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Pherogram normalization in capillary electrophoresis and micellar electrokinetic chromatography analyses in cases of sample matrix-induced migration time shifts☆

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Abstract

When analyzing bio-matrix samples using capillary electrophoresis (CE) or micellar electrokinetic chromatography (MEKC), unwanted shifts in the time axis are often observed, both between samples and standards and between samples, thus hampering identification. These shifts are caused by either or both of two sample matrix-induced effects: variations in stacking conditions (effective field strength or migration length) and variations in electroosmotic flow. Based on elementary CE principles and provided that any two peaks in the pherograms can be linked, these variations can be separately accounted and quantitatively corrected for, so that perfectly overlapping pherograms of standards and samples can be obtained after normalization. The method was validated using samples of a DNA ladder, separated in a sieving polymer. In addition, a number of data files from CE and MEKC analyses (steroids, opioids, β-blockers, amines, and inorganic anions) previously published by other authors were successfully normalized. A freeware computer programme, CEqualizer, for normalizing ASCII files of detector signals using the method described, is available to the CE community from http://www.ceyork.f2s.com.

Keywords: Peak shift; Sample matrix; Signal-processing software

1. Introduction

In bio-matrices, depending on the ionic strength, a wide range of stacking conditions can lead to different effective field strengths in the separation compartment, because the voltage drop over the injection plug compartment may be considerable, even after the initial stacking phase, because this high-resistance plug remains present in the capillary. In addition, bio-matrices often adversely affect the (local) surface characteristics in the capillary and hence electroosmosis, even in cases where the electroosmotic flow (EOF) is assumed to be completely suppressed. Both phenomena can lead to sample matrix-dependent pherogram shifts, preventing users from overlaying pherograms of different standards or samples. The phenomena also lead to incorrect peak areas, so that, for proper quantitation, peak areas have to be divided by the corresponding migration times. Peak identification in complex mixtures remains a problem, however. This contribution presents a method of normalizing pherograms to refer-

☆The corresponding author dedicates this paper to the memory of colleague Piet Leclercq (1942–2000).

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ence conditions, in order to make pherogram superimposition possible. In earlier publications it was already evident that effects such as those mentioned above can be calculated and predicted [1–3]. In the present paper we want to go a significant step further: normalizing the electropherogram with respect to reference conditions, based on the identification information contained in the time axis of the electropherograms. In another approach, Hirokawa et al. [4] normalized pherograms in such a way that the time axis was replaced by an effective mobility axis. For this approach it is necessary to identify the EOF marker migration time in each pherogram with sufficient accuracy. The latter can be a particular problem in the case of sample matrix effects, because the EOF peak, if recognizable as such, can be disturbed or obscured by neutral sample constituents. In our approach, however, any two identified peaks will suffice for the normalization procedure.

2. Experimental

2.1. Equipment

DNA experiments were carried out on a SpectraPhoresis 1000 (Thermo Separation Products, Freemont, CA, USA). The capillary (overall length 360 mm, length to the detector 280 mm, 100 μm I.D.) was coated according to the following procedure [5]: rinsing with 0.1 *M* NaOH for 10 min and with 0.1% epoxy poly(DMA), poly(dimethylacrylamide-co-allyl glycidyl ether), EPDMA for 10 min. The sieving polymer in the background electrolyte was 1.5% (w/v) hydroxy-ethyl cellulose in Taps-TRIS 100 m*M* pH 8.3.

2.2. Samples

The sample was a 123 bp DNA ladder (123 to 4182 bp in increments of 123 bp) in a storage buffer, consisting of 10 m*M* Tris–HCl pH 7.5 with 5 m*M* NaCl, DNA concentration 1 μg/μl (Sigma). The sample was injected undiluted, or after a 10-fold dilution in water.

2.3. Software

Two versions of the computer programme were made. The DOS version was written in QuickBasic version 4.5 (Microsoft, Richmond, VA, USA), and subsequently compiled using PowerBasic version 3.5 (PowerBasic, Carmel, CA, USA). The Windows version was in C, using Microsoft Visual C++ version 6.0 (Microsoft, Redmond, WA, USA).

