, they become conditional to that one particular system only (cf. Eq. (18a)). An almost 20-fold biased value of a binding constant determined in such an interacting BGE has been reported [110]. Additionally, the mobility of system peaks is affected in this way. The authors also demonstrated a complete deterioration of an otherwise promising separation as a result of these side effects. On the basis of these results it was concluded that clearly—but against the contemporary usual practice—the pH of the buffer should always be controlled after the addition of the complexation agent (even a neutral chiral selector) to reveal a possible complexation with the constituents of the buffer.

Noticeably, the complexation-induced distortion and deterioration of the BGE properties have been studied for a few neutral CDs only. The theory has not been completed for charged selectors, or mixtures of selectors. It is only partially completed for coupled complexation and acid–base equilibria. System peaks in ACE present yet another open problem.

## Viscosity

Selectors in ACE are typically large molecules, such as cyclodextrins, macrocyclic antibiotics, or even polysaccharides and polycyclodextrins [111, 112]. The addition of such molecules into the BGE undoubtedly alters electrophoretic moblities of ions (including that of the analyte and the analyte–selector complex). The related theory is reviewed, e.g., in a book by Robinson and Stokes [113] and a paper by Sadek [114]. We recapitulate the

main ideas in the ESM. In brief, a correction factor, $v$, is introduced,

$$v = \frac{\eta_s}{\eta_0}, \tag{24}$$

which provides conversion from the mobility measured in a BGE of viscosity $\eta_s$ to the reference BGE of viscosity $\eta_0$ (the so-called Walden's rule [113], $\mu \cdot \eta = \text{const}$). Consequently, Eq. (6) becomes either

$$v{\cdot}\mu_{A,eff} = \frac{\mu_{A0} + \mu_{AS}K'_{AS}c_S}{1 + K'_{AS}c_S} \tag{25a}$$

or

$$\mu_{A,eff} = \frac{\mu_{A0} + \mu_{AS}K'_{AS}c_S}{1 + K'_{AS}c_S} \cdot \frac{1}{v} \tag{25b}$$

depending on whether the model parameters are to be determined from the effective mobility data or the effective mobility is to be predicted from the model parameters, respectively. The essential problem with the viscosity correction factor, Eq. (25), is that Walden's rule is by far not ultimate. This fact apparently did not prevent this correction from becoming uncritically popular in ACE applications, which inspires us to make a relatively detailed discussion on this topic herein.

The correction factor (aka the relative viscosity) is determined in several ways in ACE: directly, by determining the individual viscosities $\eta_0$ and $\eta_S$ [115], or indirectly employing the CZE instrumentation. Typical indirect measurements consist in determination of (subscripts s and 0 refer to "with" and "without the addition of the selector", respectively): (i) $t_S/t_0$, where $t$ is the time required to push the solution through the capillary by a defined pressure [116], (ii) $I_0/I_S$, where $I$ is the current in the capillary at a constant voltage [43, 117], (iii) $G_0/G_S$ and $\kappa_0/\kappa_S$ where $G$ is conductance, $G = I/U$, at constant power, $U$ is voltage across the capillary, and $\kappa$ is conductivity of the BGE [118], (iv) $t_{MS}/t_{M0}$, where $t_M$ is a migration time of an indifferent marker [119]. When viscosity of the solution is determined by means of external instrumentation, the bulk viscosity is measured rather than its effect on the movement of ions. The same applies to method (i) above that effectively turns the CZE instrumentation into a viscometer. Using the CZE instrumentation for the viscosity measurements (in the role of a viscometer) was somewhat validated within the study by Shibukawa et al. [119] or Fanali and Boček (who weighed the vials rather than reading times $t_0$ and $t_s$) [120]. The relative viscosity generally increases with increasing concentration of the additive in a nonlinear manner [113, 115, 120]. Several studies [45, 58, 70 122, 123] nevertheless show that a linear dependence,

$$v = r{\cdot}c_S + 1, \tag{26}$$

is often obeyed with an excellent precision within the range of the selector concentrations used. In Eq. (26), $r$ is an empirical constant that has to be determined. In some studies [70, 121, 122] the intercept of Eq. (26) was also subject to the linear regression. This is indeed unrealistic ($v|_{[c_S=0]} \equiv 1$) and the intercept should always be fixed to 1 during the regression.

