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Review

Determination and regulation of the migration window in electrokinetic chromatography

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Abstract

The determination of the velocities of the mobile and the pseudostationary phases (the migration (time) window) is mandatory for the determination of physicochemical properties by electrokinetic chromatography (EKC). This review offers a detailed discussion on the definition, the importance, the determination and the regulation of the migration (time) window in EKC. An overview on the theoretical treatment of chromatographic processes in EKC is given defining EKC in comparison to the term capillary electrophoresis. Methods to determine and influence the migration window are discussed with emphasis on measures that have been taken to modify the electroosmotic flow velocity. Pseudostationary phases (or separation carriers) that are taken into consideration are anionic and cationic micelles, mixed micelles, microdroplets (microemulsions), polymeric pseudostationary phases and dendrimers.

Keywords: Reviews; Electrokinetic chromatography; Micellar electrokinetic chromatography; Microemulsion electrokinetic chromatography; Migration window; Retention factor; Resolution

Contents

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1. Introduction

Electrokinetic chromatography (EKC) is a term that has been coined by Terabe and co-workers in 1985 [1,2] denoting a capillary electromigrative separation technique employing a separation carrier, mostly an ionic micellar phase. In the past 10 years EKC has developed into a versatile and powerful technique for the separation and determination of numerous substances in many fields [3,4].

The separation carrier, also called pseudostationary phase, is an electrophoretically migrating unity (e.g. a microdroplet, a micelle, a dendrimer, or a dissolved polymer) that interacts with the solutes to be separated while its migration velocity is virtually unaffected by this interaction. This property (migration velocity is virtually unaffected by interaction with dissolved solutes) defines the difference between a pseudostationary phase and a simple complex forming agent used in capillary electrophoresis to modify the effective electrophoretic mobility. In EKC a non-charged solute will migrate either with the velocity of the electroosmotic flow or with the velocity of the pseudostationary phase. Also in chromatography the observed velocity of a solute zone is the weighed mean of two velocities (velocity of the mobile phase and "velocity" of the stationary phase) resulting from partitioning of the solute between these two phases. Consequently, the separation process in EKC can be described with chromatographic terms and the separation of neutral solutes differing in their partitioning coefficients is possible.

Already in their first papers on EKC, Terabe and co-workers [1,5] emphasized the chromatographic nature of the underlying separation process (re-) defining parameters known from chromatographic theory. Their treatment is the basis of further considerations on rational resolution optimization [6], method development [7] or experimental determination of physicochemical parameters from EKC data [8,9]. One of the peculiarities of EKC is the non-existence of a stationary phase so that the solute zone is also transported (in direction of the detector or in counter-direction) when incorporated into the pseudostationary phase. The ratio of the velocity of the mobile phase (the surrounding medium) to the observed velocity of the pseudostationary phase has a large impact on resolution and peak capacity of the separation system [1,10]. In the current literature this velocity ratio is mainly called migration (time) window reflecting the limited elution window for neutral solutes in EKC in the normal elution mode.

In this paper, we will discuss techniques used to determine the migration window (pre-requisite for the determination of retention factors and physicochemical properties), the impact of the migration window on the resolution of solute zones, the column availability, the peak capacity and the separation number, and methods developed for the enlargement of the migration window.

2. Fundamentals

2.1. Retention factor

The basic equation in EKC for neutral solutes is derived from theory in chromatography [11]:

$$v_s = \frac{1}{1+k} \cdot v_{eo} + \frac{k}{1+k} \cdot v_{sc} \quad (1)$$

where v_s is the observable velocity of solute zone, k the retention factor, v_{eo} the velocity of electroosmotic flow, v_{sc} the observable velocity of the separation carrier.

The retention factor is defined here corresponding to theory in chromatography:

$$k = \frac{v_{sc}}{v_{mob}} \cdot K \quad (2)$$

where v_{sc} is the volume of separation carrier, v_{mob} the volume of surrounding (mobile) phase, K the distribution coefficient.

Replacing the velocities in Eq. (1) by the respective ratios distance over time and rearranging results in Eq. (3) [1,5].

$$k = \frac{t_s - t_0}{t_0(1 - t_s/t_{sc})} \quad (3)$$

where t_0 is the migration time of the front of the surrounding (mobile) phase, t_s the migration time of the solute zone, t_{sc} the migration time of the front of the separation carrier.

Eq. (3) is valid in case of the so-called normal elution mode according to Vindevogel and Sandra [12]. It is now important to state that in contrast to conventional chromatography v_{eo} and v_{sc} can have opposite directions. According to theory of capillary electrophoresis a velocity (mobility) in direction to the cathode is called positive, a velocity (mobility) in opposite direction is called negative. However, it is impossible to attribute a negative value to the magnitude time.

Gareil [13] has shown that in the case that the velocity of the solute zone is opposite to v_{eo} (reversed direction mode according to Vindevogel and Sandra [12]) k has to be determined from Eq. (4):

$$k = \frac{t_s + t_0}{t_0(t_s/t_{sc} - 1)} \quad (4)$$

In that case t_s and t_{sc} can be determined simultaneously in one run while t_{eo} can be determined only after reversal of polarity or injection of a marker solution at the opposite end of the capillary. Eq. (1) has to be rewritten, provided that only absolute velocities ($v = |\vec{v}|$) are given:

$$v_s = -\frac{1}{1+k} \cdot v_{eo} + \frac{k}{1+k} \cdot v_{sc} \quad (5)$$

It can be shown that Eq. (4) can be derived from Eq. (5) after replacing the velocities in by the respective ratios distance over time.

In case that the velocity of the solute zone is opposite to v_{sc} (restricted elution mode according to Vindevogel and Sandra [12]) Eqs. (6) and (7) are valid [13].

$$v_s = \frac{1}{1+k} \cdot v_{eo} - \frac{k}{1+k} \cdot v_{sc} \quad (6)$$

$$k = \frac{t_s - t_0}{t_0(t_s/t_{sc} + 1)} \quad (7)$$

Generally, the electrophoretic mobility of the separation carrier is opposite to the electroosmotic mobility of the mobile phase, because a separation carrier of opposite charge than the surface of the capillary wall (e.g. a cationic surfactant or a cationic polymer in a negatively charged fused-silica capillary) will be adsorbed onto the surface of the capillary wall reversing the direction of the electroosmotic flow. There are two special cases: (1) v_{sc} equals zero, (2) v_{eo} equals zero. Case 1 corresponds to conventional chromatography. Case 2 corresponds also to conventional chromatography if we rename the phases.

