Review

Electrokinetic chromatography without electroosmotic flow[1]

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Abstract

This review summarizes the various aspects of conducting electrokinetic chromatography in coated columns with suppressed electroosmotic flow. The specific features of the technique will be presented and the potential applications explored. The equations of migration, resolution and zone spreading for neutral solutes will be presented, compared, and contrasted with those of conventional electrokinetic chromatography in bare-silica columns. The principle of separation is the same in electrokinetic chromatography with or without electroosmotic flow; however, there are many significant differences that will be highlighted. Published by Elsevier Science B.V.

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1. Introduction

As early as 1989, Terabe et al., who in 1984 [1,2] introduced the first mode of electrokinetic chromatography (EKC), namely micellar electrokinetic chromatography (MEKC), stated that electroosmotic flow (EOF) is not an essential parameter in EKC separations [3]. The same assertion was reiterated in a more recent review [4] and perhaps in other publications. Bulk flow is an integral part of all forms of conventional chromatography, but it is not essential in capillary electrophoresis, although it has been exploited to control the time of analysis and the direction of solute migration. Rathore and Horváth [5] made a clear distinction between separative migration, which involves selective interaction with a stationary phase and/or differences in electrophoretic mobility, and non-separative migration (bulk flow) that does not directly contribute to separation. A potentially useful feature of conducting EKC under suppressed bulk EOF is that the elution profile of the analytes is the reverse of that generated in the presence of EOF. For example, hydrophobic solutes, which are the last to elute in MEKC with EOF, are eluted first under conditions of suppressed EOF. While this technique represents a significant advantage for the rapid analysis of hydrophobic solutes, it has been almost completely neglected as is obvious from an inspection of the literature and the many excellent reviews of EKC including those of Terabe [3,4,6–10]. Studies conducted by Rasmussen and McNair [11] showing reversal of solute migration at acidic pH and those by Cole et al. [12] with organic solvents are notable exceptions. In both examples, electroosmotic flow was reduced to a value less than the electrophoretic mobility of the solutes studied but still remained significant. The first use of MEKC in coated capillaries with suppressed electroosmotic flow was reported in 1994 by Chiari et al. [13]. Neutrally-coated columns were also used by Abubaker et al. for the separation of steroids [14]. Simultaneously and independently, we reported the use of polyacrylamide-coated columns with zero electroosmotic flow for the determination of the partition coefficients of hydrophobic and slightly polar solutes in micellar–aqueous systems and suggested that these columns are potentially useful for the fast separation of hydrophobic solutes

[15]. Later we used the same technique for the micellar EKC separation of several classes of hydrophobic compounds [16] and charged cyclodextrin EKC for the separation of enantiomers and positional isomers [17].

The principle of separation in EKC is the same with or without electroosmotic flow; however, we will show in this review that there are many significant differences between the two modes of operation of EKC that in many ways parallel the differences between normal- and reversed-phase high-performance liquid chromatography (HPLC). The specific features of this technique will be highlighted and its potential applications explored, stressing the advantages as well as the limitations. The equations of migration, resolution, and zone spreading will be presented, compared, and contrasted with those of conventional EKC.

The acronym EKC was first coined by Terabe [3] to encompass separation techniques that are based on chromatographic principles but performed in capillary electrophoresis (CE) apparatus, utilizing electroosmotic flow (EOF) and electrophoretic mobility as a means of transport through the column. For example, micellar electrokinetic chromatography (MEKC) uses ionic micelles such as sodium dodecyl sulfate (SDS) and bile salts as the chromatographic separation psuedo-phases (carriers). Here again, we follow Terabe's suggestion and refer to the chromatographic pseudo-phase in EKC as a carrier [5]. Other carriers of interest are cyclodextrins (CDs) [9,18,19], ion exchangers [20], protein affinity ligands [21], hydrogen-bonding ligands [22], and complexation reagents [23,24]. Table 1 lists the different modes of EKC that have been reported to date [6].

In the course of development of the various modes of EKC, different migration equations were formulated depending on who derived them and for what mode. According to a recent critical examination by Rundlett and Armstrong [25], all the migration equations for the different modes of EKC are analogous or identical and have their origin in a 1951 publication by Alberty and King [26]. Various forms of the original Alberty and King equation have been introduced to represent the migration equation in MEKC [1,2] and the other forms of EKC [18–24], including enantioselective complexation [27–30].

Throughout this review, we will use the same

Table 1
Modes of EKC

Mode	Abbreviation	Pseudo-stationary phase	Main applicable analyte
Micellar EKC	MEKC	Micelle	Neutral, ionic drugs
Cyclodextrin-mediated MEKC	CD-MEKC	Micelle	Hydrophobic, enantiomers
Microemulsion EKC	MEEKC	Microemulsion	Hydrophobic
Affinity EKC	AEKC	Protein, polysaccharide	Enantiomers
Cyclodextrin EKC	CDEKC	Charged cyclodextrin	Enantiomers
Ion-exchange EKC	—	Polymer ion	Ionic drugs
Ligand-exchange EKC	—	Ligand+metal ion	Amino acids
Dendrimer EKC	—	Dendrimer	Hydrophobic
Resorcarene EKC	—	Resorcarene	Hydrophobic
Suspension EKC	—	Chromatographic particles	Hydrophobic

Reproduced from Ref. [6].

nomenclature and equation format that was first proposed by Terabe and co-workers [1–3]. Thus, EKC stands for electrokinetic chromatography with or without EOF. However, for clarity of presentation we refer to EKC with EOF as N-EKC where N stands for normal, and EKC under suppressed EOF as RF-EKC where RF stands for reversed-flow. Alternatively, we believe a scientifically more appropriate nomenclature would be: rheic electrokinetic chromatography for EKC with EOF and arheic electrokinetic chromatography for EKC without EOF. As mentioned by Rathore and Horváth [5], the term 'rheo' is used to distinguish capillary zone electrophoresis with EOF as part of the migration process from classical electrophoresis that is conducted under arheic conditions, i.e., without bulk flow [31].

2. Theoretical

Chromatography is defined as a method of separation based on the differential partitioning of solutes between two immiscible phases, one mobile and the other stationary. EKC defies these restrictions and broadens the classical definition of chromatography. It is not essential for EKC to have two immiscible phases. In N-MEKC, both phases are mobile, and the micelle carrier is homogeneously dispersed in the aqueous phase. In other modes of EKC, such as CD-EKC, only one phase exists with the CD carrier dissolved in the aqueous phase. In N-EKC, electroosmosis substitutes for mechanical pumps for the generation of bulk flow of the mobile phase. On the other hand, RF-EKC is conducted under arheic conditions. Despite these operational differences between EKC and classical chromatography, the separation process in EKC can be viewed as chromatographic and, hence, chromatographic formalism can be conveniently used to develop the theoretical equations of EKC.

In this section, we present the equations of migration, resolution, and zone-broadening for RF-MEKC of neutral solutes and compare them and contrast them with the corresponding equations that were derived by Terabe et al. for N-MEKC [1,2,32]. The other modes of EKC have identical equations, with the parameters referring to micelle (concentration, migration, velocity, etc.) replaced with the corresponding parameters for the carrier of interest.