## Relations among the various correction factors

Østergaard et al. [118] investigated mobility ratios, $\mu_0/\mu_S$, for various analytes (pharmaceuticals and two bile salts) in glycerol-altered BGEs. Glycerol was supposed not to interact with the analytes. We reprocessed the data and provide a graphical comparison in Fig. 2A. If Walden's rule applied, the mobility ratios would equal the relative viscosities, whereas Fig. 2A clearly displays that it is not so. Interestingly, the graphical data reveal that not only are some compounds less affected by the viscosity changes than Walden's rule suggests but other compounds are influenced to a higher extent than the mere viscosity change can account for (which is inconsistent with the underlying theory recapped in the ESM). Aware of this discrepancy, the authors attributed it to an observed decrease in temperature or possible IS effects. Alternatively though, the data may just reflect the true nature of the problem since the theory of migration under the influence of a nonelectrolyte is definitely not a closed issue yet. In any case, Fig. 2 in the original study demonstrates that the anticipated constancy of the Walden's product strongly improves if the relative viscosity or current is applied as the correction factor to the effective mobilities. Such an observation can be understood when realizing that the effective mobilities of the analytes drop roughly by 45 % (calculated as $(\mu_0 - \mu_S)/\mu_0 = 1 - (\mu_0/\mu_S)^{-1}$) as a result of the presence of glycerol, while they do not deviate from the relative viscosity trend by more than 8 % (cf. Fig. 2A).

The relative viscosity and current ratio worked comparably well in the case of glycerol in the study by Østergaard et al. The comparison between these two correction factors is depicted in Fig. 2B (we present the BGE dilution corrected values only; see the ESM for details). An agreement between the relative viscosity and current ratio was reported by other authors at low concentrations of cyclodextrins, too. A maximal relative viscosity of 1.32 (corresponding to 75 mM methylated-β-CD) was achieved in one study [43]; although this information was not available in another study [123], the hydroxypropyl-β-CD concentration did not exceed 20 mM in that case. Lemesle-Lamache et al. [117] reported a 1:1 relation between relative viscosity and current ratio up to concentrations of β-CD of

