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Journal of Chromatography A, 780 (1997) 3–40

JOURNAL OF CHROMATOGRAPHY A

Review

Micelles as separation media in high-performance liquid chromatography and high-performance capillary electrophoresis: overview and perspective

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Abstract

The use of micelles in high-performance liquid chromatography and capillary electrophoresis is reviewed. In the first part, an overview of micellar liquid chromatography (MLC) is provided. Since the first introduction of the technique by Armstrong and Henry [D.W. Armstrong and S.J. Henry, J. Liq. Chromatogr., 3 (1980) 657; D.W. Armstrong, Sep. Purif. Methods 14 (1985) 213] in 1980, the technique has received much attention due to its numerous capabilities and advantages, such as simultaneous separation of charged and uncharged solutes, rapid gradient capability, direct on-column injection of physiological fluids, unique separation selectivity, high reproducibility, robustness, enhanced luminescence detection, low cost and safety. The main shortcoming of the technique is poor chromatographic efficiency. Nevertheless, MLC is superior to ion-pair LC and ion-exchange LC for the separation of charged molecules and mixtures of charged and uncharged solutes. The roles of micelles and organic modifiers in controlling retention and selectivity in MLC is described. The differences between MLC and reversed-phase LC in terms of chromatographic behavior and scope of application are examined. A main focus of this overview is on micellar electrokinetic chromatography (MEKC). In 1984, Terabe et al. [S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando, Anal. Chem. 56 (1984) 111; S. Terabe, K. Otsuka and T. Ando, Anal. Chem. 57 (1985) 834] reported the use of micelles in buffer solutions for capillary electrophoresis (CE). MEKC was primarily developed for the separation of uncharged solutes, but it has grown far beyond its initial intent. The scope of applications covers wide groups of organic, inorganic and biochemical compounds that are of interest in various disciplines, such as pharmaceutical, clinical, biotechnological, environmental sciences and others. This is due to its unique advantages, such as high efficiency, speed, ease of method development, feasibility of incorporating various chemistries to influence retention and selectivity, small sample size and low cost. The instrumental set-up in MEKC is the same as that for CE. However, charged organized media such as micelles are incorporated in the buffer solution and act as a pseudo-stationary phase. Unchanged solutes are separated on the basis of their differential partitioning into the micellar pseudo-stationary phase. The roles of various parameters on the overall chromatographic behavior are described. Special attention is given to the characterization of selectivity of pseudo-stationary phases on MEKC.

Keywords: Reviews; Micellar liquid chromatography; Micellar electrokinetic chromatography

Contents

0021-9673/97/$17.00
PII S0021-9673(97)00610-9

1. Introduction

Over the past fifteen years, the popularity of micellar-mediated separation techniques has grown rapidly. In high-performance liquid chromatography (HPLC), micellar mobile phases have been used to control retention and selectivity [1,2]. In addition, inclusion of charged micelles in capillary electrophoresis (CE) buffer solutions has extended the capabilities of the electromigration techniques for the separation of uncharged solutes [3,4]. Micelles have also been used in other separation methods like ultrafiltration and cloud point extraction [5]. However, the focus of this paper is to provide an overview of micellar liquid chromatography (MLC) and micellar electrokinetic chromatography (MEKC) as well as of the author's perspectives of the two fields. An exhaustive review of these two fields is not intended here, thus, the list of references mainly include recent publications. The following review papers in this issue will provide more detail about various aspects of the two techniques.

2. Micelles

Surfactants are amphiphilic molecules that comprise a hydrophobic moiety and a polar or ionic head group. They can be recognized by the charge of the head group (as non-ionic, anionic, cationic and zwitterionic surfactants) or by the variations in the nature of the hydrophobic moiety (as hydrocarbon, bile salts and fluorocarbon surfactants).

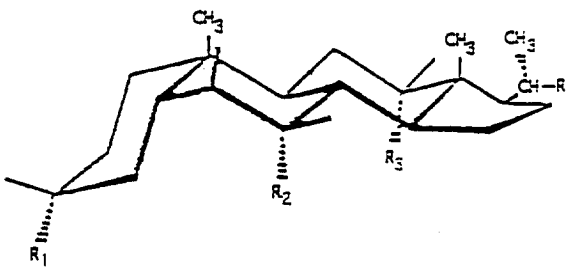
Above a critical micelle concentration (CMC), surfactants form aggregates that are known as micelles. Micelles have a dynamic structure that is the result of the rapid exchange of surfactants in the aggregated and monomeric forms.

The number of monomer surfactants in the aggregate form (called aggregation number) and the size of micelles vary greatly between surfactants. For example, surfactants with alkyl chains form roughly spherical micelles with a diameter of between 3–6 nm and aggregation numbers of between 30–100 [5–8]. On the other hand, micelles of bile salts have much smaller aggregation numbers (typically 2–10 for primary micelles), with a presumably helical structure. Bile salts are biological surfactants with a hydrophobic steroidal backbone with substituted hydrophilic groups (such as hydroxyl or carbonyl) [9–11]. The CMC and aggregation number greatly depend on a number of factors, such as ionic strength, presence of a co-solvent and temperature. Another property of surfactants is the Kraft point, which is defined as the temperature at which the solubility of surfactant is equal to its CMC. Table 1 lists the properties of some typical surfactants that can be used in MLC and/or MEKC.

Table 1
Typical surfactants for MLC and MEKC

Type	Name	CMC (m*M*)
A. Long chain surfactants		
Anionic	Sodium dodecyl sulfate (SDS)	8.1[a]
	Sodium tetradecyl sulfate	2.1[c]
	Sodium dodecyl sulphonate	9.3[c]
	Lithium perfluorooctane sulphonate (LiPFOS)	6.72[b]
Cationic	Cetyl trimethylammonium bromide	0.92[d]
	Cetyl trimethylammonium chloride	1.3[d]
	Dodecyl trimethylammonium bromide (DTAB)	15[a]
Non-ionic	Polyoxyethylene (23) dodecanol (BRIJ 35)	0.1[a]
	Polyoxyethylene [20]-sorbitane monooleate (Tween 80)	0.01[d]
	Polyoxyethylene [20]-sorbitane monolaurate (Tween 20)	0.059[d]
Zwitterionic	N-Dodecyl-N,N-dimethylammonio-3-propane sulfonate (Sulfobetain SB-12)	3.3[d]
	3-(3-cholamidopropyl)dimethylammonio-3-propane sulphonate (CHAPS)	4.2–6.3[d]

B. Bile salts



Sodium salts:	R_1	R_2	R_3	R	CMC (m*M*)
Cholate	OH	OH	OH	$-CH_2CH_2COO^-$	12.5[a]
Deoxycholate	OH	H	OH	$-CH_2CH_2COO^-$	6.4[a]
Taurocholate	OH	OH	OH	$-CH_2CH_2CONHCH_2CH_2SO_3^-$	–
Glycodeoxycholate	OH	H	OH	$-CH_2CH_2CONHCH_2COO^-$	–
Taurodeoxycholate	OH	H	OH	$-CH_2CH_2CONHCH_2CH_2SO_3^-$	–

[a] Ref. [5], [b] Ref. [6], [c] Ref. [7], [d] Ref. [8].
From: M.G. Khaledi, in M.G. Khaledi (Editor), High Performance Capillary Electrophoresis: Theory, Techniques, and Applications, Wiley, New York, 1997, Ch. 3, with permission.

For practical purposes in CE and HPLC, a suitable surfactant should have low CMC and Kraft points. A high CMC would mandate operating at high surfactant concentrations. This condition is not desirable in either HPLC (due to high viscosity of the solution) or in CE (due to high conductivity). The Kraft point should preferably be much smaller than the ambient temperature. In addition, since absorbance detectors are the most common type of detector used in both CE and HPLC, a suitable surfactant should have small molar absorptivity at the operating wavelength. Since the size of micelles is a few nanometers, they do not cause any light scattering in the operating UV–Vis range.

Another important issue is the effect of organic additives on the properties of micelles. In order to improve MLC and MEKC separations, it is often necessary to include organic modifiers in the aqueous solutions of micelles. High concentrations of organic co-solvents would disrupt micelle structure as hydrophobic effect, the main driving force for micelle formation, is reduced. The maximum allowable

concentration depends on the type of organic modifier and on the micelles and is usually not known. As a rule of thumb, the volume percentage of organic solvents should be kept below 15–20% (v/v) in MLC and MEKC separations, to ensure the integrity of micelles.

An important property of micelles is their ability to enhance the solubility of otherwise insoluble, hydrophobic organic compounds in aqueous media. However, in MLC and MEKC literature, it is often stated that micellar solubilization controls the separation process. The term "micellar solubilization" should not be confused with solubility in micellar solutions. Indeed, it is the partition coefficients between the bulk aqueous solvent and micelles, P_{mw}, (or solute–micelle binding constant, K_{mw}) that plays a key role on retention and selectivity in both techniques, and not the solubility of solutes in micellar solutions. For example, a compound such as pyrene has a significantly higher partition coefficient into SDS micelles than that of benzene, however, its solubility in SDS micellar solutions is considerably lower. On the other hand, the rate of increase in solubility (i.e. compared to that in water) is much higher for the more hydrophobic compounds, mainly due to their stronger interactions with the micelles or "micellar solubilization". Under the operating conditions of MLC and MEKC, all solutes should be dissolved in micellar solutions, nevertheless, they are separated mainly due to their different micelle–water partition coefficients. It is therefore important to achieve a better understanding of the solute–micelle interactions that occur through different mechanisms, such as surface adsorption, partitioning into the core and co-micellization. The type of mechanism determines the location of solutes in/on a micelle. Due to the heterogeneous nature of micelles, solutes experience various microenvironments with different "polarities", depending on the chemical nature of the solutes and of the surfactant. The microenvironment properties of micelles, such as "polarity", ionic strength, fluidity and acidity, are distinctly different from those of bulk aqueous media.

3. Micellar liquid chromatography (MLC)

In MLC, the mobile phase consists of surfactants at concentrations above their CMC in an aqueous solvent with an alkyl-bonded stationary phase [1,2]. In a sense, MLC is a reversed-phase LC (RPLC) system, with micelles acting as a mobile phase modifier. It is often necessary to add small concentrations of an organic modifier to the MLC mobile phases to improve the efficiency as well as to optimize the solvent strength and selectivity. These systems are often referred to as hybrid eluents.

Partitioning of solutes from the bulk aqueous mobile phase into micelles has a large effect on retention and selectivity. According to the three-"phase" model shown in Fig. 1, retention behavior in MLC is controlled by solute partitioning from the bulk solvent into micelles (P_{mw}) and into stationary phase (P_{sw}) as well as on direct transfer from the micelles in the mobile phase into the stationary phase [1,2]. While retention of more polar compounds is determined by their partitioning from the bulk aqueous phase into micelle and alkyl stationary phase, the more hydrophobic compounds might be directly transferred from micelles in the mobile phase into the stationary phase [5].

In general, the presence of micelles in the mobile phases of RPLC systems (either purely aqueous or hydro-organic) has a profound effect on the overall chromatographic characteristics that are distinctly

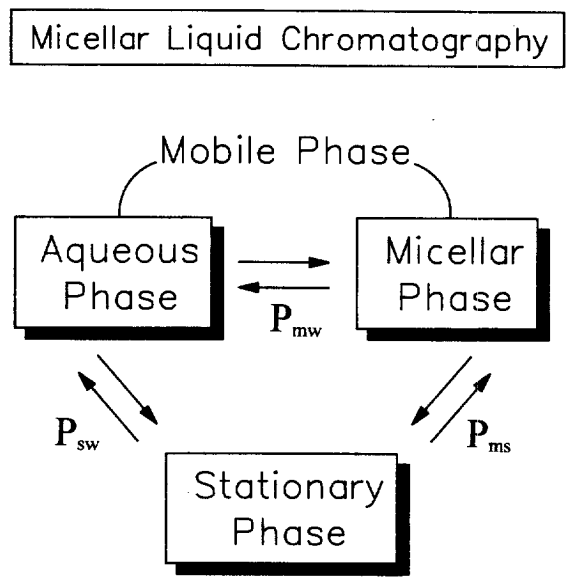


Fig. 1. Three-phase equilibria in MLC.

