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Migration time and peak area artifacts caused by systemic effects in voltage controlled capillary electrophoresis

Gunnar Johansson[a,*], Roland Isaksson[b], Valérie Harang[c,d]

[a]Department of Biochemistry, Biomedical Centre, Uppsala University, Box 576, SE-751 23 Uppsala, Sweden
[b]Department of Chemistry and Biomedical Sciences, University of Kalmar, SE-391 82 Kalmar, Sweden
[c]Product Analysis, Pharmaceutical and Analytical R&D, AstraZeneca, SE-151 85 Södertälje, Sweden
[d]Department of Pharmaceutical Chemistry, Biomedical Centre, University of Uppsala, P.O. Box 574, SE-751 23 Uppsala, Sweden

Abstract

The effect of an artificial conductivity step on the migration time for *rac*-propranolol was investigated with the purpose to give a strict physical background to the intensively studied migration time and integration variations in voltage stabilized capillary electrophoresis. The experiments verified a theoretical derivation of migration time disturbances caused by the sometimes unavoidable conductivity variations in the system. The verified equation describing migration time variations was used for simulations of a number of relevant cases, such as variation in injected sample conductivity, post-injection diffusional broadening of the sample zone, and the physical effect of a selector zone in the partial filling mode. A similar theoretical and experimental treatment of the integration errors caused by non-uniform velocity was also included with special reference to enantiomer separation in the partial filling mode. The same experiments were also used to show that the standard migration time related method for integration correction fails if the velocity of a component is not constant throughout the experiment, such as the situation when a selector is employed in partial-filling mode.
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1. Introduction

1.1. Background

Capillary electrophoresis was introduced in the early 1980s as a powerful analytical technique [1] and has since developed almost explosively. It allows an efficient way to document the purity/complexity of a sample and can handle virtually every kind of charged sample components ranging from simple inorganic ions to DNA. Individual components are often identified in, e.g., a UV detector trace from their characteristic migration times. This approach is facilitated today by the sophisticated instrumentation offering temperature-, voltage- and/or current stabilisation together with sensitive and accurate on-capillary detection.

The more or less explosive development of capillary electrophoresis since its introduction has to a great extent paralleled that of liquid chromatography. As a result, the emphasis has been put on performance data resembling those addressed in liquid chromatography, i.e., resolution, speed and sensitivity, whereas physicochemical data such as absolute mobility have not been in focus, partially due to the

*Corresponding author. Tel.: +46-18-471-4477; fax: +46-18-55-2139.
E-mail address: gunnar.johansson@biokem.uu.se (G. Johansson).

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often strong electroosmotic flow, which instead ingeniously has been employed as a unique mean to observe, in one experiment, both cationic, anionic and nonionic species in a sample [1].

Shibabi and Hinsdale [2] pointed out that the fast development in capillary electrophoresis has so far been focused on the improvement of resolution and throughput rather than reproducibility and absolute precision. One successful approach to improve also the reproducibility of both mobility and integral data has been based on internal standards [3]. The introduction of efficient capillary coating procedures [4], serving to virtually eliminate electroosmotic flow has, however, increased the interest in absolute mobility data. Specific factors influencing the reproducibility of mobility and quantitative analysis have been carefully studied. Wall adsorption effects and electroosmosis variations have been identified as important parameters, and the inconsistency in observed mobility as derived from migration time data was actually also shown to correlate with inconsistency in current [5]. Wätzig and Dette [6] developed procedures to improve reproducibility, including careful rinsing with running buffer before every run, sample introduced in running buffer, and buffer evaporation control. Field strength inconsistencies due to sample matrix or selector zone effects have received comparably little attention, but Moring et al. also reported a lack of time linearity in electrokinetic injection and actually interpreted this as due to electric field strength disturbances from the low conductivity sample zone, similar to the findings discussed herein [7,8]. In the same context it is shown that the electrokinetic injection reproducibility is poor when the sample is dissolved in a very low conductivity solution, ‘‘water’’.