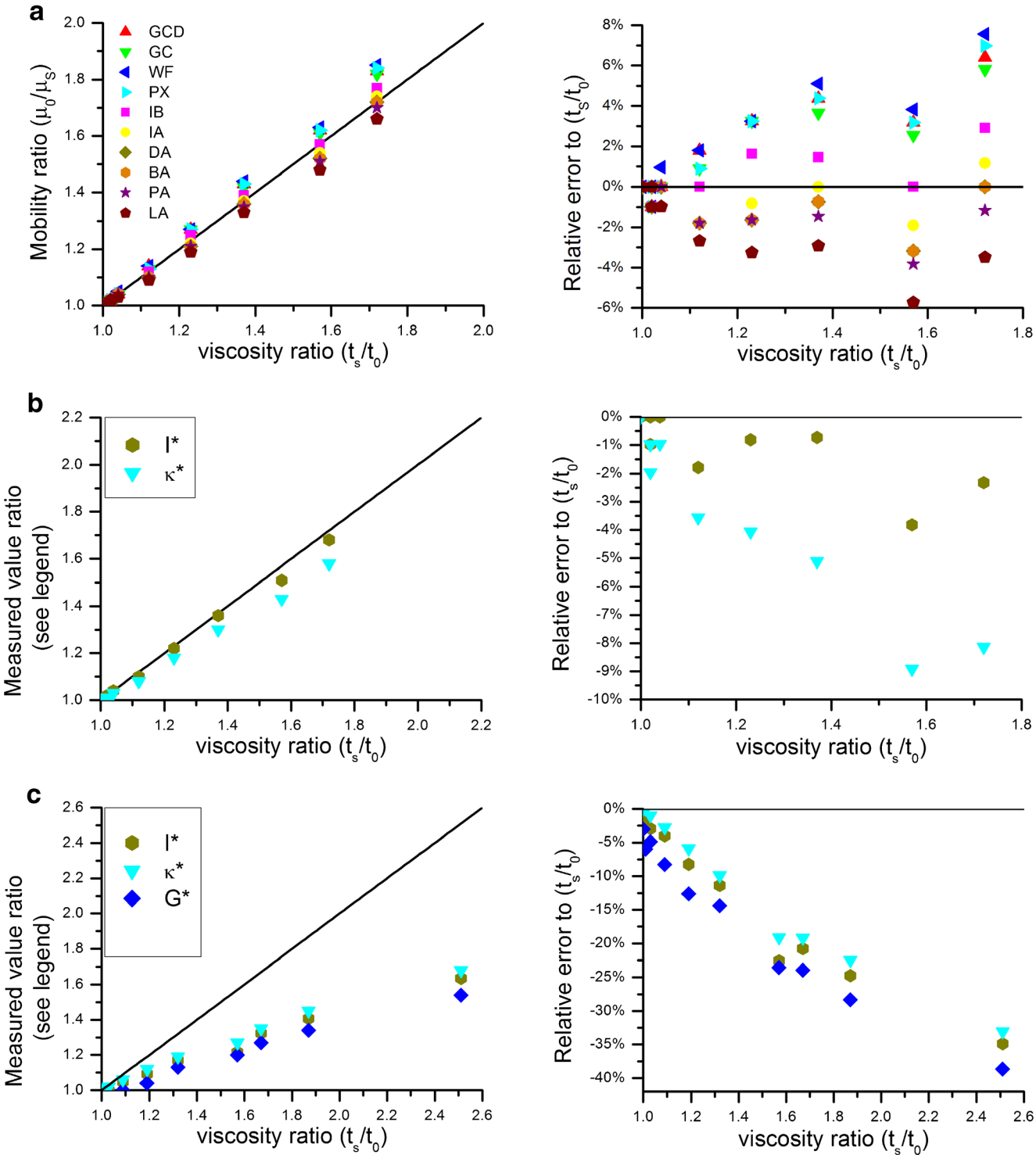


**Fig. 2** Correlation of the (BGE dilution corrected) values of relative current (*I**), relative conductivity (*k**), relative conductance (*G**); and mobility ratios of non-interacting ions, lidocain (*LA*), propranolol (*PA*), benzoic acid (*BA*), Dibucaine (*DA*), imipramine (*IA*), ibuprofen (*IB*), piroxicam (*PX*), warfarin (*WF*), glycocholate (*GC*), glycodeoxycholate (*GDC*)—see ESM for chemical structures—with the relative viscosity measured by the ratio of times needed to push the solution through the capillary by a defined pressure. **A** viscosity altered by glycerol; **B** viscosity altered by glycerol; **C** viscosity altered by cyclodextrin. *Left* absolute values, *diagonal line* shows the ideal match; *Right* relative differences to the absolute match (*diagonal line* in the *right panel*). Data reprocessed from the study by Østergaard et al. (2009)

200 mM ($v \cong 2.2$). Nevertheless, a closer look at the data plot (Fig. 3 in the original paper) reveals that the current ratio might have followed the relative viscosity well up to the values of $v \cong 1.3$ while it possibly started

deviating at the higher values of the relative viscosity (however, there are not enough data points in the study to make a solid conclusion).

These results may seduce one to a conclusion that determining the current ratio is an equivalent alternative to the relative viscosity. It may well be so but only at rather low relative viscosities (selector concentrations). Taking the $t_S/t_0$ ratio as a reference, Østergaard et al. [118] also showed that other methods (current, conductivity, or conductance ratios) provided systematically lower correction factors than the relative viscosity when a CD (instead of glycerol) was present in the BGE. The results are less optimistic than the aforementioned studies, indicating that the current or conductivity ratios approximately match the relative viscosity at the lowest CD concentrations only (cf. Fig. 2C). The opposite conclusion made for glycerol ($v$ up to 1.7; equivalent CD concentration of 100 mM) was attributed to the fact that glycerol is a much smaller molecule than the CD, comparable in size to the BGE ions. Shibukawa et al. [119] provided highly convincing results that univocally prefer the current ratio over the relative viscosity. They determined both the current ratio and the mobility ratio of (presumably non-interacting) ascorbic acid in the presence of γ-CD. The current ratio started deviating from the relative viscosity at values of approx. 1.8 and the deviation grew up to $v \cong 4.5$, $I_0/I_s \cong 3.3$ (approximate values read from data plot) at 250 mM γ-CD. To the contrary, the mobility ratio of the acid followed the relative current nearly perfectly up to the highest CD concentrations.

The fact that the relative viscosity (or rather current ratio) accounts for a big portion of the change in the electrophoretic mobility of non-interacting ions not only justifies the application of such correction factors but even makes them essential especially for the weakly interacting analytes in ACE. Penn et al. discussed in 1994 [99] that if the binding constant is of the order of 100 $M^{-1}$ or less, the relative viscosity as low as 1.02 easily overweighs the actual binding effect at low selector concentrations. At high selector concentrations the binding effect is significant enough but then so is the viscosity of the BGE [100] as well as the deviation of the effective mobility from Walden's rule [119]. Unfortunately though, the current ratio is only applicable when neutral selectors are used for obvious reasons, while the relative viscosity seems to be an appropriate choice only at low selector concentrations. Finally, Vespalec and Boček [71] pointed out that cyclodextrins are relatively small molecules (in contrast to, e.g., polymerized CDs) so that the bulk viscosity of the BGE should be sufficiently relevant. Figure 2 as well as the results by Shibukawa et al. indicate that the viscosity changes caused by the presence of CDs in the BGE do not fully correlate with mobility changes of small ions and the same can apply even to as small additives as glycerol.

