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Journal of Chromatography A, 875 (2000) 431–445

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Calculation of stability constants for the chiral selector–enantiomer interactions from electrophoretic mobilities

Radim Vespalec*, Petr Boček

Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic, Veveří 97, 611 42 Brno, Czech Republic

Abstract

Interactions of chiral selectors with enantiomers or with other chiral analytes, underlying their electrophoretic separations, are particular cases of interactions of dissolved species. The interaction model that describes these reversible, fast interactions is simple. Equations describing the model are also simple and applying equations for the calculation of respective equilibrium constants to experimental data is easy using computers. Obtaining the experimental mobility data, necessary for the calculation, is the critical step on the way to stability constants quantitating the strengths of interactions of chiral selectors with enantiomers and other analytes. These data are decisive for both the accuracy and precision of the calculated constants. The meaning and applicability of the particular constant depend on the type of the constant. The common method for the determination of stability constants from electrophoretic migration data is reasonable for low and medium stability constants. For stronger complexing, characterised by stability constants of the order of 10^4 l/mol, typical of affinity chiral selectors, the method becomes unreliable. For strong complexing giving constants of the order of 10^5 or higher the method is not applicable in its commonly used form.

Keywords: Electrophoretic mobility; Enantiomer separation; Stability constants; Binding constants; Complexation constants; Association constants; Chiral selectors

1. Introduction

The different interactions of enantiomers, forming a racemate, with a chiral selector are the only way to discriminate between them. The strengths of these interactions, and, consequently, the stability of complexes created by these interactions are expressed by the respective constants [1–3]. The magnitudes of these constants correlate with the concentration of the chiral selector in the background electrolyte (BGE) that causes the maximum possible difference in effective mobilities of the separated enantiomers [4–7]. If this difference is high enough, the constants allow calculation of the concentration of the chiral selector that is optimal for the purpose of a particular separation. The comparison of relative differences in the constants, which characterise interactions of different selectors with the analytes in question, allows the a priori selection of the chiral selector. Thus, the determination of the constants that characterise the interactions of enantiomers with chiral selectors is very important in both the research of chiral separations and in their practical utilisation.

Stability is a primary property of the complex that is created during the chiral discrimination process. Thus, the classical term stability constant [8] is preferred in this article to binding constant [9–11], complexation constant [12], association constant

*Corresponding author. Tel.: +420-5-726-8121; fax: +420-5-4121-2113.

E-mail address: vespalec@iach.cz (R. Vespalec)

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00112-6

[13,14] and to other terms that may be found in the literature. The determination of the stability constant needs a theoretical model, describing the interactions of the chiral selector with the enantiomers, a set of raw experimental data reflecting these interactions, and a calculation procedure allowing the treating of the raw data according to the model [8]. The effective mobilities of the enantiomers, measured as the function of concentration of the chiral selector in the BGE are the raw data in electrophoresis. The ease with which the mobility data may be measured and the capability of computers to fit almost any set of such data with an equation allowing to calculate the stability constant evokes the impression that the way to correct stability constants in electrophoresis is simple and straightforward. Unfortunately, this is not true.

The reversible and fast interactions of a chiral selector with enantiomers, underlying their direct electrophoretic separation, are a particular case of mutual interactions of dissolved species that have been investigated for almost a century and a half. Methods developed for their investigations include the calculations of physicochemical data, which characterize both the interaction processes and their products. These methods are based on variety of experimental techniques including electrophoresis, and are summarized in many reviews and monographs. Refs. [8,15,16] can serve as representatives from the second half of the 20th century. Good fit of experimental data with an equation that expresses the experimentally measurable data as a function of the stability constant is an important criterion for the credibility of the calculated constant. However, this fit alone is sufficient neither for the credibility of the constant nor for the determination of its usability and physicochemical meaning [8,17]. Thus, a set of rules that have to be obeyed, and a set of requirements that have to be met in the development of the model, in the selection of the calculation procedure, and, mainly, in the measurement of raw experimental data were defined [8]. The requirements on the experimental data are the most numerous and the specificity of the experimental technique utilized for their measurement needs to be accounted for.

Some of the specialities linked with the determination of stability constants from electrophoretic migration data measured by capillary zone electrophoresis have been discussed in published communications, e.g. Refs. [7,10]. However, no attempt to give a complete, summarizing analysis of the topic was made until now, to our knowledge. To fill this gap is the main aim of our article. To estimate the applicability of the commonly carried out method, and to outline a way to a robust variant of the method, capable of supplying reliable raw data without excessive demands on the number and on complexity of electrophoretic experiments are the additional aims.

2. Physicochemical backgrounds

Methods, developed for the studies of interactions of inorganic ions and complexes with metallic central ions, as well as physicochemical principles underlying these methods, hold for any type of complexes. Fast and reversible interactions only may be utilized in chiral separations [1–3].

The simplest one-to-one interaction scheme

$$A + C \leftrightarrow AC \tag{1}$$

is relevant for the vast majority of chiral separations. A is a chiral compound, usually an enantiomer, C is a chiral selector and AC is their complex [1,2,5–7]. Other interaction schemes are exceptional [13,18,19] and their descriptions have to start with the interaction scheme (1) [8]. Considering the law of the active mass action, we can define the dimensionless thermodynamic equilibrium constant, K_A^{th}, of the process (1) [8]

$$K_A^{th} = \frac{k}{k'} = \frac{a_{AC}}{a_A a_C} \tag{2}$$

The rate constants k and k' express the velocities of spontaneous formation and decomposition of AC according to Eq. (1), respectively; a_{AC}, a_A and a_C are the activities of AC, A and C, in the equilibrium, respectively. The interaction scheme (1) supposes that (i) each of the species A, C enters in the interaction in single chemical form and (ii) non of the species A, C and AC enters in any other interaction. It is evident from both the Eqs. (1) and (2) that stronger interaction between A and C increase the equilibrium active mass of their complex AC in solution; in other words, stronger interaction

increases the stability of the complex AC, and, consequently the magnitude of K_A^{th}.