Eqs. (1) and (3)–(7) are only valid for neutral solutes. In case of charged solutes or solutes that are in equilibrium with a protonated species (bases) or a deprotonated species (acids) the electrophoretic mobility of the charged species and the degree of protonation or deprotonation, respectively, have to be known if true retention factors (see Eq. (2)) are to be calculated. Alternatively, the effective mobility of the solute in the separation buffer without the separation carrier must be known. This is not a trivial task. Muijselaar et al. [14] have emphasized that mobility data obtained with capillary electrophoretic experiments should be used with caution to calculate true retention factors of charged solutes in micellar EKC. Khaledi et al. [15] and Strasters and Khaledi [16] have discussed the migration behaviour of acids and bases in micellar EKC in detail. As a consequence of the very complex situation involving several equilibria (protonation–deprotonation equilibrium, partitioning equilibria of the neutral and of the charged species, eventually ion pair equilibrium of the charged species with ionic surfactant monomers) the migration behaviour of acids and bases is mostly described with electrophoretic terms (e.g. effective electrophoretic mobility) rather than with chromatographic terms.

2.2. Definition of the migration (time) window

When introducing Eq. (3) in the expression for resolution in chromatography an equation is obtained that describes the resolution of two solute zones dependent on experimental parameters in the normal elution mode of EKC [1]:

$$R_s = \frac{\sqrt{N}}{4} \cdot \left(\frac{\alpha - 1}{\alpha}\right) \cdot \left(\frac{\bar{k}}{1+\bar{k}}\right) \cdot \left(\frac{1 - t_0/t_{sc}}{1 + (t_0/t_{sc})\bar{k}}\right) \quad (8)$$

where R_s is the resolution, N the plate number, α the selectivity factor, $\bar{k}$ the mean retention factor.

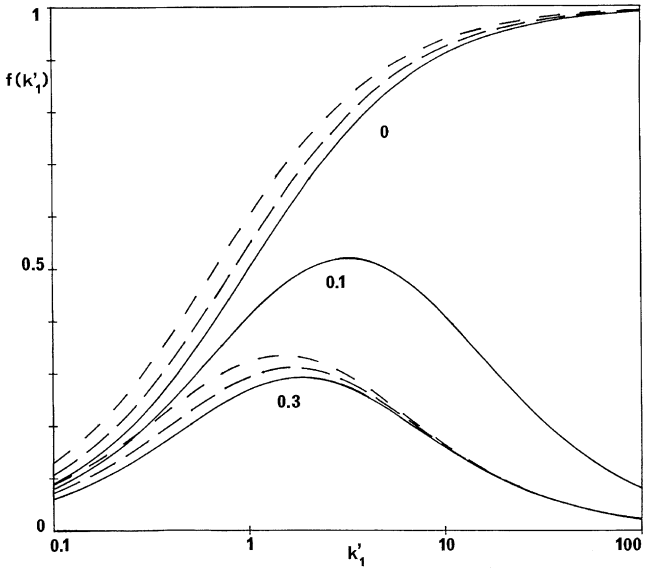

Fig. 1. Dependence of $f(k)$ on the retention factor in EKC in the normal elution mode. Full lines: selectivity factor $\alpha = 1$, long dashes: $\alpha = 1.2$, short dashes: $\alpha = 1.5$. The migration window (t_0/t_{sc}) is given directly on each full line (from [13] with permission).

Comparing Eq. (8) with the equation for the resolution of two solute zones in conventional chromatography reveals that the dependence of R_s on the mean retention factor is more complex in EKC than in conventional chromatography and that the time ratio t_0/t_{sc} has a major impact on the achievable resolution. In the normal elution mode the time span (window) in which a neutral compound can be eluted is restricted to values between t_0 and t_{sc}. Consequently, t_0/t_{sc} or its reciprocal value t_{sc}/t_0 have been mainly used in the literature to characterize the ratio of the observable velocities of the two "phases" in EKC. One widely accepted term for this time ratio is migration (time) window.

Plotting the last two factors of Eq. (8) ($f(\bar{k})$, see Eq. (9)) against $\bar{k}$ reveals that $f(\bar{k})$ goes through a maximum and that this maximum is smaller than 1 in all instances with 1 as the limiting value if t_{sc} approaches infinity (see Fig. 1).

$$f(\bar{k}) = \left(\frac{\bar{k}}{1+\bar{k}}\right) \cdot \left(\frac{1 - t_0/t_{sc}}{1 + (t_0/t_{sc})\bar{k}}\right) \quad (9)$$

Terabe et al. [1] have already recognized in their pioneering paper on micellar EKC that the smaller resolution obtained in the normal elution mode with identical N, α, and $\bar{k}$ is a disadvantage of EKC compared to conventional chromatography that can be, however, compensated by the large plate numbers achieved under routine conditions in micellar EKC (200 000–300 000). It has to be emphasized that the equation to determine the resolution of two solute zones is dependent on the elution mode (see Eqs. (3), (4) and (7) [13]).

In the reverse-elution mode Eq. (10) is valid.

$$R_s = \frac{\sqrt{N}}{4} \cdot \left(\frac{\alpha - 1}{\alpha}\right) \cdot \left(\frac{\bar{k}}{1+\bar{k}}\right) \cdot \left(\frac{1 + t_0/t_{sc}}{(t_0/t_{sc})\bar{k} - 1}\right) \quad (10)$$

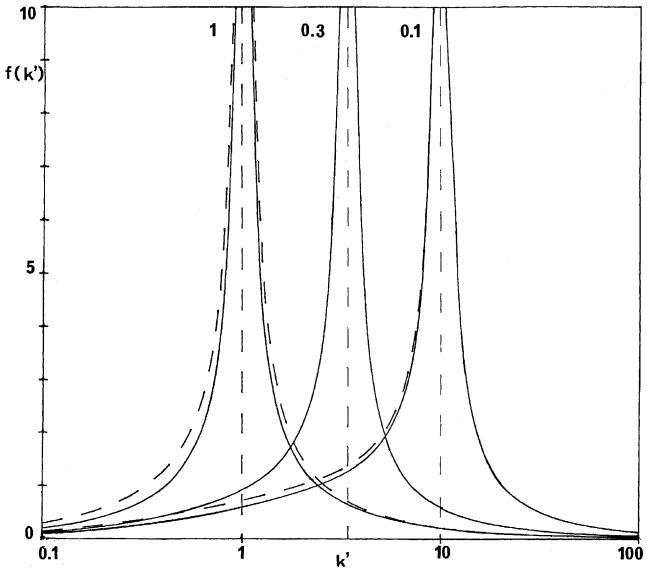

Fig. 2. Dependence of $f(k)$ on the retention factor in EKC in the reversed and the restricted elution mode. Full lines: selectivity factor $\alpha = 1$, dashed lines: $\alpha = 1.5$. The migration window (t_0/t_{sc}) is given directly on each full line (from [13] with permission).