**Using a mobility standard**

Britz-McKibbin and Chen used a non-interacting analyte as a mobility standard (MOS) for viscosity corrections in their attempt to accurately describe weak analyte–additive interactions by capillary electrophoresis [124]. When the effective mobilities were corrected to the relative viscosity only, the dependences of $\mu_{A,eff}$ vs. $c_S$ did not follow the hyperbolic pattern of Eq. (6) and the product of $\mu_A \cdot v$ was by far not constant for non-interacting analytes. The situation substantially improved by applying a correction factor $v' = \mu_{MOS,S}/\mu_{MOS,0}$, i.e., a mobility ratio analogous to that depicted in Fig. 2A. The authors attributed the difference between $v$ and $v'$ to the fact that the selector alters both the viscosity and the dielectric properties of the BGE. They originally expressed $v'$ as $v' = v \cdot v_\delta$ where $v_\delta$ is supposed to be the relative dielectric correction factor. Although this idea is consistent with the basic theory provided in the ESM and the $v_\delta$ values were reported as closely matching those obtained for glucose in the literature, the study leaves the hypothesis of altering the dielectric properties of the BGE rather open. The way in which the parameter $v_\delta$ was determined and used would be perhaps better reflected by concluding that a MOS helps in improving the results no matter what the real cause of $v' \neq v$ is. Similarly, the ascorbic acid used by Shibukawa et al. [59] was actually meant as a MOS.

Other authors realized that even the electroosmotic flow (EOF) can be used as the MOS since it is—similarly to the ion mobility (see the ESM)—inversely proportional to the viscosity of the solution,

$$\mu_{EOF} = \frac{\epsilon\zeta}{4\pi\eta}, \tag{27}$$

where  is the dielectric constant of the BGE and $\zeta$ is the zeta potential of the capillary wall. Valkó et al. [125] reported a constant Walden's product for the EOF in a phosphate buffer up to concentrations of γ-CD of 100 mM and consequently used the EOF as a MOS for determination of binding constants of mandelic acid with the CD. The constant Walden's product for the EOF is in accordance with the study by Shibukawa et al. [59] who made the same observation in the same buffer up to 250 mM of γ-CD. Interestingly, Lemesle-Lamache et al. [117] reported just the opposite. They claimed the EOF mobility ratio did not correspond to the relative viscosity (β-CD, maximal $v$ of 2.5). Unfortunately, the data are not really convincing in this case since they are rather scattered, and what manifests itself as a nonlinear relationship might be alternatively seen as a high variance caused by an unstable EOF. However, the constant Walden's product for the EOF up to the highest relative viscosities simultaneously raises doubts about using the EOF as a MOS. It rather

indicates that the EOF happens to be an excellent marker of the bulk viscosity (which seems reasonable), whereas the bulk viscosity is not always decisive for the small ions (analytes) as discussed in the previous section. This is perfectly demonstrated in the aforementioned study by Shibukawa et al. [59].

Yang, Bose, and Hage [126] showed (both theoretically and experimentally) that the reproducibility of the moblities and migration times in general CE improves significantly by considering a mobility ratio $M = \mu'_A/\mu_{MOS}$ or migration time ratio $R_t = t_A/t_{MOS}$ where the subscripts A and MOS refer to the analyte and the mobility standard, respectively. The MOS has a similar role here as in the previous cases but it is used in a different manner now. The mobility of the analyte is not multiplied by a correction factor based on the mobilities of the MOS with and without the addition of the selector, but a new parameter is introduced that is evaluated instead of the analyte's mobility. Furthermore, the correction is applied to the apparent mobility of the analyte, $\mu'_A$, not the effective one, $\mu_{A,eff}$, in the original paper by Yang, Bose, and Hage. The EOF is also applicable as a MOS in general but we already discussed that such practice is doubtful in ACE. The two ratios not only account for the viscosity effects but also do not depend on the experimental setup (capillary length, detector position, voltage, temperature), thereby making the method more robust. It is not completely independent of other effects that alter the EOF, though. The authors also stated that "*M* can be used… where it is desirable to relate… migration to fundamental parameters of the system, such as… binding to agents that have been added to the running buffer". Nevertheless, the meaning of this statement actually was—as demonstrated experimentally—that the *M* or $R_t$ parameter compensates the effects the additives have on non-interacting analytes. Kawaoka and Gomez [127] realized that the *x*-reciprocal transformation of Eq. (6) can be equivalently expressed in terms of the mobility ratio, *M*, and they determined some binding constants (though using the EOF as the MOS) in this way. We give a more general proof in the ESM showing that the *M* parameter obeys Eq. (6) exactly. The resulting binding constant, $K'_{AS}$, is identical to that obtained in the standard way but the related mobility of the free analyte, $\mu_{A0}$, and that of the analyte–selector complex, $\mu_{AS}$, result in terms of the *M* parameter. Subsequently, the original mobilities must be back-calculated from these parameters. (The situation is not so simple in the case of the $R_t$ parameter as shown in the ESM.) Two mobility standards are required to take advantage of the robustness of the *M* and $R_t$ parameters and simultaneously work with the mobilities directly [128]. Such an approach also has general applicability in CE, and the inventors demonstrated its applicability in ACE measurements, too [129, 130]. In our opinion this latter approach better accounts for various instabilities in CE, including the EOF; however, it requires two MOSs, which can cause difficulties in ACE. If the *M* parameter is to be used, we would additionally suggest to base it on the effective mobility, $\mu_{A,eff}$, rather than the apparent one, $\mu'_A$ (cf. ESM).