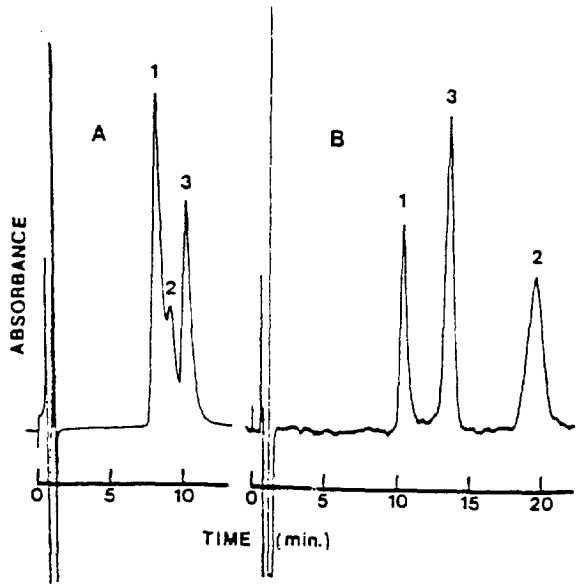


Fig. 2. Effect of surfactant head group (same chain length) on selectivity in MLC. Mobile phase: (A) 0.05 *M* SDS; (B) 0.05 *M* dodecyl trimethyl ammonium bromide, DTAB (both surfactants have a chain length of twelve carbons); solutes: (1) nitrobenzene, (2) 2-naphthol and (3) toluene. Reprinted from Ref. [13] with permission.

different from those of traditional hydro-organic systems [12,13]. Fig. 2 illustrate variations in elution order due to a change in the head group of the surfactant. Similar behavior has also been observed with changes in micelle concentration [13].

The differences between micellar and hydro-organic eluents have been demonstrated through a comparative study of the retention behavior of an homologous series in MLC and RPLC [14,15]. Usually, one would expect a linear relationship between the change in free energy in retention (as represented by log k') and the number of repeating -CH_2 units (or carbon number, N_C) due to the systematic increase in surface area of solute molecules. The slope of this line is called methylene selectivity, $\alpha(CH_2)$, and this is related to non-specific and hydrophobic interactions. In contrast to hydro-organic RPLC where the relationship between log k' and N_C is linear (Fig. 3), there exists a clear curvature in the log k' vs. N_C plot for the MLC system, which indicates the dependence of methylene selectivity on the size of homologues in the series, i.e. smaller $\alpha(CH_2)$ values are observed for

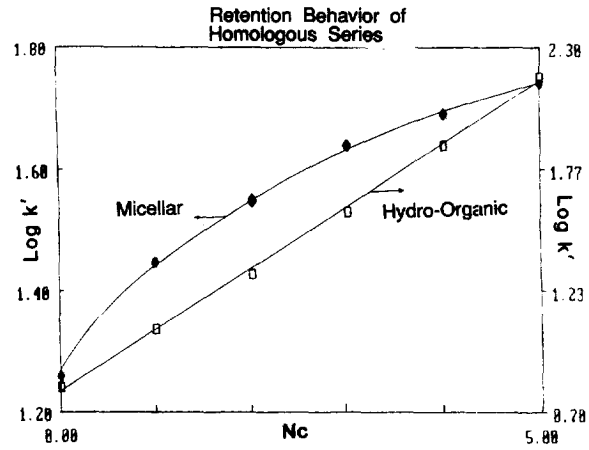


Fig. 3. Retention behavior of homologous series of *n*-alkyl benzenes by MLC (♦) with 0.072 *M* C_{16}TAB and hydro-organic RPLC (□) with 60% methanol in water. A C_{18} stationary phase was used in both cases. Reprinted from Ref. [15] with permission.

the larger, more hydrophobic members of the series. In addition, $\alpha(CH_2)$ is independent of the type of series in hydro-organic RPLC, which is not the case in MLC. This is shown in Fig. 4, where the $\alpha(CH_2)$ values obtained using alkyl phenyl ketones were consistently larger than those for alkyl benzenes at all sodium dodecyl sulfate (SDS) concentrations.

The unique retention and selectivity behavior in MLC can be attributed to two factors: The existence of competing equilibria for solute partitioning into

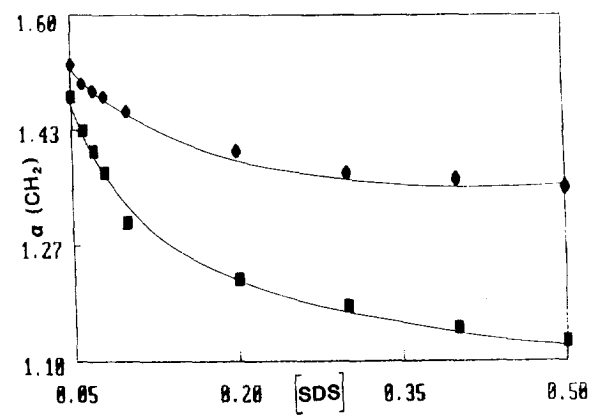


Fig. 4. Dependence of methylene selectivity in MLC on the type of homologous series and on the micelle concentration: alkyl benzenes (■), alkyl phenyl ketones (♦). Reprinted from Ref. [15] with permission.

mobile and stationary phases as well as the heterogeneous nature of the micelles.

Micelles are microscopically heterogeneous. In a given micellar eluent, solutes can be located in microenvironments with different polarities. This situation is quite different from that in homogeneous hydro-organic phases, where all compounds experience the same mobile phase polarity. In general, hydrophobic selectivity, $\alpha(CH_2)$, is inversely proportional to the microenvironment polarity of the mobile phase. For example, larger $\alpha(CH_2)$ values are observed for more polar eluents in RPLC. This principle can be used to better understand the behavior in MLC. For example, the smaller $\alpha(CH_2)$ values for larger homologues in a series (Fig. 3) show that they are located in a more hydrophobic environment of micelles. This can also be seen in Fig. 5 where carbonyl selectivity between different homologues of alkyl phenyl ketones are compared for micellar and hydro-organic eluents. Again, for the hydro-organic system, carbonyl selectivity remains constant, regardless of the hydrophobicity of the neighboring homologues. This is not the case for micellar eluents, as selectivity depends on the alkyl chain lengths of the homologous series.

Likewise, more hydrophobic series, like those of alkyl benzenes, experience microenvironments that are less polar than those for alkyl phenyl ketones (Fig. 4). As a result, methylene groups of alkyl phenyl ketones would see a different mobile phase environment than those of alkyl benzenes in MLC – such a situation does not exist in hydro-organic RPLC.

$$\alpha_{co} = \frac{k'(C_5H_6CO(CH_2)_nCH_3)}{k'(C_5H_6(CH_2)_nCH_3)}$$

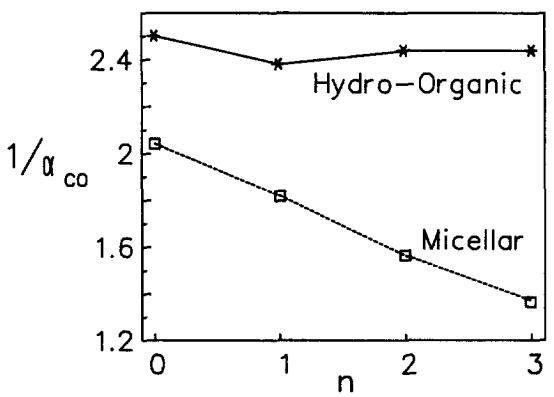


Fig. 5. Dependence of carbonyl group selectivity (α_{CO}) on the number of carbons in MLC (□) and in hydro-organic RPLC (*). The inverse of group selectivity (α_{CO}) is plotted so that selectivity values are greater than one. Reprinted from Ref. [17] with permission.

3.1. Solvent strength and selectivity

Eluent strength in MLC is inversely related to micelle concentration. A linear relationship exists between the inverse of retention factor and micelle concentration. The micelle–water partition coefficient can be determined from the slope and intercept of this linear relationship [2,16].

The concentration of an organic modifier also influences the strength and selectivity of the mobile phase. In a similar way to that found in RPLC, a linear relationship is observed between retention in MLC and the volume fraction of organic modifier, Φ_{org}, as:

$$\log k' = -S_{hyb}\Phi_{org} + \log k'_0 \quad (1)$$

where S_{hyb} is the solvent strength parameter for the hybrid system [17–19]. However, S_{hyb} is a function of micelle type and concentration. In other words, the rate of variation in retention with concentration of organic modifier changes with micelle concentration. Fig. 6a,b show the linear relationships between $\log k'$ and Φ_{org}, using propanol as the modifier for both conventional RPLC and for MLC. The different retention behaviors in RPLC and in MLC are quite evident in Fig. 6. Usually, with the RPLC system, the slope of the line (S value) depends on the solute type, with larger or more hydrophobic solutes having greater S values, i.e., their retention changes to a greater extent than does that of more polar, less retained compounds. In MLC, however, S is nearly independent of the solute type, as indicated by parallel lines in Fig. 6b. This is because of the localization of solutes and organic modifier in the micelle as well as the competing partitioning equilibria in MLC [17,18].

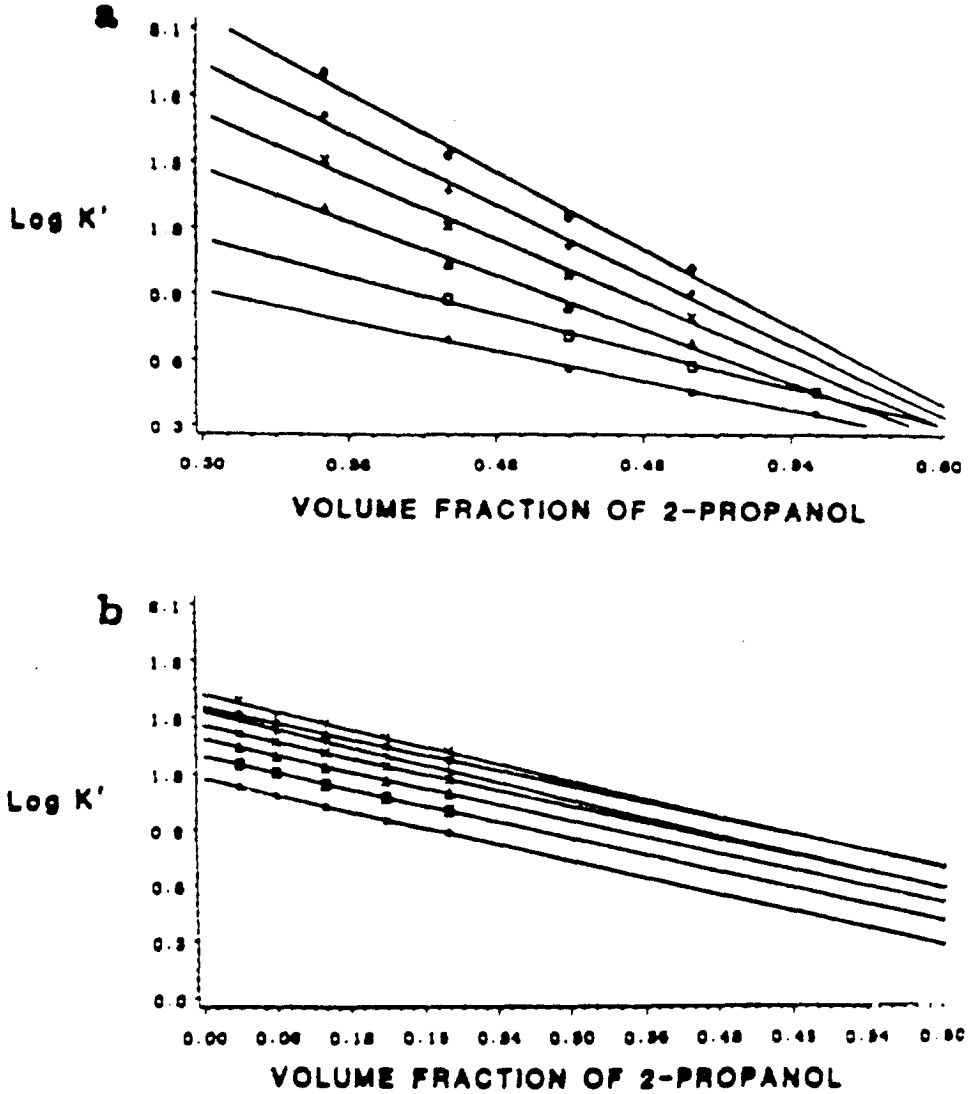


Fig. 6. Dependence of retention on the concentration of organic modifier for (a) hydro-organic RPLC and (b) MLC with 0.10 *M* CTAB in water–propanol. Test solutes are alkyl benzenes. Reprinted from Ref. [14] with permission.

The relationship between solvent strength and selectivity in MLC is different from that in RPLC. For example, selectivity in general and $\alpha(CH_2)$ in particular, decrease in RPLC as the concentration of organic modifier is increased. This is not the case, however, with the hybrid mobile phases in MLC, as $\alpha(CH_2)$ remains constant as the volume fraction of the organic co-solvent is increased [15]. For polar functional groups, there is no particular trend, as selectivity might decrease or increase [17–19].

All of these differences point out the important fact that the optimization protocol often used in RPLC would not be effective in MLC. With traditional RPLC, the concentration of organic modifier is mainly used to adjust the solvent strength; its effect on selectivity is not pronounced. As a result, a

sequential optimization strategy is used in RPLC as solvent strength is adjusted first, then selectivity is optimized through a proper selection of the type of organic modifier. In MLC, in addition to the types of surfactant and organic modifier, their concentrations can have pronounced effects on selectivity as well as on the mobile phase strength [17–19]. Due to the interactive nature of these factors, a simultaneous optimization strategy should be selected in MLC separations. In other words, they can not be optimized independent of one another.