And in the restricted-elution mode Eq. (11) is valid.

$$R_s = \frac{\sqrt{N}}{4} \cdot \left(\frac{\alpha - 1}{\alpha}\right) \cdot \left(\frac{\bar{k}}{1 + \bar{k}}\right) \cdot \left(\frac{1 + t_0/t_{sc}}{1 - (t_0/t_{sc})\bar{k}}\right) \quad (11)$$

In these modes there is no restricted time span (window) in which a neutral compound can be eluted and $f(\bar{k})$ can exceed unity. Foley [6] and Gareil [13] have shown that in the normal elution mode $f(\bar{k})$ is maximum for $\bar{k} = \sqrt{t_{sc}/t_0}$. However, for the reversed elution mode and for the restricted elution mode $f(\bar{k})$ increases dramatically, when $\bar{k}$ approaches t_{sc}/t_0 (see Fig. 2) [12,13]. Here, if $\bar{k} = t_{sc}/t_0$ then the velocity of the solute zone is zero. Consequently, in these modes very high resolutions can be obtained even for very small selectivity, however, at the expense of migration time of the solutes to be separated, e.g. Bushey and Jorgenson [17] succeeded in separating isotopically substituted compounds (dansylated methylamine and dansylated $^2H_3$ methylamine) by micellar EKC with migration times of more than 90 min.

2.3. Column availability

In 1993 Zhang et al. [10] published a paper describing phenomena in EKC based on conventional chromatography theory. They defined three new parameters. One parameter is the phase velocity ratio P_r which is identical with t_{sc}/t_0. However, they use a different definition of the migration time. Although a negative time is from a fundamental point of view not possible, they define a negative time, if the direction of migration is towards the positive electrode (the anode), and a positive time, if the direction of migration is towards the negative electrode (the cathode).

The second new parameter is the column availability A_{co} which corresponds to the last factor in Eq. (8):

$$A_{co} = \frac{P_r - 1}{P_r + \bar{k}} \quad (12)$$

The third parameter is the virtual column length L' which corresponds to the actual length of the capillary to the detector multiplied by the column availability factor. This parameter takes into account that a solute zone in case of identical directions of the observed velocities of the mobile phase and the separation carrier is in part transported by the separation carrier to the detector. In case of conventional chromatography $A_{co} = 1$ and the solute zone is only transported by the mobile phase to the detector.

Zhang et al. [10] showed that in the normal elution mode $A_{co} < 1$, in the restricted elution mode $A_{co} > 1$, and in the reverse elution mode $A_{co} < -1$ (if $P_r < (1 - \bar{k})/2$). It is important to note that A_{co} is not identical with the conventionally defined $f(\bar{k})$ (see Eq. (9)).

2.4. Peak capacity

According to Giddings [18] the peak capacity n corresponds to the maximum number of components resolvable in one chromatographic run. In deriving an equation approximating the exact number he assumed a constant plate number independent of the solute and the retention factor:

$$n = 1 + \frac{\sqrt{N}}{4} \cdot \ln\left(\frac{t_2}{t_1}\right) \quad (13)$$

t_1 is the migration (or elution) time of the first solute zone, while t_2 is the migration time of the last solute zone. In the normal elution mode in EKC t_1 is identical with t_0 and t_2 is identical with t_{sc}, because all neutral solutes have to be eluted within this range of time. Consequently, the migration window has a direct impact on the peak capacity in the normal elution mode. In other elution modes of EKC there is no fundamental restriction of the migration time.

It is, however, important to note that the requirement for Eq. (13), constant plate numbers independent of the retention factor, is not fulfilled in practice. Some authors have therefore preferred to use the separation number SN instead of the peak capacity [19,20]. The separation number is defined as the number of component peaks that can be placed between the peaks of two consecutive homologous standards (here: the homologous series of *n*-alkyl phenyl ketones) with z and $z + 1$ carbon chain atoms, separated with a resolution of 1.177. Also the separation number is dependent on the migration window. Plotting SN versus the retention factor reveals that a maximum is observed in the normal elution mode, but also if $t_{sc}/t_0 \rightarrow \infty$. This maximum is due to the diffusion of the micellar phase which is not negligible in this case.

In contrast to Eq. (13) the separation number takes into account the varying band broadening with increasing migration time. Kolb et al. [20] have therefore suggested to

calculate the overall peak capacity from the sum of separation numbers within a given *z*-range.

3. Determination of the migration window

3.1. Marker of the velocity of the mobile phase

Generally, for the determination of the electroosmotic velocity which is identical to the velocity of the mobile phase a sample is injected which contains a compound (the marker of the velocity of the mobile phase) that is not retained by the separation carrier or the capillary wall. Several polar substances have been used to this end: acetone, formamide, and thiourea. The marker must be detected by the detector in use. In case of a UV detector the baseline disturbance caused by a zone of different refractive index than the separation buffer can be used as a signal. Ahuja et al. [21] have compared the electroosmotic velocities determined by using several organic solvents as marker substances: acetone, acetonitrile, methanol, tetrahydrofuran, and 1-propanol. They showed that all these solvents are suited as marker substances with exception of 1-propanol. Obviously, 1-propanol is retained by the separation carrier (here: a micellar phase, sodium dodecylsulfate). The migration time of 1-propanol is significantly different from that of the other four solvents.