The aim of this study is to demonstrate how system properties such as conductivity variations can influence the migration time of analytes also under well controlled conditions including voltage stabilization. The integration problems coupled to the general case of non-constant analyte velocity are also addressed.

1.2. Migration time artifacts due to conductivity variations

In addition to straightforward mobility based separations, methods based on the addition of more or less specific modifying components have been developed. Variations in migration time for a certain component has here been taken as evidence for interactions. In many cases, it is an advantage to add a modifier in only a part of the capillary to avoid interference with the detection [9–11]. One can suspect, however, that an added modifier may give rise to system effects that cause variations in the migration time also in the absence of interactions. Such disturbances can easily be overlooked when precautions such as high precision voltage stabilisation are taken. We will here demonstrate that pure system effects can give rise to considerable variations in retention time. The earlier free electrophoresis techniques such as the moving boundary technique [12,13] and rotating tube free zone electrophoresis [14,15] allowed repeated monitoring of the whole system, thus providing correct information about non-uniform migration velocity at deliberate time intervals.

1.3. Shortcomings of the integration correction

Quantitative analysis of peaks in the historical techniques has been straightforward, since the whole separation compartment is viewed in real time by the schlieren optics [13] or the UV scanning system [15], respectively.

The relative integration of components in chromatographic peaks using post-column detection does generally not represent any problem either, since all components migrate with the speed of the liquid flow once they have left the separation compartment.

Capillary electrophoresis with on-capillary detection represents a fundamentally different case since the residence time of different components in the detector area depends on their migration velocity [16]. As a consequence, a correction factor must be applied to cancel out these effects. The only approach readily available here is, however, to use a compensation factor based on the observed migration time, which in the basic case of zone electrophoresis with uniform migration velocity throughout the process is inversely proportional to the effective velocity and, also, mobility of a component. The corrected peak area can then be calculated from raw data by means of the migration time, since the

velocity in the detector region in this case is equal to the average velocity [17]:

$$A_{\mathrm{corr}} = \frac{A_{\mathrm{obs}}}{t_{\mathrm{mig}}} \quad (1)$$

Loebe and Roeckel [18] developed an empirical correlation procedure to minimize integration errors, since they found that also the time-corrected peak area procedure failed to work properly. They also reported a better reproducibility in automated injection compared to manual procedures.

The use of specific selectors introduced in partial-filling mode introduces complications also in properly carried out experiment with carefully balanced conductivities, since the partial-filling technique implies that the selector is not reaching the detector area, and, conclusively, the velocity in the detector region is different from the average velocity. In this work we demonstrate theoretically and experimentally why the correction algorithm above is insufficient for such applications.

2. Materials and methods

2.1. Materials

Rac-propranolol and bis-tris [bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane] were purchased from Sigma (St. Louis, MO, USA). Acetic acid and phosphoric acid were from Prolabo (Briare le Canal, France). All other chemicals were purchased from Merck (Darmstadt, Germany). The water used was of Millipore quality (Watford, UK). All chemicals were of analytical grade.

2.1.1. Buffer preparation

A buffer stock solution (0.05 *M* bis-tris–acetate, pH 6.5) was made by diluting an appropriate mass of acetic acid in approximately 80 ml of water. The pH of the solutions was adjusted to 6.5 using 0.50 *M* bis-tris and then made up to volume (100.0 ml) with water. The background electrolyte (BGE) (0.015 or 0.030 *M* bis-tris–acetate, pH 6.5) was made by diluting the buffer stock solution. All solutions were filtered through a Gelman GHP Bulk Acrodisk 13 syringe filter 0.45 μm (Ann Arbor, MI, USA).

2.1.2. Sample preparation

A sample stock solution containing 150 μ*M* of *rac*-propranolol was dissolved in water. The sample (15 μ*M* *rac*-propranolol) was prepared by diluting the sample stock solution with water and buffer. The buffer concentration in the sample was always 10 times lower compared to the BGE.