The activity of any solution constituent correlates with its concentration in solution. For the species A, C and AC, participating in the equilibrium (1), it holds that [8]

$$a_A = \frac{[A]}{c_A^0} \cdot \gamma_A \quad (3a)$$

$$a_C = \frac{[C]}{c_C^0} \cdot \gamma_C \quad (3b)$$

$$a_{AC} = \frac{[AC]}{c_{AC}^0} \cdot \gamma_{AC} \quad (3c)$$

where [A], [C] and [AC] are the equilibrium concentrations in mol/l. The terms c_A^0, c_C^0 and c_{AC}^0 are arbitrary reference concentrations of the species A, C and AC at the standard state conditions. These concentrations are usually chosen equal to 1 mol/l [20]. The temperature of 25°C and the pressure of 0.1 MPa are the standard temperature and pressure. The dimensionless terms γ_A, γ_C and γ_{AC}, called earlier activity coefficients [8], are the activities of single ions of the species A, C and AC, respectively, at the standard state conditions [20]. However, the more common classical term, activity coefficient, will be used throughout this text for γ.

In analogy to the thermodynamic stability constant K_A^{th}, the stoichiometric stability constant [8], K_A, and the normalized stoichiometric constant, K_A^0, may be defined for the interaction (1) in the forms

$$K_A = \frac{[AC]}{[A][C]} \quad (4)$$

$$K_A^0 = \frac{[AC]}{[A][C]} \cdot \frac{c_A^0 c_C^0}{c_{AC}^0} = K_A \cdot \frac{c_A^0 c_C^0}{c_{AC}^0} \quad (5)$$

With respect to Eqs. (2)–(5), it holds

$$K_A^{th} = K_A^0 \cdot \frac{\gamma_{AC}}{\gamma_A \gamma_C} = K_A \cdot \frac{c_A^0 c_C^0}{c_{AC}^0} \cdot \frac{\gamma_{AC}}{\gamma_A \gamma_C}$$

$$= \frac{[AC]}{[A][C]} \cdot \frac{c_A^0 c_C^0}{c_{AC}^0} \cdot \frac{\gamma_{AC}}{\gamma_A \gamma_C} \quad (6)$$

The stoichiometric equilibrium constant is based on equilibrium concentrations of the species A, C and AC at the used experimental conditions only. Its magnitude depends on temperature, pressure and on ionic strength of the solution. This constant is the easiest to measure. Obviously, the calculated value of K_A holds for the given experimental conditions only. The dimension of the concentration scale used for [A], [C] and [AC] gives the dimension of the constant. The molar scale is recommendable for the sake of comparisons with other stability constants. Because of its dimension, neither the decadic nor the natural logarithm of K_A may be calculated [21]. For such calculations, the normalized stoichiometric stability constant K_A^0 that is dimensionless should be used. Because of the use of standard concentrations c_A^0, c_C^0 and c_{AC}^0 equal to 1 mol/l in the calculation of K_A^0, the equilibrium concentrations [A], [C] and [AC] have to have the same dimension. The normalization of equilibrium concentrations [A], [C] and [AC] to their respective standard concentrations eliminates the dependence of K_A^0 on pressure. Its dependencies on temperature and on the ionic strength of the liquid medium survive. The ionic strength dependence is eliminated in the K_A^{th} (Eqs. (2) or (6)). Unfortunately, this constant is usually difficult to determine. The absence of knowledge of activity coefficients of the species participating in the equilibrium (1) is the main difficulty in the calculation of thermodynamic stability constant [8].

There is, however, another big problem here. As mentioned above, Eq. (1) supposes implicitly that none of the species A, C, and AC enters into another equilibrium. This supposition is scarcely met in complex solutions used in electrophoretic chiral separations. Interactions of the chiral selector with the buffer constituents or with another additive of the BGE [22–27] are the most frequent side interactions that cannot be eliminated in electrophoretic chiral separations, as a rule. If some of the species A, C and AC participates in a side interaction, then Eqs. (2) and (6) do not give true thermodynamic constants. The so-called conditional thermodynamic constant is calculated [8]. Such a constant is valid for a given composition of the solution only. Evidently, the same applies for K_A and K_A^0.

The course of more complex interactions causing the chiral discrimination, e.g., the interaction of two chiral selectors with an enantiomeric compound having two chiral centers [13], may be described as

the sequence of elementary steps complying with Eq. (1). In this case, the interaction (1) is called step interaction, and the constant characterizing it is the step constant. The over-all stability constants, β, β^0 and β^{th}, characterizing the over-all complexing process, may be calculated either from the experimentally measurable raw data or from the respective step constants. For example, the overall stoichiometric constant β, which characterizes a complexing process that consists of n elementary equilibrium steps, quantified by the stoichiometric step constants $K_1, K_2, \ldots K_n$, is [8]

$$\beta = K_1 K_2 \cdot \cdot \cdot K_n = \prod_{}^{n} K_n \tag{7}$$

3. Complexing in chiral separations

The effect of fast equilibria among the solution constituents on the electrophoretic migration of an ionising species is described by the concept of effective mobility by Tiselius [28] that is commonly utilized in electrophoresis, see, e.g., Li [2] or Foret et al. [29]. The effective mobility of a species, u_{eff}, present in the solution in i coexisting forms which are bound one to another by fast equilibria, equals to the sum of the mobility contributions of each of the coexisting forms that are normalised to the total analytical concentration of the species, c

$$u_{eff} = \sum_i u_i \cdot \frac{c_i}{c} = \sum_i u_i x_i \tag{8}$$

u_i is the ionic mobility of the ith form of the species, present in the solution in the equilibrium concentration c_i. Evidently, $c_1 + c_2 + c_3 + \ldots = c$. For the interaction (1), in which [A] and [AC] are the equilibrium concentrations of the free and the complexed forms of the compound A, respectively, it holds

$$c = [A] + [AC] \tag{9}$$

If u_A and u_{AC} are ionic mobilities of the free and complexed compound, respectively, the effective mobility of A, $u_{eff,A}$ may be written in the form

$$u_{eff,A} = \frac{[A]}{c} \cdot u_A + \frac{[AC]}{c} \cdot u_{AC} \tag{10}$$

which, by using Eqs. (4) and (9), becomes

$$u_{eff,A} = \frac{1}{1 + K_A[C]} \cdot u_A + \frac{K_A[C]}{1 + K_A[C]} \cdot u_{AC} \tag{11}$$

For A consisting of enantiomers R and S, Eq. (11) gets the forms

$$u_{eff,R} = \frac{1}{1 + K_R[C]} \cdot u_A + \frac{K_R[C]}{1 + K_R[C]} \cdot u_{AC} \tag{12a}$$

$$u_{eff,S} = \frac{1}{1 + K_S[C]} \cdot u_A + \frac{K_S[C]}{1 + K_S[C]} \cdot u_{AC} \tag{12b}$$

for the following reasons. The mobilities of enantiomers R and S, u_R and u_S, respectively, are identical in the absence of C in the solution and are equal to u_A.