Fuguet et al. [22] investigated for several micelle-forming surfactants the signals produced by potential markers of the electroosmotic velocity: dimethyl sulfoxide, thiourea, formamide, *N*,*N*-dimethylformamide, methanol, acetone, acetonitrile, propan-1-ol, and tetrahydrofuran. The surfactants taken into their investigations are sodium dodecylsulfate, lithium dodecylsulfate, lithium perfluoooctane sulfonate, sodium cholate, sodium deoxycholate, tetradecyltrimethylammonium bromide, and hexadecyltrimethylammonium bromide. It is interesting that the suitability of the marker is dependent on the micellar system. Methanol, acetonitrile and formamide were well suited (not retarded by the micellar phase) with all micellar systems studied. They also conclude that any solvent can be used as marker if t_0 is measured by the first disruption of the baseline, because this disruption corresponds to the solvent not partitioned in the micellar medium. According to Fuguet et al. this seems to be the most accurate method to determine t_0.

3.2. Marker of the velocity of the separation carrier

If a substance (the marker of the velocity of the separation carrier) is available which is exclusively transported by the separation carrier and not transported by the mobile phase ($k \rightarrow \infty$) and this substance can be detected by the detector in use, then the velocity of the separation carrier can also be determined with a sample containing a marker. In the beginning of EKC very non-polar azo dyes (Sudan III, Sudan IV) [1,23], dodecanophenone [24] or polycyclic aromatic hydrocarbons [25] have been employed as marker substances. Thorsteinsdóttir et al. [26] used a peptide as marker of the micellar separation carrier.

It must be stated that it is inconvenient to work with these non-polar substances because they are difficult to dissolve in solvent mixtures compatible with EKC conditions. When using negatively charged micellar phases as separation carrier Terabe et al. have therefore suggested to employ positively charged compounds with a non-polar structure unit [27]. One of these substances is timepidium bromide [28]. Another substance employed to this end is quinine hydrochloride [7,29]. It has to be emphasized that these positively charged marker substances may be only used with negatively charged separation carriers (especially negatively charged micelles). Obviously, the inclusion of the hydrophobic structure unit into the hydrophobic core of the micelle is possible while the positively charged part of the molecule interacts very strongly with the oppositely charged surfactant head groups.

Comparing separations obtained by micellar EKC and microemulsion EKC Terabe et al. [30] report that phenanthrene and *p*-amylphenol were eluted in the normal elution mode after timepidium bromide if negatively charged microdroplets are the separation carrier. They conclude that timepidium bromide is not suitable as marker of the velocity of the separation carrier in case of microemulsion EKC.

Determinations of the velocity of the separation carrier with a simple marker should be used with caution. As will be shown in the next section, careful investigations have shown that these data can be misleading, especially if mobile phases are used that contain a considerable volume fraction of an organic solvent.

3.3. Iterative procedure

Bushey and Jorgenson [17,31] succeeded in separating isotopically substituted compounds by micellar EKC. In order to improve resolution, it was necessary to use a separation electrolyte containing a volume fraction of methanol of 20%. When attempting to employ 9-methylanthracene as marker of the velocity of the separation carrier, they noticed that these results were misleading compared to the migration time of dansylated dodecylamine. They therefore concluded that the results of (micelle) markers are unreliable with high volume fraction of an organic solvent in the separation electrolyte and they suggested a new iteration procedure to determine the separation carrier migration time in these media.

According to the Martin equation there is a linear relationship between the logarithm of the retention factor and the carbon number of the members of a homologous series. Muijseaar et al. [32] have verified for the homologous series of *n*-alkylbenzenes and *n*-alkyl phenyl ketones as solutes and buffers containing sodium dodecylsulfate, decyltrimethylammonium bromide, or hexadecyltrimethylammonium bromide that this linear relationship is also valid in EKC with micellar separation carrier. Bushey and Jorgenson

investigated the migration times of dansylated *n*-alkylamines in micellar EKC. They took the migration time of the longest chain dansylated amine (dansylated dodecylamine) as approximation of the separation carrier migration time, calculated the retention factors of the shorter chain dansylated *n*-alkylamines with this approximated separation carrier migration time, calculated the regression line with these data, calculated a new value for the retention factor of the longest chain member of the series by extrapolation of the regression line, calculated a new value for the separation carrier migration time from the extrapolated value of the retention factor for the longest chain member of the series, and continued this procedure until the difference between a new value for t_{sc} and the value calculated in the last circle is below a threshold value.

This general procedure has been used by many working groups to estimate the migration time of the separation carrier with different mobile phases and different pseudostationary phases: micelles [33–40], microdroplets [41], starburst dendrimers [42] and polymeric pseudostationary phases [43]. Following homologous series were used: *n*-alkyl phenyl ketones, *n*-alkylbenzenes, phenyl-*n*-alkyl alcohols and dansylated *n*-alkylamines.

Shi et al. [44] have determined t_{sc} in micellar EKC with separation electrolytes containing 20 mmol l^{-1} sodium dodecylsulfate and various volume fractions of methanol. They compared data obtained with the marker (Sudan III) method and the iteration method according to Bushey and Jorgenson. The homologous series in their experiments was benzene to butyl benzene. They showed that the relative difference between results of the two methods is increasing with increasing volume fraction of methanol and that the values obtained with the marker method are systematically lower than those obtained with the iteration method.

Kuzdzal et al. [45] have suggested a procedure for the simultaneous determination of the mobile phase velocity and the separation carrier velocity based solely on the migration times of solutes belonging to a homologous series. They employed the homologous series of parabenes and, alternatively, alkylbenzenes. The determination of t_{eo} and t_{sc} is based on a grid search algorithm. First the program determines the range of possible t_{eo} and t_{sc} that will be included in the search.

With the estimated values retention factors are calculated for the members of the homologous series. The linear regression correlation coefficient for plotting $\log k$ against the carbon number is taken as a measure of the goodness of fit. They emphasize that this method is especially useful for pseudostationary phases which do not allow a simple determination of phase migration velocities, e.g. dendrimers. In order to show the validity of their approach, they verified for micellar EKC with aqueous mobile phase that estimates of t_{eo} and t_{sc} obtained by this (grid search) procedure are in accordance with those determined with markers known in the literature (formamide and Sudan III).