An effective strategy for the simultaneous optimization of mobile phase parameters in MLC is the iterative regression procedure [20]. This is an interpretive method that is based upon building empirical linear models to describe retention as a function of parameters using a minimum number of initial experiments. For example, for simultaneous optimization of concentrations of micelle and organic cosolvent, Strasters et al. [20] successfully predicted the retention behavior of all solutes in a mixture of phenols over the parameter space, based on five initial experiments. Excellent correlations were observed between the observed and the predicted optimum chromatograms, as shown in Fig. 7. These results indicate that retention in MLC varies linearly with these two parameters. The iterative regression study was based on the linear relationships between the logarithm of the retention factor (log k') and the two parameters. Other studies have intensively investigated other forms of empirical models to describe retention as a function of these two important parameters, especially organic modifier [21–27]. In addition to the predictable behavior, Fig. 7 shows that the retention behavior is robust and reproducible, considering the fact that the data for the predicted chromatogram were obtained on a column that was different from that used for observed measurements (both columns were from the same manufacturer). This is of great significance in method development.

A large majority of MLC studies have been performed on alkyl-bonded phases. The usefulness of a fluorocarbon stationary phase has also been investigated [28,29]. The selectivity for polar molecules on a fluorinated phase was quite different than that for alkyl-bonded phases. However, the overall general behavior, such as the effects of micelles and organic

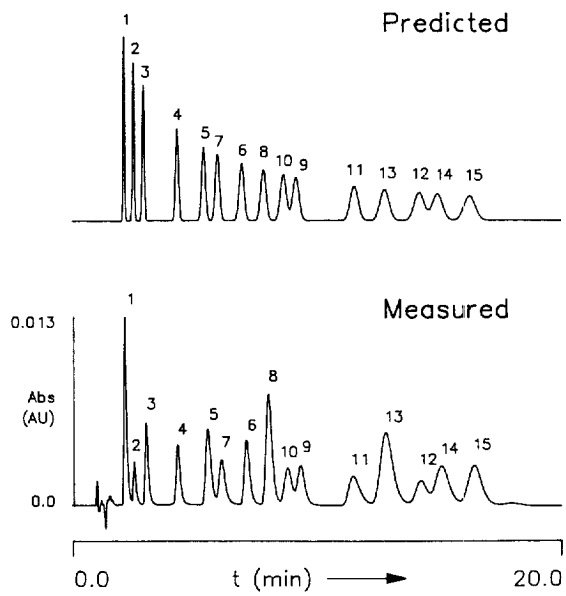


Fig. 7. Comparison of the predicted and observed optimum isocratic separation of a mixture of fifteen phenols by MLC. (Top) The chromatogram predicted on the basis of the linear retention model used in the iterative regression optimization, assuming 4000 plates and equal areas for all components. (Bottom) The measured chromatogram was obtained on a different column (the same type of packing). Optimum conditions: 0.11 *M* CTAB, 10% propanol. Reprinted from Ref. [20] with permission.

modifiers, the relationship between solvent strength and selectivity, as well as predictable and robust retention behavior, were similar. Higher chromatographic efficiency was observed with the fluorinated stationary phase.

3.2. *Efficiency*

Column efficiency is another important aspect of chromatographic separations. Poor column efficiency is the single, most important drawback of MLC. Compared to traditional hydro-organic eluents, the efficiency of an alkyl-bonded phase column is reduced by a factor of two or three with micellar mobile phases.

The possible causes for lower efficiency include slow mass transfer from the stationary phase as well as slow exit rates of hydrophobic solutes from micelles in the mobile phase [30–37]. Slow stationary phase mass transfer can be attributed to poor "wetting" of the stationary phase with a purely

aqueous mobile phase as well as to adsorption of monomer surfactants that change the characteristics of the alkyl-bonded stationary phases [31,33–37]. In order to improve the stationary phase mass transfer, the addition of a small percentage of propanol (~3%) and using a higher column temperature (~40°C) has been recommended [30,31]. The presence of the organic modifier should improve the "wetting" problem, as 3% propanol covers about 97% of the stationary phase, while higher temperatures increase the kinetics of mass transfer. In general, operating under these conditions would enhance column efficiency, however, the column plate counts are still considerably low compared to those in MLC.

3.3. Gradient elution in MLC

In order to perform gradient elution in MLC, one can increase the concentration of micelles and/or organic modifier during the course of the separation. For HPLC analysis of complex mixtures, the use of gradient elution is often necessary to solve the general elution problem. Enhanced peak resolution, faster analysis times and better detectability are the advantages of gradient elution. A shortcoming of solvent programming is that the composition of the stationary phase changes with that of the mobile phase. As a result, there is a need for column regeneration at the end of gradient elution and before the next analysis. The additional re-equilibration step increases the overall analysis time. For certain techniques, such as ion-pair chromatography, column regeneration can be prohibitively long. In MLC, the re-equilibration period at the end of a gradient run is not typically needed for a micelle concentration gradient and it is very short for organic solvent gradients. This is because the composition of stationary phase remains nearly constant with changes in the concentration of micelles [38,39]. The alkyl stationary phase is modified with monomer surfactants in MLC. In micellar solutions, the concentration of monomer surfactants is nearly constant and equals the CMC, thus, the composition of the stationary phase would not change with variations in the micelle concentration in the mobile phase [38,39]. This is a different behavior than that found in RPLC, where the composition and conformation of the alkyl-bonded phase depend on the composition of the hydro-organic eluents. One can even perform an organic modifier gradient, or simultaneous micelle and organic modifier, with minimum variations in the stationary phase composition [40,41]. This is due to the small overall concentration range of organic modifier that is used in MLC in order to maintain the integrity of micelles.

On the basis of the gradient elution theory in traditional RPLC, equations have been derived for the prediction of gradient elution times in micelle concentration gradients and organic modifier from isocratic data [40,41]. Using these equations, micelle–water partition coefficients can be estimated from two micelle concentration gradient runs. Fig. 8 shows an example of an organic modifier gradient in MLC.

3.4. Detection capabilities

Localization of solutes in micelles at a molecular level would influence their photophysical pathways. This could sometimes lead to improvements in detection capabilities. The fluorescence intensity of certain compounds in micellar media can be dramati-

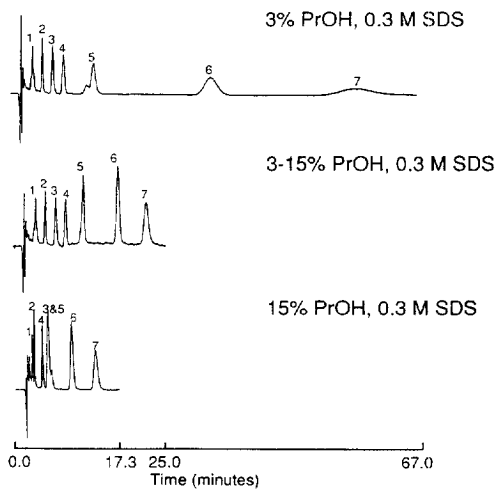


Fig. 8. Isocratic and gradient separation of a mixture of amino acids and small peptides in MLC. Mobile phase: 0.30 *M* SDS, phosphate buffer, pH 2.5, with propanol (PrOH) (top and bottom) and isocratic separations were performed with 3 and 15% 2-propanol, respectively. (Middle) gradient separation with 3 to 15% propanol. 1=DF, 2=F, 3=KF, 4=FF, 5=FFF, 6=FFFF, 7=FFFFF. Reprinted from Ref. [41] with permission.

cally increased due to micellar solubilization [42–44]. Solutes that are localized in the anisotropic media of micelles experience a microenvironment with different polarity and higher viscosity than those of the bulk aqueous solvent. As a result, their freedom of movement is limited in the micelles and results in the shielding of compounds from non-radiation deactivation and/or to an increase in quantum efficiency. Consequently, fluorescence signals are often intensified in the presence of micelles. Even room temperature phosphorescence has been observed in ionic micellar solution with heavy atom counter-ions [43].

3.5. Direct injection of physiological fluids

The ability of micelles to selectively bind (or solubilize) solutes with a wide range of polarities is advantageous in the purification and isolation of compounds of interest. Micelles provide electrostatic and hydrophobic sites of interaction with solutes. As a result, the use of micellar solutions for the extraction of metal ions, organic compounds, biological substances or agricultural materials has received much attention [5]. Obviously, micellar-mediated extraction can be used as a step for sample treatment prior to the chromatographic experiments.

A fascinating feature of certain types of micelles, such as SDS, is their ability to solubilize proteins. This capability has been effectively exploited for the direct injection of untreated biological fluids onto RPLC columns. A major drawback of HPLC methods for the routine analysis of protein-based biological samples is the need for a sample preparation step prior to injection, in order to remove proteinaceous materials. This is necessary to prevent irreversible adsorption to the packing and column plugging by the background protein. Protein precipitation is tedious, time consuming and can cause sample dilution or loss of material. In fact, many of the commonly used techniques for protein precipitation are incompatible with trace analysis by HPLC. DeLuccia et al. [45] and Arunyanart and Cline Love [46] were the first to demonstrate that micellar mobile phases can provide a unique opportunity for the direct injection of physiological fluids, such as urine, serum and plasma, without protein precipitation or analyte extraction steps [45,46]. The protein matrix of a biological sample is solubilized by certain micelles, such as SDS or Brij-35, and is eluted with the solvent front. Direct injection of physiological fluids is one of the important capabilities of MLC that is particularly useful in therapeutic drug monitoring. The popularity of this application of MLC has grown rapidly in recent years [47–63]. Fig. 9 illustrates a recent example of the use of a direct injection for the analysis of sulfonamides in milk.

3.6. Scope of applications

From the early publications, micelles were viewed as mobile phase modifiers that can replace organic co-solvents in RPLC. The capabilities of MLC have often been compared to those of traditional hydro-organic RPLC. While such comparisons are useful in achieving a better understanding of the new technique, they resulted in a misconception that the two techniques have the same scope of applications, that is, for the separation of moderately to highly hydrophobic solutes. In addition to the high efficiency of RPLC, hydro-organic mobile phases provide a wide range of solvent strengths and selectivities for the separation of a variety of uncharged solutes. Versatility is one of the key reasons behind the popularity of RPLC.

On the other hand, MLC is a poor choice for such applications, when various chromatographic parameters are considered. For hydrophobic solutes, the problem of poor efficiency in MLC is even more pronounced. Micellar eluents are generally weaker than typical hydro-organic phases. The use of higher

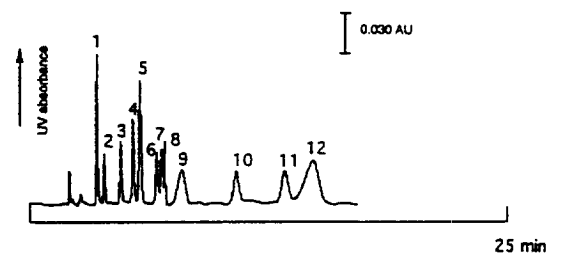


Fig. 9. Direct injection of a milk sample for analysis of sulfonamides by MLC. Mobile phase: 70 m*M* SDS, 6.0% 1-propanol, pH 3.0. Stationary phase: C_{18}. Reprinted from Ref. [63] with permission.

micelle concentrations in combination with an organic co-solvent can solve the problem, however, the higher viscosity of the mobile phase results in lower plate counts. Nevertheless, the retention behavior and separation of hydrophobic compounds, such as polyaromatic hydrocarbons, by MLC have been extensively studied [64–73].