The use of a MOS may seem the best idea to cope with the side effects caused be the presence of the selector. Unfortunately even this cannot be applied without caution. The problem with using the EOF as a MOS has been already discussed. The selector may further alter the zeta potential of the capillary wall [131], which would make the entire approach of no use. If an internal standard is used as the MOS, it should resemble the analyte as much as possible, which contradicts the requirement of the MOS not to interact with the selector. Additionally, another interesting finding results from the study by Østergaard et al. [121]. The visual similarity of tested compounds does not imply their similar sensitivity to the presence of an additive. When data in Fig. 2A are compared to chemical structures of the compounds (available in the ESM) it turns out that, for example, benzoic acid (orange hexagons) does not follow ibuprofen (violet squares), its closest molecule by visual comparison, but it perfectly overlaps with dibucaine (green diamonds), a molecule that looks somewhat different. Similarly, benzoic acid is the smallest of the tested compounds but lidocaine (brown pentagons) is the least affected by the viscosity changes whereas ibuprofen, the smallest molecule but one, approaches the largest ones. Several observations of this kind can be made. Only the very large molecules (triangles) somewhat cluster together, but again piroxicam and warfarin (rightward and leftward triangles) are the most sensitive to the viscosity changes even though they are apparently smaller than the bile salts (upward and downward triangles). In fact, using, e.g., benzoic acid as a MOS for ibuprofen (under the conditions of Fig. 2A) would even worsen the situation compared to the simple relative viscosity correction as benzoic acid deviates systematically negatively while ibuprofen deviates positively from the relative viscosity.

## Other approaches

Barták et al. [132, 133] took advantage of the linearization of Eq. (6) and applied an advanced statistical approach to the evaluation of binding constants in ACE. A perfect linear dependence would result after linearization if the effective mobility of an analyte was influenced by nothing but the mere interaction with the selector. Further, only a slight deviation from linearity is expected if the effective mobility is not disturbed by the viscosity (or other) effects too much. On the other hand, real experimental data are always of a random nature so that the nonlinearity cannot be easily uncovered and compensated. The statistical approach introduced by Barták et al. allows for estimating the extent of nonlinearity of experimental data on the basis of the expected function (the linearized form of Eq. (6)), which leads to corrected

estimates of the parameters, i.e., $\mu_{A0}$, $\mu_{AS}$, and $K'_{AS}$. Although the method can handle only minor nonlinearities [71] and it requires rather sophisticated mathematical treatment, it can be advantageous for evaluation of the corrected binding constants. On the other hand, it leaves the question of the source(s) of such nonlinearity open and thus it is not useful for predicting the effective mobility in the separation system, e.g., for the purpose of further method optimization.

Inspired by the fact that the viscosity changes caused by large selectors should mostly influence similarly sized moving particles, we adopted a practice of correcting only the mobility term of the complex in some of our studies [45, 70],

$$\mu_{A,\mathrm{eff}} = \frac{\mu_{A0} + \frac{\mu_{AS}}{\nu} K'_{AS} c_S}{1 + K'_{AS} c_S}. \tag{28}$$

Equation (28) requires an explicit formula for the relative viscosity, $\nu$, as a function of the selector concentration, $c_S$, (cf., e.g., Eq. (26)) and can no longer be linearized. This correction is also implemented in the CEval software [58]. Although this idea is becoming rather dubious in the light of the just reviewed evidence that small ions are affected by the presence of large additives as well, applying the relative viscosity in Eq. (28) provided a complete match between the fitting function, Eq. (28), and the experimental data, further supported by a perfect agreement between the binding constants determined in this way and those determined independently by NMR [70]. To the contrary, biased fitting curve and binding constants resulted without any viscosity correction. Perhaps, the best practice would be to use different correction factors for the two mobilities on the right-hand side of Eq. (6) (alike Eq. (28)). Such an approach has not been tested in the literature yet. As a final note let us point out that the $\chi$-conditional mobility, Eq. (19) or generally Eq. (21), can be corrected in the same way only under the—yet most likely reasonable—assumption that the correction factor, $\nu$, is common to all selectors in the mixture.