Certainly, the marker method is more convenient the the iterative procedure. Several researchers have shown that in case of purely aqueous mobile phases the results for t_{sc} obtained from the migration time of a suitable marker can be equivalent to that obtained by the iterative procedure. Bailey and Dorsey [46] emphasize that small errors in determining the migration time of the marker can lead to drastic errors in the calculated retention factor. They evaluated decanophenone, Sudan IV, Sudan III, Orange OT and Yellow AB as potential markers. From these decanophenone has been selected as the most suitable due to its solubilty properties and its high absorbance coefficient. Muijselaar et al. [32] observed for an aqueous buffer containing sodium dodecylsulfate small differences between the migration time of the marker Sudan III and the calculated t_{sc} employing the iteration procedure. Because of the small differences they conclude that both methods can be used for the determination of t_{sc} with aqueous separation buffers.

Fuguet et al. [22] evaluated following solutes as potential markers of the velocity of the separation carrier: anthracene, phenanthrene, Sudan III, octylbenzene, and dodecanophenone. The separation carriers taken into these investigations have been listed in Section 3.1. They also calculated t_{sc} by the iterative method employing either *n*-alkyl phenyl ketones or *n*-alkylbenzenes as homologous series. It is important to state that they only tested aqueous separation buffers. In all cases t_{sc} calculated with the iterative procedure is very close to the migration time of dodecanophenone. The authors conclude that dodecanophenone can be regarded as the best marker of the velocity of the separation carrier.

4. Regulation of the migration window

4.1. Anionic micellar pseudostationary phases

4.1.1. Sodium dodecylsulfate

To-date most of the work in EKC was done with anionic micellar pseudostationary phase, especially with separation electrolytes containing sodium dodecylsulfate (SDS) in aqueous solutions in a concentration above the critical micellar concentration. Considerations outlined in the theoretical section of this paper show that the migration window has an important impact on resolution and peak capacity. Assuming a constant efficiency and selectivity, the resolution is influenced by the migration window and by the retention factors of the solutes.

The migration window reflects the velocity ratio of the two "phases" involved. While it is difficult to modify the efficient electrophoretic mobility of the pseudostationary phase, the electroosmotic mobility generated with a native fused-silica capillary can be easily modified by changing the pH of the separation electrolyte. For neutral solutes, the pH has no impact on the retention factor. Rasmussen and McNair [47] showed that the elution order for *n*-alkylparabenes can easily be reversed by decreasing the separation buffer pH from 7.0

to 3.37. This corresponds to a change in the elution mode (normal to reversed elution mode). In a second paper the same working group discussed optimization of resolution in micellar EKC taking the electroosmotic mobility as the decisive parameter [48].

Otsuka and Terabe [49] investigated in detail the effect of the pH of the separation buffer on the velocities of the mobile and the micellar phase. The pH range investigated was from 7.0 to 3.0. The electroosmotic velocity decreased dramatically with a decrease in pH below 5.5, while the electrophoretic velocity of the micellar phase was almost constant throughout the pH range investigated. At a pH of 5.0 the absolute velocity of the micellar phase is identical to the absolute electroosmotic velocity. These results were corroborated by investigations performed by Muijselaar et al. [33]. They also showed that retention factors for neutral compounds are virtually independent of pH and ionic strength of the separation buffer. It is, however, important to note that this only applies for neutral solutes hence the retention factor is strongly influenced via the degree of dissociation for acidic and basic solutes.

Organic additives have also been used to modify the migration window. Those modifiers comprise glucose [7,50], urea [7,27,33,40,51], acetonitrile and methanol [25,33,35,36,40,52], tetrahydrofuran [34,35,40], dimethyl sulfoxide and acetone [53], *n*-propanol and *n*-butanol [54], 2-propanol [40], and alcohols of various chain lengths [24]. With exception of glucose [7] all these modifiers not only expand the migration window but also have an impact on the retention factor. Generally, a decrease in the velocity of the mobile and the pseudostationary phase is observed with increasing concentration of the organic modifier. This decrease is due to changes in the viscosity and the dielectric constant of the separation electrolyte and modifications of the micellar structure. The fact that several parameters influencing migration time of the solutes and resolution are simultaneously changed when changing the concentration of an organic modifier in the separation electrolyte renders method development in EKC to a complex task that makes computer-assisted method development desirable.

Pyell and Bütehom [7,51] have shown that urea has only a modest influence on the retention factors of neutral solutes, while the migration window can be substantially increased. They also showed that the assumption that the separation process in EKC can be modelled by a chromatographic process having two "phases" migrating at constant velocity (not influenced by the solute zone) can be successfully used for computer-assisted resolution optimization.

While for polar solutes a drastic decrease in retention factors is not desirable, it is a pre-requisite for the separation of non-polar solutes. Bütehorn and Pyell [36] have shown that the simultaneous addition of urea and acetonitrile to the separation electrolyte can be used to dramatically reduce the retention factors of non-polar solutes and expand the migration window, while the velocity of the mobile phase is only moderately reduced. The retention factor for dansylated hexylamine dependent on the molar concentration of urea and the volume concentration of acetonitrile has been determined. The retention factor for this non-polar solute is reduced from 185 to 1.26. Chen et al. [35] emphasize that with acetonitrile as modifier and SDS as separation carrier the migration window can be extended without any significant increase in the migration time of the marker of the electroosmotic flow.

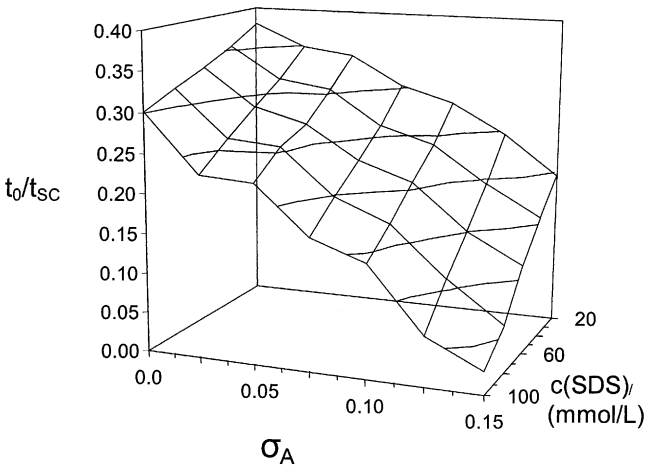

Fig. 3. Dependence of the migration window (t_0/t_{sc}) on the molar concentration of SDS and the volume concentration of acetonitrile on the separation buffer. Fused-silica capillary, 565 (500) mm × 75 μm i.d., buffer c($Na_2B_4O_7$) 10 mmol/l, c(H_3BO_3) = 10 mmol/l voltage 25 kV, temperature 25 °C (from [52] with permission).