2.1. The migration equations

The separation principal in RF-MEKC is illustrated in Fig. 1A. Electropherograms in RF-MEKC differ from those generated by N-MEKC in that the migration range is infinite, as illustrated in Fig. 1B. The first solute to appear is the micelle marker (a solute that is fully partitioned in the micelle) followed by analytes in order of decreasing hydrophobicity. The aqueous marker (a solute that is fully partitioned in the aqueous phase) will never appear because the aqueous phase is immobile in the absence of electroosmotic flow. In this respect RF-MEKC is very similar to micellar liquid chromatography, albeit with some theoretical and operational differences. For example, having a mobile micellar phase and a stationary aqueous phase is a unique
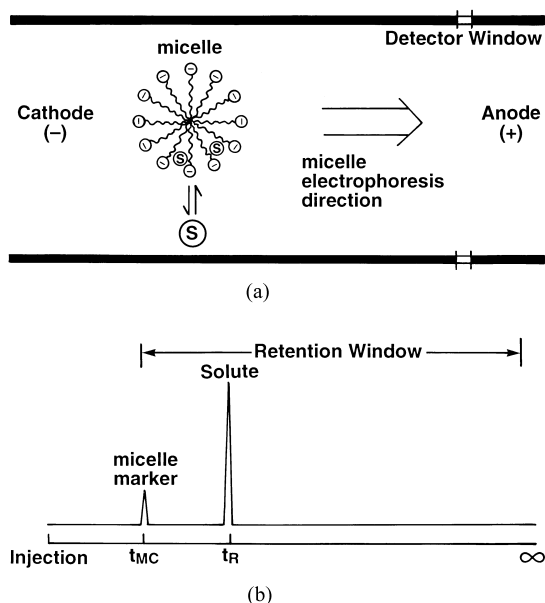
(a)

(b)

Fig. 1. (A) Schematic of the separation principle of RF-MEKC with negatively-charged micelles. (B) Schematic electrophero-gram in RF-MEKC.

situation that cannot be operationally duplicated by conventional chromatographic techniques.

The equations presented here for RF-MEKC are only valid for neutral solutes that only migrate when associated with the micelles. While the equations of migration for charged solutes in N-MEKC were fully developed [7,10], the same thing could not be said about the corresponding equations for charged solutes in RF-MEKC. The theory developed by Khaledi [7] and Foley and Ahuja [10] could be readily extended to charged solutes in RF-MEKC but, to our knowledge, this has not been attempted.

The equation of migration in RF-MEKC is derived by following basic chromatographic theory. The migration time (t_R) of a neutral solute is given by:

$$t_R = t_{mc} + t_{aq} \tag{1}$$

where t_{aq} is the time the analyte spends in the immobile aqueous phase and t_{mc} is the migration time of a micelle marker. It follows that:

$$t_R = t_{mc} + t_{mc}\frac{t_{aq}}{t_{mc}} = t_{mc} + t_{mc}\frac{n_{aq}}{n_{mc}} \tag{2}$$

where (n_{aq}/n_{mc}) (the ratio of the number of moles of solute in the stationary aqueous phase to that in the mobile micellar phase) is the capacity factor (k'), defined the same way as in elution chromatography. Thus:

$$t_R = t_{mc}(1 + k') \tag{3}$$

For a solute that is fully partitioned in the micelle, $k' = 0$ and hence, the micelle marker in RF-MEKC serves the same purpose as the dead-time marker in elution chromatography.

This simple equation is contrasted with the equation of migration in N-MEKC [1,2]:

$$t_R = \frac{1 + \tilde{k}'}{1 + \left(\dfrac{t_o}{t_{mc}}\right)\tilde{k}'}\, t_o \tag{4}$$

where $\tilde{k}'$ of Eq. (4) is, by definition, the inverse of k' of Eq. (3). Throughout this paper, k' refers to the capacity factor for RF-MEKC and $\tilde{k}'$ refers to the capacity factor for N-MEKC.

It is to be noted that Terabe [4] arrived at Eq. (3) from Eq. (4) by allowing t_o to go to infinity. Alternatively, Eq. (3) also can be arrived at, based on the electrophoretic migration principles, as follows:

The velocity of the solute (v_R) is equal to the fraction of solute in the electrophoretically migrating micelle times the velocity of the micelle (v_{mc}):

$$v_R = \left(\frac{n_{mc}}{n_{aq} + n_{mc}}\right) v_{mc} \tag{5}$$

Realizing that $v_R = (l/t_R)$ and $v_{mc} = (l/t_{mc})$, where l is the injector-to-detector column length, we have:

$$\frac{l}{t_R} = \left(\frac{n_{mc}}{n_{aq} + n_{mc}}\right)\frac{l}{t_{mc}} \tag{6}$$

Simple algebraic rearrangement yields Eq. (3).

2.2. The resolution equation

The resolution equation in RF-MEKC need not be derived, because it is identical to that of elution chromatography:

$$R_s = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'_2}{k'_2 + 1}\right) \tag{7}$$

where N is the number of theoretical plates and α, the separation factor for two closely-eluting solutes 1 and 2 is equal to $k_2'/k_1' = (t_{R2} - t_{mc})/(t_{R1} - t_{mc})$. In contrast, the resolution equation in N-MEKC [1,2] is given by:

$$R_s = \frac{\sqrt{N}}{4} \left(\frac{\alpha - 1}{\alpha} \right) \left(\frac{\tilde{k}_2'}{\tilde{k}_2' + 1} \right) \left(\frac{1 - (t_o/t_{mc})}{1 + (t_o/t_{mc})\tilde{k}_1'} \right) \tag{8}$$

Here, $\tilde{k}'$ implicitly depends on the magnitude of the electroosmotic flow through its explicit dependence on t_o Eq. (4).

Both equations have their origin in Giddings' equation of resolution for chromatography and electrophoresis [33]:

$$R_s = \frac{\sqrt{N}}{4} \frac{\Delta v}{\bar{v}} \tag{9}$$

where $\Delta v / \bar{v}$ is the relative velocity difference between the two migrating zones of interest. $\Delta v / \bar{v}$ is a measure of the system selectivity (separation power; degree of separation). It translates to the last two terms of Eq. (7) for RF-MEKC and the last three terms of Eq. (8) for N-MEKC. It could be easily shown, by simple algebraic manipulation; that $\Delta v / \bar{v}$ is equal to $\Delta t / \bar{t}$, the relative migration time difference.

In chromatography as well as in RF-MEKC it is convenient to assume that the three terms: N (column efficiency), α (separation factor), and k (retention parameter) are independent of each other, although this is not strictly true. This allows for the systematic optimization of resolution by sequentially optimizing each parameter separately. Optimization of resolution in N-MEKC is more complicated because of the coupling of the selectivity terms.

In this review we argue that the resolution is better in RF-MEKC compared to N-MEKC for the following reasons. In comparing Eq. (7) to Eq. (8), we note that α is the same and the $(\alpha - 1/\alpha)$ term is the same for both. However, the retention term for RF-MEKC $(k'/(1+k'))$ is larger than the retention contribution to resolution in N-MEKC (the last two terms of Eq. (8)) for all values of k'. The term $k'/(1+k')$ monotonously increases with increasing k' and asymptotically approaches 1. In contrast, the retention term for N-MEKC increases with increasing

$\tilde{k}'$ to a maximum at some value of $\tilde{k}'$ and (t_o/t_{mc}), then it decreases with a further increase in $\tilde{k}'$. It does not exceed 0.5, even for the best value of (t_o/t_{mc}) that is experimentally feasible. This is very clearly illustrated in Fig. 8 of Ref. [2]. $\tilde{k}'$ increases with increasing micelle concentration and (t_o/t_{mc}) decreases with increasing micelle concentration [2] and, therefore, optimization of selectivity in N-MEKC is a complex procedure that is compounded by the fact that different solute pairs in the same sample require different micelle concentrations for optimum separation.

In contrast, selectivity in RF-MEKC increases with increasing k' for all solutes. Selectivity is maximized for large k' values; however, large values of k' result in excessive analysis time and wide solute zones that become increasingly difficult to detect. The adjustment of k' into an optimum range of $1 < k' < 10$ is desirable, and easily accomplished by changing micelle concentration. The effect of carrier concentration on selectivity will be further discussed in a later section.

2.3. Partition coefficients

From chromatographic theory, k' in RF-EKC is related to the partition coefficient of the solute (P_{wm}) between the stationary phase (aqueous) and the micellar phase (mobile) by the relationship:

$$k' = \frac{P_{wm}}{\beta} \tag{10}$$

where, $\beta = (\text{vol}_{mc}/\text{vol}_{aq})$, and vol_{mc} and vol_{aq} are the volumes of the micellar phase and the aqueous phase respectively, inside the column. Vol_{aq} is strictly the volume of the column (vol_c) minus the volume of the micelle (vol_{mc}), however, since $\text{vol}_c \gg \text{vol}_{mc}$, it can be reasonably assumed that $\text{vol}_{aq} = \text{vol}_c$.