3. Principle of computation

Based on the fact that the effective mobility in electrophoresis is a unique property of the sample components under well-defined experimental conditions, the model used in the computation directly follows from elementary electrophoretic principles. The migration time of a component *i* under reference conditions is given by

$$t_{iR} = \frac{L_d L_t}{V(u_i + u_{eof})} \quad (1)$$

Here, L_d is the capillary length to the detector, L_t the total capillary length, V the voltage over the capillary, u_i the effective mobility of component i, and u_{eof} the electroosmotic mobility. In the case of micellar electrokinetic chromatography (MEKC), separation is based on selective partition, rather than selective migration. In that case it is more appropriate to call u_i the pseudo-effective mobility. The former and all subsequent equations, however, remain valid.

The migration time of the same component under non-reference conditions can be denoted as

$$t_i = \frac{L_d L_t}{\alpha V(u_i + u_{eof} + \beta)} \quad (2)$$

In this equation, two corrections are included: a non-unity factor α and a non-zero term β. The factor α takes into account situations where the effective voltage drop over the separation compartment is decreased due to a high resistance sample compartment, and also the situation that the sample components are stacked in front of a large injection plug, thus decreasing their effective migration length to the

detector, L_d. The term β takes into account the fact that the electroosmotic flow can deviate from the reference conditions, due to the fact that the inner capillary surface characteristics are altered. In order to calculate the two unknowns, α and β, two identifiable, corresponding peaks in the pherograms are required.

The effective mobility in both cases is, of course, the same, and can, for component 1, be written as

$$\frac{L_d L_t}{V t_1} - u_{eof} = \frac{L_d L_t}{\alpha V t_{1R}} - u_{eof} - \beta \quad (3)$$

The same can be written for component 2:

$$\frac{L_d L_t}{V t_2} - u_{eof} = \frac{L_d L_t}{\alpha V t_{2R}} - u_{eof} - \beta \quad (4)$$

From the above two equations, the unknowns α and β can now be calculated. Subtracting Eqs. (3) and (4) yields factor α:

$$\alpha = \frac{(1/t_1) - (1/t_2)}{(1/t_{1R}) - (1/t_{2R})} \quad (5)$$

It can be seen that the dimensionless factor α must be a (non-zero) positive number around unity. Addition of Eqs. (3) and (4), combining Eq. (5), yields term β:

$$\frac{2V\beta}{L_d L_t} = \frac{(1/t_1) + (1/t_2)}{\alpha} - \left(\frac{1}{t_{1R}} + \frac{1}{t_{2R}}\right) \quad (6)$$

The left-hand side of Eq. (6) is now denoted γ, which is also dimensionless. It will be shown that it is not necessary to calculate β explicitly, which means that in order to apply the algorithm to measured signals, instrumental parameters such as V, L_d and L_t need not be known. Values for β (and consequently for γ) can be both positive and negative. An equation for the apparent mobility under non-reference conditions can be obtained from Eq. (2):

$$u_i + u_{eof} = \frac{L_d L_t}{\alpha V t_i} - \beta = \frac{L_d L_t}{\alpha V t_i} - \frac{L_d L_t \gamma}{2V} \quad (7)$$

The equations for α and γ are now substituted into Eq. (7), and the resulting equation for $u_i + u_{eof}$ is substituted into Eq. (1):

$$t_{iR} = \frac{1}{(1/\alpha t_i) - (\gamma/2)} \quad (8)$$

Eq. (8) directly converts experimental migration times t_i to migration times under reference conditions t_{iR}, provided that two peaks in both pherograms can be positively identified as being from the same component.

A local compression factor C_t can be defined for each time element as

$$C_t = \frac{t_i}{t_{iR}} = \frac{1}{\alpha} - \frac{t_i \gamma}{2} \quad (9)$$

A special case is where C_t is unity; the corresponding peak will not shift, but this will only occur at non-unity α values. The local compression factor has exactly the same effect as dividing the peak area by the migration time in non-normalized pherograms.