Measuring the retention factors for various neutral solutes at constant surfactant concentration Muijselaar et al. [33] obtained a linear relationship between the logarithm of the retention factor and the modifier (methanol, urea) concentration whereas for acetonitrile a second order relationship was obtained. A linear relationship between the logarithm of the retention factor and the modifier (urea) concentration had been already determined by Terabe et al. [27] and Pyell and Bütehom [7,51].

At high concentration of SDS (100 mmol l^{-1}) and a volume concentration of acetonitrile of 15% in the separation buffer an infinite elution range was approached with a borate-phosphate buffer in native fused-silca capillaries, i.e. the absolute velocity of the micellar phase was about the absolute velocity of the mobile phase [52]. The same holds true for a borate buffer, c(SDS) = 20 mmol l^{-1}, c(urea) 3–5 mol l^{-1} and volume concentration of acetonitrile = 20% [36]. In Fig. 3 the migration window dependent on the SDS concentration and the volume concentration of acetonitrile is given. Liu et al. [40] classified the organic modifiers investigated into two different groups: one group includes urea and methanol for which the ability to expand the migration window is relatively weak, the other group includes acetonitrile, dioxane, tetrahydrofuran, *n*-propanol and 2-propanol for which the ability to expand the migration window is very high.

4.1.2. Other anionic surfactants

If it is desired to vary the migration window without change in the electroosmotic mobility, the electrophoretic mobility of the separation carrier has to be influenced. One possibility to achieve this goal is to change the composition of the pseudostationary phase.

Tanaka et al. [55] and Harino et al. [56] report the use of double chain surfactants (surfactants with two ionic groups and two lipophilic chains) in micellar EKC. These surfactants show a different selectivity and a wider migration window (at identical pH of the separation buffer) compared to SDS. Takeda et al. [57] employed sodium *n*-acyl sarcosinates of various chain lengths as micellar media in EKC. They showed that the migration window is widened with a decrease in the alkyl chain length of the surfactant at identical pH of the separation buffer and identical surfactant concentration.

Cai and El Rassi [58] introduced a surfactant forming micelles with adjustable surface charge density. This surfactant is octyl β-D-glucopyranosid (a polyol) forming reversibly complexes with borate ions. With in situ charged micelles the surface charge density and consequently the electrophoretic mobility of the micelles can be varied over a wide range by changing the borate or boronate concentration and/or the pH of the separation buffer. Higher polyol-borate complex forming constants were obtained with surfactants with linear sugar moieties: *N*-D-gluco-*N*-methylalkylamides [59].

4.1.3. Coated capillaries

Janini et al. [60,61] have shown that with polyacrylamide-coated capillaries hydrophobic solutes can be separated with high efficiency in a short run time. With these capillaries the electroosmotic flow is almost completely suppressed [62], so that the elution mode corresponds to Special Case 2 of Section 2.1. In this case the separation carrier takes over the role of the mobile phase and the surrounding medium can be regarded as equivalent to the stationary phase of conventional chromatography. Consequently, the column availability factor is 1 and there is no restricted migration window. Obviously, there is no band broadening induced by the polyacrylamide coating (see Fig. 4).

Coatings can be also used to reduce the electroosmotic velocity maintaining a normal elution mode regime [63,64]. However Muijselaar et al. [33] report for one uncoated and three different coated capillaries a pronounced decrease in efficiency when working with a coated capillary, probably due to solute-wall interactions. Landmann et al. [65] have chosen another approach: by coating the inner wall of the separation capillary with a negatively charged polymer the electroosmotic velocity becomes independent of the pH of the separation buffer. Micellar EKC separations with SDS and a separation buffer of low pH in the normal elution mode become possible.

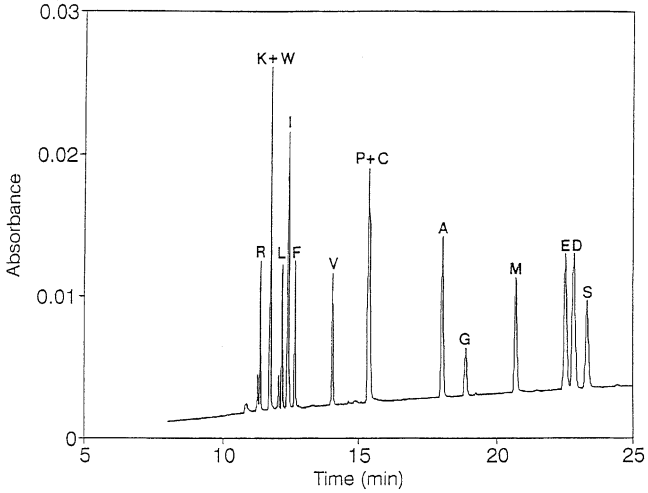

Fig. 4. Reversed-mode micellar EKC separation of dansylated amino acids (identification with one-letter abbreviations). Linear polyacrylamide-coated fused-silica capillary, 570 (500) mm × 75 μm i.d., buffer c(Na acetate) 25 mmol/l, c(SDS) = 25 mmol/l, pH = 4.2, voltage −15 kV (from [60] with permission).

4.2. Mixed micellar pseudostationary phases

Selectivity is influenced largely by the identity of the surfactant. Characterization of surfactant selectivity in micellar EKC has therefore been an important topic of several investigations [66–69]. One approach for optimizing the selectivity in micellar EKC is therefore the use of mixed micelles, particularly mixtures of ionic and neutral surfactants [70] or mixtures of bile salts with SDS [71,72]. Of these, mixtures of SDS with polyoxyethylene [23] lauryl ether (Brij 35) have been used by several working groups. The separation selectivity can be well controlled by adjusting the mixing ratio of the two surfactants [73]. There is, however, a marked narrowing in the migration window with increasing fraction of the non-ionic surfactant in the normal elution mode due to a decrease in the charge density of the mixed micelles formed [48,67,74].