## Ionic strength

The IS influences the effective mobility of an analyte in ACE in two ways: (i) it governs the complexation and the acid–base equilibria, (ii) it affects the movement of ions in the BGE

### Complexation and acid–base equilibria

We advise the reader to refer to the review by Vespalec and Boček [71] who summarized the related physicochemical concept of apparent vs. true thermodynamic binding equilibrium in a highly educative way. The true thermodynamic binding constant is related to the apparent one through the activity coefficients, $\gamma$, according to

$$K^{\mathrm{th}}_{AS} = K'_{AS} \frac{\gamma_{AS}}{\gamma_{A0}\gamma_{S0}} = K'_{AS} \cdot K^{\gamma}_{AS}, \tag{29}$$

where we use the superscript *th* to emphasize the thermodynamic constant and the subscripts AS, A0, and S0 in their usual meanings. The activity coefficients can be expressed from the given IS of the BGE using the Debye–Hückel extended law,

$$-\log\gamma_i = \frac{0.509 z_i^2 \sqrt{I}}{1 + 3.287 a \sqrt{I}}, \tag{30}$$

where the two numerical constants are valid for water solutions at 25 °C, $z_i$ is the charge of the species $i$ (AS, A0, or S0), parameter $a$ represents the distance of closest approach of ions (in nanometers), and $I$ is the IS, $I = \sum c_i z_i^2$, where the summation extends over all ions in the system and the concentrations are expressed in moles per cubic decimeter. The logarithm of the activity coefficient quantifies (but not equals) an extra potential energy that the species $i$ attains in the solution owing to its interactions with surrounding molecules (including those of the species $i$ itself). It is generally accepted that electrostatic interactions are the prime source of such energy in electrolyte solutions as reflected by Eq. (30). At this point we have to distinguish among three cases. The first one is a neutral analyte interacting with a charged selector. The potential energy of neutral analytes is not affected by the electrostatic interactions and thus its activity coefficient equals one (the logarithm being zero). The analyte–selector complex bears the same charge as the selector itself and it is reasonable to assume that the two are of similar sizes as well, thus sharing the same value of the parameter $a$. Consequently, the activity coefficients of the selector and the complex are likely similar. Under all these assumptions, $K^{\gamma}_{AS} \cong 1$, and, $K^{\mathrm{th}}_{AS} \cong K'_{AS}$ [134]. The opposite case happens when a charged analyte interacts with a neutral selector. Then the activity coefficient of the (neutral) free selector is equal to one. The parameter $a$ of small ions is generally close to 0.5 nm, which gives the McInnes approximation of the product $3.287a \cong 1.5$. Taking the same value for the analyte–selector complex is not appropriate for most selectors, which are generally much bigger than the "small ions", but the correct value is hardly available and so one is left with using a rough estimate (cf. [70]). Alternatively, the value can be estimated from the dependency of the effective mobility of an analyte (either the free ion or the selector) on the IS [45, 135]. The final case is that of both charged analyte and selector. The McInnes approximation can be used for the analyte, and the same parameter $a$ would possibly apply to a large selector and its complex with the analyte. The activity

coefficients of the free selector and the complex do not cancel each other this time since the free selector and the analyte–selector complex have different charges. As far as we are concerned, no data have been published that would deal with the IS correction in such systems.

Next, let us consider the $\chi$-conditional binding constant, Eq. (19c), of a (presumably commercial) mixture of selectors. In the case of neutral analytes, $K'_{AS^{[q]}} \cong K^{th}_{AS^{[q]}}$, and consequently, $K'^{\chi}_{AS} \cong K^{th\chi}_{AS}$, regardless of the nature of the selectors used. In the case of a mixture of neutral selectors, the constant, $K^{\chi}_{AS}$, reduces to, $\gamma_{AS}/\gamma_{A0}$, which factors out of the summation in Eq. (19c) only if all the selectors in the mixture have similar sizes, i.e., they share a common parameter $a$. The same applies to a mixture of selectors that all have the same charge (and a charged analyte) if additionally the size of the complex is comparable to the size of the selector ($a_{AS} \cong a_{S0}$), which is often a reasonable assumption, though. Lastly, the $\chi$-conditional binding constant cannot be corrected to the IS as if it were a binding constant of a single selector when the mixture consists of selectors of different charges (and the analyte is also a charged molecule). Instead, an individual correction constant, $K^{\gamma}_{AS^{[q]}}$, applies to each individual selector that cannot be separated out of the summation, Eq. (19c). Unfortunately, this is often the case with commercial mixtures of CDs and so attention must be paid to this fact when analyzing charged solutes. Combinations of charged and neutral CDs in dual systems are also common but often employed for neutral analytes or the individual constants, $K^{\gamma}_{AS^{[q]}}$, can be utilized since the composition of such artificial mixtures of selectors is known (unless being composed of commercial mixtures of selectors, for which the above stated limitations apply).