Incorporating the above relationship into Eq. (3) and rearranging gives the expression:

$$\tilde{t}_R = 1 + \frac{\text{vol}_c}{\text{vol}_{mc}} \cdot \frac{1}{P_{mw}} \tag{11}$$

where $\tilde{t}_R = (t_R/t_{mc})$, or the migration time of the solute relative to that of the micelle, and P_{mw} is the reciprocal of P_{wm}.

By introducing C_{mc}, the molar concentration of the

micelle that is equal to the total analytical surfactant concentration minus the critical micelle concentration (CMC), and Vol_{mc} as the partial molar volume of the micelle, into Eq. (11) and rearranging, we obtain:

$$\tilde{t}_R = 1 + \frac{1}{P_{mw} \, \text{vol}_{mc} \, C_{mc}} \tag{12}$$

Since $(\tilde{t}_R - 1) = k'$ (from Eq. (3)), Eq. (12) rearranges to:

$$k' = \frac{1}{P_{mw} \, \text{vol}_{mc} \, C_{mc}} \tag{13}$$

On the other hand, the capacity factor in N-MEKC is related to P_{mw} by the following expression [1,2]:

$$\tilde{k}' = P_{mw} \text{vol}_{mc} C_{mc} \tag{14}$$

Note that all the terms of Eqs. (13) and (14) are defined similarly except that $k' = (n_{aq}/n_{mc})$ of Eq. (13) is the reciprocal of $\tilde{k}' = (n_{mc}/n_{aq})$ of Eq. (14). Operationally, k' is obtained from the electropherogram according to the relationship $k' = (t_R/t_{mc}) - 1$, while $\tilde{k}'$ is obtained from the relationship [1,2]:

$$\tilde{k}' = \frac{t_R - t_o}{t_o(1 - t_R/t_{mc})} \tag{15}$$

It is to be emphasized that to arrive at Eq. (13), we assumed that EOF=0. This must be kept in mind when trying to extract physico-chemical data from Eq. (13). Any residual EOF will introduce an error in the determination of k' and the parameters derived from it. Residual EOF should not, however, affect separations obtained with RF-MEKC as long as the electroosmotic mobility is only a small fraction of the apparent electrophoretic mobility of the slowest moving analyte.

2.4. Zone broadening effects

The factors contributing to zone broadening in N-MEKC have been qualitatively [34] and quantitatively [32] analyzed. These are broadly subdivided into extracolumn and intracolumn contributions. Extracolumn zone broadening processes in MEKC are the same as those found in capillary zone electrophoresis. These factors can cause zone broadening [32,34,35] but are rendered negligible or minimized by the proper choice of injection tech-

nique and on-column detection. Sepaniak and Cole [34] as well as Terabe et al. [32] subdivided intracolumn zone broadening into five additive contributions, namely [32]:

$$H_{col} = H_1 + H_{mc} + H_{aq} + H_t + H_{ep} \tag{16}$$

where H_{col} is the overall column plate height, and H_1, H_{mc}, H_{aq}, H_t and H_{ep} are plate height contributions generated by longitudinal diffusion (H_1), sorption–desorption kinetics (H_{mc}), intermicelle mass transfer in the aqueous phase (H_{aq}), radial temperature gradient effect on electrophoretic velocity (H_t), and electrophoretic dispersion of the micelles (H_{ep}), respectively.

The temperature gradient effect resulting from Joule heating and the electrophoretic dispersion effect resulting from the electrophoretic movement of the micelle are the same in N-MEKC and RF-MEKC; however, the presence of EOF in N-MEKC and its absence in RF-MEKC results in different expressions for the other three contributions.

The reader is referred to Terabe et al. [32] and references therein for a comprehensive discussion of this subject; however, in this section we will present expressions for the effects that are unique to RF-MEKC and different from N-MEKC.

2.5. Longitudinal diffusion

Solutes in RF-MEKC are subjected to longitudinal diffusion when affixed in the stationary aqueous phase and also when incorporated in the micelle. The latter contribution can be considered as that due to the axial diffusion of the micelles.

Starting with the Einstein equation for diffusion:

$$\sigma^2 = 2Dt \tag{17}$$

where σ^2 is the total distance variance ($\sigma^2_{aq} + \sigma^2_{mc}$) due to axial diffusion, D is the diffusion coefficient, and t is the time period over which diffusion occurred, and realizing that $H_1 = \sigma^2/l$, when l is the injector-to-detector length, we have:

$$H_1 = \frac{2D_{aq}t_{aq}}{l} + \frac{2D_{mc}t_{mc}}{l} \tag{18}$$

Since t_{aq}, the time solute spends in the aqueous phase is equal to the total migration time (t_R) times

the fraction of solute in the aqueous phase; $k'/(1 + k')$, we have:

$$H_1 = \left[2D_{aq}\left(\frac{k'}{1+k'}\right)t_R + 2D_{mc}t_{mc} \right]\frac{1}{l} \qquad (19)$$

Also realizing that $t_R = (1 + k')t_{mc}$ (Eq. (3)) we arrive at:

$$H_1 = [2D_{aq}k't_{mc} + 2D_{mc}t_{mc}]\frac{1}{l} \qquad (20)$$

which rearranges to:

$$H_1 = \frac{2}{v_{mc}}[k'D_{aq} + D_{mc}] \qquad (21)$$

The corresponding expression (Terabe et al.) for H_1 for N-MEKC (Eq. (15) of Ref. [32]) is:

$$H_1 = \frac{2(D_{aq} + \tilde{k}'D_{mc})}{1 + (t_o/t_{mc})\tilde{k}'}\frac{1}{v_{eo}} \qquad (22)$$

where v_{eo} is the electroosmotic velocity. The complexity of Eq. (22) in comparison to Eq. (21) results from the fact that in N-MEKC both phases are mobile while in RF-MEKC the aqueous phase is stationary. Also, unlike RF-MEKC, in which all solutes spend the same length of time in the mobile micellar phase, different solutes spend different lengths of time in each of the two phases in N-MEKC.

2.6. Sorption–desorption kinetics

For a solute (S) to partition between an aqueous phase and a micellar phase, the following equilibria is established:

$$S_{aq} \overset{k_a}{\underset{k_d}{\leftrightarrow}} S_{mc} \qquad (23)$$

where S_{aq} denotes solute in the aqueous phase and S_{mc} denotes solute in the micellar phase, k_a is the association rate constant and k_d is the dissociation rate constant. Giddings [33] derived the contribution H_{mc} for solute partitioning between the mobile and stationary phase in conventional chromatography:

$$H_{mc} = 2R(1-R)\frac{v}{k_d} \qquad (24)$$

where v is the flow velocity of the mobile phase and R is the fraction of solute in the mobile phase.

Following Giddings approach, we derived H_{mc} for RF-MEKC, where the micelle is the mobile phase and obtained the following expression:

$$H_{mc} = \frac{2k'}{(1+k')^2}\frac{v_{mc}}{k_a} \qquad (25)$$

The corresponding expression (Terabe et al.) for N-MEKC is (Eq. (21) of Ref. [32]):

$$H_{mc} = \frac{2(1 - t_o/t_{mc})^2\tilde{k}'}{(1 + (t_o/t_{mc})\tilde{k}')(1 + \tilde{k}')^2}\frac{v_{eo}}{k_d} \qquad (26)$$

According to Terabe et al. [32] the sorption–desorption kinetic contribution to zone broadening is not significant unless the kinetics of the process are very slow. Since desorption rates parallel solubility in water [36], slow kinetics are expected for hydrophobic solutes. For these types of solutes, RF-MEKC offers the advantage of short analysis time (small k'). Also, in Eq. (23) for RF-MEKC, we are dividing by k_a, the sorption rate constant that is greater for hydrophobic solutes than k_d, the desorption rate constant, which appears in Eq. (24) for N-MEKC.