Ahuja et al. [75] discussed the possibility to control (increase) the migration window by adjustment of the electrophoretic mobility of the micelles by employing a nonionic/anionic mixed micellar system (Brij 35 and SDS). They showed that variation of the concentration of the two surfactants effected the electroosmotic mobility and the electrophoretic velocity of the micelles in a different way so that the migration window could be substantially widened. By fine-tuning of pH (6.2) and surfactant concentration even a nearly infinite elution range was obtained corresponding to a “quasistationary” pseudostationary phase. They also showed that with silanized fused silica capillaries quasistationary conditions can be obtained at higher pH. However, adsorption of Brij 35 onto the silanized fused silica walls resulted in a marked decrease in efficiency.

4.3. Cationic micellar pseudostationary phases

Otsuka et al. [76] were the first to employ a cationic surfactant in micellar EKC. They obtained for the surfactant dodecyltrimethylammonium bromide (DTAB) a substantially smaller migration window than for SDS in the normal elution mode. They state that this is probably the main reason why SDS gave a better resolution as a whole than DTAB for the separation of 22 phenylthiohydanthoin amino acids. According to Poole and Poole [67] hexadecyltrimethylammonium bromide (CTAB) has a complementary selectivity to the other anionic surfactants investigated but provides only a small migration window.

With cationic micelles as separation carriers in EKC with bare fused-silica capillaries the direction and the velocity of the electroosmotic flow are determined by the hemimicellar layer formed at the capillary–liquid interface. Dworschak and Pyell [77,78] investigated in detail factors influencing the migration window in EKC with cationic micelles. Those factors comprise: selection of surfactant, variation of pH, volume fraction of organic modifiers, concentration of metal salts, concentration of cationic additives and dynamic coating of the capillary wall. In accordance with Crosby and El Rassi [79] a small increase in the migration window with increase in the length of the alkyl group of the surfactant was observed.

A reduction of the pH of the separation electrolyte has only a small influence on the electroosmotic velocity, hence does not substantially improve the migration window. Also the organic modifiers tested do not improve the migration window in a sufficient manner, although they can be employed for the adjustment of retention factors. By addition of inorganic salts with divalent metal cations to the separation electrolyte a strong decrease and a substantial improvement of the migration window was obtained [77], especially at low pH of the separation electrolyte. Divalent metal cations are known be effective additives reducing strongly the electroosmotic velocity in fused silica capillaries [80,81]. Fig. 5 shows the improvement in the separation of nitrotoluenes obtained through addition of $CaCl_2$ to the separation electrolyte.

Also cationic additives have been shown to be very efficient in reducing the electroosmotic velocity and improving substantially the migration window [78]. According to Dworschak and Pyell the cationic additives employed alter the structure of the hemimicelles formed on the inner capillary surface by competing with the surfactant monomers for ion-exchange positions on the fused-silica surface. Dynamic coating of the capillaries with hydroxypropylmethyl cellulose was of limited use in EKC with cationic micelles due to a strong decrease in efficiency of the separation system observed with dynamically coated capillaries [77].

4.4. Microemulsions

Oil-in-water (o/w) microemulsions have been introduced as separation carriers in EKC by Watarai [82–84]. Watarai

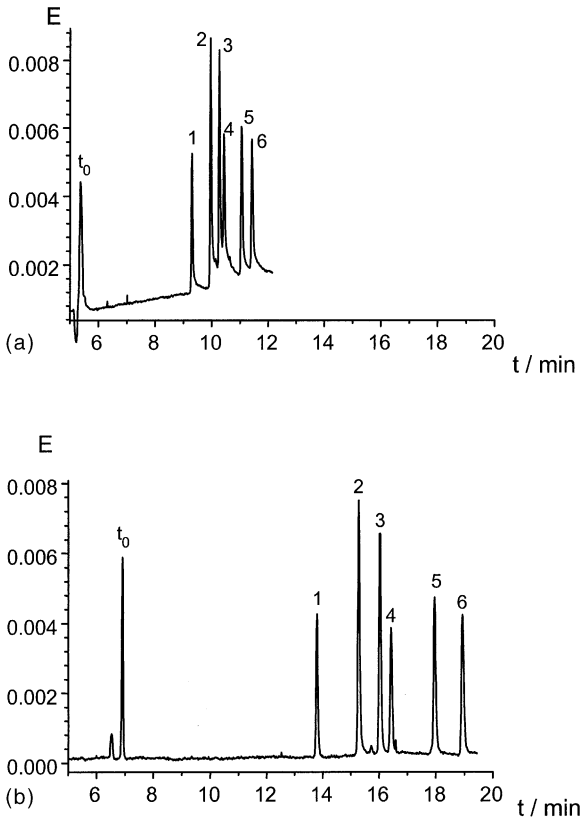

Fig. 5. Separation of nitrotoluenes with a separation electrolyte containing (a) 0 mmol/l, (b) 30 mmol/l $CaCl_2$. Fused-silica capillary 565 (500) mm × 75 μm i.d., buffer c(Na acetate) 10 mmol/l, c(acetic acid) = 10 mmol l, c(tetradecylammonium bromide) = 40 mmol/l, pH = 4.6, voltage 15 kV, temperature 25 °C. Peak identification: (1) 2,4,6-trinitrotoluene, (2) 2,5-dinitrotoluene, (3) 2,4-dinitrotoluene, (4) 2,6-dinitrotoluene, (5) 3,4-dinitrotoluene, (6) 2,3-dinitrotoluene (from [77] with permission).

and other researchers showed that the separation mechanism with neutral compounds is very similar to that of ionic micelles and that the migration window can be effectively extended by changing the composition of the microemulsion [30]. The larger migration window obtained with o/w microemulsions compared to SDS micellar solutions is obviously due to a higher electrophoretic mobility of the microdroplets compared to SDS micelles [85]. The electrophoretic mobility of the microdroplets and hence the migration window can be regulated by changing of the surfactant concentration [86]. However, plate heights in microemulsion EKC were reported to be about twice those in micellar EKC [30] while other authors report separations obtained by microemulsion EKC with good efficiency [87]. Microemulsion EKC has been also applied for the evaluation of the hydrophobicity of various substances [41]. To this end a correct determination of the migration window is mandatory.