The difference in mobilities of complexes RC and SC, u_{RC} and u_{SC}, respectively, may result from the difference in their shapes only. This difference is maximum if the chiral centres of the selector and of the complexed enantiomer are bound to each other. Simultaneously, the chiral selector C has to have its molecular size comparable to that of the enantiomers R, S. Such a complexing is typical of indirect chiral separations where the kinetically stable complexes are employed [3]. The number of indirect electrophoretic chiral separations, realised in achiral background electrolytes, is very low since these separations suffer usually from too low separation selectivity. The minimum measurable mobility difference, which is usually sufficient for the baseline separation, ranges from 0.1 to $0.2 \cdot 10^{-9}$ $m^2\,V^{-1}\,s^{-1}$. It reveals that the shape difference alone can only seldom produce a mobility difference of this magnitude even at optimum conditions. Chiral selectors utilised in direct separations do not exist as enantiomers and are much greater in size than the separated enantiomers. It eliminates the chance that the shape difference of enantiomers complexed by a chiral selector for direct separations may result in a measurable mobility difference. Thus, $u_{RC} = u_{SC} = u_{AC}$ holds for direct chiral separations with an error lower than $0.1 \cdot 10^{-9}$ $m^2\,V^{-1}\,s^{-1}$.

It is evident from Eqs. (12a) and (12b) that different stability constants of the enantiomers R and S with a chiral selector, $K_R \neq K_S$, are the reason for, and the way to, the direct chiral separation of enantiomers. At the chiral selector concentrations

approaching zero, the effective mobilities of the enantiomers *R*, *S* approach the same value, $u_R = u_S = u_A$. At concentrations of the chiral selector going to infinity, the enantiomers are fully complexed and have the same mobility, $u_{AC} \neq u_A$, which is lower then u_A as a rule. Consequently, there must be a chiral selector concentration between these two extremes, C_{max}, called also optimum concentration, which causes (corresponds to) a maximum difference in effective mobilities of the enantiomers *R* and *S*, $u_{eff,R}$ and $u_{eff,S}$, respectively (Fig. 1). If $u_R = u_S = u_A$ and $u_{RC} = u_{SC} = u_{AC}$, C_{max} correlates simply with stoichiometric stability constants K_R and K_S [4–7]

$$C_{max} = (K_R K_S)^{-0.5} \quad (13)$$

4. Calculation procedures

The presented model of complexing of enantiomers with a chiral selector [5,6] offers Eq. (11) for the calculations of individual stoichiometric stability constants. Any of the published calculation procedures utilises either this equation or some of its algebraic equivalents. The point-by-point calculation, linearized plots of raw experimental data and graphical calculations based on them [9,12,30], and the non-linear computer fitting procedures have been applied for the calculations of *K*. With respect to the calculating power of present computers, the computer fitting of experimental mobility data may be recommended as the most effective way to stability constants. The conversion of *K* to either the normalized stoichiometric constant, K^0 (Eq. (5)) or to the thermodynamic stability constant, K^{th}, given by Eq. (6), are additional steps. Thus, they are discussed at the end of the chapter.

The most simple interaction scheme, see Eq. (1), and the model starting from it suppose that the species A, C, and AC interact exclusively with each other, and only in the manner expressed. Let us consider that this is the case; or, more exactly, that their behaviour in the investigated system approaches this assumption. In this case, any of the calculation procedures mentioned above gives the correct *K* value. If, in addition, the calculation method supplies another magnitudes, e.g., u_A, or u_{AC}, they are correct, too. For such a set of 'ideal-like' data (in other words, for data that are both accurate and precise), the non-linear computer fitting of Eq. (11), or of any of its analogs, is the best choice. Three effective mobilities of each enantiomer, measured at different concentrations of the chiral selector, are sufficient for the calculation. Mathematically, the system of three equations containing three unknowns *K*, u_A, and u_{AC} is solved and their (accurate and precise) values are displayed.

The experimental data always suffer from random errors. For such accurate but not precise data that are sometimes denoted as good data, usually from six to ten pairs of raw data $u_{eff} = f([C])$ are necessary to reach reasonable precision by computer fitting.

For biased raw data that suffer from both systematic and random errors (for non-precise data whose accuracy is neither known nor secured) the fit again follows mathematical criteria that improve the precision of the fit. The fit is optimised disregarding the agreement of any of the 'adjustable constants' with its accurate (real) value. In other words, the 'adjustable constants' *K*, u_A and u_{AC} are optimised in this case for the formal mathematical fit of the raw data with the selected equation, not for the agreement of *K*, u_A and u_{AC} with their magnitudes in the investigated system. It is evident from Eq. (11) or from its any equivalent form, e.g., Eq. (16) given below, that the manipulation of u_A or u_{AC} affects the calculated *K* value. In this way, the adjustment of u_A or u_{AC} may improve of the precision of calculated *K* at the cost of possible deviations of *K*, u_A, and u_{AC} from their correct magnitudes. The credibility of *K* increases if correct u_A and u_{AC} are used for the calculation. Thus, the accuracy of two or more 'adjustable constants', obtained from one calculation run, has to be either secured or checked by other than mathematical means. There are two possibilities for it, in principle: (i) cross-check on the calculated constant or (ii) cross-checks on u_A or u_{AC} obtained simultaneously.

The consideration analogical to the presented one is possible for any of the linearization or graphical calculation procedures including those summarised in reviews [9,30].

