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Selectivity manipulation in micellar electrokinetic chromatography*

SHIGERU TERABE

Faculty of Science, Himeji Institute of Technology, Kamigori, Hyogo 678-12, Japan

Abstract: Micellar electrokinetic chromatography (MEKC) permits the separation of electrically neutral analytes by chromatographic principles in a capillary electrophoresis system. The most effective way to obtain high resolution in MEKC is to increase the separation factor, as in conventional chromatography. The separation factor in MEKC depends on the molecular structure of the micelle and hence on the surfactant used, the pH of solution, and the nature of any additives to the micellar solution. The hydrophilic moieties of surfactant molecules generally affect selectivity more than do the hydrophobic moieties. Chiral surfactants enable the enantiomeric separation of mixtures of chiral solutes to be achieved. Mixed micelles consisting of ionic and nonionic surfactants display different selectivity from that of single ionic micelles. Additives such as cyclodextrins, ion-pair reagents, urea, organic solvents and metals can also serve as useful modifiers of the micellar solution for improving separation. In particular, cyclodextrins are useful for the separation of aromatic isomers and enantiomers. A general introductory guide to the design of successful separations by MEKC is proposed, based primarily on the author's work.

Keywords: *Micellar electrokinetic chromatography (MEKC); separation factor α in MEKC; mixed micelle; additives to micellar solutions; enantiomeric separation; steroids; optimization in MEKC; surfactants; cyclodextrins.*

Introduction

Micellar electrokinetic chromatography (MEKC) is a mode of capillary electrophoresis that enables the separation of electrically neutral analytes by electrophoresis in a manner which is analogous to the principles of chromatography [1–3]. An ionic micellar solution is used in MEKC, by contrast with the simple buffer solution conventionally used in capillary zone electrophoresis (CZE). The micelle migrates at a different velocity from that of the surrounding aqueous phase, because the micelle is subject to the process of electrophoresis as well as to electroosmosis.

An analyte injected into the micellar solution is distributed between the micelle and the surrounding aqueous phase. The distribution coefficient of the solute is directly related to its migration velocity, v_R, which has an intermediate value between that of the micelle migrating at a lower linear velocity, v_{mc}, and the faster electroosmotic velocity of the surrounding aqueous phase, v_{eo}, as discussed below.

When an anionic surfactant in solution above pH 6 is used in a fused silica capillary, the electroosmosis of the bulk solution (with intrinsic mobility $+\mu_{eo}$, flowing from positive to negative) is greater than the electrophoretic mobility of the negatively charged anionic micelle ($-\mu_{ep,mc}$, migrating from negative to positive). Thus the net mobility of the micelle, μ_{mc}, becomes:

$$\mu_{mc} = \mu_{eo} + (-\mu_{ep,mc}). \quad (1)$$

The ionic mobility, μ, is directly related to the linear ionic velocity, v, in a constant electrical field, E (generated by voltage V applied across a capillary of length L), by the well-known formula:

$$v = \mu \cdot E = \mu \cdot V/L. \quad (2)$$

Therefore in a fused silica capillary above pH 6, when the relationship between the linear electroosmotic velocity, v_{eo}, and the electrophoretic velocity of the micelle, $v_{ep,mc}$, is:

$$|v_{eo}| > |v_{ep,mc}| \quad (3)$$

the micelle will migrate in the same direction as the electroosmotic flow, but at a lower net velocity, $|v_{mc}|$:

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$$|v_{mc}| = |v_{eo}| - |v_{ep,mc}|. \quad (4)$$

Thus, for an electrically neutral analyte, if it is completely excluded from the micelle it migrates at the fastest velocity, v_{eo}, while if it is totally incorporated into the micelle it migrates at the slowest velocity, v_{mc}, as indicated in Fig. 1(A). A solute distributed between the micellar and the aqueous phase will migrate at an intermediate velocity, v_R, such that $v_{eo} > v_R > v_{mc}$, for which the corresponding migration times will be $t_0 < t_R < t_{mc}$. This is precisely analogous to the concept of separation and retention time in chromatography, as illustrated in Fig. 1(B). In MEKC the chromatogram is obtained under these conditions by detecting the bands as they migrate past a defined observation zone near the end of the capillary.

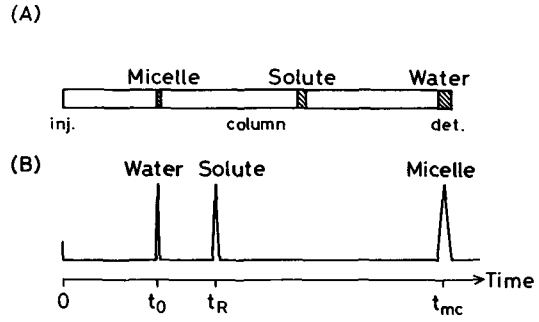

Figure 1
Diagrammatic representation of the principles of micellar electrokinetic chromatography (MEKC) (reprinted with permission from *Anal. Chem.* [2]). (A) Schematic diagram of zone migration in MEKC; (B) illustrative chromatogram. Key: inj., point of sample introduction into capillary column; det., zone of detection on column; column, capillary filled with aqueous background electrolyte; t_0, the electroosmotic migration (or 'break-through') time, corresponding to the minimum migration time for an analyte (e.g. water) that is excluded from the micelle; t_R, migration time for a solute distributed between micellar and aqueous phases; t_{mc}, micelle migration time.