For diagnostic purposes, it is useful to formulate separate equations for migration time shifts originating from the effects represented by the factor α and the term β. The former simply amounts to $1/\alpha$; the latter is different for each peak and equals $\alpha t_i / t_{iR}$. The normalization calculates the latter by averaging the values for both reference peaks.

4. Results and discussion

The effect of sample-induced migration time shifts is best illustrated by using electropherograms generated by CE simulation software [6,7]. In this program, an arbitrary test mixture was analyzed at 25 kV with the zeta potential of the capillary wall set at 0 mV, corresponding to a coated capillary with suppressed EOF (Fig. 1A). It is assumed that this is the electropherogram referred to as the reference. The effective field strength can be decreased in the case of injection of a large volume with a low conductivity, because the effective field strength over the majority of the migration length will decrease. In addition, when anionic components in such an injection compartment are stacked at the front of this compartment (at negative inlet polarity; this stacking can take place within a fraction of a second), the

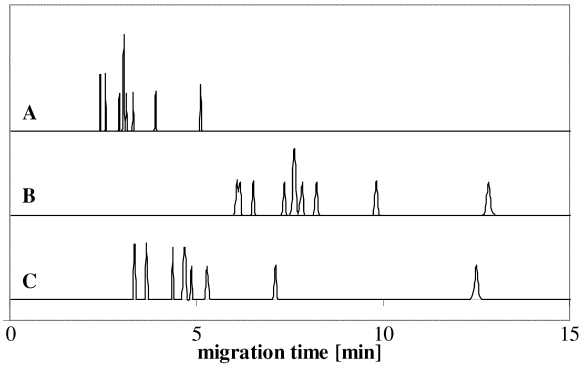

Fig. 1. Simulated electropherograms [7,8], artificially illustrating the isolated effect of (B vs. A) a drastic decrease in effective field strength (from 25 to 10 kV) and (C vs. A) a significant change in capillary wall zeta potential (from 0 to −25 mV), resulting in a corresponding change in EOF. In practice, both effects can occur simultaneously.

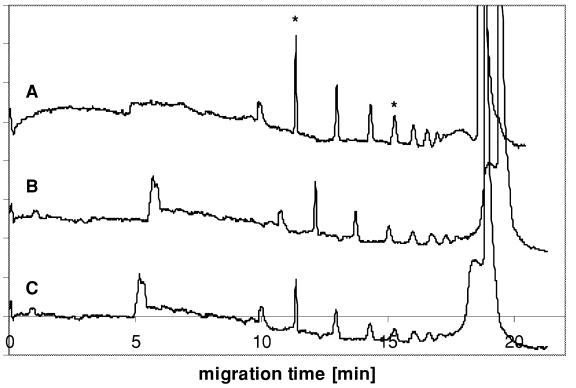

Fig. 2. Example of the separation of part of a DNA ladder (123–4182 bp) of a 3 s injection of standard solution (A) and a 30 s injection after 10-fold dilution in water (B). The last electropherogram can be normalized with respect to the first, using the peaks denoted by an asterisk for identification (C).

effective migration length to the detector is decreased. This is not the case with sample ions of opposite sign (cations at negative inlet polarity): these are stacked at the beginning of the sample compartment. The net result in all cases is an α value less than unity in Eq. (2). In order to mimic this situation, the simulated analysis was repeated at a combination of 15 kV and a zeta potential of 0 mV (Fig.1B). In other cases, samples may induce EOF changes, either because the capillary coating is gradually washed away, or due to the presence of surface-active components (detergents, proteins) in the samples; in Eq. (2), a non-zero β term. This can be illustrated by changing the zeta potential to −25 mV, at 25 kV (Fig. 1C).

Consider electrophoretic practice: peak shifts in figures like the above cannot easily be interpreted intuitively. One observes a ''normal'' trace like Fig. 1A, comparing it with something intermediate between Fig. 1B and 1C. Peak normalization is the solution, but as a diagnostic tool it also yields information on the main cause of the shifts: as mentioned, effects corresponding to α and β are calculated and presented by the programme.