One area where MLC should perform better than other HPLC methods is in the separation of charged compounds or mixtures of charged and uncharged compounds. MLC is a powerful alternative to ion-pair chromatography (IPC) and ion-exchange chromatography (IEC). The chromatographic capabilities of MLC and IPC have been systematically compared in a recent study [74]. Several advantages of MLC were demonstrated, as summarized in the following. First of all, column efficiency is often equivalent in these three techniques. Secondly, MLC has a more reproducible retention behavior. This is mainly due to the unchanging composition of the stationary phase in MLC. With both IPC and IEC, reproducibility can be a major problem. In IPC, for example, the concentration of ion-pairing reagent that is adsorbed onto the stationary phase is directly related to that in the mobile phase. Any small changes in the mobile phase composition lead to variations in the characteristics of the stationary phase (e.g. charge density) and, subsequently, affect the retention behavior. Thirdly, method development in MLC is much faster and easier. Again, due to the fixed composition of the stationary phase, one can rapidly scan various mobile phase compositions with little or no need for stationary phase equilibration. This can drastically reduce the time needed for method development. On the contrary, column equilibration times in IPC, with variations in mobile phase composition, can be long and time consuming. In addition, gradient elution in MLC is quite feasible, while the gradient capabilities of IPC are limited due to the long column regeneration times. Finally, due to the reproducible and predictable retention behavior in MLC, the optimization of mobile phase conditions is more feasible. Following an optimization process using an iterative regression strategy, the separation of a group of amino acids and small peptides using MLC and IPC was compared. As shown in Fig. 10, for the IPC separation, there is poor agreement between the predicted and observed

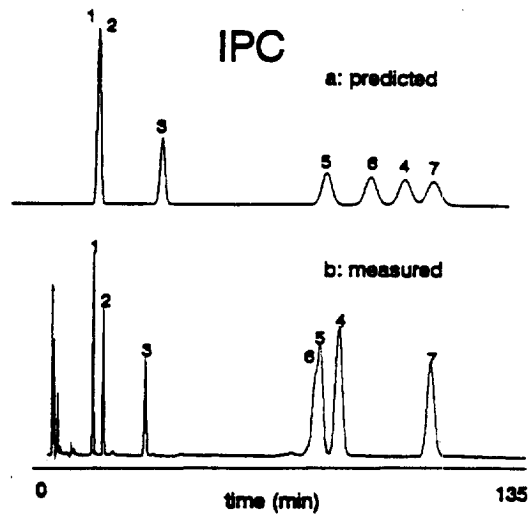


Fig. 10. Separation of a group of seven amino acids and small peptides by ion-pair chromatography using an iterative regression optimization procedure. (a) Predicted and (b) observed. Mobile phase: 2 m*M* SDS and 16% 2-propanol. Reprinted from Ref. [74] with permission.

separation. The analysis time is very long and gradient elution is needed to effectively resolve this mixture with a reasonable analysis time. On the other hand, excellent agreement was observed between the predicted and observed values in MLC. Fig. 11 illustrates a more complex mixture of amino acids and peptides that has been completely separated by MLC in a much shorter analysis time.

The influence of prototropic equilibria on the retention of monoprotic molecules was initially quantitatively described by Arunyanart and Cline Love [75]. The model was later corrected and extended to include polyprotic and zwitterionic compounds by Rodgers et al. [76]. The usefulness of the retention models for the simultaneous optimization of pH and micelle concentration was demonstrated [77]. Due to the presence of micelles, the ionization constants of ionizable compounds would be different from those in aqueous media [78]. The magnitude of the micelle-induced pK_a shift is proportional to the solute–micelle binding constants and can be quite significant. This can be seen in Fig. 12, where the pK_a of several amino acids and small peptides in an aqueous, an ion-pairing and a micellar mobile phase are compared. The pK_a shifted con-

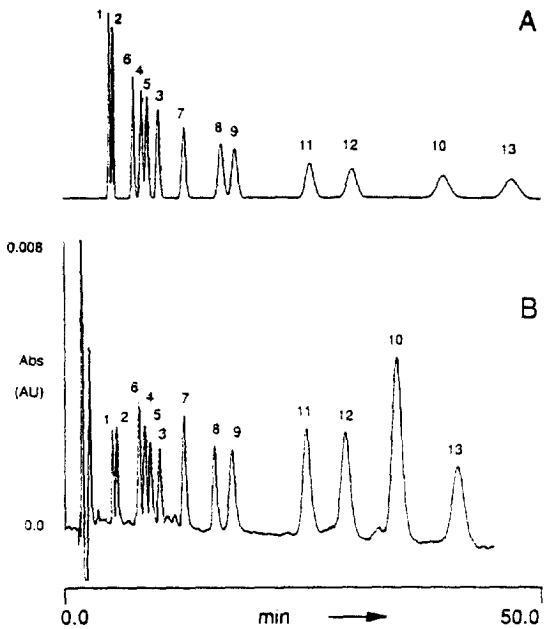


Fig. 11. Separation of a group of 13 amino acids and small peptides by MLC using the iterative regression optimization procedure. (A) Predicted and (B) observed. Mobile phase: 0.16 *M* SDS and 12% 2-propanol. Reprinted from Ref. [20] with permission.

siderably to higher values (i.e. weaker acids) as the anionic ion-pairing reagent and anionic micelles are included. The magnitude of the shift in the ion-pairing solution is nearly the same for all solutes, while the micellar-induced pK_a shift varies for different compounds, depending on the extent of interaction with micelles. The pK_a values for the three amino acids or for the two peptides are nearly identical in the aqueous and ion-pairing eluents. They are significantly different in the micellar eluent. This can have a significant effect on retention and selectivity in MLC, as well as MEKC (vide infra). Therefore, higher selectivity can be observed in MLC for solutes with similar pK_a values in aqueous media. Another interesting observation is that the pK_a values of acids in anionic micelles shift to higher values, i.e. acids are weaker in the presence of SDS micelles. This is important in LC using the alkyl silane stationary phases that have a limited operating pH range of 2–8. In other words, the pK_a of relatively strong acids can shift to higher values that are within the operating pH range of the column, thus prototropic equilibria can be incorporated into optimization schemes.

In addition to uncharged hydrophobic solutes,

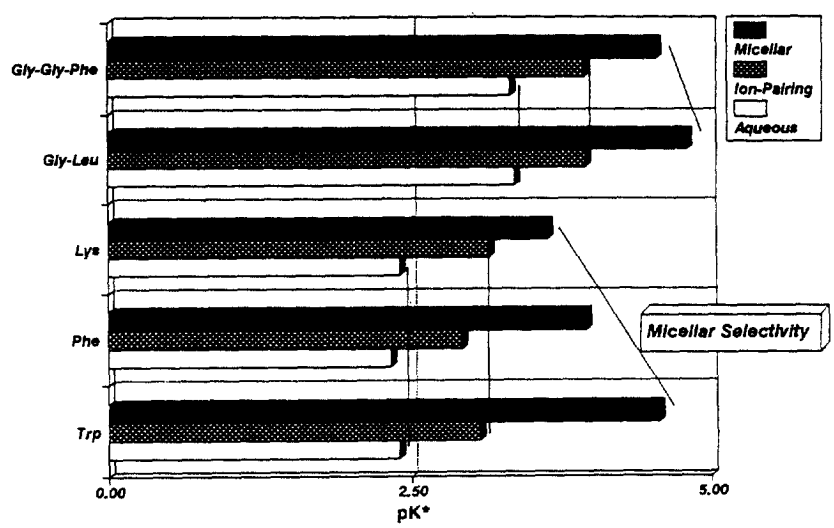


Fig. 12. Comparison of the ionization constants of five amino acids and small peptides in aqueous, ion-pairing and micellar mobile phases. Reprinted from Ref. [76] with permission.

MLC has been used in a variety of other applications [45–63,79–101]. Direct injection has been used for the analysis of therapeutic drugs [45], theophylline in serum [47] and urine [55], acyclovir in serum and plasma [48], the anti-neoplastic drug, teniposide, in plasma [49], an enzyme inhibitor and its metabolite in urine [50], mitomycine [51], corticosteroids [52], sulfonamides in urine [53] and milk [63], antipyrine metabolites in plasma [54], caffeine and theobromine in urine [55], bilirubin species in urine [56], nicotine and cotinine in urine [57], dope control in sports [58], acetaminophen in urine [59], catecholamines in urine [60], cephalosporins [61] and cortisol in urine [62].

Other types of applications include the analysis of recombinant human growth hormone in *Escherichia coli* fermentation [79], the analysis of dihydropyridines [80], maleic hydrazide in tobacco [82], flavonol-2-O-glycosides [83], sun-screen agents in cosmetics [84], diuretics [85], environmental applications [86], plant growth regulators [87], catecholamines [88–90], amino acids and peptides [20,91–93], pharmaceutical compounds [94,95], enantiomeric separations [96–98] and organometallic compounds [99–101].

4. Micellar electrokinetic chromatography (MEKC)

Micellar electrokinetic chromatography (MEKC) is a mode of CE that is capable of separating uncharged compounds. The technique has also been referred to as micellar electrokinetic capillary chromatography (MECC) in the literature. Since its first introduction by Terabe et al. [3,4] over ten years ago, MEKC has become widely popular and hundreds of studies and applications have been reported. MEKC uses the same instrumental set-up as CE, however, charged organized media, such as micelles, are incorporated, and these act as the separation medium for uncharged solutes. Charged micelles migrate in the electric field at an electrophoretic velocity that is proportional to their charge-to-size ratio. Uncharged solutes with different micelle–water partition coefficients, P_{mw}, can then be separated. Since the separation mechanism is based on differential partitioning, MEKC is viewed as a chromatographic technique with migrating charged micelles (or other types of organized media) acting as pseudo-stationary phases. Consequently, a limited elution window exists in MEKC. All uncharged solutes have to be separated between the elution time of an unretained solute, t_{eo}, and the migration time of micelles, t_{mc} (Fig. 13). MEKC can be viewed as a hybrid of RPLC and capillary zone electrophoresis (CZE), as the separation process incorporates hydrophobic and polar interactions, a partitioning mechanism and electromigration. However, MEKC offers a combination of the features of CZE and RPLC, such as high efficiencies, rapid analysis time, small sample size, small solvent consumption and the versatility of incorporating chemical selectivity in the separation process. MEKC is the only CE technique that is capable of separating mixtures of charged and uncharged molecules. It offers higher efficiency than RPLC and capillary electrochromatography (CEC). The number of theoretical plates in a MEKC separation can be ten or more times greater than that in RPLC. Another major advantage over conventional

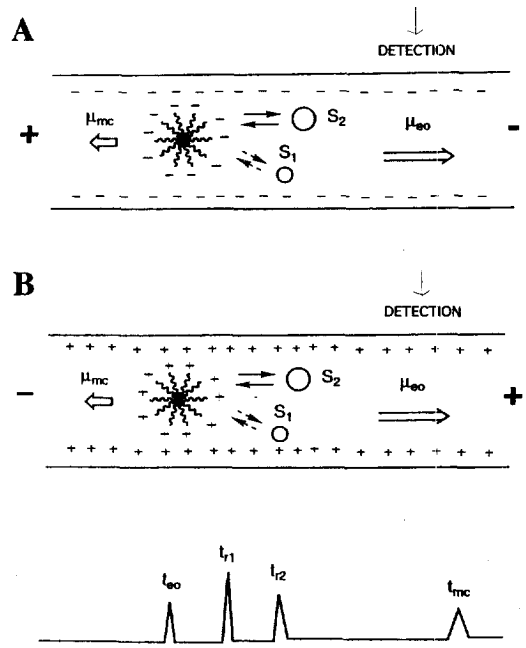


Fig. 13. Migration of uncharged compounds in MEKC using (A) anionic- and (B) cationic pseudo-stationary phases. The separation of solutes S1 and S2 was achieved due to their differential partitioning into the pseudo-stationary phase. The uncharged solutes are eluted within an elution window (t_{mc}/t_{eo}).

chromatographic techniques as well as CEC is the flexibility and ease of changing the chemical composition of the pseudo-stationary phases. For example, the type and/or composition of the micellar solution can be easily modified or replaced by simply rinsing the capillary with a new type of pseudo-stationary phase. The equilibration times are often very rapid. This inherent flexibility of controlling key parameters leads to enhanced separations and greatly facilitates the process of method development.

4.1. Migration and resolution

Fig. 13 illustrates typical migration schemes for uncharged compounds in MEKC using an anionic surfactant and a cationic surfactant in an uncoated fused-silica capillary. Anionic micelles migrate in the opposite direction to the electroosmotic flow (EOF) in an uncoated capillary (Fig. 13A). Typically, the EOF velocity is stronger than the electrophoretic velocity of anionic micelles under "normal" conditions (e.g. an uncoated capillary and a pH value greater than six). As a result, the anionic micelles are carried towards the cathode. Using cationic micelles, the capillary wall is coated with the positively charged surfactants and, often, this leads to a reversal in the direction of the EOF. It is therefore necessary to reverse the polarity of the electrodes in the CE set-up to ensure the elution of the cationic micelles and, consequently, of the uncharged solutes through the detection window (Fig. 13B).

Two extremes that define an elution window in MEKC exist. Analytes that do not interact with micelles ($P_{mw} \sim 0$) spend all of their migration time in the bulk aqueous phase and migrate at the electroosmotic mobility. These are typically uncharged polar molecules, like methanol or acetonitrile, which are EOF markers and elute at t_{eo}. The other end is defined by the elution of analytes that interact so strongly with the micelles ($P_{mw} \sim \infty$) that they spend all of their migration time with micelles. The t_{mc} markers are typically very hydrophobic compounds that are sparingly soluble in the aqueous media, with reported examples being Sudan III and dodecanophenone. The elution times for these analytes coincide with the micellar migration time, t_{mc}. The existence of an elution window limits the peak capacity in MEKC, as all uncharged solutes have to be separated between the migration time of an unretained solute, t_{eo}, and that of a fully retained solute, t_{mc}. The size of the elution window can be enhanced by using organic modifiers or mixed micelles, or by modifying the capillary walls (vide infra).