In MEKC the capacity factor, k', is defined as:

$$k' = \frac{n_{mc}}{n_{aq}}, \quad (5)$$

where n_{mc} is the number of analyte molecules incorporated into the micelle and n_{aq} represents the number of free molecules in the aqueous phase. As indicated in outline below [2], the capacity factor can be directly related to the migration time of: the analyte, t_R; the aqueous phase or bulk solution, t_0; and the micellar phase, t_{mc}.

The *free fraction* of molecules in the aqueous phase, i.e. the so-called 'retention ratio', R_r, may be defined as:

$$R_r = \frac{n_{aq}}{n_{aq} + n_{mc}} = \frac{1}{1 + k'}. \quad (6)$$

Thus, the net linear velocity, v_R, of an analyte can be expressed as the sum of the contributions from electroosmosis and micellar migration:

$$v_R = R_r \cdot v_{eo} + (1 - R_r)v_{mc}. \quad (7)$$

Given the inverse relationship between linear velocity and migration time noted above, combination of (6) with (7) readily yields:

$$t_R = \frac{1 + k'}{1 + (t_0/t_{mc})k'} \cdot t_0 \equiv \quad (8)$$

$$(1 + k')t_0 \cdot \frac{1}{1 + (t_0/t_{mc})k'}$$

or its equivalent:

$$k' = \frac{t_R - t_0}{t_0 (1 - t_R/t_{mc})} \equiv \quad (9)$$

$$\frac{t_R - t_0}{t_0} \cdot \frac{1}{1 - t_R/t_{mc}}.$$

These equations correspond to the conventional formulae in chromatography, with an additional term reflecting the fact that in MEKC the range of migration times is restricted to the window between t_0 and t_{mc} for electrically neutral analytes. If the micellar phase were stationary, with $t_{mc} = \infty$, these additional terms would reduce to unity, giving the relationships found in classical chromatography. The treatment is rather more complex for ionic solutes [4, 5] and it is not considered further here, since the primary purpose of the present discussion is to consider the manipulation of selectivity, rather than the strict description of the phenomena involved.

The resolution equation in MEKC for neutral solutes migrating closely together as peaks of comparable efficiency, N, is similar to that in conventional chromatography [2]:

$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{\alpha - 1}{\alpha} \cdot \frac{k_2'}{1 + k_2'} \cdot \frac{1 - t_0/t_{mc}}{1 + (t_0/t_{mc})k_1'}, \quad (10)$$

where R_s is the resolution, N the performance in terms of plate number and α is the separation factor, which is defined classically as the ratio k_2'/k_1'. Equation (10) differs from that for conventional chromatography by virtue of the last term on the right-hand side, which arises from the limited migration-time window. Thus for a truly *stationary* phase, where $t_{mc} = \infty$, this equation reduces to that for classical chromatography.

Equation (10) suggests that the higher the plate number and the higher the separation factor, the higher the resolution. The optimum value of the capacity factor depends on the ratio t_0/t_{mc} and is given mathematically by equation (11), as derived by Foley [6]:

$$k'_{opt} = \sqrt{t_{mc}/t_0}, \qquad (11)$$

where t_{mc}/t_0 is assumed to be positive. The dependence of N on k' has been investigated both theoretically and experimentally [7], confirming that there is a small but significant dependence. From the practical point of view, the recommended range of k' is between 1 and 5 for optimum performance, when the pH is above 6.

Manipulation of the capacity factor close to its optimum value or into its optimum range is straightforward, because k' can be related to the surfactant concentration, C_{sf}, as follows [2]:

$$k' = K\bar{v}(C_{sf} - \mathrm{CMC}), \qquad (12)$$

where K is the distribution coefficient, $\bar{v}$ is the partial specific volume of the micelle and CMC is the critical micelle concentration. Equation (12) suggests that once a capacity factor has been obtained, the concentration of surfactant required to give a specified value of k' can be calculated, provided that the CMC is known, because $K\bar{v}$ is independent of C_{sf}. However, if the calculated concentration is too low or too high, from a practical viewpoint such conditions are not recommended. Solutions to such problems are discussed below.

As is evident from equation (10), high plate numbers yield high resolution; the maximum plate numbers reported in MEKC approach 500,000 and in most cases, values have been reported between 100,000 and 200,000. A high applied voltage usually produces high plate numbers, unless Joule heating causes an adverse effect. In general, plate number increases with the length of the capillary, although there is no explicit theoretical relationship between capillary length and plate number. Extracolumn effects are, however, predictable and can be reasonably eliminated by proper instrument design [7].

Although negative time may seem unnatural, a negative value of the ratio t_{mc}/t_0 can be assumed in equation (10), when the linear velocity of migration of the bulk solution, v_{eo}, and that of the micelle, v_{mc}, are in opposite directions because of high micellar velocity due to electrophoresis, $v_{ep,mc}$ [8, 9]. In this case, resolution would theoretically approach infinity when k' approaches $-(t_{mc}/t_0)$ after a considerable time. Although the adjustment of t_{mc}/t_0 to its optimum value may be a powerful technique for increasing resolution, the manipulation of these values is not always feasible and is beyond scope of the present paper.