A number of DNA standard mixtures were analyzed in a coated capillary (see Experimental). The DNA sample was injected undiluted for 3 s at 0.75 p.s.i. (Fig. 2A). After 10-fold dilution in water, a 30 s injection was applied (Fig. 2B). Significant peak shifts occurred. The latter pherogram was normalized (Fig. 2C) with respect to the former, using the two peaks designated with an asterisk for reference purposes. With respect to the use of the normalization software as a diagnostic tool: in the DNA example given, field strength/migration length effects were responsible for a 13% increase in the migration time, overshadowing a slight EOF difference between the runs. Mathematically: the α factor predominates over the effect of the β term in Eq. (2).

With respect to the choice of reference peaks: it is preferred that they are as far apart as possible, because then normalization of the electropherogram is obtained by interpolation. One could, of course, take the first two distinguishable peaks, the normalized pherogram to the right of the second peak consequently being obtained from extrapolation. The result may be that the peaks in the normalized and the reference electropherogram do not exactly superimpose. Whichever two peaks are chosen, the normalized electropherogram always better resembles the reference than does the non-normalized in terms of peak migration times.

At this point we would like to emphasize that it is not our intention to use the normalization software in cases where it can be assumed that capillary equilibration with the background electrolyte was not obtained. This and the following examples are

merely used to illustrate the principle and the magnitude of the migration time shift that the normalization program is capable of dealing with. As an additional point in this example it should be mentioned that, naturally, within-run differences in EOF should be absent, as they were not incorporated in the model presented in the Introduction.

In the next example, different migration lengths and detectors were used with otherwise identical equipment [8]. The analysis in Fig. 3 depicts a mixture of inorganic anions in a closed system, with suppressed electroosmosis. One of the capillaries (Fig. 3A) was provided with a 300 μm I.D. fluoroplastic capillary, with a distance of 250 mm to the detection sensor, an electrolyte solution mediated contact detector (ESMC). The other capillary (Fig. 3B) was provided with a 300 μm I.D. capillary tube with a length of 160 mm to the reference contact detector. In this example, normalization of Fig. 3B with respect to Fig. 3A was successfully carried out, in this case using the first and last peaks (Fig. 3C and D). In contrast to the example in Fig. 2, here the α coefficient in Eq. (2) is mainly responsible for the difference in the two runs, because of different migration and overall capillary lengths. The β term was close to zero. Although normalization in this example yielded satisfactory results, the procedure should be used with extreme caution in cases where runs from different capillaries or equipment are combined, even when the same background electrolyte is used. A slight difference in experimental conditions may result in different operating temperatures in the separation capillary. The equations on which the normalization is based assume constant effective mobilities of all sample components in all runs used in the normalization process.

A standard mixture of opioids was analyzed with CE–UV, in a study [9] also using CE–MS for identification. An aqueous volatile buffer of 25 m*M* ammonium acetate was used, adjusted to pH 9 with a 1 *M* ammonia solution. At positive inlet polarity in an uncoated capillary, a significant EOF was observed, and eight of the analytes in a test mixture (Fig. 4A) migrated as cations before the EOF marker. A 1:1 spiked urine sample (Fig. 4B) displayed decreased migration times for all peaks. The urine trace was subsequently normalized (Fig. 4C) with respect to the aqueous standard, using the first and seventh peak for identification (the eighth is probably shouldering against the off-scale EOF event). The cause of the peak shift for this urine sample is two-fold: a 2% migration time decrease due to field strength/migration length effects and a 2% decrease due to EOF changes.

In addition to sample matrix effects on the migration of analytes, the injection technique, especially the injection volume, can play a significant role in migration time shifts.