2.7. Intermicelle diffusion

Intermicelle resistance to mass transfer was discussed qualitatively by Sepaniak and Cole [34] and quantitatively by Terabe et al. [32]. While Cole and Sepaniak considered this contribution to zone broadening to be substantial, Terabe et al. argued that it is not significant. Terabe et al. used the random walk theory to derive an expression for the plate height contribution of intermicelle diffusion in the aqueous phase for N-MEKC (Eq. (25) of Ref. [32]):

$$H_{aq} = \left(\frac{\tilde{k}'}{1 + \tilde{k}'}\right)^2\frac{(1 - t_o/t_{mc})^2}{1 + (t_o/t_{mc})\tilde{k}'}\frac{d^2v_{eo}}{4D_{aq}} \qquad (27)$$

where d is half the intermicelle distance and the rest of the terms are as defined earlier. We followed the same formalism employed by Terabe et al. [32] to arrive at the corresponding expression for RF-MEKC:

$$H_{aq} = \frac{d^2}{4D_{aq}}\frac{k'}{(1+k')^2}v_{mc} \qquad (28)$$

3. Column and carrier attributes for reversed-flow micellar electrokinetic chromatography

The RF-EKC column must be coated with a neutral material, preferably polymeric, that completely covers the silanol groups on the fused-silica surface. The coating material must be chemically stable over a wide range of pH so as not to be stripped by chemical degradation. The coating must be anchored to the surface so as not to be extruded from the column by mechanical forces. Finally, the coating must be non-adsorptive to the carriers and the analytes.

In order for a substance to be potentially useful as a carrier in RF-MEKC, it must meet these minimum requirements.

(a) The substance must be charged, and it must retain its charge, whether positive or negative, over a wide pH range; neutral solutes will not migrate through the column.

(b) The substance must not adsorb to the column coating; otherwise, the charged surface would create electroosmotic flow in a direction opposite to the electrophoretic migration of the carrier.

(c) The substance must be selective, i.e., it should exhibit differential complexation, for a broad range of solutes.

(d) Solute–carrier complexation kinetics must be rapid (otherwise, the advantage of high column efficiency is lost to slow sorption–desorption zone broadening mechanisms.

In contrast, selectors in N-MEKC do not necessarily have to be charged or be non-adsorbing to the column surface, but they must also meet the third and fourth conditions.

Fig. 2 shows the separation of three amino acids on four different carriers. The runs on each carrier were performed using the same 10% linear polyacrylamide-coated column under the same experimental conditions except that the voltage was reversed for CTAB. The fact that the peaks were eluted in a short time indicates that each of these carriers can potentially be used in RF-MEKC mode with this particular coated column. Although Fig. 2 shows differences between the different carriers for this set of solutes, the experiment was not intended as a comparative study and for that reason the separation conditions were not optimized for each carrier.
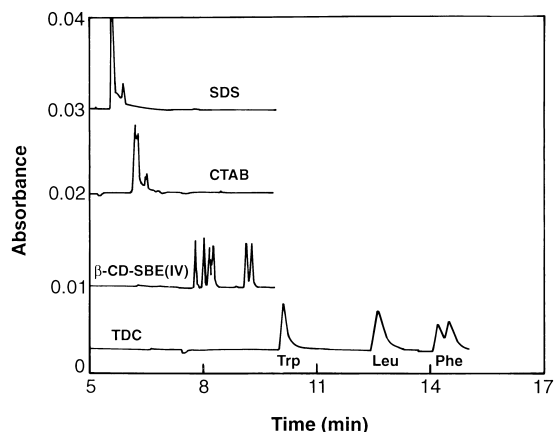

Fig. 2. Electrophoretic profile of three dansylated amino acids on different carriers. Column, 10% linear polyacrylamide-coated fused-silica; column, 47 cm×50 μm I.D.; instrument, Beckman Model P/ACE 5500; voltage, −15 kV; buffer, 10 mM phosphate + 1% carrier, pH 3.0. SDS, sodium dodecyl sulfate; CTAB, hexadecyltrimethylammonium bromide; β-CD-SBE(IV), tetrakis[6-O-(sulfobutyl)]-β-cyclodextrin, sodium salt; TDC, taurodeoxycholic acid, sodium salt.

It is known that CTAB adsorbs to bare fused-silica surfaces and reverses EOF [34]. CTAB similarly adsorbs to C_8-coated columns [37] which renders this particular combination of column and carrier unsuitable for RF-MEKC applications. In contrast, our in-house 10% polyacrylamide-coated columns are non-adsorptive to CTAB or the other carriers shown in Fig. 2 and are stable over extended periods of time as will be illustrated in the next section.

4. Determination of aqueous micelle partition coefficients

The partition coefficients of solutes in micelle-aqueous systems are a useful indicator in the research areas of oil recovery, micellar catalysis, enzymes, and biological membranes [38]. Various techniques have been used to measure this indicator, including partitioning in octanol–micellar solutions [39] and micellar chromatography [40,41]. The octanol–micellar solution system [39] is limited because the aqueous phase is not a pure micellar system; some octanol is solubilized in water and in the micelle as a co-component. The micellar chromatography method [40,41] suffers from interrelated, multiple equilibria and solute adsorption on active

sites on the chromatographic stationary phase that might adversely affect the measurement.

Partition coefficients can, alternatively, be determined by N-MEKC according to Eq. (14). This method, however, requires accurate determination of the migration times of the electroosmotic flow (EOF) marker and the micelle migration marker. The EOF marker must: (1) be neutral; (2) have UV absorbance; and (3) not partition in the micelle. Unfortunately, no such compound is available, and methanol is often used for this purpose [2,7]. The marker for micelle migration should, on the other hand, be totally partitioned into the micelle; Sudan III is often used for this purpose. Unfortunately, measurement of the migration time of Sudan III is not straightforward. Its serious problems include inaccurate determination of the peak position because of low solubility, resulting in poor detection and the broadness of the peak caused by long migration times [2,7]. By applying RF-MEKC for this determination according to Eq. (12) and Eq. (13), the advantages of MEKC are retained while the complications related to the measurements of the migration time of Sudan III and EOF are avoided [15]. In comparison with other published methods [2,7,39–41], RF-MEKC is, theoretically and experimentally, the most straightforward for this application because it only requires the measurement of the migration times of the solutes of interest and Sudan III in buffers with different concentrations of micelle. The method was tested using 10% neutral polyacrylamide-coated columns, SDS surfactant and benzene, toluene, naphthalene, and phenol solutes [15]. With the detector at the positive electrode, Sudan III is the first to elute, followed by other solutes which elute in order of decreasing hydrophobicity. The partition coefficients are easily obtained from the slopes of plots of solute t_R values versus micelle concentration. This is illustrated in Fig. 3. The fact that the plots in Fig. 3 are linear with correlation coefficients >0.99 and that the plots converge to an intercept near unity is a strong indication of the validity of this theoretical treatment and experimental arrangement. If SDS adsorbs on the coating, the solutes will not migrate towards the positive electrode, and if EOF was present, the plots in Fig. 3 will not extrapolate to unity. The experiments were conducted at pH 7, and no signs of column deterioration were observed. There appears
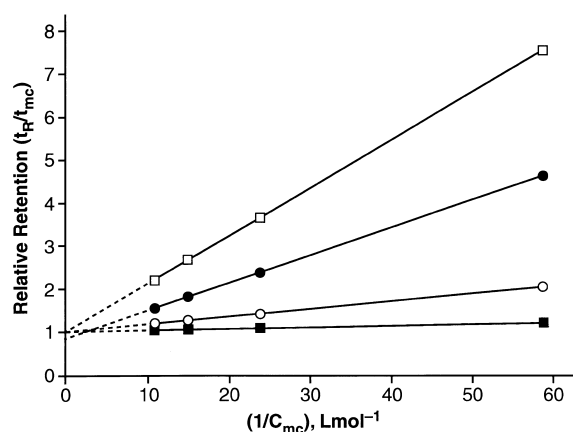

Fig. 3. Plots of relative migration against reciprocal micelle concentration in 5 mM phosphate buffer at pH 7.0. Solutes: ■, naphthalene; ○, toluene; ●, benzene; and □, phenol. $C_{mc} = ([SDS] - CMC)$. From Ref. [15].

to be a reasonable degree of concern about the stability of polyacrylamide-coated columns at basic pH and high operating voltage [16,42]. Fortunately, these extreme conditions are not necessary for RF-EKC applications. The separation of neutral hydrophobic solutes can be conveniently conducted at low pH with buffers of low ionic strength. Under these conditions the polyacrylamide coating is stable over an extended period time [16].