Although MEKC provides very high efficiency, the most important factor for improving resolution is the separation factor, α, as indeed is the case in high-performance liquid chromatography (HPLC). It can be calculated that even when $\alpha = 1.01$, it is possible to obtain an R_s value of 1, if N, k', and t_0/t_{mc} are reasonably assumed to be 290,000, 2 and 0.25, respectively. In the case of many difficult separations, however, α is often less than 1.01. It is thus essential to increase α values to enhance resolution, so in this paper, a general strategy for improving separation by increasing α values is presented.

Selectivity Manipulation

The micelle in MEKC corresponds to the stationary phase in conventional chromatography and the surrounding aqueous phase to the mobile phase; therefore, the type of micellar phase and the aqueous phase can be selected in order to modify selectivity. Another key parameter that affects selectivity is temperature. The choice of micelle and of modifier added to the aqueous phase is the most effective and important means of enhancing selectivity. Each experimental factor is discussed separately below.

Choice of the micelle

Solubility of the surfactants. The micelle used in MEKC must be ionic, that is, it must consist of an ionic surfactant or a mixture of ionic and

nonionic surfactants. Various kinds of surfactants are commercially available but the Kraft point of the surfactant must be lower than the operating temperature used in MEKC. The Kraft point is the temperature below which an ionic surfactant does not dissolve in water sufficiently to reach the concentration required to form a micelle. It should be noted that the counter-ion of an ionic surfactant affects its Kraft point. For example, the Kraft point of sodium dodecyl sulphate (SDS) is 16°C, but that of potassium dodecyl sulphate is about 35°C; therefore, care must be taken not to use a buffer containing potassium ion when preparing SDS solutions.

The effect of the ionic surfactant's molecular structure on selectivity (choice of surfactants). The following discussion assumes that the surfactant possesses a long alkyl chain as the hydrophobic group and an ionic group as the polar group. It is also assumed that the micelle formed by such a surfactant is spherical in shape, with the polar groups being located in the outer zone of the micelle and the alkyl groups constituting a hydrophobic core (Fig. 2).

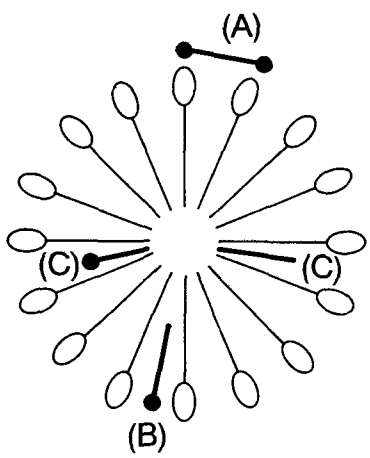

Figure 2
Schematic of the interaction between three types of solute and an ionic micelle. Open circles represent the polar group of the surfactant and closed ones that of the solutes A, B and C.

When a solute is incorporated into the micelle, three types of interaction are possible as shown in Fig. 2: (A) the solute is adsorbed onto the surface of the micelle by electrostatic or dipole interaction; (B) the solute behaves as a co-surfactant by participating in the formation of the micelle; (C) the solute is incorporated into the core of the micelle. The effect of the surfactant's molecular structure on the separation selectivity will differ according to the type of interaction involved.

Highly polar solutes will be mainly adsorbed onto the surface of the micelle [Fig. 2(A)], unless the solutes are considerably hydrophobic as a whole. In this case, the surface of the micelle or the nature of the polar group of the surfactant has a greater effect on the separation selectivity than does the core of the micelle or the surfactant's hydrophobic group. Polar solutes having both a polar and a hydrophobic group in the molecule may behave as co-surfactants [Fig. 2(B)].

SDS and sodium tetradecyl sulphate (STS) give almost identical distribution coefficients for polar solutes such as resorcinol, phenol, *p*-nitroaniline and nitrobenzene (Table 1). Resorcinol and *p*-nitroaniline probably interact with the micelle [Fig. 2(A)], as do phenol and nitrobenzene [Fig. 2(B)]. By contrast, SDS and sodium dodecanesulphonate (SDDS) display considerably different selectivity among the polar solutes. These results indicate that the different polar groups of various surfactants will show different selectivity for polar solutes, even if the surfactants have identical alkyl chain groups. Thus, it may be helpful to use another surfactant with a different polar group, when polar analytes are not resolved with a particular surfactant solution. A typical example which demonstrates the change in selectivity obtained for different surfactants is illustrated in Fig. 3 [10].

Table 1 also shows that STS gives larger distribution coefficients than SDS and SDDS for hydrophobic analytes such as toluene and 2-naphthol. These results suggest that these hydrophobic analytes are incorporated into the hydrophobic core of the micelle as shown in

Table 1
Distribution coefficients at 35°C

Solute	Distribution coefficient		
	SDS*	STS†	SDDS‡
Resorcinol	21.6	20.8	27.7
Phenol	52.1	52.3	56.1
p-Nitroaniline	103	100	84.3
Nitrobenzene	135	138	111
Toluene	318	345	288
2-Naphthol	656	789	698

*Sodium dodecyl sulphate.
†Sodium tetradecyl sulphate.
‡Sodium dodecanesulphonate.
Source: *Anal. Chem.* [2].