2.2. Theoretical

2.2.1. Mobility/migration time errors arising in voltage stabilised mode

First, regard electrophoresis carried out in a capillary with total length d_{tot} at constant voltage U. The average field strength E_{a} is obtained as:

$$E_{\mathrm{a}} = \frac{U}{d_{\mathrm{tot}}} \quad (2)$$

The total migration time t_0 with an apparent mobility μ:

$$t_0 = \frac{d_{\mathrm{tot}}}{v} = \frac{d_{\mathrm{tot}}}{\mu E_{\mathrm{a}}} \quad (3)$$

However, with a conductivity κ_1 in the fraction X of the total capillary length at the sample application end (Fig. 1) and a different conductivity κ_2 in the remaining fraction $1-X$, while the total voltage U is maintained, a total migration time longer than t_0 will always be observed. The reasons for this will be given below.

Variations in conductivity and field strength are interdependent:

$$E_1 \kappa_1 = E_2 \kappa_2 \quad (4)$$

and thus

$$E_2 = \frac{\kappa_1}{\kappa_2} \cdot E_1 \quad (5)$$

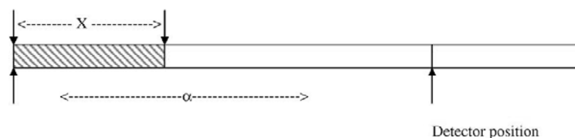

Fig. 1. Definition of the relative distances X and α used in the equations and calculations. The total length of the capillary is taken as unity.

If one should maintain a constant voltage it is necessary that

$$E_1X + E_2(1 - X) = E_a \tag{6}$$

Eqs. (5) and (6) are combined to determine E_1 and E_2:

$$E_1 = \frac{E_a}{X + \frac{\kappa_1}{\kappa_2} - \frac{\kappa_1}{\kappa_2} \cdot X} \tag{7}$$

$$E_2 = \frac{E_a}{X \cdot \frac{\kappa_2}{\kappa_1} + 1 - X} \tag{8}$$

The total migration time for the component will thus, provided that the mobility is constant, depend on the parameter X and the conductivities as:

$$t = d_{tot} \cdot \left(\frac{X}{\mu E_1} + \frac{1 - X}{\mu E_2} \right) \tag{9}$$

When we define E_1 and E_2 according to Eqs. (7) and (8) and rearrange we obtain the resulting ‘‘retention’’

$$t - t_0 = \frac{d_{tot}}{\mu E_a} \cdot \left[X \cdot (1 - X) \cdot \left(\frac{\kappa_1}{\kappa_2} + \frac{\kappa_2}{\kappa_1} - 2 \right) \right] \tag{10}$$

which is >0 for $0<X<1$ and $(\kappa_1/\kappa_2) \neq 1$, with a maximum at $x=0.5$

Going from general theory to the more typical case with the detector positioned at a fraction α (Fig. 1) of the total capillary length, Eq. (3) changes to

$$t_0 = \frac{\alpha d_{tot}}{v} = \frac{\alpha d_{tot}}{\mu E_a} \tag{11}$$

and for a proper use of the partial filling technique, i.e., $X<\alpha$, and defining E_1 and E_2 according to Eqs. (7) and (8) we obtain a modified version of Eq. (9):

$$t = \frac{d_{tot}}{\mu E_a} \cdot \left[X \cdot \left(X + \frac{\kappa_1}{\kappa_2} - \frac{\kappa_1}{\kappa_2} \cdot X \right) + (\alpha - X) \cdot \left(X \cdot \frac{\kappa_2}{\kappa_1} + 1 - X \right) \right] \tag{12}$$

If we take into account that the mobility usually is influenced by the environment and thus not equal in the two sections of the capillary, Eq. (12) changes to the generally valid

$$t = \frac{d_{tot}}{E_a} \cdot \left[\frac{X}{\mu_1} \cdot \left(X + \frac{\kappa_1}{\kappa_2} - \frac{\kappa_1}{\kappa_2} \cdot X \right) + \frac{(\alpha - X)}{\mu_2} \cdot \left(X \cdot \frac{\kappa_2}{\kappa_1} + 1 - X \right) \right] \tag{13}$$

which has been employed in the comparison between theory and experiment.