The fundamental resolution equation for uncharged solutes in MEKC, Eq. (2), has the same format as that for conventional chromatography, as it indicates that resolution depends on three terms related to efficiency, selectivity and retention [4]. The fourth term is unique to MEKC and represents the existence of an elution window.

$$R = \left(\frac{N^{1/2}}{4}\right)\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'_2}{1 + k'_2}\right)\left(\frac{1 - \left(\frac{t_{eo}}{t_{mc}}\right)}{1 + \left(\frac{t_{eo}}{t_{mc}}\right) k'_1}\right) \quad (2)$$

Again, if micelles were truly stationary (i.e. $t_{mc} \sim \infty$), the fourth term would drop out and the equation would be identical to that in conventional chromatography. The size of the elution window has a significant impact on MEKC separations. In general, better resolution is achieved at wider elution windows (i.e. $t_{eo}/t_{mc} \rightarrow 0$). Another difference between MEKC and conventional LC is the effect of retention (k') on resolution. In conventional LC, there is an initial rapid increase in resolution, with increasing retention of shortly retained solutes. Eventually, resolution reaches a plateau value beyond which there is not much gain in the quality of separation. In MEKC, an optimum range of k' (around one–five) exists, within which maximum resolution can be achieved. Outside this optimum range (i.e. for poorly and strongly retained solutes), resolution decreases rapidly. This is mainly due to the limited elution window, as strongly retained compounds are pushed to the end of the chromatogram and elute close to or at the t_{mc}. It is therefore of prime importance to optimize retention in MEKC and increase the width of the elution window. These can be achieved through proper adjustment of solution parameters.

As in chromatography, better resolution is achieved at higher efficiency. The disadvantage of the limited elution window is mostly compensated

for by the large number of theoretical plates that are routinely achieved in MEKC.

4.2. Retention and selectivity

Since the introduction of the technique, it has been widely believed that retention in MEKC depends on the hydrophobicity of the solute. Fig. 14a–d shows the relationships between retention in MEKC (log k') using four different micellar systems and the logarithm of the octanol–water partition coefficient (log P_{ow}), a widely accepted scale for hydrophobicity. The best single correlation was observed for the sodium cholate (SC) system. For SDS and cetyl trimethyl ammonium bromide (CTAB), different relationships exist for various groups of congeneric solutes. The worst correlation was observed for the lithium perfluorooctane sulfonate (LiPFOS) system. The large differences in correlations indicate the existence of various retention behaviors in MEKC among various types of pseudo-stationary phases [102]. There is no doubt that hydrophobic interaction plays a major role in solute–micelle interactions and consequently it affects retention in MEKC. However,

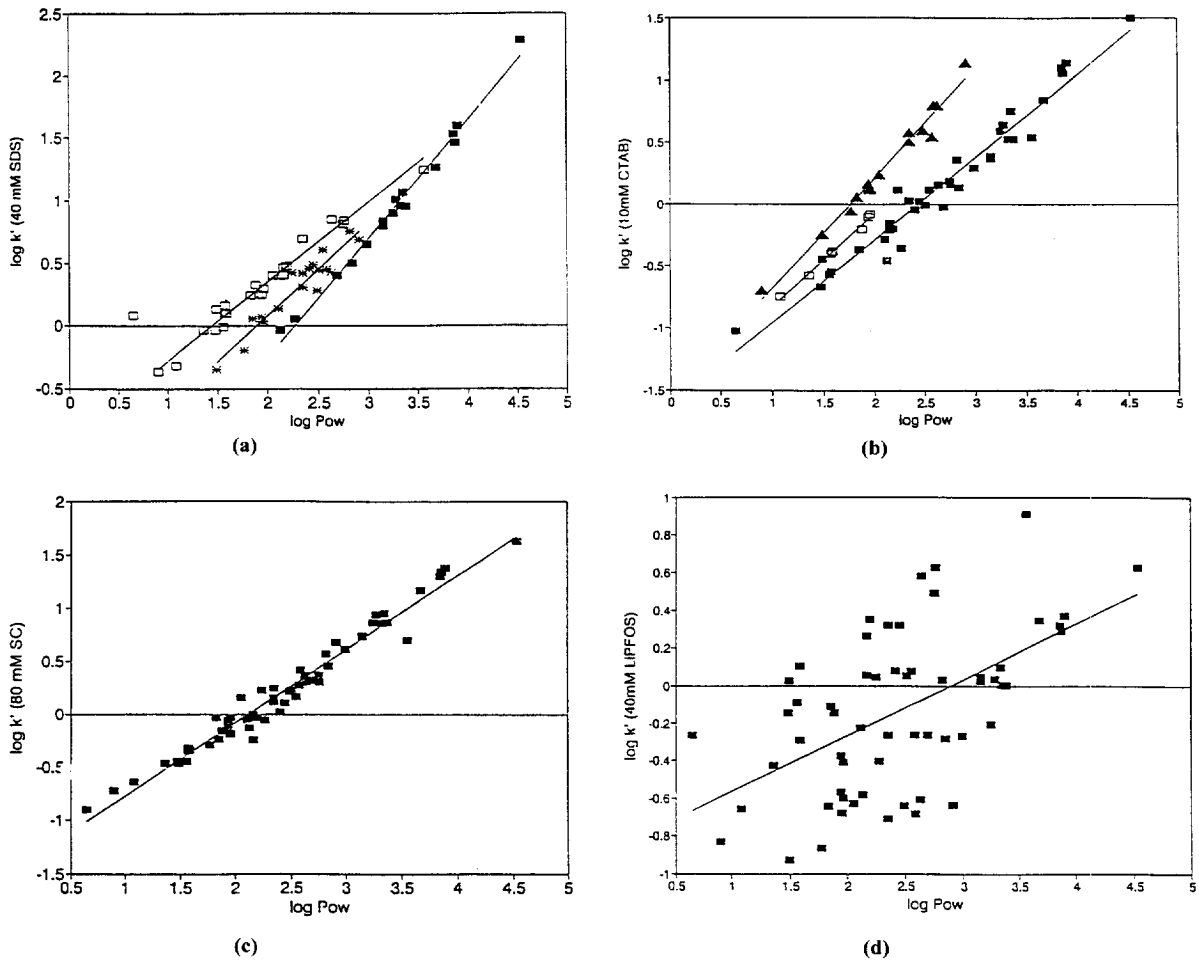


Fig. 14. Relationships between log k' and log P_{ow} for a group of 60 compounds in different MEKC systems: (a) 40 mM SDS; log $k'=(0.60\pm0.01)$; log $P_{ow}=-0.95$; $n=60$; $r=0.9301$; S.E.$=0.189$; (b) 10 mM CTAB; log $k'=(0.60\pm0.01)$; log $P_{ow}=-1.31$; $n=60$; $r=0.8877$; S.E.$=0.248$; (c) 80 mM SC; log $k'=(0.69\pm0.00)$; log $P_{ow}=-1.46$; $n=60$; $r=0.9829$; S.E.$=0.103$; (d) 40 mM LiPFOS; log $k'=(0.30\pm0.02)$; log $P_{ow}=-0.86$; $n=60$; $r=0.5595$ and S.E.$=0.352$. Reprinted from Ref. [102] with permission.

one would observe identical selectivity for different micellar systems if retention in MEKC was dependent solely on hydrophobicity. This is not the case, as large variations in migration patterns are often observed for different types of pseudo-stationary phases. Fig. 15a–f illustrates an example of the large variations in selectivity observed using four micellar and one polymeric pseudo-stationary phases, a triblock co-polymer known as Elvacite 2669 [103]. The chromatograms shown in Fig. 15 clearly indicate three important points. First, micelle concentration has no effect on selectivity (Fig. 15a,b), second, the overall separation patterns in MEKC strongly depend on the type of surfactant and third, solutes do not necessarily elute according to their hydrophobicity. In fact, in systems such as SDS and LiPFOS, the two most hydrophobic solutes, benzene (peak 1) and anisole (peak 5) are the least retained. Note that surfactant and polymer concentrations were selected to provide equivalent elution times for all five systems, thus, there are some overlapping peaks. However, the main purpose was to demonstrate selectivity changes rather than resolving the five test solutes.

The availability of a wide variety of pseudo-stationary phases with different selectivities is quite advantageous in method development. The large number of choices, however, would make the process of selecting the appropriate type of pseudo-stationary phase difficult. This problem is particularly pronounced for the separation of complex mixtures, where operating under optimum conditions is crucial. Presently, due to a lack of knowledge about the exact chemical nature of solute–micelle interactions, selection of the chemical composition of the pseudo-stationary phase in MEKC is based on trial and error or on the experience of the analyst. As a result, a general understanding of the properties of various surfactants would be useful in selecting the optimum conditions for MEKC separations.

The methodology of linear solvation energy relationships (LSER) can provide valuable information about the nature of underlying solute–micelle interactions that lead to selectivity differences in MEKC systems. Yang and Khaledi [104,105] reported the use of Kamlet–Taft's linear solvation energy relationships (LSER) [106–110] to quantitatively describe retention in MEKC according to the structural properties of the solute, V, π^*, β and α as:

$$\log k' = SP_0 + mV_1/100 + s\pi^* + b\beta + a\alpha \quad (3)$$

where V is the molar volume of the solute, π^* is the measure of the dipolarity/polarizability of the solute, β is the hydrogen bond acceptor strength (basicity) of the solute and α is the hydrogen bond donor strength (acidity) of the solute. The three parameters π^*, β and α have been derived based on solvatochromic comparison methods that were first developed by Kamlet, Taft, and co-workers. SP_0 is the regression constant and contains information about the chromatographic phase ratio, e.g. it varies with micelle concentration.

Using a group of aromatic test solutes, the LSER modeling of retention in MEKC was investigated for various micellar pseudo-stationary phases. The coefficients m, s, b and a in the LSER equation represent the properties of the micellar systems and the values for representative systems are listed in Table 2. The data suggest that solute size plays the most important role in retention, as m is the largest coefficient in the LSER models. The positive sign for m indicates that bulkier molecules are retained longer in MEKC (i.e stronger solute–micelle interaction). The mV/100 term in the LSER model is related to hydrophobic interaction, as it represents an unfavorable energy term for the formation of a properly sized cavity in the solvent system for solute accommodation. The next largest coefficient in most systems is b (with the exception of the fluorocarbon micelles of LiPFOS), which indicates that type A hydrogen bonding (solute acceptor–solvent donor) is the second most important type of interaction that influences retention in nearly all MEKC systems (Table 2). The negative sign for b indicates that stronger hydrogen bond acceptor solutes (i.e. larger β values) would have less interaction with the micelles and are retained less. This is reasonable, since water is a stronger hydrogen bond donor than micelles, thus, more basic solutes would have a stronger interaction with the bulk aqueous media than with micelles. A combined effect of these two factors mainly determines solute retention in MEKC. For example, compared to benzene, a substituted aromatic compound, such as nitrobenzene, is bulkier and is a stronger hydrogen bond acceptor. The larger size of the substituted aromatic compound causes stronger interaction with micelles than found for the parent benzene molecule. However, the increase in