5. Applications of reversed-flow micellar electrokinetic chromatography

Fig. 4 is an illustration of solute elution order in RF-MEKC. Sudan III (the micelle marker) is eluted first, followed by solutes in order of decreasing hydrophobicity. The separation was carried out at pH 4.2.

N-MEKC separations are usually conducted in neutral and acidic buffers. Separations at low pH are impractical because the electroosmotic flow is slowed down and, at some point (about pH 5), it is counterbalanced by the micelle electrophoretic mobility in the opposite direction, resulting in excessively long migration times. However, this is not the case in RF-MEKC where it is possible, and in certain applications preferable, to conduct the separation at acidic pH. This is clearly illustrated in Fig. 5, which
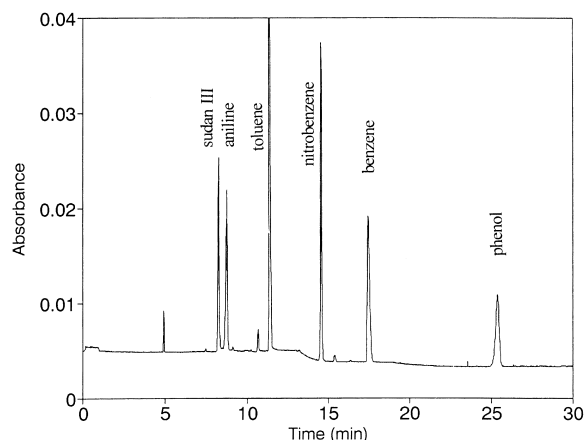
Fig. 4. Order of migration versus solute hydrophobicity in RF-MEKC mode. Column, 10% linear polyacrylamide-coated fused-silica. Column dimensions: L_{total} = 57 cm; $L_{detector}$ = 50 cm; I.D. = 75 μm. Buffer, 10 mM acetate and 50 mM SDS, pH 4.2; applied voltage, −20 kV; current, 30 μA; detection, 214 nm. From Ref. [16].

shows the separation of a set of 15 dansylated amino acids at pH 4.2.

The order of migration can be explained largely in terms of differences in solute hydrophobicity. The positively charged and the more hydrophobic solutes are eluted first, and the negatively charged and least hydrophobic solutes are the last to elute. The ability
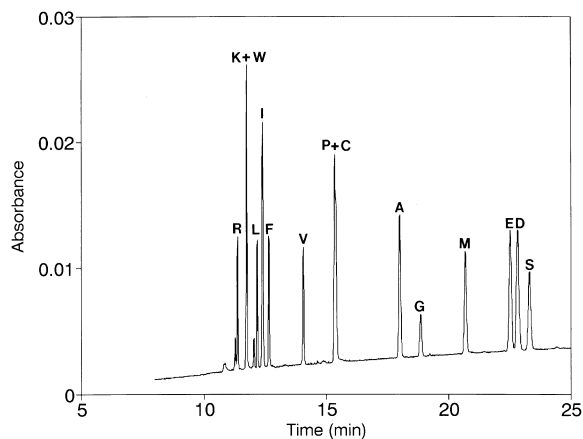

Fig. 5. RF-MEKC separation of 15 dansyl amino acids. Column, as in Fig. 4. Column dimensions: L_{total} = 57 cm; $L_{detector}$ = 50 cm; I.D. = 75 μm. Buffer, 25 mM acetate and 25 mM SDS, pH 4.2; applied voltage, −15 kV; current, 27 μA; detection, 214 nm; solute concentration, 10 μg/ml each. The peaks are identified with one-letter abbreviations. From Ref. [16].

to conduct the separations at low pH allows for subtle manipulation of the selectivity by taking advantage of differences in the ionization constants of the carboxylic groups. In a recent review of MEKC methods for the separation of amino acids [43], only one out of 17 methods listed is conducted at acidic pH. This was reported by Waldron et al. [44], who compared the separation of DABTH–amino acids at pH 2.5 with separations at higher pH, and concluded that the separation of amino acids at low pH in bare-silica is not ideal because of long migration times and excessive peak broadening.

MEKC is particularly suited to the analysis of polycyclic aromatic hydrocarbons (PAH), and their derivatives and metabolites from biological samples, because they are present at trace amounts in small volumes. Fig. 6 gives the separation of three polycyclic hydrocarbon compounds using a γ-CD-containing buffer. Laser-induced fluorescence was used for detection in this example to illustrate a unique advantage of RF-MEKC over N-MEKC, namely the capability of producing relatively sharp peaks (*N* for anthracene = 170 000 plates/m) for extremely hydrophobic solutes, with a relatively low SDS concentration. Extremely hydrophobic solutes, including
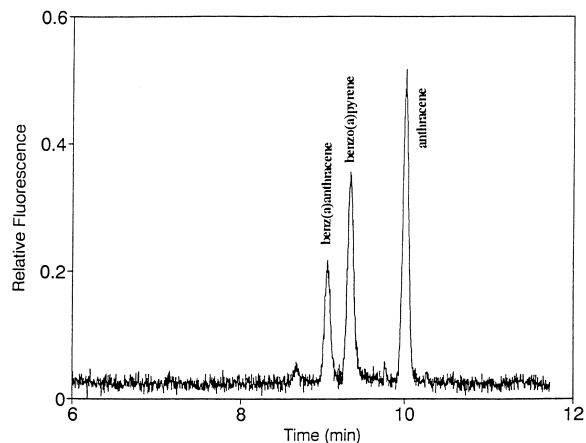

Fig. 6. Electropherogram of the separation of three polycyclic aromatic hydrocarbons using γ-CD shape selector. Column, as in Fig. 4. Column dimensions: L_{total} = 60 cm; $L_{detector}$ = 53 cm; I.D. = 75 μm. Buffer, 10 mM phosphate, 25 mM SDS, 15 mM γ-CD, pH 2.8; applied voltage, −18 kV; current, 39 μA; detection, LIF (ex. 248, em. 400±20 nm); solute concentration, 0.1 μg/ml. Concentration limit of detection for benzo[a]pyrene [B(*a*)P] in 5 ppb. From Ref. [16].

those of Fig. 6, have been separated by N-MEKC only by using a high concentration of SDS and other additives such as organic solvents, urea and cyclo-dextrins, among others [45–48]. However, as pointed out by Yan et al. [49], the use of large amounts of SDS and other additives results in excessive fluores-cence background and reduced sensitivity. The order of migration in Fig. 6 does not seem to strictly follow hydrophobicity because benzo[a]pyrene (five-membered ring) is larger and more hydrophobic than benz[a]anthracene (four-membered ring). This rever-sal of migration may, however, be explained on the basis that benzo[a]pyrene forms a stronger inclusion complex with the γ-CD cavity and consequently resides longer than benz[a]anthracene in the im-mobile phase. The separation of geometric isomers of polycyclic aromatic hydrocarbons is hard to achieve without the use of shape selectors as addi-tives to the buffer. Copper and Sepaniak [47] used γ-CD and Szolar et al. [48] used a mixture of neutral and anionic β-cyclodextrins for the separation of benzo[e]pyrene from benzo[a]pyrene. We achieved this very difficult separation (Fig. 7) by using γ-CD (unpublished results). As expected, the order of migration is the reverse of that reported by Copper and Sepaniak [47]. Fig. 7 also shows the simulta-neous separation of three- and four-membered PAH isomers.

An interesting application of RF-MEKC separa-tion of clinically relevant steroids was reported by Abubaker et al. [14]. The structures of the steroids are given in Fig. 8 and the separation is shown in Fig. 9. The authors used neutral eCAP (Beckman) capillaries at pH 6.0 with a buffer containing 20% acetonitrile. The addition of an organic modifier to the buffer enhanced the solubility of the hydrophobic steroids in the aqueous phase and improved the separation of the steroids.