2.2.2. Shortcomings of the integration correction due to non-uniform migration velocity

Using an approach directly based on velocities

$$t_{mig} = \frac{\alpha d_{tot}}{v_{ave}} \tag{14}$$

For different velocity in two sections:

$$t_{mig} = d_{tot} \cdot \left(\frac{X}{v_1} + \frac{\alpha - X}{v_2} \right) \tag{15}$$

we find that with v_2 representing the velocity in the detector region

$$\frac{\alpha}{v_{ave}} = \frac{X}{v_1} + \frac{\alpha - X}{v_2} \tag{16}$$

which rearranges to the following ratio between the true velocity in the detector region and the average velocity employed for the standard integral correction:

$$\frac{v_2}{v_{ave}} = 1 + \frac{X}{\alpha} \cdot \frac{v_2 - v_1}{v_1} \tag{17}$$

which is $\neq 1$ whenever $v_2 \neq v_1$.

As a result, the integral based on the average velocity is incorrect by a factor f, where

$$f = \frac{v_{ave}}{v_2} \tag{18}$$

2.3. Apparatus and methods

Experiments were performed on a Hewlett-Packard ^{3D}Capillary Electrophoresis system (Agilent Technologies, Waldbronn, Germany) using Chemstation (version A.06.01) for system control, data collection and data analysis. UV detection was carried out at 210 nm. The sample solution and background electrolytes were hydrodynamically injected at the anode using a pressure of 34.5 mbar. All

runs were made using double or triple injections and the mean value of each response was calculated.

Separation was performed on poly(vinyl alcohol) (PVA)-coated capillaries (Agilent Technologies) of 33 cm (effective length 24.5 cm)×50 μm I.D. The ratio α between the effective length and the total length of the capillary used herein was thus 24.5/33=0.742. A coated capillary was chosen to minimize the EOF. New capillaries were flushed with water for 10 min, 0.01 *M* H_3PO_4 for 20 min and water for 5 min. The capillaries were preconditioned before each injection with water for 3 min, 0.01 *M* H_3PO_4 for 5 min, water for 3 min and BGE for 5 min. All solutions were filtered through a 0.45-μm syringe filter. The applied voltage was set to 12 kV and the temperature was 22 °C.

To achieve constant plug lengths, the application time, i.e., the time for a solution to reach the detection window and give a UV response, was determined by applying a pressure of 34.5 mbar to a vial containing 15 μ*M* *rac*-propranolol in the BGE. The pressure was then applied to a vial containing only BGE and the time for the UV absorbance to drop to zero was measured. This procedure was repeated twice and an average application time could then be calculated. The application time was determined for every BGE employed.

The partial filling technique was applied to introduce plugs with lower or higher conductivity compared to the BGE. The principle is sketched in Fig. 1. The plug lengths varied between 0 and 100% of the effective capillary length. In the case of a completely filled capillary, the equivalent plug length was 135% of the effective capillary length. Two series of experiments were made, one using 0.015 *M* bis-tris–acetate, pH 6.5, as BGE and 0.030 *M* bis-tris–acetate, pH 6.5, introduced as the plug, and one with the same two buffers reversed. The first series were repeated a few months later by another person. The experiments within one series were made in random order to avoid systematic errors. In all experiments the sample load corresponded to 2.0% of the effective capillary length and the BGE introduced after the sample corresponded to 0.8%.

2.4. Calculations

Calculations of relative migration time in comparison to experimental data were carried out using Eqs. (13) and (11), whereas the simulated data for sample zone conductivity effect, sample zone dilution and selector zone dilution were based on Eq. (12).