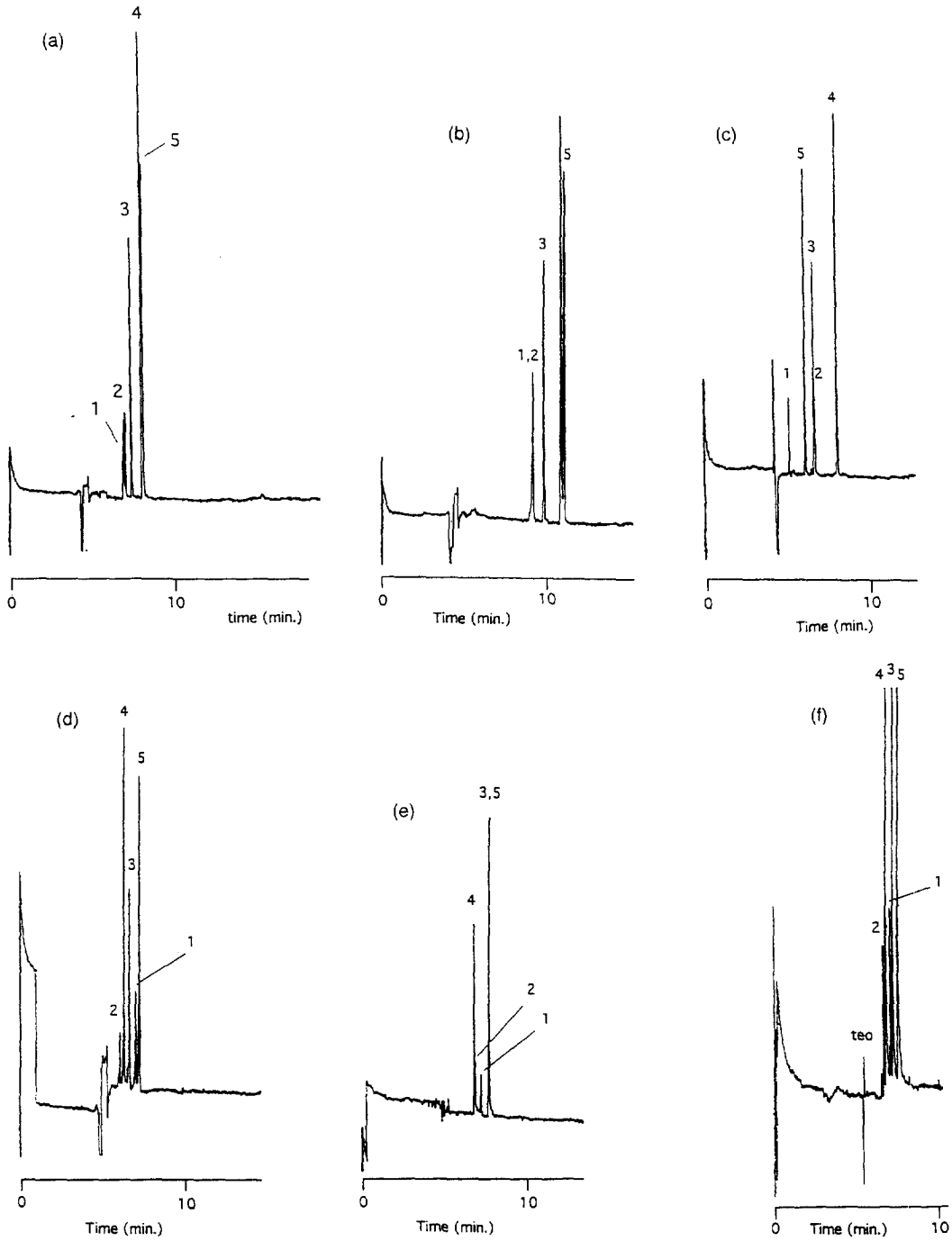


Fig. 15. Influence of surfactant type on elution pattern and selectivity in MEKC. (a) 40 m*M* SDS, (b) 60 m*M* SDS, (c) 40 m*M* LiPFOS, (d) 100 m*M* SC, (e) 4% Elvacite 2669 and (f) 25 m*M* C_{14}TAB. Peaks: 1 = benzene, 2 = benzonitrile, 3 = nitrobenzene, 4 = acetophenone and 5 = anisole. From ref. [103] with permission.

Table 2
LSER modeling of retention in MEKC with different pseudo-stationary phases (Eq. (3))

MEKC system[a]	SP_o	m	s	b	a
0.04 *M* LiPFOS	−1.51	2.44	−0.25	0.16[a]	−0.98
2% Elvacite 2669[b]	−1.55	3.00	0.09[c]	−2.33	0.24
0.06 *M* SC	−1.62	3.89	−0.27	−2.88	0.23
0.08 *M* SC	−1.38	3.82	−0.32	−2.85	0.18
0.01 *M* C_{14}TAB	−1.78	3.96	−0.26	−2.75	0.99
0.02 *M* SDS	−1.87	4.00	−0.25	−1.79	−0.16[a]
0.04 *M* SDS	−1.49	3.95	−0.26	−1.80	−0.18[a]
log P_{ow}	0.17	5.62	−0.66	−53.90	0.14

[a] Buffer: 50 m*M* phosphate, pH 7.0.
[b] Buffer: 100 m*M* CAPS, pH 10.00.
[c] Values are not significant at the 95% confidence level.
In all cases, $n=60$ and correlation coefficients for the regression of better than 0.95 were observed. See Refs. [104,105].

its basicity reduces its interaction with the micelle. Subsequently, the relative retention of these two solutes is mainly determined by the net effect of these two opposing factors and can vary greatly among various micellar systems, depending on their interactive properties (vide infra).

The m coefficient is related to the cohesiveness of the micellar phase. The LiPFOS micelles are the most cohesive (smallest m coefficient), while the hydrocarbon micelles of SDS, CTAB and SC are the least cohesive phases (large m coefficients) [105]. As a point of reference, recall that water is the most cohesive solvent and hydrocarbon liquids are among the least cohesive phases. According to Table 2, the m values are very similar for the hydrocarbon micelles (i.e., C_{14}TAB, SDS and SC). This suggests that cavity formation energy has a minor effect on the differences in chemical selectivities among hydrocarbonaceous surfactants in MEKC. However, differences in selectivities between LiPFOS or Elvacite 2669 and the hydrocarbonaceous surfactants can partly be due to the cavity formation term.

Coefficient b represents the relative strength of the micellar phase as hydrogen bond donors (HBDs) or acidity. The larger b value means that the micellar phase is a stronger hydrogen bond donor [105]. Based on Table 2, the relative HBD strength of the micellar systems can be ranked as: LiPFOS>SDS>Elvacite 2669>C_{14}TAB>SC>1-octanol. This suggests that an LiPFOS–MEKC system is the strongest HBD system, followed by the SDS–MEKC system. The acidities of Elvacite 2669 and C_{14}TAB are between those of SDS and SC. The values of b are very different in these five MEKC systems, ranging from −2.88 to +0.16, therefore, type A hydrogen bonding interactions contribute significantly to the chemical selectivity differences among these five systems. The negative b values indicate that the micelles are weaker HBD than the bulk aqueous phase.

On the other hand, the term ($a\alpha$) corresponds to a type B hydrogen bond interaction, which involves solutes acting as HBDs (acids) and solvents as hydrogen bond acceptors (HBAs) (bases). The coefficient, a, is a measure of the relative strength of micellar phases as HBAs (i.e. basicity). The larger value for the a-coefficient refers to the higher HBA strength of the micellar phase. According to Table 2, the HBA strength of the micellar systems can be ranked as: C_{14}TAB>Elvacite 2669>SC>1-octanol>SDS>LiPFOS. This means that the C_{14}TAB MEKC system is the strongest HBA (basic) system among these five MEKC systems, followed by Elvacite 2669. The values of the a-coefficient are also very different for these five MEKC systems (−0.98 to 0.99), which suggests that type B hydrogen bonding interactions also contribute significantly to the chemical selectivity of these five MEKC systems.

The term $s\pi^*$ represents the dipolar interactions between solutes and solvents. Due to the fact that s values are small in magnitude and are similar for the systems studied, it can be concluded that dipolar interactions have little or no effect on retention and selectivity in these MEKC systems.

In summary, the LSER results indicate that hydro-

gen bonding is a major reason for the selectivity differences among different surfactants. In general, the fluorocarbon micelles of LiPFOS have the strongest HBD strengths among the five MEKC systems. This conclusion is consistent with the observed elution behavior, as HBA solutes interact strongly with the LiPFOS micelles. One such example is given in Fig. 15c, where the five test solutes elute according to their basicity (i.e. β values) [103]. On the other hand, SC and CTAB, which are viewed as HBA micelles, have much stronger interactions with HBD solutes, such as phenols and alcohols, while they exhibit weaker affinities for solutes with HBA functionalities. The elution pattern in the SDS system (Fig. 15a) is based mainly on solute size and the HBA strength of the solute. For instance, the two early-eluting peaks in the SDS system, benzene (peak 1) and benzonitrile (peak 2), have the smallest sizes. Acetophenone (peak 4) is bulkier than anisole, which favors more retention. However, the effect of the larger size of acetophenone on retention is somewhat offset by its greater HBA strength than that of anisole. As a result, it elutes slightly earlier than anisole. According to the LSER model, retention in the SDS system decreases as the basicity of the solute increases, due to the negative b coefficient. Based on the LSER results, SDS has intermediate properties in terms of hydrogen bonding than have LiPFOS on the one hand and SC, Elvacite and CTAB on the other hand. It is a stronger HBD than SC, CTAB and polymeric Elvacite, but it is weaker than LiPFOS, while as a HBA, it is weaker than SC, CTAB and Elvacite, but is stronger than LiPFOS.

The LSER results also shed some light on the relationships between MEKC retention and log P_{ow}. Micelles have a more cohesive environment than octanol, as indicated by the smaller m values. The three hydrocarbon-based micelles, SDS, CTAB and SC, have nearly identical m coefficients. Thus, the cavity term does not contribute to the differences in the retention–log P_{ow} relationships for the three micellar systems [102].

The congeneric behavior between retention and log P_{ow} for the SDS and CTAB micelles (as evident by the existence of different lines for various groups of solutes in Fig. 14) can also be interpreted in terms of the hydrogen bonding characteristics of the two systems. SDS micelles are stronger HBDs than 1-octanol, thus, they exhibit more selective interactions towards HBA solutes. In fact, one can recognize a trend in the grouping of various solutes in the three log k' vs. log P_{ow} lines, depending on their HBA strengths (as measured by the Kamlet and Taft solvatochromic b values). For example, the first subgroup (Fig. 14a, bottom line with filled squares) consists of weak HBAs, such as hydrophobic-substituted aromatic compounds like alkylbenzenes, halogenated benzenes and polycyclic aromatic hydrocarbons (PAHs) ($\beta \leq 0.20$). The second subgroup (middle line with asterisks) contains HBA compounds with intermediate strengths, such as aromatic ethers and some substituted nitrobenzenes ($0.20 \leq \beta \leq 0.35$). The third subgroup (upper line with open squares) contains strong HBA compounds like alkyl aromatic ketones, benzonitrile, aromatic esters, aromatic alcohols, anilines and some other nitrobenzenes ($\beta \geq 0.35$).

The congeneric behavior for CTAB (Fig. 14b) can be interpreted in terms of the HBD strengths of the three subgroups. Since CTAB micelle is a stronger HBA than octanol, it selectively interacts with solutes with HBD functional groups. In Fig. 14b, the first subgroup (bottom line with filled squares) consists of non-HBDs and weak HBDs ($\alpha \leq 0.17$), such as anilines. The second subgroup (middle line with open squares) contains solutes with intermediate HBD strength, like benzyl alcohols ($0.33 \leq \alpha \leq 0.40$), while the upper line (filled triangles) includes phenols that are strong HBDs with $\alpha \geq 0.54$.

The interactive properties of the LiPFOS micelles are very different from that of octanol, as is evident from the large differences in the LSER coefficients. The LiPFOS micelles are the most cohesive (smallest m coefficient), the strongest HBDs (largest b coefficient) and the weakest HBAs (smallest a coefficient) of the five systems. On the other hand, octanol has a lower cohesive character, is a weaker HBD and is a stronger HBA than LiPFOS. Consequently, a poor correlation was observed between retention in LiPFOS–MEKC and log P_{ow}. On the basis of the LSER coefficients, one can conclude that SC micelles have closer interactive properties to octanol than to the other three micellar systems. It is therefore not surprising that the best correlation between retention and log P_{ow} was observed for the SC micelles [102].

One should also consider the differences in the micelle structures for alkylated surfactants like SDS and CTAB systems and for bile salts such as SC. The SDS and CTAB micelles are roughly spherical, with large aggregation numbers (between 60–80), while bile salt micelles, such as SC, are much smaller, with aggregation numbers of around two–ten. There is a greater degree of heterogeneity in the SDS and CTAB micelle structures than in SC micelles. This leads to larger variations in the locations and microenvironment polarities in the alkyl chain micelles than in the bile salt micelles.

4.3. Effects of chemical composition of micellar solutions

In MEKC, resolution is a function of retention (k'), selectivity (α), efficiency (N) and the size of the elution window (t_{mc}/t_{eo}). In turn, these four terms are influenced by experimental parameters such as surfactant type and concentration, type and percentage of modifiers, pH, temperature, ionic strength and applied field strength. In the following sections, the roles of various parameters in MEKC separations are discussed.

4.3.1. Surfactant concentration

The primary role of surfactant concentration is to adjust the retention factor to within the optimum range, in order to achieve better resolution. According to Terabe et al. [4], the relationship between the retention factor, k', and the surfactant concentration can be described as:

$$k' \cong P_{mw} v(C_{sf} - \mathrm{CMC}) \quad (4)$$

where v is the surfactant molar volume, C_{sf} is the total surfactant concentration, CMC is the critical micelle concentration and P_{mw} is the partition coefficient of a solute between an aqueous phase and micelles. One can then determine the P_{mw} and CMC values for a given surfactant using MEKC. A comparative study of P_{mw} values obtained by MEKC and by MLC showed that both techniques provide equivalent results [111]. Using Eq. (4), one can predict the retention of solutes with known P_{mw} values in a given micellar system. Unfortunately, the P_{mw} is not known for the majority of compounds. One can then appreciate an effort for the compilation of P_{mw} data [112]. The determination of P_{mw} using modified capillaries and in the absence of EOF has recently been reported [113].