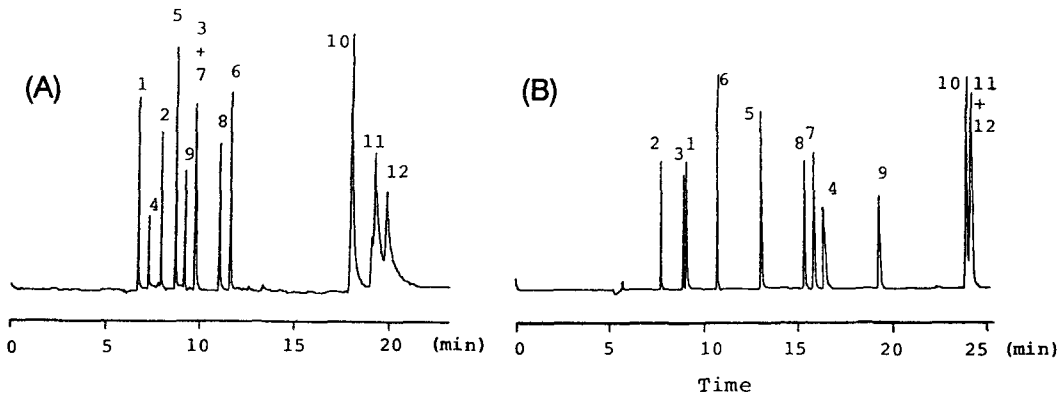

Figure 3
Comparison of selectivity between surfactants possessing different polar groups: 1, caffeine; 2, acetaminophen; 3, sulpyrin; 4, trimetoquinol; 5, guaiphenesin; 6, naproxen; 7, ethenzamide; 8, phenacetin; 9, isopropylantipyrine; 10, noscapine; 11, chlorpheniramine; 12, tipepidine. Conditions for separation solution: (A) 0.1 M sodium trioxyethylene alkyl (C_{12}–C_{14}) ether acetate (ECT) in 20 mM phosphate–borate buffer (pH 9.0); (B) 0.1 M SDS in the same buffer as for (A). Capillary, 50 μm i.d. × 65 cm (effective length 50 cm); applied voltage, 20 kV; detection, UV absorbance at 210 nm. (Reprinted with permission from *J. Pharm. Sci.* [10].)

Fig. 2(C). Thus the alkyl-chain length affects selectivity among hydrophobic solutes.

Although MEKC is advantageous for the electrophoretic separation of neutral compounds, it is also useful to improve the separation of ionic compounds, especially when separation by CZE is not successful [11]. Small ions having the same charge as that of the micelle will not interact with the micelle due to electrostatic repulsion, but will migrate with their own mobility. On the other hand, ions having the opposite charge will interact with the micelle to a considerable extent depending on the ionic charge number. Therefore, the charge on the micelle has a substantial effect on selectivity for ionic analytes. The use of ion-pair reagents is also a useful technique for manipulating the selectivity of ionic analytes, as discussed below.

Cationic micelles such as cetyltrimethylammonium bromide (CTAB) show substantially different selectivity among compounds as well as ionic solutes, compared with anionic micelles, because of the different polar group on this surfactant [12]. It should be mentioned that a cationic surfactant *reverses* the direction of electroosmotic flow due to adsorption of the cationic surfactant on the capillary wall, which therefore creates a positive surface [12]. The migration order, however, still follows the order of increasing capacity factor, as in the case of anionic micelles, because the cationic micelle is subject to electrophoresis in the opposite direction to that of electroosmotic flow. Thus the cationic micelles migrate at a lower net velocity in the same direction as the electroosmotic flow (from negative to positive). It should also be noted that since most cationic surfactants have halide ions as counter ions, they produce corrosive halogen at the anode.

Thus it can be concluded that, in general, different surfactants will give different separation selectivity. It is therefore recommended that another surfactant be tried if a particular separation is unsuccessful. It is very easy to change the surfactant solutions in MEKC.

Bile salt surfactants. Bile salts are a group of natural steroidal surfactants. These surfactants are considered to form a helical micelle with a reversed micelle conformation [13]. Bile salts display two characteristic features that differentiate them from long alkyl-chain surfactants: they have a relatively low solubilizing power, and they are capable of chiral discrimination. Hydrophobic compounds are not usually well separated by MEKC with long alkyl-chain surfactants, because of excessively large capacity factors. However, bile salt micelles have been successfully employed to solve this problem by taking advantage of their low solubilizing effect [13, 14].

Bile salts are chiral and can be used for enantiomeric separation [15–17]. In this respect deoxycholate and its taurine conjugate have shown successful results. Taurodeoxycholate can be used even under acidic conditions above pH 3, but deoxycholate must be

used under neutral or alkaline conditions to keep the carboxyl group ionized. It should be noted that the deoxycholate solution tends to gelate, when a relatively high concentration buffer solution is employed to prepare the micellar solution.

Other chiral surfactants. Amino acid derivatives such as sodium *N*-dodecanoyl-L-valinate (SDVal) are readily available chiral surfactants. These surfactants have also been successfully used for the enantiomeric separation of amino acid derivatives [18–20]. Addition of SDS, urea and methanol have improved peak shapes and resolution [20]. Digitonin is a natural nonionic surfactant and has been used for the enantiomeric separation of dansylated DL-amino acids as a mixed micelle with SDS [21]. It has been found in the author's laboratory that glycyrrhizic acid and β-escin are also promising agents for enantiomeric separation.