The theoretical values for the integration error factor *f* were calculated from Eqs. (17) and (18).

3. Results and discussion

3.1. Migration time artifacts

3.1.1. verification of the derived equations

Eq. (13) was tested by a series of experiments where a selected length in the beginning of the capillary had twice or half the conductivity of the background electrolyte in the remaining part, which included the detector position. The results are shown in Fig. 2 and demonstrate a good agreement between theory and practice, allowing us to employ Eqs. (12) or (13) for the simulations of other cases below.

3.1.2. Effect of sample conductivity

Fig. 3 shows the effect on the migration time when the conductivity of the sample zone, corresponding to 1% of the total capillary length, was varied from 0.01 to 20 times that of the background electrolyte. The customary use of low conductivity in

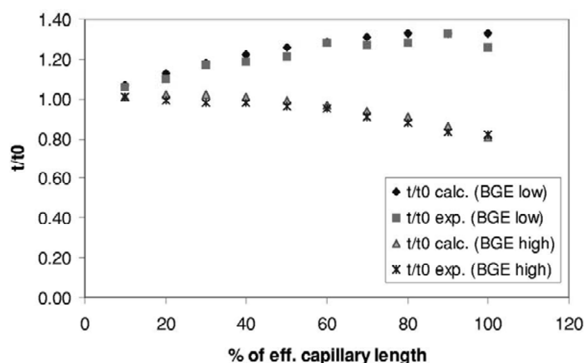

Fig. 2. Effect of conductivity variations in the capillary. The background electrolyte was replaced in a certain fraction of the effective capillary length by the same buffer with twice (BGE low) or half (BGE high) the concentration of the background electrolyte. Data calculated using Eqs. (13) and (11) are compared with the experimental results.

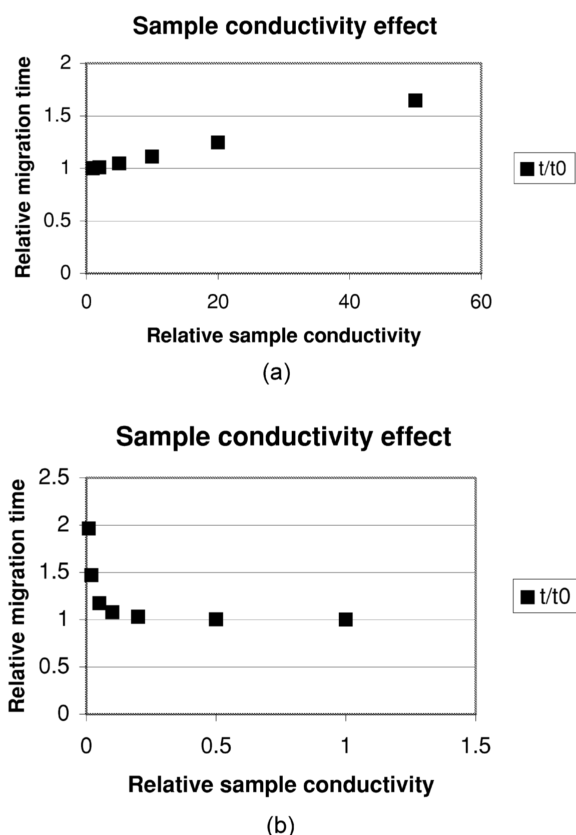

Fig. 3. Simulation by Eq. (12) of the effect of sample conductivity. (a, top) The effect on the migration time in voltage stabilised mode when the relative conductivity of a sample zone corresponding to 1% of the total capillary length varies between 1 and 50. (b, bottom) The effect on the migration time in voltage stabilised mode when the relative conductivity of a sample zone corresponding to 1% of the total capillary length varies between 0.01 and 1.

the sample zone to achieve an initial sharpening does obviously represent a considerable error source, asking for exact standardization [6–8,18].