Eq. (13) defines, in fact, the geometrical mean of the stoichiometric stability constants, K_{mean} [7,31]

$$K_{mean} = (K_R K_S)^{0.5} = C_{max}^{-1} \quad (14)$$

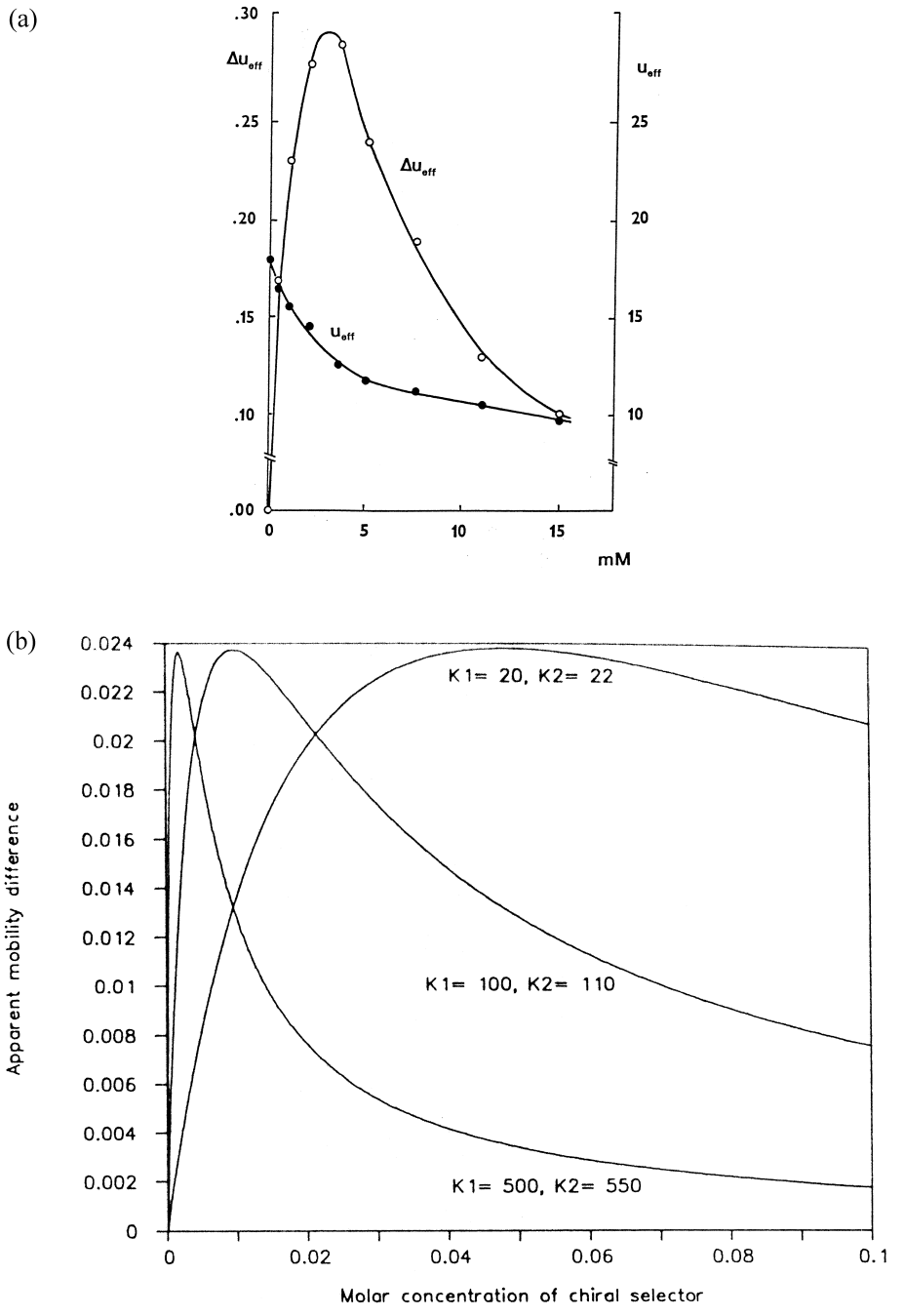

Fig. 1. Illustrative comparisons of (a) changes in the effective mobility of the *R* enantiomer, ($u_{eff} = u_{eff,R}$), and the difference in effective mobilities of enantiomers *R* and *S*, $\Delta u_{eff} = u_{eff,R} - u_{eff,S}$, as the function of the chiral selector concentration for the stoichiometric stability constants $K_R = 336$ l/mol and $K_S = 374$ l/mol, and (b) shapes of the dependencies $\Delta u_{eff} = f([C])$ for different magnitudes of stability constants of the enantiomers 1 and 2, K_1 and K_2, respectively, given as parameter. (a) Represents the chiral separation of *N*-*t*-BOC-D,L-tryptophan with β-cyclodextrin, described in detail in Ref. [76]. Mobilities are given in 10^{-9} m^2 V^{-1} s^{-1} units. (b) Taken from Ref. [5], was computer generated; here, mobilities are given in 10^{-4} cm^2 V^{-1} s^{-1} units.

The magnitude of K_{mean} depends on the location of the maximum of the function $\Delta u_{eff} = u_{eff,R} - u_{eff,S} = f([C])$ in the scale of [C] only. It is independent of u_{AC}, of the absolute magnitudes of $u_{eff,R}$, $u_{eff,S}$ and of their difference, as well as of possible distorting effects caused by varying experimental conditions, e.g., by the concentration of chiral selector or by the electroosmotic flow. The agreement of K_{mean}, calculated from K_R and K_S that were obtained by computer fitting or by other means, with K_{mean} determined from the dependence $\Delta u_{eff} = u_{eff,R} - u_{eff,S} = f([C])$, is therefore the check for both the correctness of the experimental raw data and stability constants derived from them by the computer fitting with Eq. (11).

The mobility of the non-complexed and fully ionised enantiomer, u_A, is accessible experimentally, in principle. Unfortunately, this is not true for u_{AC}. The reason is that, strictly speaking, u_{AC} is the mobility of the compound in the solution containing the infinitely high concentration of the chiral selector. For comparison, the concentrations of solid cyclodextrins are approximately 1 mol/l [31]. Thus, any calculation procedure requires a method for the determination or, at least, for the estimation of u_{AC}. This method also affects the number of necessary experiments. The simultaneous determination of u_{AC} with K from a linearized plot in graphical calculation methods is one such method.

The estimation of K_A from single experiment at $[C] \neq 0$ is possible only if u_A is known and u_{AC} may be approximated by the mobility of the chiral selector. For a compound of molecular mass of ≈ 300, bearing one elemental charge, and for a relative error in the estimated u_{AC} not exceeding 5%, a chiral selector having the molecular mass of $5 \cdot 10^4$ at least is necessary [31]. Evidently, the large biopolymers only can meet this moderate requirement. Any other method for the estimation of u_{AC} requires a set of the effective mobility data.