Micelle concentration determines the phase ratio of the system. The retention factor in MEKC should be optimized primarily through micelle concentration [4,114]. The size of the elution window and efficiency can also be influenced by this factor, however, it has little, if any, effect on the selectivity of uncharged solutes. This can be seen from Fig. 15a,b, and is also evident from the LSER results, where only the regression constant, SP_0, changes, while the other four coefficients, m, s, b and a, are independent of micelle concentration. Note that selectivity between a pair of solutes is simply defined as the ratio of the retention factors, which is approximately equal to the ratio of micelle–water partition coefficients at low surfactant concentration, according to Eq. (5), i.e.:

$$\alpha = k'_2/k'_1 \cong P_{mw,2}/P_{mw,1} \quad (5)$$

4.3.2. Type of pseudo-stationary phase

Various types of pseudo-stationary phase have been used in MEKC over the past ten years. They can be categorized into two general groups; the first and most widely used are charged micelle (i.e. dynamic aggregates of charged surfactants) and the other group consists of covalently bonded or polymerized charged organized assemblies. Variations in the hydrophobic moiety, the ionic head group or the type of counter-ion can influence retention, selectivity, the size of the elution window and efficiency in MEKC.

4.3.2.1. Anionic alkyl chain surfactants

Anionic alkyl-chain surfactants, especially SDS, have been the most widely used surfactant type [115–147]. The popularity of SDS can be attributed to its high aqueous solubility, low CMC, low Kraft point, low UV molar absorptivity (even at low wavelengths), its availability and cost. Serendipitously, SDS might provide the "right" type of selectivity for many solute mixtures. LSER studies indicate that SDS is a stronger HBD than most other surfactant systems studied thus far, such as bile salts, cationic CTAB surfactants and methacrylate-based copolymers. Consequently, SDS should be a better surfac-

tant type in many situations, considering that the great majority of small solutes that are separated by MEKC contain a HBA functional group, such as nitro, carbonyl, cyano, etc. Even HBD solutes, such as phenols or alcohols, have hydrogen bond accepting characteristics. One such example can be seen in Fig. 16, where a mixture of 24 explosive chemicals, most of which are nitroaromatic molecules, is separated in approximately 10 min with a SDS micellar solution [142]. It has been reported that the type of counter-ion (e.g. Na^+, Li^+ or K^+) for dodecyl sulfate micelles can greatly influence the efficiency, elution pattern, retention and the size of the elution window in MEKC [148].

4.3.2.2. Bile salts

As an alternative to SDS, the use of bile salt surfactants in MEKC separations has become popular [145,149–164]. Various types of bile salts have been used as pseudo-stationary phases in MEKC. They provide different selectivities than SDS. As mentioned previously, bile salt surfactants also have aggregation properties and structures that are very different from those of SDS micelles. They can tolerate relatively higher concentrations of organic modifier (30% organic solvent) than can SDS micelles, without disrupting their structural integrity. Bile salt micelles are generally considered to be more "polar" than SDS micelles. This arises from the general observation that most solutes have a stronger interaction with the SDS micelles. This perception is somewhat misleading, as "polarity" is defined within a broad context. According to the LSER results, however, both SDS and SC micelles have nearly identical *m* (i.e. cohesiveness) and *s* (dipolarity/polarizability) coefficients. SDS micelles are stronger HBDs, while SC micelles are stronger HBA micelles. This trend was also consistent for another bile salt surfactant, sodium deoxycholate (SDC). Therefore, one can expect that HBA solutes would have a stronger interaction with the SDS micelles, while HBDs would have a greater affinity for the bile salt micelles. As mentioned earlier, the majority of solutes bear a HBA functional group that has lead to the general notion of stronger interaction with the SDS micelles. In addition, retention is influenced by the phase ratio of the micellar system.

4.3.2.3. Fluorocarbon surfactants

In general, the use of fluorinated surfactants in MEKC separations has been very limited. This has been due mostly to the lack of availability of MEKC-compatible fluorocarbon surfactants of high purity. Ye et al. [165] reported the first application of the fluorocarbon micelles of LiPFOS for the MEKC separation of a group of small peptides. They

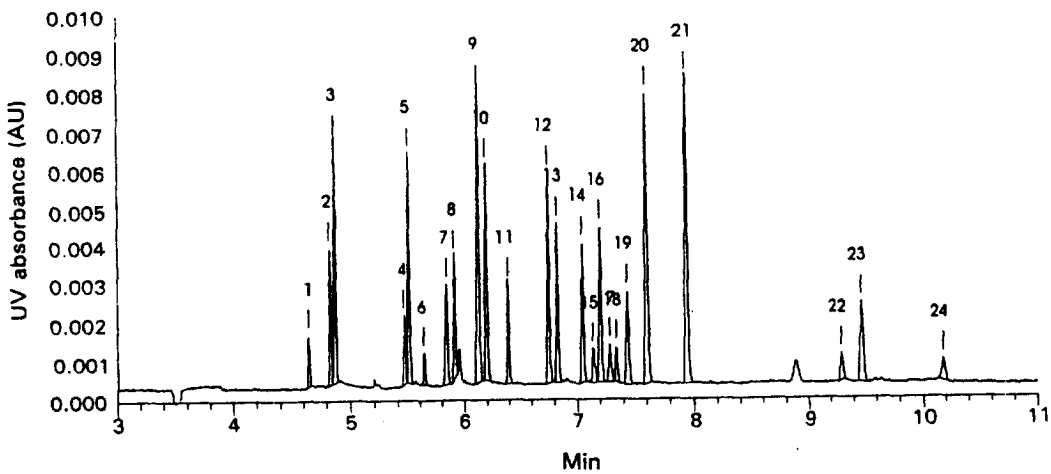


Fig. 16. MEKC separation of a mixture of 24 nitroaromatic compounds. MEKC conditions: 50 m*M* SDS micelles in borate buffer, applied voltage = +25 kV, λ = 230 nm, total length = 60 cm. From Ref. [142] with permission.

observed that the selectivity and elution pattern for LiPFOS is dramatically different from that obtained with typical anionic alkyl chain surfactants like SDS or LiDS (lithium dodecyl sulfate). As mentioned earlier, LiPFOS micelles are more cohesive than SDS, as evident from the smaller m coefficient. This results in a smaller solute interaction with LiPFOS micelles. On the other hand, LiPFOS is a stronger HBD than other micellar systems that have been studied thus far. Consequently, a stronger interaction between solutes with HBA functional groups and LiPFOS micelles is observed. In principle, a HBD micelle should provide better selectivity for a mixture of HBA solutes. Fig. 17 shows the separation of a group of seventeen corticosteroids using LiPFOS micelles. The separation of this group of corticosteroids was very challenging, due to the high structural similarity of the solutes and the complexity of the mixture [145]. These corticosteroids are bulky molecules with a steroidal backbone and several hydrogen bonding functional groups, such as carbonyl or hydroxyl groups. Using SDS, the majority of these solutes eluted near, or at, the t_{mc}. This is consistent with LSER results, as the large m coefficient indicates that a strong interaction occurs between the bulky molecules and the micelles and, consequently, leads to excessive retention. The quality of separation improves considerably by using bile salt micelles and/or inclusion of various modifiers, such as an organic co-solvent, cyclodextrins or urea. However, none of these systems were suitable for achieving a complete separation of all seventeen solutes. As mentioned earlier, bile salts are weaker HBDs (larger negative b coefficient) than SDS, thus, less retention is observed for solutes such as corticosteroids that have several HBA groups. Nevertheless, several peaks overlapped due to the lack of selectivity and/or the narrow elution window. With the LiPFOS micelles, the m coefficient is much smaller than for SDS, therefore, the problem of excessive retention is resolved. On the other hand, the stronger HBD nature of LiPFOS micelles provides better selectivity for HBA functional groups. As shown in Fig. 17, the majority of peaks are resolved. The resolution can be even further improved by optimizing the micelle concentration. As shown in a following section, the use of mixed micelles of bile salts and alkyl surfactants can also lead to enhanced separation of this mixture, due to near optimum retention and a very wide elution window.

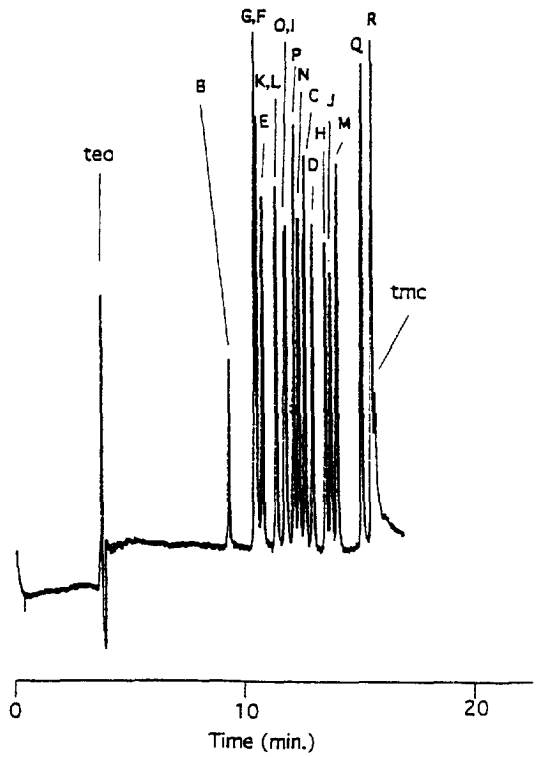


Fig. 17. MEKC separation of a mixture of seventeen corticosteroids using a fluorocarbon surfactant, 40 mM LiPFOS. MEKC conditions: Applied voltage = +15 kV, phosphate–borate buffer, λ = 254, total length = 62 cm. Peak identification: (A) t_{eo}, (B) triamcinolone, (C) prednisone, (D) cortisone, (E) fludrocortisone, (F) hydrocortisone, (G) prednisolone, (H) prednisone acetate, (I) fludrocortisone acetate, (J) cortisone acetate, (K) prednisolone acetate, (L) hydrocortisone-21-acetate, (M) corticosterone, (N) triamcinolone acetonide, (O) fluocinolone acetonide, (P) 6α-methyl prednisolone, (Q) deoxycorticosterone, (R) progesterone and (S) t_{mc}. From Jefferson G. Bumgarner, Ph.D. Dissertation, North Carolina State University, 1996.

4.3.2.4. *Cationic surfactants*

Cationic surfactants generally interact with the negatively charged silica capillary wall and reverse the direction of the EOF. As a result of the reversed EOF, the polarity of the electrodes would have to be reversed in order to elute solutes through the detector window (Fig. 13B). According to LSER analysis, the cationic micelles of CTAB have a very different

interactive behavior than have SDS micelles. The CTAB micelles are stronger HBAs.

4.3.2.5. Novel and chiral surfactants

Other anionic surfactants that have been examined are in situ borate-complexed surfactants such as the N-D-gluco-N-methylalkanamide (MEGA) series and N,N-bis-(3-D-gluconamidopropyl)-cholamide and -deoxycholamide (Big CHAP and deoxy Big CHAP). Charge density on the micelles and consequently the size of the elution window can be controlled through proper adjustment of the pH value and the borate concentration. These surfactants have been used for the MEKC separation of compounds such as herbicides, barbiturates and amino acids [176–178].

Due to their chiral functionalities, Big CHAP and deoxy Big CHAP have also been applied for the chiral separation of compounds such as troger's base and Silvex herbicide by MEKC. Other novel anionic chiral surfactants such as *R*- and (*S*)-N-dodecoxycarbonylvaline and sodium N-dodecanoyl-L-valinate as well as polymerized chiral micelles have been reported for the separation of chiral molecules [179–183]. Many of these chiral surfactants are synthetic and consist of a hydrophobic alkyl chain tail and an amino acid or a carbohydrate head group with a chiral center.

4.3.2.6. Non-ionic and zwitterionic surfactants

In order to separate uncharged molecules in MEKC, charged surfactants must be used. Surfactants with a zero net charge, such as non-ionic and zwitterionic surfactants, can be used along with an ionic surfactant as mixed micelles. They can also have a great influence on the MEKC separation of charged molecules. Both non-ionic and zwitterionic surfactants have been used alone for the separation of charged compounds such as amino acids and polypeptides [184,185]. Non-ionic surfactants and mixtures of anionic/non-ionic surfactants have been shown to provide different selectivities, reduced currents and changes in elution window sizes compared to anionic surfactant systems. Zwitterionic surfactants have been used alone and in conjunction with SDS in MEKC. Because these surfactants do not increase the conductivity of the buffer, they can be used at high concentrations while still allowing the use of high voltages and large I.D. capillaries.

4.3.2.7. Mixed micelles

As mentioned above, different retention behaviors and selectivities can be observed for various types of surfactants. For certain complex mixtures of structurally similar solutes, however, one might not be able to find a suitable surfactant type that provides adequate resolution. This is typically due to a lack of selectivity and/or a narrow elution window. In these situations, the use of mixed micelles can lead to enhanced separations. Mixing surfactants with different interactive properties can lead to great changes in selectivity for a given mixture. Selection of the optimum composition would then be crucial for improving the quality of the separation. The size of the elution window for certain mixed micellar systems is often larger than for the single constituent surfactants. Retention factors and selectivity change with the mole fraction of surfactants in the mixed micelles. Efficiency in the mixed micellar systems is often not much different from that in single surfactant systems, however, in a few cases, loss of efficiency has been observed.