Use of mixed micelles. It is known that mixed micelles are formed when more than two different surfactants are dissolved in a solution. The mixed micelle consisting of an ionic and a non-ionic surfactant is particularly useful in MEKC. Because such a mixed micelle has a larger size and lower surface-charge density than the ionic micelle, it gives a larger capacity factor and a narrower migration-time window and also has a different separation selectivity [22]. Most nonionic surfactants have polyoxyethylene groups as hydrophilic moieties, therefore the surface structure of the mixed micelle and hence selectivity will be significantly different from that of the ionic micelle. An example is given in Fig. 4, taken from Y. Ishihama's results in the author's laboratory.

Choice of the buffer solution

Constituents or components of the buffer do not generally affect the distribution coefficient. However, the pH of the buffer is very important for the separation of ionizable analytes, just as it is in CZE, because ionic analytes show remarkably different affinity with respect to ionic micelles, depending on the ionic charge status, as mentioned above. For example, when the analytes are acids, an increase in pH promotes ionization, leading to reduced interaction with an ionic micelle such as SDS [23]. Although the capacity factor therefore decreases with increasing pH in this case, the net migration time is not always short, as one might at first expect, because the ionized acid migrates electrophoretically in the same direction as the anionic micelle (from negative to positive) due to the negative charge on the molecule.

It should be noted that the pH has a remarkable effect on the electroosmotic flow velocity, especially in the region below pH 6 [24]. The electroosmotic flow velocity itself does not contribute to the change of separation selectivity observed, but it does affect the migration time window and hence resolution. From a practical viewpoint, the easiest way of finding the optimum pH is to run the sample under a few different conditions of pH.

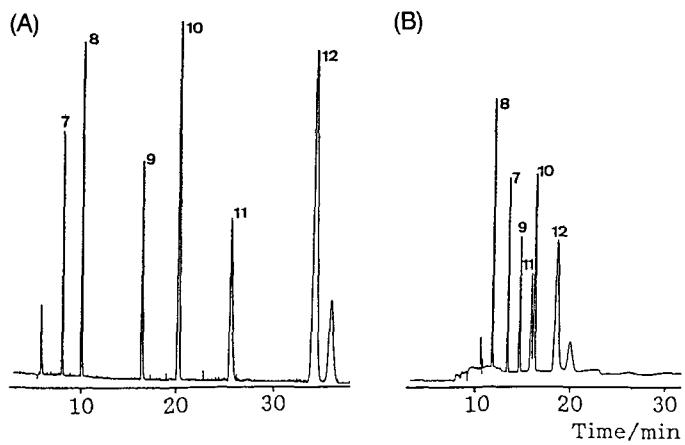

Figure 4
Effect of addition of nonionic surfactant: 7, acetaminophen; 8, caffeine; 9, guaiphenesin; 10, ethenzamide; 11, isopropylantipyrine; 12, trimetoquinol. Conditions: (A) separation solution, 100 mM SDS in 50 mM phosphate–100 mM borate buffer (pH 7.0); (B) 30 mM Tween 60 added to the same solution as in (A); capillary, 75 μm i.d. × 57 cm (effective length 50 cm); applied voltage, 18 kV; detection, UV absorbance at 214 nm.

Although theoretical prediction of pH is possible, provided some parameters are known [4, 5], the empirical approach is valuable in practice.

Additives to the aqueous phase

The aqueous phase in MEKC corresponds to the mobile phase in reversed-phase liquid chromatography (RPLC) and therefore the various mobile phase modifiers developed in RPLC are also applicable in MEKC. The following additives, cyclodextrins (CDs), organic solvents, ion-pair reagents, urea and metal ions are particularly useful, as described below.

Cyclodextrins. CDs are now widely utilized in analytical chemistry, especially for the purpose of chiral discrimination. CDs have also been successfully employed for enantiomeric separation in CZE [25, 26]. Inclusion complex formation of an ionic analyte with a CD reduces the electrophoretic mobility, due to the apparent increase in molecular size. Therefore it is the difference in inclusion complex formation constants between enantiomers in a racemic compound that leads to enantiomeric separation.

CD derivatives with ionic group substituents can be used as a pseudo-stationary phase instead of the micelle in electrokinetic chromatography (EKC) for the separation of a number of neutral compounds, including aromatic isomers and racemic mixtures [27, 28].

When CD is added to a micellar solution, the analyte is distributed among three phases: micelle, CD and water (aqueous phase). From the viewpoint of electrophoretic separation, CD and water migrate at identical velocities, equal to the electroosmotic velocity. However, CD exerts a remarkable effect on the apparent capacity factor (or distribution coefficient) between the micellar and nonmicellar (i.e. CD and water) phases. The technique that employs CD with an ionic micelle is described as CD-modified MEKC (CD/MEKC) [29]. CD/MEKC has extended MEKC to two useful application domains: separation of hydrophobic compounds [29] and enantiomeric separation of neutral racemic mixtures [30, 31].