The approximation of u_{AC} by the limit approached by effective mobilities at increasing [C] is standard procedure in biochemical studies on 'binding' constants, e.g. [11]. Simple calculation, based on Eq. (4), shows that the stoichiometric stability constant of $1 \cdot 10^3$ l/mol requires a 20 mmol/l chiral selector concentration if 5% of non-associated A is tolerated. This 5% allowance gives the 5% error in the estimated u_{AC} again. For the 1% error in u_{AC}, the 100 mmol/l concentration of the selector is necessary if $K = 1 \cdot 10^3$ l/mol.

If $\Delta u_{eff} = u_{eff,R} - u_{eff,S} \neq 0$ at C_{max}, u_{AB} may be calculated from these effective mobilities and from the mobility of the enantiomeric analyte, A, in the absence of the selector in the BGE, u_A. It holds [31]

$$u_{AC} = (u_{eff,R,max} + u_{eff,S,max}) - u_A \quad (15)$$

The calculated u_{AC} allows, except others, the point-by-point calculation of the K_R and K_S from Eq. (11), rearranged for convenience to the form

$$K_A = \frac{1}{[C]} \cdot \frac{u_A - u_{eff,A}}{u_{eff,A} - u_{AC}} \quad (16)$$

The $\Delta K = K_R - K_S$ may be obtained directly from the difference of effective mobilities of *R* and *S*, $u_{eff,R}$, $u_{eff,S}$ [31]

$$\Delta K = \frac{1}{[C]} \cdot \frac{(u_A - u_{AC})(u_{eff.R} - u_{eff,S})}{(u_{eff,R} - u_{AC})(u_{eff,S} - u_{AC})} \quad (17)$$

There are two checks for the accuracy of the magnitude of u_{AC} calculated from Eq. (15). The agreement of the geometrical mean of K_R and K_S calculated from, e.g., Eqs. (11) or (16), using u_{AC} from Eq. (15), with that derived from the location of C_{max} is the first check. The independence of both K_R and K_S on the chiral selector concentration in the point-by-point calculation is the second check. If the u_{AC}, used in the calculation of K, suffers from an error, the apparent dependence of the calculated data on the chiral selector concentration is observed. This apparent dependence is diagnostic in respect to both sign and magnitude of the error. For a positive error in u_{AC}, the calculated stability constants are greater than the correct value and exhibit an apparent increase with the equilibrium concentration of the chiral selector (Fig. 2). The greater is the positive error, the greater is the difference between the correct and the calculated values of the stability constant at a particular equilibrium concentration of the chiral selector; simultaneously, the apparent increase in the calculated constants with the equilibrium concentration becomes more pronounced. For negative errors in u_{AC}, the consequences are the opposite. The calculated values are lower than the

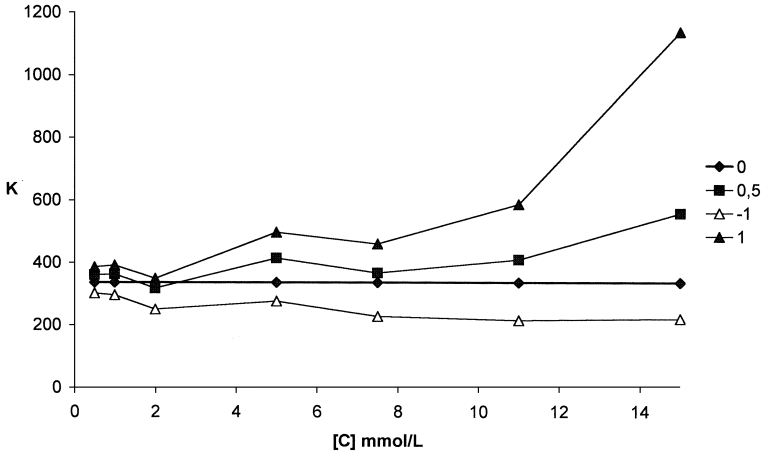

Fig. 2. Effect of the error in magnitude of u_{AB}, given in 10^{-9} $m^2 V^{-1} s^{-1}$units, used for the calculation of the stoichiometric stability constant, *K*, on the magnitude of *K* obtained from Eq. (20) at various concentrations of the chiral selector in solution. Effective mobilities, u_{eff}, from Fig. 1a, and correct $u_{AB} = 8.4 \cdot 10^{-9}$ $m^2 V^{-1} s^{-1}$, taken from [31], were used in the calculation.

correct ones and exhibit apparent decrease with the increasing equilibrium concentration of the selector in the system. [32].

The apparent mobilities of the enantiomers *R* and *S*, u_R, u_S, respectively, correlate uniquely with their migration times at the used voltage, *V* given in volts, t_R and t_S, respectively [29]

$$u_{app} = \frac{1}{t} \cdot \frac{Ll}{V} \tag{18}$$

L and *l* are the total and detection lengths of the capillary, respectively. If the ramp time of the high voltage unit at the used voltage, *V*, is t_r, the experimentally measured migration time, t_e, must be corrected. It holds

$$t = t_e - \frac{t_r}{2} \tag{19}$$

In the absence of the electroosmotic flow (and of any other macroscopic flow of the liquid inside the capillary, as common in electrophoresis), $u_{app} = u_{eff}$. If the electroosmotic flow is present

$$u_{eff} = \left(\frac{1}{t} - \frac{1}{t_0}\right) \cdot \frac{Ll}{V} \tag{20}$$

where t_0 is the migration time of the electroosmosis marker, corrected for the ramp time using Eq. (19). Thus, it holds for the $\Delta u_{eff} = u_{eff,R} - u_{eff,S}$

$$\Delta u_{eff} = \left(\frac{1}{t_R} - \frac{1}{t_S}\right) \cdot \frac{Ll}{V} \tag{21}$$

eliminating the magnitude of the electroosmosis in the particular experiment. The total (analytical) concentration of the selector in the background electrolyte, *c*, correlates with [C] uniquely, too. Thus, the $K_{mean} = C_{max}^{-1}$ may be obtained directly from the dependence $(1/t_R - 1/t_S) = f(c)$ [32].