The following example illustrates this effect, and the potential use of pherogram normalization in

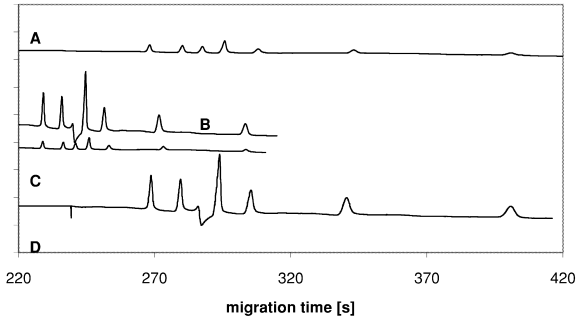

Fig. 3. Comparison [9] of detection with the aid of (A) electrolyte solution mediated contact detection and (B) reference contact detection in the CE separation of a test mixture (30 μmol/l each) of, respectively, chloride, nitrate, sulfate, nitrite, iodide, fluoride and phosphate. Either of the two electropherograms can be normalized with respect to the other using the first and last peak for identification, as seen in signals (C) and (D).

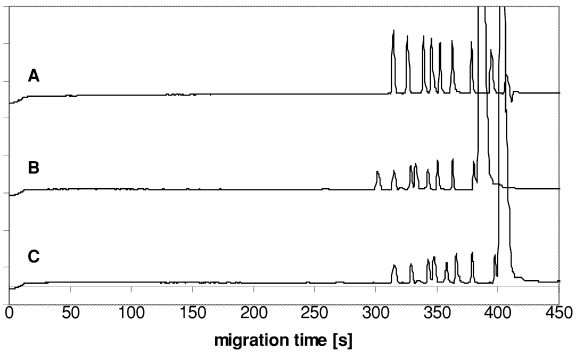

Fig. 4. CE–UV electropherograms [10] of (A) a test mixture (10 μg/ml each) of eight opioids, respectively NDHC, NCOD, NDHM, DHC, NMOR, DHM, COD and MOR, (B) a two-fold diluted spiked urine and (C) the electropherogram (B), normalized with respect to (A), using the first and seventh peak for identification.

MEKC. A mixture of steroids was analyzed using MEKC in the sweeping injection mode [10], where the sample solution, not containing SDS, was adjusted to the same ionic strength as the BGE. The initial field strength over the whole capillary length can consequently be assumed to be uniform. Two injections were compared, a 0.57 mm length of a 100 ppm mixture (Fig. 5A), and a 62.7 mm length of 1 ppm (Fig. 5B). The SDS entering the sample plug will sweep the analytes to the front of the plug, thus reducing their effective migration length considerably. This can be seen by comparing Fig. 5A and 5B. When normalizing Fig. 5B with respect to Fig. 5A as reference electropherogram, using the first (cortisone) and last peak (testosterone), the second peak (hydrocortisone) in the normalized pherogram (Fig. 5C) coincides exactly with the hydrocortisone peak in the reference electropherogram (Fig. 5A). The sensitivity enhancement of sweeping MEKC, reported to amount to factors of the order of 10^3 [11,12], thus far had to be paid for with significant peak shifts. In this example it is illustrated that the normalization procedure is capable of dealing with these shifts, thus producing consistent electropherograms over a broad sample concentration range. The mechanism seems quite complicated, because it has been calculated that a 10% migration time decrease due to field strength/migration length effects comes on top of a 13% reduction due to EOF changes. The latter is likely caused by the absence of detergent in the relatively large sample compartment.

Large volume sample stacking is often applied in CE. In the first of such examples, a test mixture of β-blockers was analyzed under low reversed electroosmotic flow, induced by a cationic surfactant (2 m*M* CTAB) at acidic pH [13]. Acetonitrile (20%) was added to the BGE. After 120-fold dilution of the sample, and injecting a sample plug length as large as 80 mm, considerable migration time shifts were observed (Fig. 6A and B). The field strength/migration length effect predominates, amounting to a factor of 1.44. The cations will stack at the front of the sample zone. This alone would result in shorter migration times. However, the large low conductivity sample volume consumes most of the voltage drop, especially during the initial phase of the analysis (this length in the capillary in this case shortens during analysis because the EOF is directed towards the inlet). The effect of the EOF-induced shift was calculated to be negligible in this case. Using the first and last peaks, Fig. 6B could successfully be normalized with respect to Fig. 6A (see Fig. 6C).