Highly hydrophobic compounds tend to be almost totally incorporated into the micelle because of their low solubilities in water. CD is water-soluble and capable of including hydrophobic compounds into its hydrophobic cavity. The inclusion complex formation process depends on how the analyte fits into the CD cavity. Thus a fraction of the hydrophobic analyte will be included into the CD cavity, even in the presence of the micelle. Therefore the migration time or capacity factor of a hydrophobic analyte that forms an inclusion complex with a CD will decrease with an increase in CD concentration. Separation selectivity among highly hydrophobic analytes depends solely on the difference in their partition ratio between CD and the micelle, because such hydrophobic analytes can be assumed to be insoluble in water. An example of the utility of CD/MEKC for the separation of hydrophobic compounds is shown in Fig. 5 [32].

CD/MEKC is useful not only for the separation of highly hydrophobic compounds but also for the separation of closely related isomers and racemic mixtures, as noted above. In the case of partially water-soluble compounds, the distribution coefficient between the micelle and water can be altered by the addition of CD. CDs are known to discriminate among aromatic isomers, such as positional isomers of cresols, xylenols, or xylenes, [33, 34]. Various racemic compounds have been successfully resolved by CD/MEKC [30, 31]. An example of enantiomeric separation by CD/MEKC is given in Fig. 6 [31]. Although β-CD or its derivatives have been mainly employed in HPLC for enantiomeric separation, γ-CD has been found to be generally more effective in CD/MEKC. This is probably attributable to the presence of a long alkyl-chain surfactant in the solution, which will be co-included into the CD cavity together with the analyte. Thus co-inclusion would be expected to require a larger cavity size, such as that of γ-CD. The use of a chiral additive has been shown to give enhanced chiral discrimination in some cases [30].

Ion-pair reagents. Ion-pair reagents have often been employed in RPLC for the separation of ionic compounds. As the micelle is ionic, ionic analytes having the same charge as the micelle will not be incorporated into the micelle, or if so it would be only slightly. On the other hand, ionic analytes having a different charge will strongly interact with the micelle.

An ion-pair reagent will strongly modify the

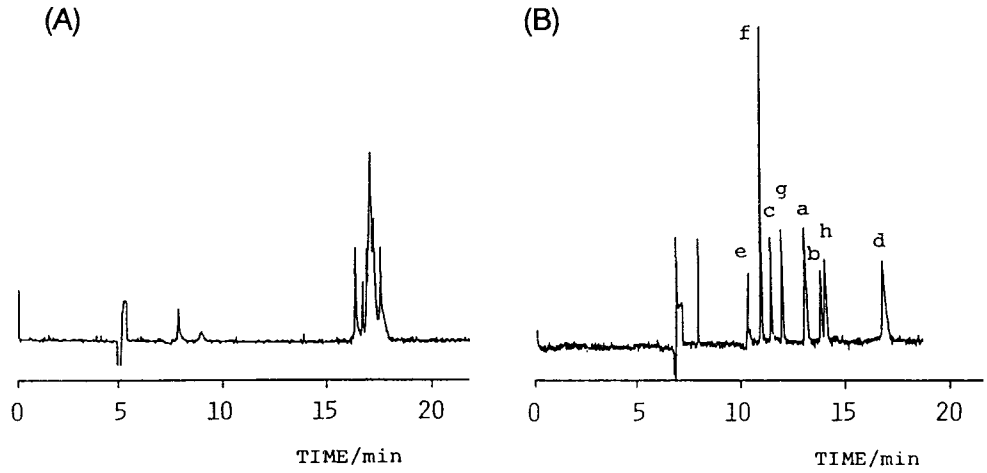

Figure 5
CD/MEKC separation of eight corticosteroids: a, hydrocortisone; b, hydrocortisone acetate; c, betamethasone; d, cortisone acetate; e, triamcinolone acetonide; f, fluocinolone; g, dexamethasone acetate; h, fluocinonide. Conditions: (A) separation solution, 50 mM SDS in 20 mM phosphate–borate buffer (pH 9.0); (B) 15 mM γ-CD and 4 M urea added to the same buffer solution as in (A); capillary, 50 μm i.d. × 65 cm (effective length 50 cm); applied voltage, 20 kV; detection, absorbance at 210 nm. (Reprinted with permission from *J. Liq. Chromatogr.* [32].)