The recalculation of *K* to K^0 consists in the correction of *K* for the deviations of equilibrium concentrations of A, C and AC, [A], [C] and [AC], respectively, from their values at the standard state conditions. In electrophoresis utilising aqueous solutions, the concentration shifts caused by the thermal expansion of water cause these differences only. The cubic expansion coefficient of water, $\alpha = (1/V)(\partial V/\partial T)_p$, extrapolated from data for aqueous solutions of sodium chloride [33], is approximately $2.5 \pm 0.5 \cdot 10^{-4}$ at 25±5°C. For temperature deviations from the standard temperature of 25°C, amounting up to 20°C, the relative concentration changes not exceeding 1% may be expected. The same relative change in the magnitude may be expected at the recalculation of *K* to K^0 with respect to Eq. (5). The elimination of the constant dimension is therefore the sole practical consequence of the recalculation of *K* to K^0 with respect to the common precision of the determination of stability constants.

The transformation of K^0 to K^{th} is the last step to the thermodynamic stability constant which is independent of the experimental conditions except of temperature. Activity coefficients of A, C and AC in the selected BGE at the used temperature and pressure are necessary for it usually. Unfortunately, there is no generally applicable way to activity coefficients of the complex constituents and of the complex. There are many calculation methods, recommended either for the complexes of particular composition and concentrations or for particular experimental techniques used in the measurement of raw experimental data or for particular experimental conditions of these measurements [8]. Basically, there are two methods applicable often to electrophoretic chiral separations. The first one relates to the case where only one of the complex constituents is charged and the complex bears the same charge. In this case, the K^{th} may be obtained by extrapolating the stoichiometric constants, determined at a series of ionic strengths, to infinite dilution. The plots of log K against ionic strength, I [34], or against $I^{1/2}$ [35–38], are linear.

The ionic strength of background electrolytes that are used in electrophoresis exhibit often the ionic strength $I \leq 1 \cdot 10^{-2}$ mol/l. These values exceed only moderately the limit 10^{-3} mol/l for the applicability of the simple limiting Debye–Hückel relationship [39]

$$\log \gamma_{\pm} = -\frac{az_1z_2\sqrt{I}}{1 + bw\sqrt{I}} \quad (22)$$

allowing the calculation of the mean ionic activity coefficient, $\gamma_{\pm}$ for the 1:1 complexes [40,41]. The coefficients a and b in Eq. (22) are dependent on the temperature an on the dielectric constant of the liquid medium, and may be found in tables [42]; z_1 and z_2 are the charge numbers of the ions forming the electrolyte, w is the ion size parameter. The ionic strength, I, is defined as

$$I = \frac{1}{2}\sum_i c_i z_i^2 \quad (23)$$

where c_i and z_i are the concentration and the charge number of the ith ion in the solution, respectively. It is commonly assumed that the individual activity coefficient, γ, equals to the mean activity coefficient, $\gamma_{\pm}$, in diluted solutions [8]. Thus, for diluted aqueous background electrolytes at 25°C, whose ionic strength is of the order of millimoles, the limiting Debye– Hückel relationship for the calculation of the activity coefficient if an ion gets the form [43,44]

$$\log \gamma = -\frac{0.585z^2\sqrt{I}}{1 + 3.28w\sqrt{I}} \quad (24)$$

where z is the ion valency. The magnitude of the effective hydrated diameter of the ion, w, given in nanometers, is known seldom. The approximate value of 0.5 nm is recommended as generally applicable for small ions and is used in the calculations of dissociation constants of weak electrolytes from effective mobilities measured by capillary zone electrophoresis [43,44]. This value may be considered acceptable for enantiomeric analytes. However, for chiral selectors whose molecular mass approaches 1000 or is even higher, w needs to be increased. For the calculations of K^{th} from the data measured in the background electrolyte of the ionic strength exceeding markedly the limit of 10^{-3} mol/l, some of the extended forms of the Debye–Hückel relationship is recommendable [8].

5. Experimental data

There is a principal requirement on experimental raw data serving for the calculations of stability constants. These data, and their changes with the concentration of a chiral selector, must result from the mutual interactions of the complex constituents only; if not, the deviations must be corrected for either preliminary or in the calculation procedure. Data meeting this principal requirement must be measured at the constant ionic strength, at known, constant temperature and in the widest possible range of the chiral selector concentrations [8]. Only if both these conditions are met, accurate stability constants may be obtained disregarding both the experimental technique, which supplies raw data, and the selected calculation procedure. Meeting of the principal requirement in electrophoresis needs (i) the absence of side-effects that may follow from the varying concentration of the chiral selector, namely, changes in viscosity, ionic strength, electroosmosis and tempera-

ture, which is affected by the Joule heat production; (ii) the absence of competing processes and equilibria of other solution constituents with the selector, enantiomers and their complexes; (iii) the absence of interactions of the chiral selector, enantiomers and their complexes with the capillary wall, and (iv) negligible changes in the concentration of the selector, which is dissolved in the BGE filling the capillary, by the migrating zones of enantiomers [10].

The viscosity changes that affect mobilities are common with any chiral selector and cannot be eliminated experimentally. The calculated stoichiometric stability constant is overestimated if the viscosity effect is not corrected for properly. The macroscopic viscosity, measured by the classic viscosimetry, is relevant for the correction if the low-molecular-mass chiral selectors like cyclodextrins [5,7,14,45] are used. If a high-molecular-mass constituent like protein, polymerised cyclodextrin or polyvinylpyrrolidone [46] is present, the macroscopic viscosity of the solution is not decisive for the electrophoretic transport of low-molecular-mass ions. The analysis with the chiral selector incorporated in the three-dimensional gel, polymerised inside the capillary and having infinite macroscopic viscosity [47], may serve as an example here. The fact that the viscosity of the solvent in the closest surrounding of the dissolved ion, called microscopic viscosity, is decisive for its electrophoretic transport is the explanation. For high-molecular-mass constituents, the microscopic and the macroscopic viscosities are different; for cyclodextrins and compounds of comparable molecular mass are identical.

The correction for the increase in the microscopic viscosity, based on the current that passes through the capillary at the constant driving voltage, is a reasonable method [7,10]. Unfortunately, it may be applied only if the selector is uncharged. The correction utilising simultaneous injection of a mobility standard, which interacts neither with the chiral selector nor with the capillary wall is the only possibility here. The correction based on two mobility standards [48] given below decreases the number of necessary experiments and makes the method more robust [49]. However, the necessity to find two mobility standards not interacting with the chiral selector is the principal difficulty with the application of the method. A chemometric method for the correction of the viscosity effects, which are caused by the varying concentrations of a chiral selector, during the computation of stability constant was proposed recently [12]. The selected mathematical conception of the method, and the used algorithm guarantee the elimination of additional, simultaneously occurring non-linearities, supposing they are not too large. Large non-linearities, unexpected interactions, deviations from starting experimental conditions and the deviations from the one-to-one interaction model disable the linearization according to the hitherto checking of the method [50].