3.1.3. Effect of sample dilution

When a sample zone is applied, it is inevitable that it mixes with the background electrolyte to some extent, partially by longitudinal diffusion, but also, due to an initial parabolic flow profile, combined with radial diffusion. To investigate this effect, we applied Eq. (12) to simulate the situation where a sample zone with a relative conductivity of 0.1 or 5 was mixed into a fraction X of the total capillary length, thus gradually approaching the conductivity of the background electrolyte. We made the approximation that the conductivity change was colinear with the fraction of sample in the mix. As shown in Fig. 4 the effect of the initial sample conductivity will, due to dilution effects, not always be as dramatic as shown above. On the other hand, the mixing may not be exactly predictable, especially if the application–start sequence is not exactly standardized. As a consequence of that, we may face not only systematic errors but also a poor reproducibility. It is earlier reported that automated sample loading improves the reproducibility [18].

3.1.4. Effect of selector zone dilution

As was mentioned before, the attractive use of selectors for, e.g., chiral resolution in the partial filling mode may give rise to migration anomalies due to system effects. Preliminary observations have suggested that the specific effect of a selector is

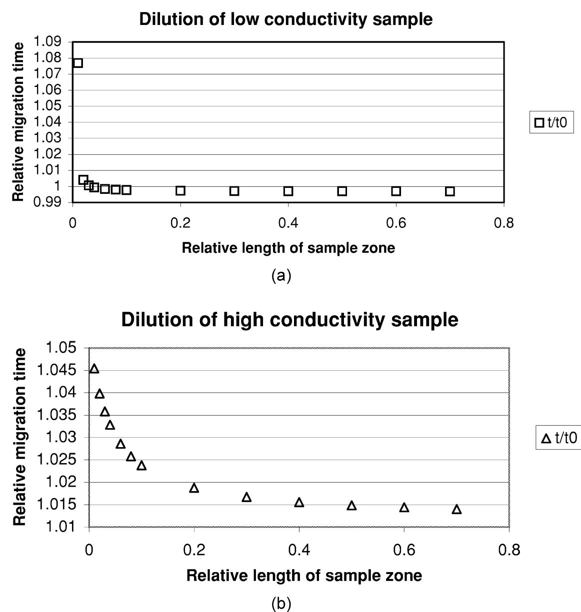

Fig. 4. Simulation by Eq. (12) of the effect of sample dilution. (a, top) The effect on the relative retention time when a sample with a relative conductivity of 0.1 and filling 1% of the capillary is diluted with background electrolyte to a relative length X. (b, bottom) The effect on the relative retention time when a sample with a relative conductivity of 5.0 and filling 1% of the capillary is diluted with background electrolyte to a relative length X.

mainly due to the absolute amount used, but independent of its distribution. The result obtained when Eq. (12) was used to simulate the non-specific effect on the migration time caused by dilution of a selector zone initially occupying 10% of the capillary length into a larger part X of the capillary is shown in Fig. 5. We find that the system-dependent retention decreases smoothly as the selector is spread out in a larger part of the capillary, suggesting that, when the partial filling strategy is necessary, a given amount of selector should still occupy the largest possible fraction of the capillary length for optimal results.

3.2. Integration artifacts

The experimentally determined error factor f in the time-corrected integral is compared with values calculated from Eqs. (17) and (18) (see Fig. 6). It is clear that the theoretical prediction reproduces the experimental pattern well, although with some underestimation of the effects. The remaining difference between predicted and actual values can possibly be ascribed additional disturbances from the low-conductivity sample application zone.