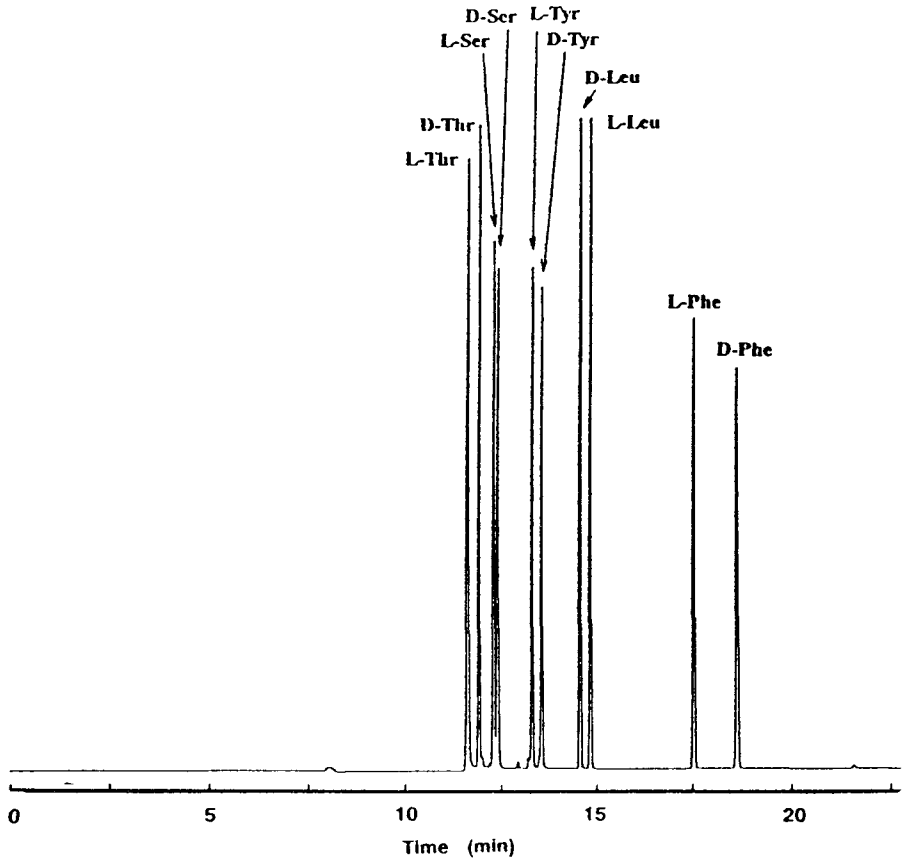

Figure 6
CD/MEKC separation of a mixture of five CBI (1-cyano-2-substituted-benz[*f*]isoindole)-DL-amino acids. Conditions: separation solution, 50 mM SDS, 10 mM γ-CD in 100 mM borate buffer (pH 9.0); capillary, 50 μm i.d. × 70 cm (effective length 50 cm); applied voltage, 15 kV; detection, laser-induced fluorescence (λ_{ex} = 442 nm, λ_{em} = 490 nm). (Reprinted with permission from *Anal. Chem.* [31].)

above-mentioned interaction between ionic analytes and an ionic micelle. For example, a cationic ion-pair reagent such as tetraalkylammonium salts will enhance the interaction between an anionic analyte and an anionic micelle by forming the ion-pair with the analyte or with the micelle. On the other hand, a cationic ion-pair reagent will retard the interaction between a cationic analyte and an anionic micelle by competing with the analyte for combining with the micelle. The effect of the ion-pair reagent on the migration time or separation selectivity depends on the molecular structure of the ion-pair reagent itself [35].

Urea. Although urea is not often used in RPLC, it is useful for the separation of hydrophobic compounds by MEKC. A high concentration of urea added to an SDS solution significantly reduces the capacity factor [36]. The same mixture of steroids shown in Fig. 5 was also successfully resolved with the addition of 6 M urea and 50 mM SDS, although the elution orders are considerably different [36]. It should also be mentioned that relatively high concentrations of urea, such as 1 M, often improve peak symmetry or peak shape [20].

Organic solvents. Water-miscible organic solvents such as methanol and acetonitrile can be used in MEKC, just as they are in RPLC. These organic solvents reduce the capacity factor and alter separation selectivity. A high concentration of organic solvent may break down the micellar structure and therefore it is recommended that the concentration should not exceed 50%. The addition of the organic solvent to a micellar solution usually reduces the electroosmotic velocity, thereby extending the migration-time window [37, 38] and increasing resolution. A solvent programming operation, where the content of the organic solvent was changed with time, has also been developed to improve resolution [39, 40].

Metal ions. Magnesium, zinc, and copper ions are known to form complexes with nucleotides. The addition of these metal ions to the SDS solution has been shown to improve the resolution of oligonucleotides [41] and may have further uses in this and other contexts.

Temperature. The dependence of the distribution coefficient on temperature follows the van't Hoff relationship:

$$\ln K = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R}, \qquad (13)$$

where ΔH° is the enthalpy change associated with micellar solubilization, ΔS° is the corresponding entropy change, R is the gas constant, and T is absolute temperature. The distribution coefficient is related to the capacity factor according to equation (12). Both ΔH° and ΔS° are thermodynamic quantities characteristic of the analyte, and therefore the dependence of the capacity factor on temperature would be expected to differ among analytes.

The differences, however, are usually not remarkable and the separation selectivity is not significantly dependent on temperature. It should be noted that in MEKC the temperature should be strictly controlled in order to obtain reproducible results, because migration time is seriously dependent on temperature due to the viscosity change when operating at a constant voltage.

An Introductory Guide to Optimum Separation

Figure 7 shows a general guide to successful separation by MEKC. This flow chart has been prepared for the separation of small molecules. A set of standard operating conditions using a 50 mM SDS solution, as given in Fig. 7, is proposed for the initial stage in developing a MEKC separation.

If a sample consists of a few components, these standard conditions may be successful. When the resolution is more than that required, the migration time can be reduced by using a shorter capillary or by filling it with a more dilute SDS solution. If the separation is not satisfactory, it is necessary to know the approximate values of the capacity factors, according to equation (9). To evaluate the capacity factor, t_0 and t_{mc} must be known. Experimentally, methanol or formamide is often used as a marker for t_0 for migration of the bulk solution, while Sudan III or Sudan IV are used as a tracer for the micelle for t_{mc}. The marker or tracer is usually added to the sample solution. The peak of the micelle corresponding to t_{mc}, is not always readily observed due to the low solubility of the tracer. In such cases, t_{mc} can be assumed to be three to four times t_0.