The possible additional interactions of the chiral selector or the enantiomer with other solution constituents are given by both their nature and the solvent used. The number of these interactions is large, in general and the general discussion of the ways to their elimination is therefore impossible. The capability of cyclodextrins to host in their cavities the majority of organic compounds [51], the affinity of albumins to both organic compounds and very simple inorganic ions like chloride and sodium [52] and the dependence of enantioselective capability of macrocyclic antibiotics [53] on the composition and concentration of the background electrolyte are the examples of side-effects taking place in systems for chiral separations. Thus, conditional constants are measured, as a rule. In this case, the calculated constants hold exactly for the solution of the used composition only [8]. In the solutions of another composition they may serve as the estimates of the stability constant only. The role of the solvent cannot be passed over.

The side interactions that may be predicted and controlled effectively are the protolytic equilibria of enantiomers and chiral selectors. The acidobasic properties of compounds are either tabulated, e.g., [54–60], or may be measured easily by capillary zone electrophoresis [43,44,49,61–63]. If the analyte is a weak electrolyte, the interactions of the selector with its acidic and basic forms are different, in principle. The so-called duoselective separations with cyclodextrins [64,65] are the relevant example. In this case, particular stability constants describe the interactions of each of the forms of an enantiomer with the selector. The same holds for the interaction of a selector being weak electrolyte with an enantio-

mer, which is present in the system in single chemical form. Consequently eight equilibria, described by up to eight different stability constants, are relevant for the interaction of a partially ionised chiral analyte with a partially ionised chiral selector. This fact stresses the strong need for a good buffering of chiral separation systems and for the careful selection of experimental conditions. The pH dependence of calculated constants evidences the participation of protons in their side-interactions with the complex constituents.

In the considerations of the electrophoretic transport in bulk solutions and in tubes of I.D.$>$1 mm, the influence of the vessel walls is neglected as a rule. However, this is not possible in CZE where long capillaries of small I.D., usually $<$0.1 mm, are used. The effects of the adsorption of a species on the capillary wall are triple. Any adsorption affects usually decreases electroosmosis (see below). Adsorption of the selector, both reversible and irreversible, increases the mean concentration of the selector inside the capillary above its starting concentration in BGE and, in this way, affects effective mobilities of chiral analytes. Anomalous peak width and, mainly, the tailing of the peak are usually the consequences of, and the evidences for, the adsorption of any of species A, C and AC on the capillary wall supposing the time constant of the detection system, t, is reasonably low. Analysis of sorption effects and of their electrophoretic appearances, which accounts for the roles of particular species in the chiral discrimination process, to their charges, mobilities and effects on electroosmosis is out of the scope of this article. However, careful checks on possible adsorption effects, highly probable with selectors of biological or biochemical origin, like proteins or macrocyclic antibiotics, and possible with chiral analytes containing large hydrophobic moieties, are highly recommendable, in our experience.

The time constant is the time in which the response of a measuring transducer (detector electronics) to a stepwise input change c_0 reaches 0.632 c_0 [66,67]. The detector response time, called also detector rise time, characterises the speed of the response of the whole detector to a stepwise concentration change of an analyte in the column effluent [67]. Being given by the detector time constant and slightly affected by the filling of the detector cell with the analyte, the detector rise (response) time approximately equals its time constant. A time constant of 0.04 s [2] and a detector rise time of 0.05 s [68] are recommended for common electrophoretic analysis. The correlation

$$\tau = \sqrt{\frac{0.2Dt^3}{l}} \tag{25}$$

gives detector rise time that decreases the separation efficiency, N, by 10% at the most [68] and has negligible influence on the migration time of an analyte peak and on its shape. Diffusion coefficients of the detected species, D, may be found in Refs. [69–72] or calculated [73,74]. Large time constant, e.g., 1 s, shifts the migration time, t, to higher values and makes the peaks lower, broader and tailing [67]. Noisier baseline is the charge for the short detector rise times.

The effect of a species adsorbed on the capillary wall on electroosmosis results from its influence on the electric double layer existing in the interface between the capillary wall and the background electrolyte. More specifically, these effects are given by the changes of the zeta potential [2,29,75]. Zeta potential is caused by the charge of the solid surface (of the capillary wall) at such a distance from the surface in which the liquid, adhering firmly to the surface, starts to move by the action of the applied driving voltage. The magnitude of zeta potential, ζ, together with the permittivity of the solvent, ϵ, and with its viscosity, η, determine the magnitude of the electroosmotic coefficient, u_{eo}, and, therefore, the velocity of the electroosmotic flow, v_{eo} [2,29,75]

$$v_{eo} = u_{eo} \cdot \frac{V}{L} = -\frac{\epsilon\zeta}{\eta} \cdot \frac{V}{L} \tag{26}$$

Evidently, the adsorption of any charged species, including charged chiral selector, affects the electroosmotic flow much more than the adsorption of uncharged solution constituents. The electroosmotic flow superimposes with the electrophoretic transport of the chiral analytes and must be therefore measured during the whole set experiments until its absence is proved or secured.

The coating of the inner wall of the capillary with a polymer guarantees neither the absence of electroosmosis nor the absence of adsorption of a

selector on the wall. For example, native cyclodextrins are retained so strongly on the polyacrylamide coating that they cannot be washed off completely by water or by aqueous electrolytes [32]. Thus, the capillary that had been exposed to a native cyclodextrin cannot be used for the measurement of u_A. The adsorbed selector increases the mean effective concentration of the selector in the capillary above its analytical concentration in the BGE. Consequently, the effective mobilities of strongly interacting analytes, measured with submillimolar or even millimolar concentrations of cyclodextrins, may be distorted markedly. This distortion may become particularly serious if the effective mobilities of analytes have been measured in the narrow concentration range of the selector and the inverse linearization that overestimates data measured at low chiral selector concentrations [30] had been selected for the calculation of *K*.

For the calculation of mobility data of enantiomers from their migration times, the injection of two mobility standards, readily detectable and not interacting with the selector, is recommendable [48]. From mobilities of such standards, determined at the used temperature in the selected BGE at the absence of the selector, and from migration times of standards and enantiomers measured at the presence of the selector, effective mobilities of enantiomers may be calculated with high precision. No other input data, like voltage and total and separation lengths of the capillary are necessary. The mobilities calculated in this way are corrected for many side effects including the viscosity increase caused by the increasing concentration of the selector. If one of the mobility standards is uncharged, it serves for the monitoring of electroosmosis simultaneously.