The effect of the addition of organic solvents to the buffer in capillary zone electrophoresis is well studied [50]. Typically, the addition results in an increase in buffer viscosity and a decrease in electro-osmotic flow. In MEKC, the situation is more complicated because of the effect of the additive on the micelle and on solute partitioning between the micelle and the aqueous phases. In general, the addition of organic solvents renders the aqueous phase more hospitable to hydrophobic solutes. This
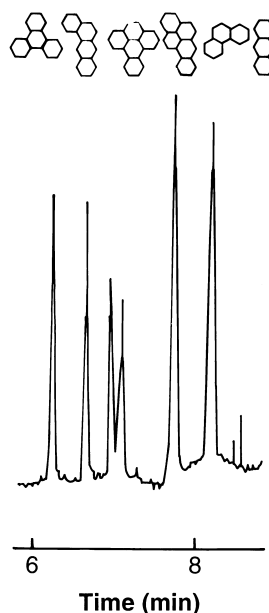

Fig. 7. RF-MEKC separation of PAH positional isomers using a γ-CD shape selector. Column, 10% linear polyacrylamide-coated fused-silica. Column dimensions: $L_{total} = 47$ cm; $L_{detector} = 40$ cm. Buffer, 10 mM phosphate 1% SDS + 2% γ-CD, pH 3.0; applied voltage, −15 kV. Unpublished results.

should speed up the migration of solutes in N-MEKC. However, since electroosmotic flow is slowed down to a larger extent, the net effect of these two opposing factors is a decrease in migration time with an increase in the percentage of organic modifier. The situation is different in RF-MEKC because the effect of electroosmotic flow is negli-gible. Here, the addition of organic solvents favors partitioning of the hydrophobic solutes in the im-mobile aqueous phase, resulting in longer solute migration times and a higher degree of selectivity. This is clearly illustrated in Fig. 10, which shows the effect of the addition of acetonitrile (ACN) on the separation of aflatoxins B_1, B_2, G_1 and G_2. A significant improvement in the resolution is achieved with the addition of a relatively small amount of ACN (4%).

RF-MEKC was also utilized by McLaughlin et al. [51] for the analysis of linear alkyl benzene sul-fonates (LAS). The separation was conducted using a hydrophilic coated fused-silica column, Fig. 11.
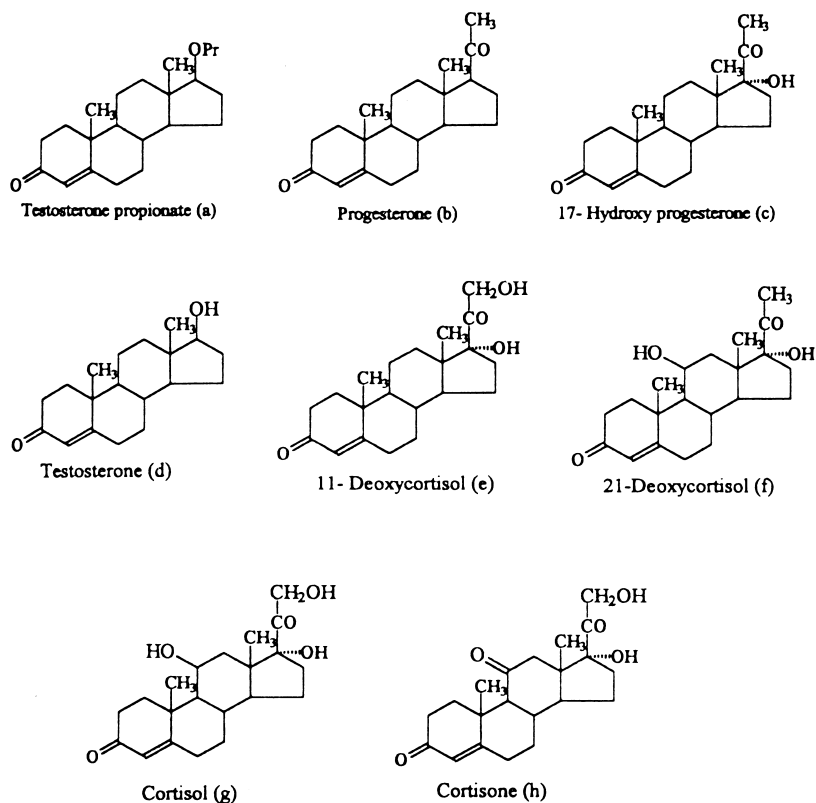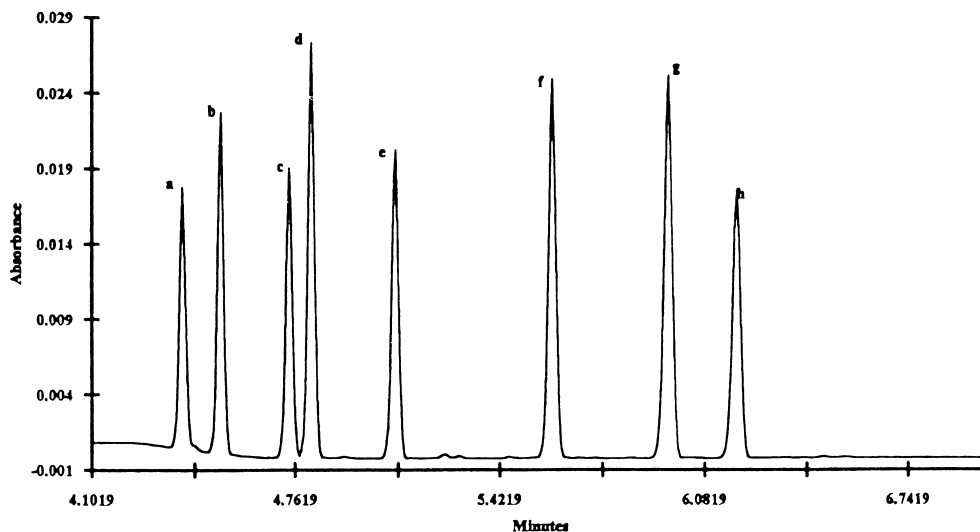
Fig. 8. Structures of steroids. From Ref. [14].

Fig. 9. Separation of steroids on neutral eCAP capillary. Buffer, 100 mmol/1 SDS, 20% (v/v) acetonitrile and 20 mmol/1 ME, pH 6; voltage applied, 15 kV; temperature, 16°C. Peaks are identified in Fig. 8. From Ref. [14].
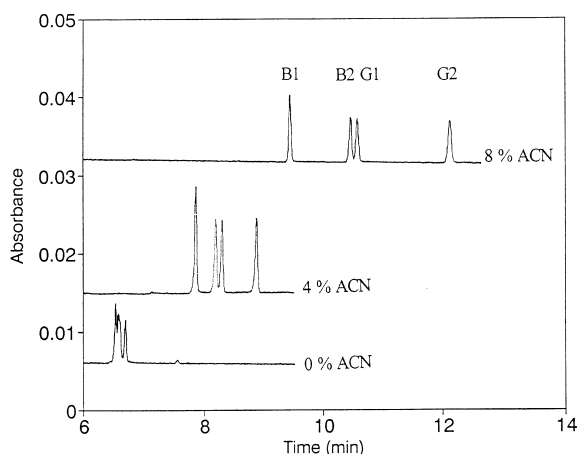
Fig. 10. Effect of acetonitrile concentration on the RF-MEKC separation of aflatoxins. Column, as in Fig. 4. Column dimensions: $L_{total}=47$ cm; $L_{detector}=40$ cm; I.D.=75 μm. Buffer, 10 mM phosphate, 50 mM SDS, pH 3.0; applied voltage, −15 kV; current, 25 μA; detection, 214 nm; solute concentration, 2–5 μg/ml. From Ref. [16].