4.5. Polymeric pseudostationary phases

Several types of synthetic ionic polymers have been employed as separation carriers in EKC [88,89]. These

polymeric pseudostationary phases comprise micelle polymers (polymerized surfactants), linear amphiphilic polymers and charged dendrimers. Investigations have shown that these polymeric materials provide high efficiency separations comparable to that obtained with micellar pseudostationary phases. The advantages of polymeric materials over conventional micellar and microemulsion pseudostationary phases are seen in the possibility to use these separation carriers dissolved in organic media, which is desirable for the separation of hydrophobic compounds, in the possibility to obtain unique selectivities (e.g. shape selectivity, chiral selectivity), and in advantages when coupling with electrospray ionization MS is needed.

It can be shown that the migration window and the selectivity can be fine-tuned by variation of the weight ratio when mixing two different pseudostationary phases [43]. Electrophoretic mobilities of the synthesized polymeric separations carriers can be also fine-tuned by variation of the monomer ratios [90]. Methods to determine the velocities of the mobile phase and the separation carrier are identical to those being developed for micelles and microdroplets [42,43].

5. Gradients

Terabe et al. [1] have compared the migration times observed in micellar EKC in the normal elution mode with the retention times obtained under gradient elution in conventional liquid chromatography. A very similar relationship between the elution times and the retention factors has been obtained in micellar EKC under the conditions of calculation and for conventional liquid chromatography having a concave gradient from water to methanol. These calculations illuminate one of the advantages in micellar EKC in the normal elution mode: the fact that neutral solutes have to be eluted within a restricted migration time window corresponds to an inherent “gradient type” elution. Run times in this mode can be kept very short. The disadvantage of lower column availability is mitigated by a higher efficiency compared to conventional liquid chromatography.

Balchunas and Sepaniak [91] and Sepaniak et al. [92] have demonstrated that the migration window can be widened (and the “inherent gradient” can by lowered) by using step-wise or linear gradients of the separation buffer composition (increase in the volume fraction of the organic modifier). By changing the volume fraction of an organic modifier in the separation buffer in the inlet vial during the chromatographic run, the retention factors are decreased during the run. The operation conditions are chosen so that all solutes of interest are eluted under optimum conditions. In order to optimize solvent gradients in EKC, a model for predicting retention times has been developed [93]. However, solvent gradients correspond to sections of different electroosmotic velocity in the separation capillary resulting unavoidably in increased band broadening due to a non-ideal liquid flow profile as a consequence of intersegmental pressure [94]. This might be the reason why an attept to implement the method of Balchunas and Sepaniak on an automated CE apparatus failed [95]. Band broadening due to intersegmental pressure will be mitigated in very narrow separation channels. Kutter et al. [96] realized successfully solvent programming in micellar MEKC with a microchip device having a separation channel of 36.3 mm length, 9 μm depth and 50 μm width.

A second possiblity to alter the retention factors of the solutes during the chromatographic run is temperature programming. From liquid chromatography studies it is known that temperature largely effects retention [97]. Temperature gradients can be very elegantly implemented with modern CE instrumentation [98]. Linear temperature gradients from 20 to 60 °C with a ramp rate of 3 °C/min were realized.

By applying radial electric potential gradients across the capillary wall, the direct control of the ζ-potential and the electroosmotic mobility, hence the velocity of the mobile phase, is possible [29]. Widening of the migration window by reducing the mobile phase velocity could be demonstrated. An immediate change in the mobile phase velocity over the entire length of the capillary was obtained when the radial electric potential gradient was varied. Consequently a gradient of the mobile phase velocity could be generated. The application of such a gradient for resolution optimization was demonstrated.

The velocity of the mobile phase can be also reduced by applying a counter-pressure. Kolb et al. [20] have shown that this measure results in an increase of the overall peak capacity for micellar EKC in the normal elution mode, although the efficiency is reduced due to increased resistance to mass transfer in the mobile phase due to the generation of a partly parabolic velocity profile (see Fig. 6).

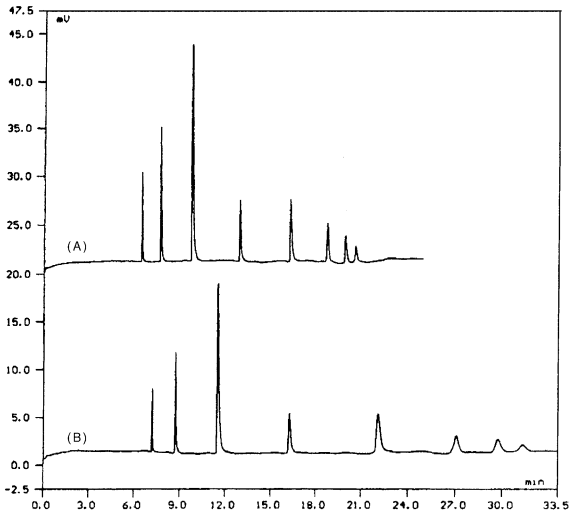

Fig. 6. Comparison of electropherograms for the separation of a homologous series of *n*-alkyl phenyl ketones without (A) and with 9.8 mbar (B) counter-pressure, voltage 30 kV (from [20] with permission).

6. Concluding remarks

The determination of the migration window (the ratio of the velocities of the two "phases" involved in the separation process) in mandatory for the determination of physicochemical properties with electrokinetic chromatography. While the use of a marker seems to be reliable for the determination of the electroosmotic velocity, the determination of the velocity of the separation carrier with a marker is prone to systematic errors. To-date, the iteration method according to Bushey and Jorgenson [17,31] seems to be the most reliable method. With aqueous mobile phases (not containing an organic modifier) dodecanophenone has been shown to be a reliable marker of the velocity of the separation carrier (anionic and cationic micelles).

Varying the migration window is beside regulation of the retention factor one of the most important parameters for resolution optimization in EKC. The electroosmotic mobility and the electrophoretic mobility of the separation carrier are the key parameters to be controlled. Measures that can be taken to vary the electroosmotic mobility in fused-silica capillaries can be considered to be very different for anionic separation carriers compared to those taken for cationic separation carriers, due to the altering of the capillary wall zeta-potential by adsorption of the cationic separation carrier onto the negatively charged fused-silica wall.

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