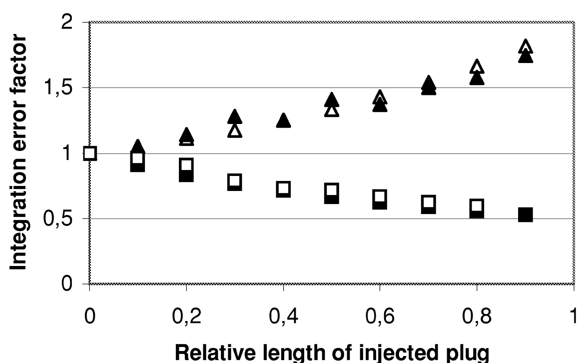

Fig. 6. Shortcomings of the standard retention time based integration correction function. Experimental error factors obtained from the same experiments as in Fig. 2 were compared with factors calculated by Eqs. (17) and (18). (■) Theoretical values for low conductivity BGE; (□) Experimental values for low conductivity (BGE); (▲) Theoretical values for high conductivity BGE; (△) Experimental values for high conductivity BGE.

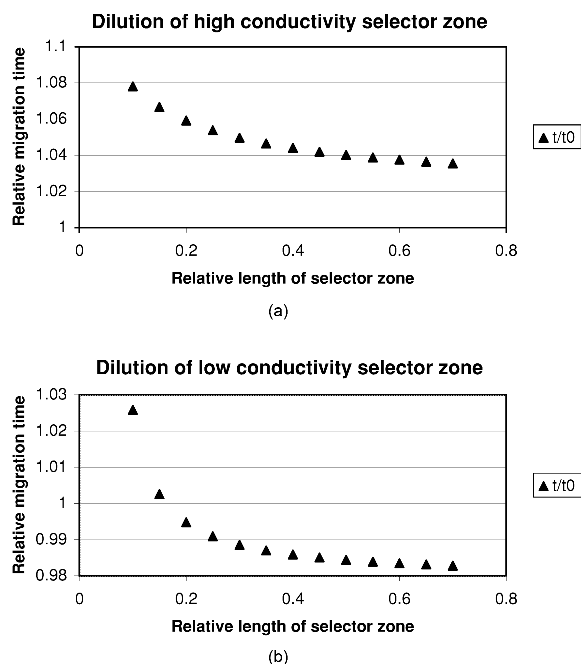

Fig. 5. Simulation by Eq. (12) of the system effect when a selector zone disturbs the conductivity. (a, top) System effect on the relative migration time when a selector zone initially occupying 10% or the total capillary length and with a relative conductivity of 2 is diluted with background electrolyte to a relative length X. (b, bottom) System effect on the relative migration time when a selector zone initially occupying 10% or the total capillary length and with a relative conductivity of 0.5 is diluted with background electrolyte to a relative length X.

Although the velocity differences here are produced by a conductivity step in the system, the same integration disturbances will be found in, e.g., enantiomer separations using a selector in the partial filling mode [9–11]. The recorded migration time and thus the average velocity will differ for the two enantiomers, resulting in different correction factors despite the fact that they really migrate with the same velocity in the detector region, where no selector is present. Conclusively, the standard migration time based correction algorithm is generally unsuitable for the partial filling technique. For complex samples, it may be necessary to determine correction factors in separate experiments using the plain buffer, whereas the important special case of enantiomer analysis can be handled simply by disabling the correction function. One general solution of the problem has been described [19], namely to employ a multipoint detection system in constant current mode where the migration time for each

sample component *between* the detector positions should serve as an individual correction factor.

4. Concluding remarks

The results presented here may explain some reproducibility problems that have been disturbing in the past. Furthermore, the observations and conclusions by Moring et al. [7,8] concerning field strength influence from the sample matrix are in full accordance with Eq. (13). The procedures described by Wätzig and Dette [6] actually help to keep the field strength more constant throughout the length of the capillary, thus diminishing the effects discussed herein. The important observations by Smith et al. [5] that an inconsistency in migration time correlated with inconsistency in current can also be explained by our results. Taken together, we find that today's high-performance electrophoresis may suffer from errors due to conductivity variations within the system that are more than superficially resembling the classical mobility and concentration anomalies of the Tiselius method.

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