6. Applications of reversed-flow cyclodextrin-modified electrokinetic chromatography

The first use of cyclodextrins (CDs) in capillary electrophoresis (CE) was reported by Terabe et al. [3,52] for the separation of positional isomers by CD-modified MEKC. Later, Guttman et al. [53] reported chiral separation via CDS incorporated within polyacrylamide gel-filled columns and Fanali [54] obtained chiral separation in CE by adding CD to the running buffer. Since then, much progress has been achieved and a large number of enantiomers have been separated as detailed in recent reviews [55–57]. The first use of charged CDS for chiral separation was reported by Terabe [3], who utilized 6[(3-aminoethyl)amino]-6-deoxy-β-CD for the separation of dansylated amino acids. Earlier, Terabe et al. [52] used carboxymethyl-β-cyclodextrin (a charged CD derivative) for the separation of positional isomers. Schmitt and Engelhardt [58] later used the same CD for interesting chiral separations. Sulfobutyl ether β-cyclodextrin (β-CD-SBE(IV)), a polyanionic sulfobutyl ether derivative of β-CD, was first introduced by Tait et al. [59] and shown to produce superior enantioseparations to those achieved with neutral selectors such as β-CD and its neutral derivatives. To date, this chiral selector has been used by several research groups [17,60–63]. Other sulfated cyclodextrins also were reported to affect enantiomeric separations [65].
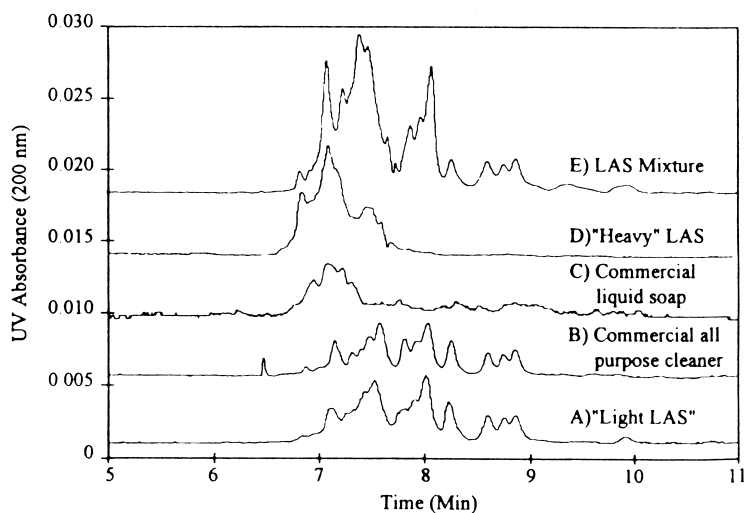
Charged enantiomers can be separated by N-EKC

Fig. 11. Separation of LAS samples and commercial cleaners using MEKC, coated capillary, and reversed polarity. Capillary, 42.8 cm (L_d)×50 cm (L_t)×75 μm I.D. hydrophilic coated FS; buffer, 8 mM CAPS–20 mM SDS–40 mM sodium octylsulfonate in water–acetonitrile (80:20, v/v); voltage, 25 kV; temperature, 22.5°C; detection, UV (224 nm); injection, pressure (5 s, 3.45 kPa). Concentration: (A,D,E) 2 mg/ml total in water; (B,C) 100 mg/ml commercial all purpose cleaner and liquid soap total in water. Peaks: (A) light LAS mixture; (B) commercial all purpose cleaner; (C) commercial liquid soap; (D) Heavy LAS mixture; (E) LAS formulation A235. From Ref. [51].

using a background electrolyte containing native cyclodextrins or their neutral derivatives [55–57]. Neutral enantiomers, on the other hand, require the use of a charged CD to effect the separation [63]. Most EKC separations were conducted in bare-silica columns [62,63], but coated columns with modified EOF were also used by several groups [17,58,64–67]. Both positively charged [3,67] and negatively charged [17,58–65] CDs have been utilized for interesting separations. The separation principles of cyclodextrin electrokinetic chromatography have been most recently reviewed by Tanaka et al. [68].

In what follows we present examples of chiral and positional isomer separations conducted in the RF-MEKC mode with β-CD-SBE(IV), a negatively charged cyclodextrin.

Fig. 12 shows the enantiomeric separation of a racemic mixture of dansylated amino acids [17]. The electropherogram shown in the figure was obtained in a coated column using a concentration of 1% β-CD-SBE (IV) at pH 3.1. The dansylated amino acids are either neutral or weakly negative at this pH, which facilitates their approach to the cyclodextrin hydrophobic interior. The more hydrophobic residues are expected to form stronger complexes with the CD. This trend is observed in the figure, where
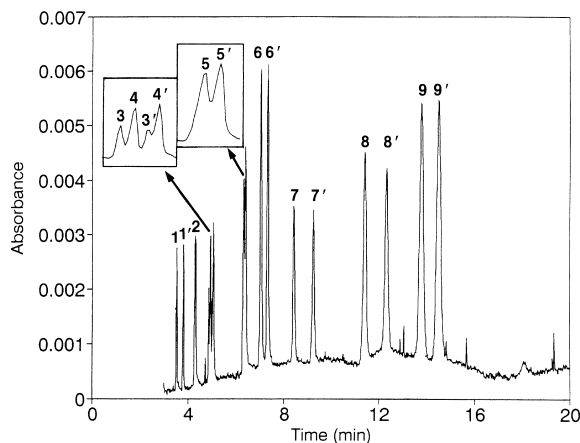

Fig. 12. RF-CD-EKC separation of a racemic mixture of dansylated amino acid at pH 3.1. Column, 10% linear poly-acrylamide-coated fused-silica. Column dimensions: $L_{total}=27$ cm; $L_{detector}=20$ cm; I.D.=50 μm. Instrument, Beckman Model P/ACE System 5500; buffer, 10 mM phosphate and 1% β-CD-SBE(IV), pH 3.1; applied voltage, −10 kV; current, 20 μA; detection, 214 nm. Solutes: 1, Trp; 2, Phe; 3, nor-Leu; 4, Leu; 5, Met; 6, nor-Val; 7, Val; 8, Glu and 9, Asp. From Ref. [17].
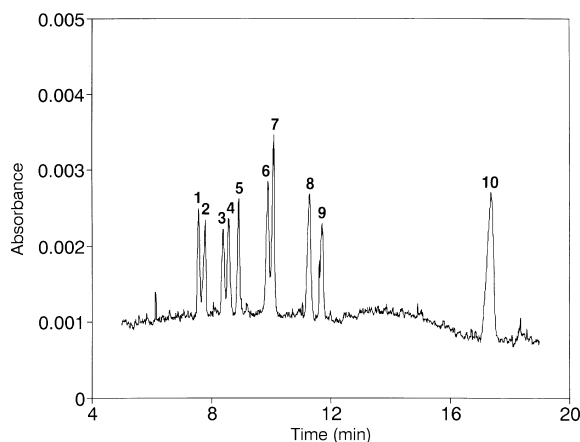

Fig. 13. Separation of chlorophenols and phenol by RF-CD-EKC at pH 3.1. Column dimensions: $L_{total}=37$ cm; $L_{detector}=30$ cm. Applied voltage, −10 kV; current, 14 μm; other experimental conditions, As in Fig. 12; solute concentration, 1–5 μg/ml each. Solutes: 1, 2,3,5-TCP; 2, 2,4,5-TCP; 3, 2,4,6-TCP; 4, 2,3,6-TCP; 5, 2,5-DCP; 6, 4-CP; 7, 3-CP; 8, 2,6-DCP; 9, 2-CP; 10, phenol. From Ref. [17].

dansylated Trp is the first to elute because of its strong association with the CD, and the more polar solutes are retained longer. In all cases, the L-isomer migrated faster than the D-isomer as confirmed by spiking. The excellent separation presented in Fig. 12 is achieved because the dansylated amino acids are almost fully protonated at pH 3.1 and, therefore, better accommodated inside the CD cavity. The relatively short analysis time is obtained because the column is coated and, therefore, there is no EOF opposing the migrating of solutes towards the detector. The separation of positional isomers is illustrated in Fig. 13, which gives the separation of nine chlorinated phenols and phenol at pH 3.1 where the solutes are neutral and their selective interaction with the interior of the cyclodextrin maximized.