In another example using the same experimental setup [13], a mixture of organic amines was analyzed (Fig. 7A). Here, also, after 100-fold sample dilution and injecting a 76.8 mm plug length (Fig. 7B), significant migration time shifts were observed. The field strength/migration length effect also predominates, amounting to a factor of 1.26, but here the EOF is changed, leading to a 13% average reduction

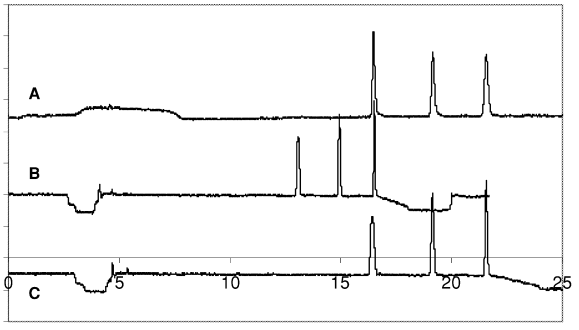

Fig. 5. Sweeping MEKC analysis of test steroids [11], respectively cortisone, hydrocortisone and testosterone, (A) 0.57 mm injection of 100 ppm each, (B) 62.7 mm injection after 100-fold dilution, and (C) the electropherogram in (B), normalized with respect to that in (A), using the first and third peak for identification.

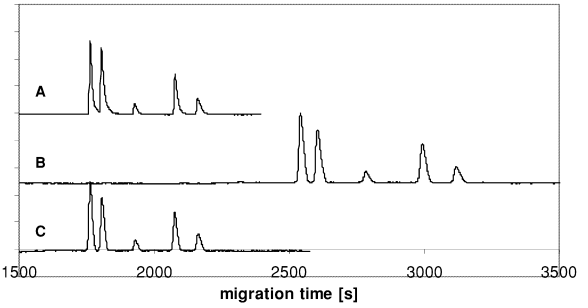

Fig. 6. Large volume stacking in CE of a test mixture of β-blockers [13], respectively bunitrolol, propranolol, metoprolol, procaterol, and acebutolol, (A) 0.64 mm injection of ca. 200 ppm each, (B) 80 mm injection after 120-fold dilution and (C) the electropherogram (B) normalized with respect to (A), using the first and last peak for identification.

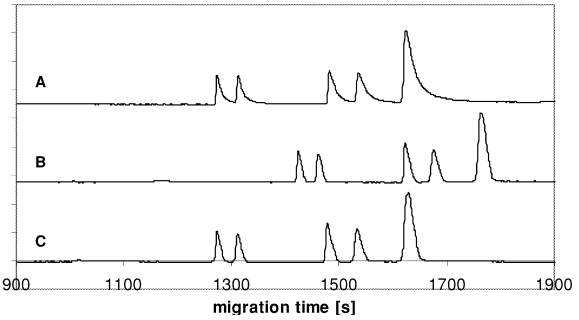

Fig. 7. Effect of large volume stacking in CE of a test mixture of aromatic amines [13], respectively aniline sulfate, benzyl amine HCl, 3,5-dimethylaniline, L-(−)-1-phenylethylamine, and naphthylamine, (A) 0.64 mm injection of ca. 100 ppm each, (B) 76.8 mm injection after 100-fold dilution and (C) the electropherogram in (B), normalized with respect to that in (A), using the first and last peak for identification.

in migration time. The net result is increased migration times, but not as large as in the previous example. In Fig. 7C it is shown that the pherogram in Fig. 7B can also be successfully normalized with respect to the reference pherogram in Fig. 7A, using the first and last peak for identification purposes.

5. Conclusions

It has been shown that migration time shifts between electropherograms, induced by a number of different sample matrix effects, can be readily identified. In order to be able to do this, both electropherograms should contain at least two corresponding peaks for identification. The CEqualizer software subsequently quantifies the migration time shift factors from two independent origins: field strength and/or migration length differences and EOF differences between runs. Finally, the software can normalize with respect to time axis information any signal to any other signal, provided they were obtained under instrumental and background electrolyte conditions guaranteeing constant effective mobilities. The software can be obtained from the website http://www.ceyork.f2s.com.

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