The correct determination of the mobility from the migration time by means of standard equations [2,29] and equations based on them, e.g., [48], requires a constant migration speed of the analyte throughout the capillary. In the case of the analyte present in two different chemical forms, the ratio of the coexisting forms must be constant throughout the run. In chiral separation it requires constant concentration of the chiral selector, undisturbed by the migrating zone [10] at any point of the capillary. In other words, the migration of the analyst must not change the chiral selector concentration inside the capillary. It is possible only if the concentrations of the injected enantiomers are much lower than the concentration of the selector in the BGE [10]. The equilibrium concentration of the selector, [C], approximates its total (analytical) concentration in such experiments.

At the measurement of effective mobilities of enantiomers, the concentration of the chiral selector in the BGE has to range around its optimum concentration (Fig. 1), which is inversely proportional to the geometrical mean of the stability constants of enantiomers with the selector [13]. For the purpose of our discussion, this geometrical mean may be replaced (approximated) by any of the stability constant of the respective enantiomers. For the stoichiometric stability constants up to 1000 l/mol, $C_{opt} = 1$ mmol/l. Concentrations of the injected enantiomers are usually close to 0.05 mmol/l from detection reasons. At such both concentrations of enantiomers and values of stability constants, the concentration of the selector in migrating zones of analytes differs negligibly from its starting analytical concentration. However, for high stability constant, e.g., $1 \cdot 10^5$ l/mol, the optimum selector concentration, 0.01 mmol/l, may be several times lower than the concentrations of the injected enantiomers. With respect to it, and to the high stability constant, the equilibrium concentration of the selector in the migrating zones of enantiomers has to differ markedly from its starting concentration in the BGE. Evidently, the starting concentration of the selector in the BGE stops to be undisturbed by the migrating zone of the second complex constituent, and the precondition necessary for the applicability of the method [10] is not fulfilled. The consequences of this have not been investigated yet. However, it is evident that the commonly utilised method of the determination of stability constants is not applicable for the stability constants of the order of 10^5 l/mol or even higher. Distorted data have to be expected if the constants are of the order of 10^4 l/mol.

If electroosmosis participates in the transport of enantiomers through the capillary, its contribution to the apparent migration speed (apparent mobility) of the injected complex constituent, quantified by the electroosmotic mobility, u_{eo}, has to be constant during the run. In uncoated fused-silica capillaries that are usually preferred in the measurement of migration data, the electroosmotic mobility varies monotonously after stepwise changes of the BGE

pH. The most pronounced changes are observed after flushing the capillary with sodium hydroxide between the runs, followed by water and then by the introduction of a buffering BGE. Depending on the BGE concentration, composition and pH, the stabilisation of the electroosmosis may last up to several hours in our experience. Thus, the electroosmotically-stabilised systems in which the run-to-run systematic shifts in electroosmosis are absent must be used.

In accordance with general requirements on experimental raw data [8], the effective mobilities must be measured at the constant ionic strength of the BGE and at constant temperature. From the viewpoint of the stability constant determination, the standard temperature of 25°C inside the capillary is recommendable.

The effect of the ionic strength is doubled in electrophoresis. First, it affects ionic mobilities of all ions in the solution [2,29]. Second, it affects the discrimination process itself by influencing the activities of the interacting species (Eqs. (2) and (6)). Keeping the ionic strength constant is very important. BGEs of sufficient buffering capacity secure a constant ionic strength if uncharged chiral selectors are used. With a charged selector, the changes in ionic strength caused by variations in its concentration have to be counterbalanced by varying concentrations of an indifferent electrolyte. The contribution of the selector to the ionic strength is easy to determine for charged cyclodextrins and alike simple selectors. However, for proteins and other polyelectrolytes such a determination has not been solved satisfactorily yet, to our knowledge.

The requirements concerning the temperature are more difficult to meet. Electrophoretic process always produces Joule heat, which must be transported out of the capillary. Both the instrument and the capillary give the conditions of the heat transport. The constant production of the Joule heat inside the capillary guarantees the constant mean temperature inside it supposing the initial equilibration period is negligible with respect to the duration of the experiment. Experiments with constant electric power in the capillary are therefore the best way to keep a constant temperature inside the capillary during the whole set of experiments. The adjusted voltage has to be measured for the mobilities calculation. If two mobility standards are used for the calculation of effective mobilities of enantiomers [48], the measurement of the adjusted voltage is not necessary. With instruments lacking the constant power facility, the manual adjustment of the voltage is necessary in order to approximate the constant Joule heat; the current passing through the capillary has to be measured in addition in this case. The initial equilibration period may be minimised by zeroing of the ramp time of the high voltage unit. The magnitude of the electric power has to be specified in the description of experiments.

The determination of the temperature at which the chiral discrimination process takes part is still more complicated task. The temperature inside the capillary is never the same as the temperature of the thermostating medium because of the finite temperature gradient necessary for the transport of the Joule heat out of the capillary. The gradient has to be minimised as far as possible and its magnitude must be determined in some way, e.g., [2,29]. For instruments equipped with an active motion of the thermostating fluid around the capillary, the Joule heat production up to approximately 0.15 W per meter of capillary causes the temperature rise roughly up to 0.5°C, in our experience. To minimise the temperature increase, low concentrated buffers consisting of low mobility ions are recommendable; if an inert electrolyte is added in the system in order to compensate for the effects of varying concentrations of a charged selector on the ionic strength, the same requirements hold for it. The selected buffer concentration must be a compromise between reasonable buffering capacity and low electric conductivity. If the buffer concentration cannot be decreased sufficiently, the lowering of the driving voltage is often a reasonable solution. The facts that the Joule heat is proportional to the square of the driving voltage and that the migration time is inversely proportional to it substantiate this solution. However, such a decrease in the Joule heat production is paid for by an increase in migration times.

Acknowledgements

The authors acknowledge the support of the study by the Grant Agency of the Czech Academy of Sciences, grant No. 4031703, by the Grant Agency of the Czech republic, grant No. 203/99/0044 and

by the Ministry of Education of the Czech republic, grant No. VS 96021.

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