Concerning the mixed protonation constant, it results [66] that

$$K^{th}_{prot} = K^{mix}_{prot} \frac{\gamma_{A^{[+H]}}}{\gamma_{A^{[-H]}}}. \tag{31}$$

The activity coefficients can be calculated according to Eq. (30), e.g., using the McInnes approximation. The two activity coefficients do not cancel each other because of obviously different charges of the two protonated states of the analyte.

## Effective mobility

Correction of the ionic moblities to the IS poses a convoluted problem that involves recursive formulas as results from the theory by Onsager and Fuoss [136] (cf. namely Eqs. (4.6.8), (4.7.11), (4.7.12), (4.8.1)–(4.8.3) in the reference). The formulas must be solved numerically as implemented, e.g., in our software PeakMaster [137] (available for free at http://echmet.natur.cuni.cz/download), though only for non-interacting analytes and BGE constituents. The equations have not been solved for analytes interacting with a selector in the BGE yet. The IS mobility correction is considerably simplified when only two species (an ion and its counterion) are present in the solution (cf. Eq. (4.9.1) in [136]). Then,

$$\mu^0_A - \left(0.7853 \cdot \mu^0_A |z_A z_B| \frac{q}{1+\sqrt{q}} + 3.138 \cdot 10^{-8} \cdot |z_A|\right) \frac{\sqrt{I}}{1 + 3.287 a \sqrt{I}} \tag{32a}$$

where $z_A$ is the charge of the ion of interest, $z_B$ is its counterion, and the numeric constants are valid for water solution at 25 °C if the IS is expressed in moles per cubic decimeter, the parameter $a$ in nanometers, and the electrophoretic mobility in meters squared per volt per second. $\mu^0_A$ is the limiting electrophoretic mobility of the ion A (at infinite dilution of the solution) and

$$q = \frac{|z_A z_B|}{|z_A| + |z_B|} \frac{\mu^0_A + \mu^0_B}{|z_A|\mu^0_A + |z_B|\mu^0_B}. \tag{32b}$$

Specifically, $q = 0.5$, for uni-univalent electrolytes. One may be aware of a simplified version of Eq. (32a), which takes only the square root of the IS on the right-hand side, thus not requiring the parameter $a$. Unfortunately, this simplification is not appropriate in CE [135]. Electrophoretic mobility of an analyte, A, can be corrected to the IS of the BGE using Eq. (32) while the counterion of the BGE is taken for the ion B. This results in a somewhat hybrid approach when only analyte–counterion interaction is assumed on the one hand, but all present ions are accounted for in the calculation of the IS on the other hand. Nevertheless, such an approximation is preferred over not performing the IS correction at all [138]. It has been further justified experimentally in a rather unique study by Beneš et al. [45]. The authors studied electromigration of a neutral analyte in the presence of a defined monovalent charged CD. When the simple Eq. (6) was employed, the fitting curve did not match the experimental points very well but the situation improved dramatically when viscosity and IS corrections were applied. Additionally, the data were obtained in two ways. First the IS was kept constant by decreasing buffer concentration while increasing the (charged) selector concentration. Second, the IS was not adjusted manually but the IS correction, Eq. (32a), was employed to account for it. The parameter $a$ of the selector was estimated experimentally in the study and the authors introduced a simple verification that the same value applies to the analyte–selector complex, too. A perfect agreement between binding constants determined at the constant IS and the variable IS was reported. Equation (32a) has been applied by other authors even in methanol/water solvent systems (after accommodating the numerical constants properly) [139]. Other approaches to the IS

correction can be found in the literature [135] but they have not been apparently transferred to ACE.

When a highly charged selector is employed in the separation system, it influences the IS of the BGE to a great extent and simultaneously the mobility of the (highly charged) complex is especially sensitive to the IS (cf. Eq. (32a)). In such systems, the dependence of the effective mobility of the analyte on the selector concentration may completely violate the hyperbolic pattern prescribed by Eq. (6). Instead it may exhibit a local maximum, which would be much unexpected if not observed for several highly charged CDs ($z > 7$) by the group of Vigh [140–143]. The authors explained their observation by the mechanism that we reproduce in the ESM. Additionally, the large multiply charged selectors (as typical for several widely used CD derivatives) definitely do not satisfy assumptions required for the validity of the Onsager–Fuoss theory. As a matter of fact, the entire concept of the IS is questionable in such systems. So even the compensation for the increasing IS is not fully justifiable, notwithstanding the usually unknown composition of commercial mixtures. Whatever else might be said about it, the problem of large highly charged selectors and their impact on the (effective) mobilities of ions remains an open issue.