7. Coated versus bare-silica columns in reversed-flow electrokinetic chromatography

Under favorable conditions, RF-EKC can be conducted in bare-silica columns at low pH where EOF is slower than solute apparent electrophoretic mobility [11,17,65]. Fig. 14 compares the separation obtained at pH 3.1 in a coated column versus a bare-
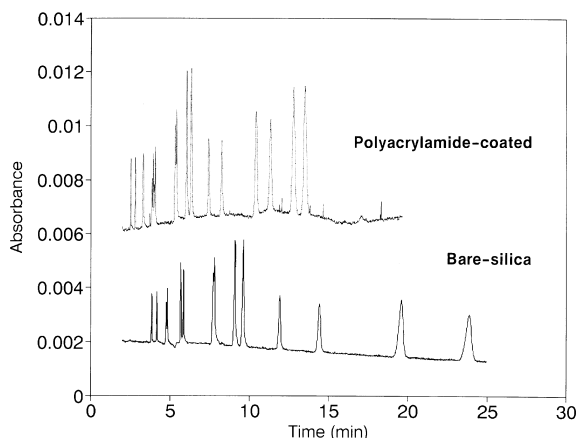
Fig. 14. Comparison of bare-silica and polyacrylamide-coated capillaries for RF-CD-EKC chiral separation of amino acids at pH 3.1. Bare-silica column dimensions: $L_{total} = 27$ cm; $L_{detector} = 20$ cm. Other experimental parameters, as in Fig. 12. From Ref. [17].

silica column using experimental conditions as in Fig. 12. The order of migration is the same in both columns, but solutes migrate slower in the bare-silica column because of the presence of EOF. Solutes that are strongly associated with the CD (fast migrating) are relatively less affected by EOF than solutes that are weakly associated with the CD. The advantages of the coated-column are shorter analysis time and better migration reproducibility. It has been observed that bare-silica columns suffer from instability and irreproducibility at acidic pH below 5.0 [59,66,69]. Furthermore, weakly associated solutes that have long migration times in coated-columns may not arrive at the detector if their forward mobility is counterbalanced by EOF in bare-silica columns. One advantage that is often cited for bare-silica columns is that EOF can be manipulated to control migration time and separation, but the same effect also could be achieved in coated columns by varying the column length.

8. Effect of carrier concentration

A very subtle difference exists between RF-EKC on the one hand and N-EKC and conventional chromatography on the other. In RF-EKC, solute residence time in the selective (separative) carrier phase (t_{mc} in Eq. (1)) is the same regardless of the amount (or concentration) of the selective carrier phase in the column. Different solutes have different migration times because their residence times in the non-selective aqueous phase are different. In contrast, solutes in N-EKC and conventional chromatography have different migration times because they reside for different periods of time in the selective phase. As a consequence of this, selectivity is enhanced with increasing concentration of the selective phase in chromatography and N-EKC, while the reverse is true for RF-EKC. The dependence of selectivity in RF-EKC on carrier concentration can be rationalized according to the resolution equation (Eq. (7)). The separation factor (α) is a thermodynamic quantity that is not affected by changes in carrier concentration; however, k', and consequently, the retention term (the third term in Eq. (7)) increase with decreasing carrier concentration, resulting in improved selectivity. This is clearly illustrated in Fig. 15 which gives electropherograms for the separation of selected dansylated amino acids at different concentrations of β-CD-SBE (IV).

The mathematical relationship between the retention term ($k'_2 / 1 + k'_2$) and carrier concentration is simply derived from Eq. (13) [17]:

$$\frac{k'_2}{k'_2 + 1} = \frac{1}{1 + C[K_2 + 1]} \tag{29}$$

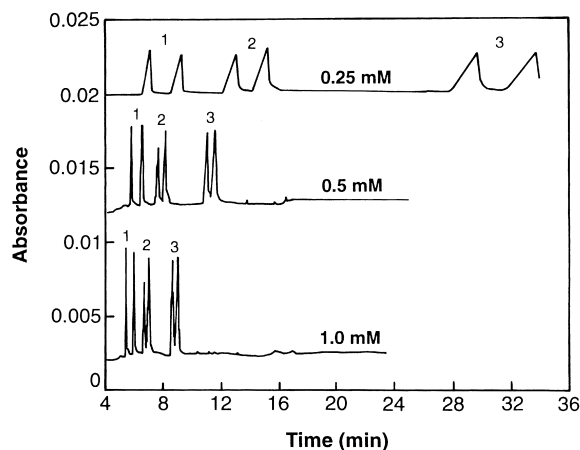where K_2 is the solute–carrier binding constant. At

Fig. 15. Effect of β-CD-SBE(IV) concentration on migration time of selected dansylated amino acids in RF-CD-EKC. Experimental conditions, as in Fig. 12. Solutes: 1, Trp; 2, Phe; 3, Leu. Unpublished results.
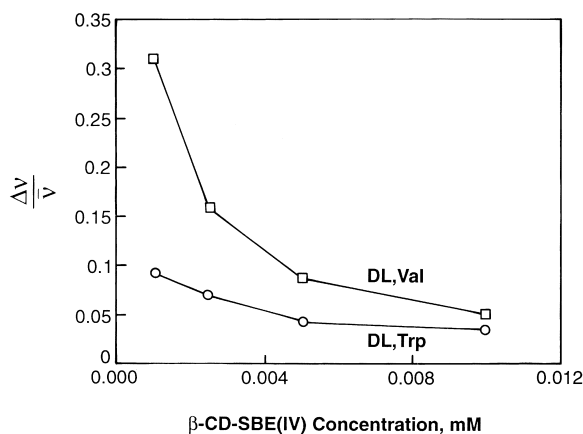
Fig. 16. Dependence of selectivity on carrier concentration in RF-EKC. (Unpublished results).

Fig. 17. Experimentally determined apparent mobility difference (10^{-4} cm^2/V s) for different β-blockers at different MeBCD concentrations. ○, propranolol; ■, atenolol; ●, metoprolol; □, oxprenolol. From Ref. [27].

constant K_2, selectivity increases continuously with decreasing carrier concentration until at a sufficiently large k_2' value, $(k_2'/k_2'+1)$ approaches 1 and thus the term plays no further part in improving the separation. This is clearly illustrated in Fig. 16 with data derived from Fig. 15.

On the other hand, the dependence of selectivity on carrier concentration in N-EKC was theoretically developed and experimentally verified by Wren and Rowe [27,28]. The theory was developed for chiral separations, but it is equally applicable to all modes of N-EKC. According to this theory, the apparent mobility difference for a pair of solutes increases with increasing carrier concentration, plateaus at a given concentration, then decrease with further increase in carrier concentration. The theory also predicts that the optimum carrier concentration is different for different pairs of solutes depending on the strength of their binding to the carrier. These trends were experimentally verified for a set of solutes as shown in Fig. 17. Clearly, the trend presented in Fig. 17 for N-EKC is significantly different from that shown in Fig. 16 for RF-EKC. If one considers that the underlying principles for both modes of EKC are the same, except for EOF, one concludes that the maxima in the selectivity versus carrier concentration curves in N-EKC are an artifact of the presence of EOF. It is interesting to note that this fact has been observed by Terabe et al. (see Fig. 8 of Ref. [20]).
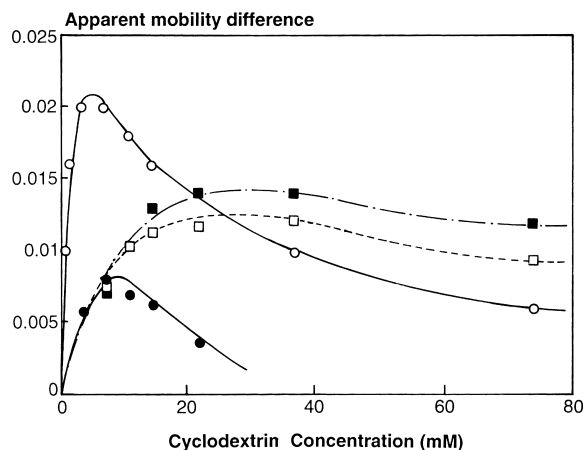
9. Conclusion

RF-EKC shares the same principle of separation as N-EKC, but there are several significant differences that were highlighted in this review. RF-MEKC provides excellent separations for hydrophobic solutes in short analysis time. The elution order correlates with decreasing hydrophobicity. RF-CDEKC provides excellent chiral and positional isomer separations of hydrophobic compounds. In contrast to N-EKC, solute elution order correlates with decreasing solute hydrophobicity and solute migration time increases with decreasing carrier concentration.

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