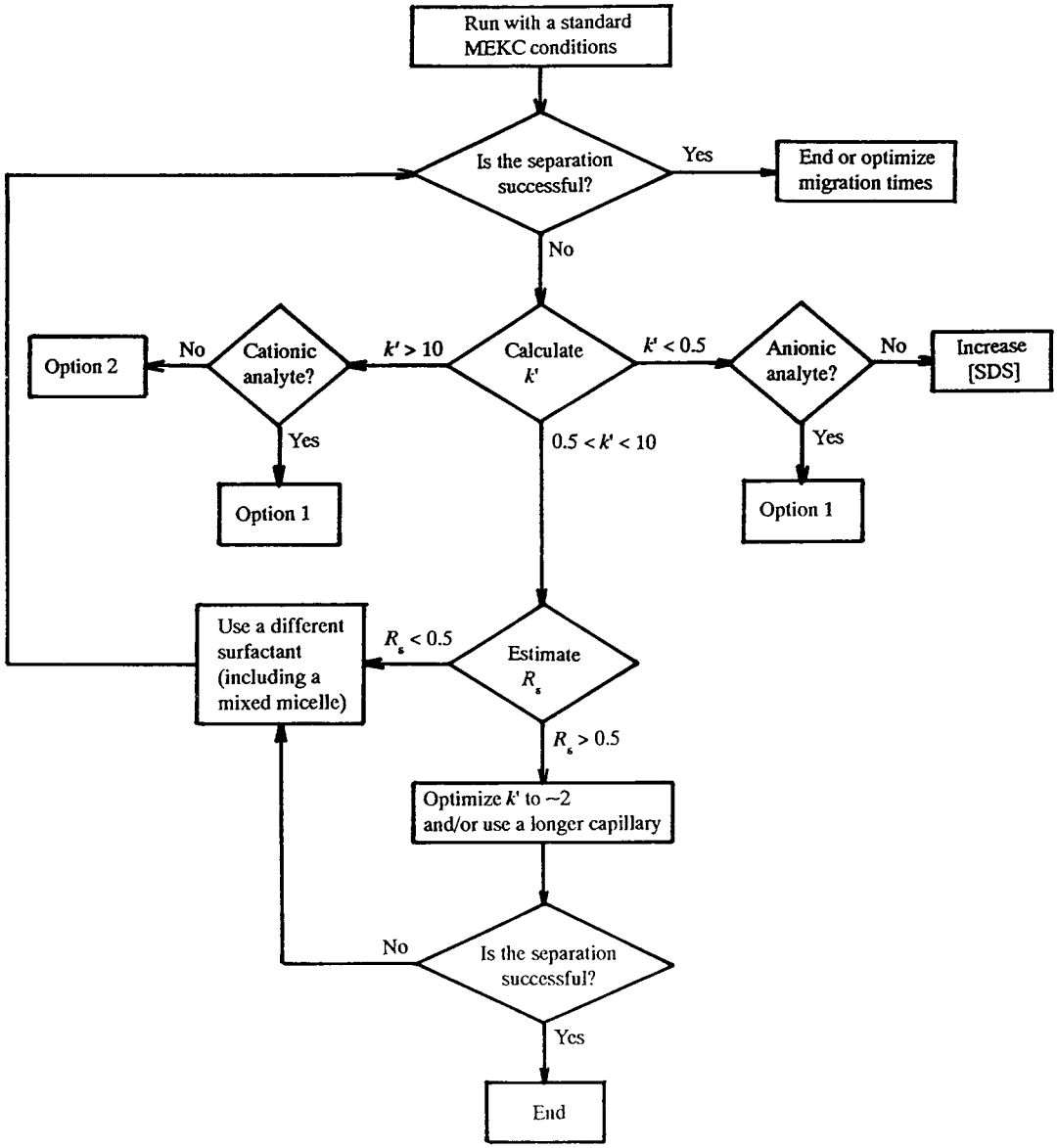

Figure 7
Introductory guide to the development of an optimum separation. Standard MEKC conditions: running solution: 50 mM SDS in 50 mM borate buffer (pH 8.5–9.0); capillary: 50–75 μm i.d. × 30–50 cm (from the injection end to the detector); applied voltage: 10–20 kV (keep current below 50 μA); temperature: 25°C or ambient; sample solvent: water or methanol; sample concentration: 0.1–1 mg ml^{-1}; injection end: the positive end or anodic end; injection volume: below 2 nl (or less than 1 mm from the end of the capillary); detection: 200–210 nm (depending on the sample). Option 1: use an ion-pair reagent (an ammonium salt) or a cationic surfactant. Option 2: use bile salts instead of SDS, add CD to an SDS solution (CD/MEKC), or add an organic solvent or urea to an SDS solution.

For analytes having capacity factors that are too large or too small, either option 1 or option 2 are recommended, according to the flow chart. In particular, for hydrophobic compounds, there is a wide range of choices as noted in option 2.

When peaks are partially resolved, further refinement of the conditions will often lead to a successful separation. However, when the separation is unsuccessful with SDS solutions, other surfactants should be explored.

The flow chart given in Fig. 7 is an example of the strategy that can be adopted for developing a successful separation on the basis of the author's experience. A few papers describing the problems of optimization in MEKC have been published, based on chemometric approaches or on mathematical calculations [6, 42, 43].

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