## Conclusion

Various equations describing the dependence of the effective mobility of an analyte on the selector concentration, pH, and composition of a mixture of selectors have been reviewed and their mutual relationships explored. Equation (6) is the fundamental equation in ACE, originally developed for interactions with a single selector in systems where neither the analyte nor the selector is involved in the acid–base equilibria. Several authors extended this formula in an attempt to account for the pH dependence of the effective mobility of a monovalent weakly acidic/basic analyte interacting with a neutral or permanently charged selector. The resulting expressions are not always easily seen as being actually identical but they are all covered by Eq. (13). If pH is kept constant, the analyte obeys Eq. (6) with the pH-conditional parameters regardless of its actual ionization state. If the selector is present at a constant amount, the analyte behaves as a standard weakly acidic/basic compound possibly with a complexation-induced shifted p$K_A$ value.

The most recently published multi-analyte multi-selector model discloses that Eq. (6) with conditional parameters, $\mu_{A0}^{M_A M_S}$, $\mu_{AS}^{M_A M_S}$, $K_{AS}^{'M_A M_S}$, i.e., Eq. (21), retains its validity if (i) the external conditions (typically pH) are maintained so that the distribution of the analyte into its possible (typically ionic) forms does not change; and (ii) a mixture of selectors of constant composition is employed. The pH-conditional and the $\chi$-conditional parameters can be further combined into the $M_A M_S$-conditional ones. Specifically, in any of the previously published models any mixture of selectors can be treated as a single selector with the $\chi$-conditional parameters, including the case when $\chi$-conditional parameters of several mixtures of selectors are combined into subsequent $\chi$-conditional parameters of yet another mixture. This justifies the usual practice of treating commercial mixtures of selectors as a single one. Nonetheless, the $\chi$-conditional parameters may (or may not) lose their validity when the selectors alter the viscosity of the BGE or when it comes to the ionic-strength-related effects. Also, attention must be paid to the fact that the fundamental Eq. (6) as well as any of its various conditional alternatives are always additionally IS-conditional.

Apart from the 1:1 complexation stoichiometry several further requirements apply, namely fast equilibrium rate, sufficiently abundant amount of the selector in comparison with the amount of the analyte, and no interaction between the selector and the BGE components. Violation of these requirements has diverse consequences. Slow equilibrium makes the peak wider, while it can eventually split into two. Low selector concentration does not alter the effective mobility of the analyte but rather leads to an unexpected dispersion of its migrating zone. The Haarhoff–Van der Linde (HVL) function seems to describe the dispersed peak well. Finally, if the selector interacts with any of the BGE components, conditional parameters valid in the particular separation system only appear in Eq. (6). Such side interactions can also considerably alter the basic BGE properties such as its pH. pH of the buffer should always be checked after the addition of a selector (even a neutral one). If the buffer pH changes upon addition of the selector, it indicates undesirable side interactions of the BGE component(s) with the selector (but the stabile pH does not guarantee a lack of such side interactions).

Both viscosity of the BGE and its ionic strength have a significant impact on the effective mobility of the analyte. None of these effects can be treated in an ultimately exact way. Viscosity effects are unavoidable in ACE but few studies can be found in the literature on this topic, sometimes with contrary conclusions. Different ions exhibit different sensitivity to the addition of large particles (selectors) into the BGE, perhaps regardless of their visual structural resemblance on paper. An observation has been made that large ions are influenced by the addition of glycerol to a larger extent than the bulk viscosity can account for, which is in contradiction with the standard theory. Using the current ratio (neutral selectors only) as a "viscosity" correction factor or adopting different correction factors for the mobility of the free analyte and the

mobility of the complex, perhaps employing a mobility standard (MOS), seems the best practice in the light of current studies. Further investigations are needed, though. Nonetheless, using the EOF as the MOS is not advisable. Few studies examined the IS-related effects in ACE. The binding constant is (under reasonable assumptions) not affected by the IS if a neutral analyte interacts with any selector or their mixture. Otherwise it can be approximately recalculated to the thermodynamic one, but mixtures of charged selectors of unknown composition cannot be generally treated in this way. IS correction of the effective mobility is a complex task that has not been fully accomplished in the literature yet. The Onsager–Fuoss formula for two interacting ions (the free analyte and the BGE counterion and/or the complex and the BGE counterion) was reported to work with sufficient precision for this purpose. In general, it is best to keep the IS constant in the separation system. The BGE can be appropriately diluted upon addition of a well-defined charged selector. This practice is, however, hard to meet specifically in systems with highly charged selectors or a mixture of selectors. A suitable treatment of the IS remains an open problem there.

When (possibly conditional) complexation parameters are to be extracted from the experimental data, linearization of Eq. (6)—as often applied in practice—is neither advisable nor needed with current computational power and tools. We kindly encourage the reader to download our recently published freeware CEval (http://echmet.natur.cuni.cz/download) for this purpose. The software accounts for HVL distortion of the peaks, viscosity corrections, nonlinear regression of Eq. (6), and statistical evaluations.

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**Compliance with ethical standards**

**Conflict of interest** All authors declare no conflicts of interest.

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