A good example of the usefulness of mixed micelles is the separation of the mixture of the seventeen corticosteroids mentioned earlier. Fig. 18a shows the separation of the mixture using an equimolar mixture of the two bile salt surfactants, glycodeoxycholate (GDC) and taurocholate (TC). Even with this mixed micellar system, there are a number of overlapping peaks. Note that the size of the elution window in the bile salts systems is small, which might be due to the typically small aggregation number of these systems and to subsequent small values in the charge-to-size ratio. Extending the size of the elution window should lead to increased separation. This can be achieved by incorporating an alkyl chain ionic surfactant with the bile salts. As illustrated in Fig. 18b, incorporation of SDS into the mixed bile salts systems has resulted in a significant increase in the size of the elution window (from around three in the bile salts to about 4.4 in the ternary system of the two bile salts and SDS). However, several peaks still overlap and/or coelute, apparently due to the loss of selectivity and/or to a strong solute interaction with the SDS in the ternary

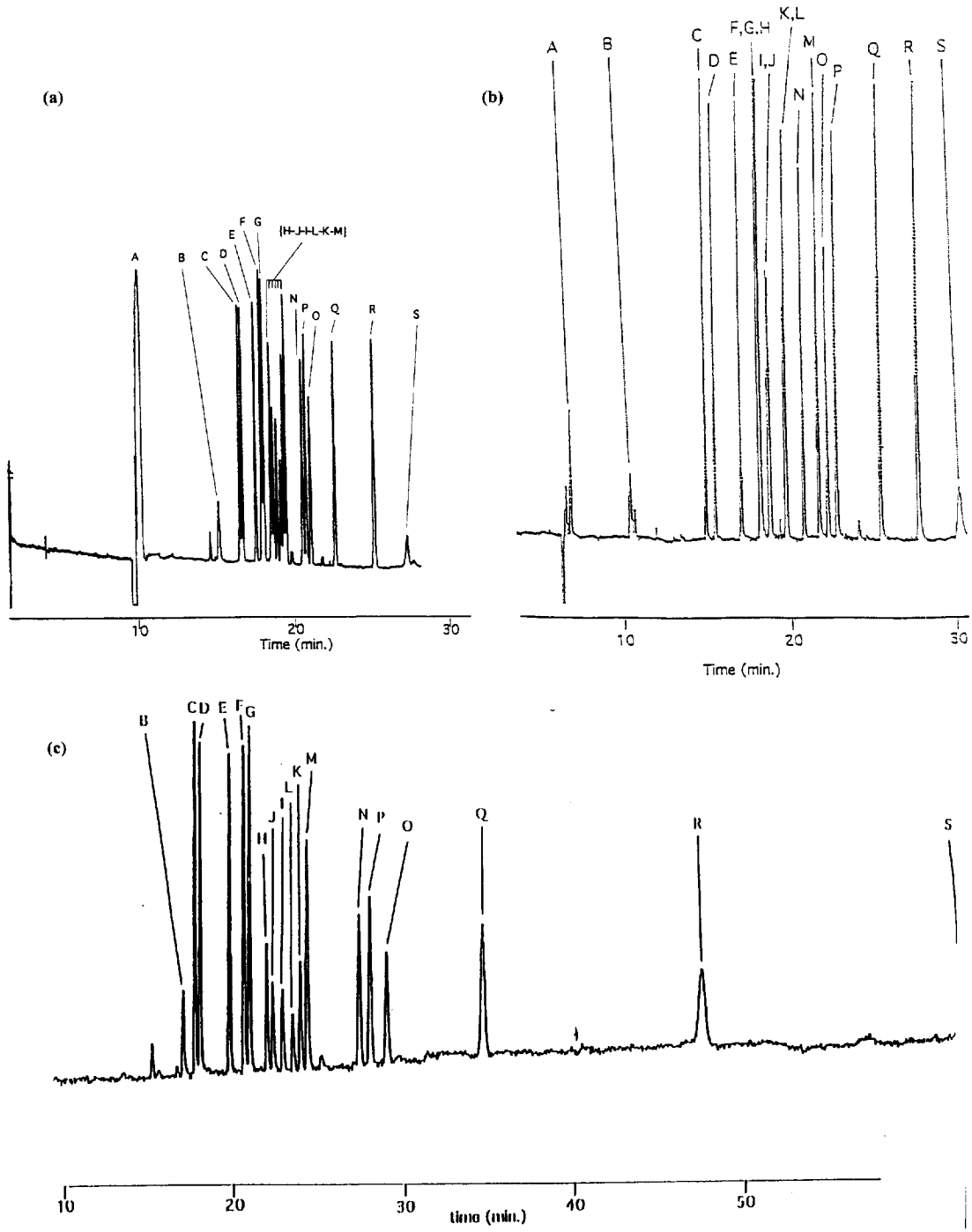


Fig. 18. Mixed micellar electrokinetic chromatography of the mixture of seventeen corticosteroids. (a) Binary mixed micelles of two bile salts: 50 m*M* glycodeoxycholate and 50 m*M* taurocholate; (b) ternary mixed micelles of the two bile salts and SDS: 33 m*M* glycodeoxycholate, 33 m*M* taurocholate and 33 m*M* SDS; (c) mixed micelles of the two bile salts and a short chain alkyl surfactant: 33 m*M* glycodeoxycholate, 33 m*M* taurocholate, 70 m*M* butanesulphonate. Peak identification is the same as in Fig. 17. From Ref. [186], with permission.

mixed micellar system (i.e. k' is too large). In order to maintain the wide elution window without sacrificing selectivity, an anionic surfactant with a short alkyl chain length, such as butane sulfonate (BuS), can be added to the mixture of bile salts. Butane sulfonate does not form micelles and, therefore, has little effect on the overall retention behavior and selectivity. As shown in Fig. 18c, the ternary system of GDC–TC–BuS has a very wide elution window (~seven) and baseline separation of all peaks has been achieved. This increase in the size of the elution window is mainly due to the increase in the migration time of the micelles (i.e. t_{eo} remained almost constant). Bile salt monomers have bulky steroid backbones with only one charged head group. The aggregation number of the bile salt micelles are between two and ten, which means that their charge-to-mass ratio is small. The anionic alkyl surfactants are attracted to the hydrophobic portion of the bile salt micelles, thus increasing the charge-to-mass ratio of the mixed micelles. This increase in the negative charge density would enhance the mobility of the mixed micelles in the opposite direction to that of EOF and causes the micelles to elute at a later time. The size of the elution window initially increases with the concentration of the alkyl surfactant before reaching a plateau. For BuS, this is achieved at 70 m*M*. It is worth noting that the efficiency of the GDC–TC–BuS system (Fig. 18c) is considerably lower (by a factor of four–five) than that achieved with the GDC–TC–SDS system (Fig. 18b). This is probably due to the joule heating that occurs as a result of using high concentrations of BuS. In spite of the lower efficiency, however, much better resolution is achieved in the BuS system, due to the wider elution window, higher overall selectivity and, perhaps, near optimal retention.

Various combinations of different surfactants, such as bile salt/anionic [145,186], anionic/zwitterionic [142,187], cationic/zwitterionic [188], fluorocarbon/anionic [165], anionic/non-ionic [187,192,194,196–198], cationic/cationic [189,190], anionic/cationic [191], bile salt/bile salt [145,193] and non-ionic/non-ionic (for charged solutes) [195] have been used in MEKC. The use of mixed micelles of non-ionic Brij 35 and anionic SDS has resulted in an infinite elution window at a specific Brij 35 concentration [108]. Typically, the addition of Brij 35 yields a narrower elution window and large changes in selectivity. However, at a specific composition, the mobility of anionic micelles equals the electroosmotic mobility, thus making the charged micelles effectively stationary.

4.3.2.8. Polymeric pseudostationary phases

In recent years, there has been a great deal of interest in the use of polymeric pseudo-stationary phases. Three different types of polymeric phases have been reported: (1) Polymer micelles where surfactant monomers are polymerized and covalently bonded together [199,200], (2) cascade macromolecules like starburst dendrimers [201–204] and (3) ionic block co-polymers [205–207]. The primary advantage of using polymeric phases is the stability of their structure in the presence of large concentrations of organic modifiers. Conventional micelle-forming surfactants, such as SDS and bile salts, can tolerate up to 20–30% organic modifier before micelle formation is inhibited. The use of high concentrations of organic modifier is necessary for the separation of highly hydrophobic solutes that interact strongly with the micelles. Therefore, the majority of the reported applications for the polymeric pseudo-stationary phases include hydrophobic solutes. Fig. 19 illustrates the first reported separation of fullerenes by a CE method using a tri-block co-polymer of poly(methyl methacrylate–ethyl acrylate–methacrylic acid) (commercially known as Elvacite 2669). As shown, the C-60 and C-70 peaks are easily separated, due to sufficient differences in the size of the two molecules using an organic-rich buffer.

4.3.3. Modifiers

Different types of modifiers such as organic solvents, cyclodextrins and urea are typically incorporated in the aqueous buffers of MEKC in order to reduce the retention factors of strongly bound solutes to micelles. Their presence can also lead to wider elution ranges and/or higher selectivities.

4.3.3.1. Organic solvents

Organic modifiers, such as methanol and acetonitrile, have been extensively utilized for improving resolution in MEKC [208–230]. In RPLC, organic modifiers play an important role in controlling

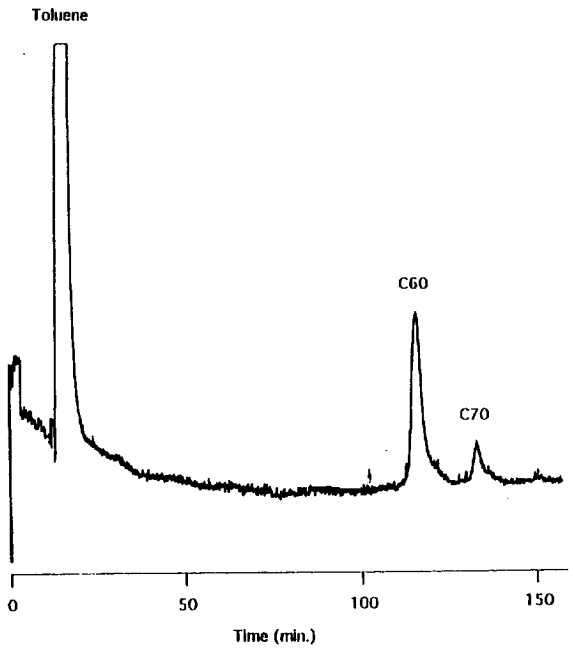


Fig. 19. Separation of fullerenes in a fullerite sample using a polymeric pseudo-stationary phase; 2% Elvacite 2669 polymer, 84% methanol in water. The fullerite sample was dissolved in toluene. MEKC conditions: Applied voltage = +28 kV, λ = 260, total length = 62 cm, temperature = 40°C, CAPS buffer. From: Jefferson G. Bumgarner, Ph.D. Dissertation, North Carolina State University, 1996.

retention and selectivity for a wide variety of compounds. Their use in MEKC separations, however, has been mostly for improving the separation of hydrophobic compounds that interact strongly with micelles and elute at, or near, the migration time of micelles. The main role of organic modifiers in MEKC has been to reduce the retention factors of highly hydrophobic solutes to within, or near, the optimum range. Typically, inclusion of organic solvents leads to an increase in the size of the elution window. As a result, resolution in MEKC can be enhanced at the expense of longer analysis times. Their influence on the selectivity of partitioning into micelles for a wide range of molecules with polar functional groups is not clear. The concentration of organic solvents would have to be limited (typically ≤20%) in order to maintain the integrity of micelles. The use of polymeric phases will provide an opportunity to investigate the role of organic solvents over a wider range of concentrations. Chromatographic behavior with more hydrophobic modifiers can be different from those for polar solvents. This is because polar modifiers, like methanol or acetonitrile, have little or no interaction with the micelles, while the more hydrophobic ones, such as longer chain alcohols, are incorporated into micelles.

Aiken and Huie [215] examined the effects of the addition of 1-alkanols (C_4–C_8) in MEKC. In general, the size of the elution window increased upon addition of the long chain alcohols, which was attributed to the increase in mobility of the SDS micelles as the EOF remained relatively constant. Interestingly, higher retention factors were observed when longer chain modifiers, such as octanol, were used. This increase was more pronounced for the hydrophobic solutes. For example, the retention factors of alkyl phenols in 100 m*M* SDS increased upon addition of 50 m*M* long chain alcohols, from butanol to octanol. Inclusion of the long chain alcohol, however, only caused a small increase in retention of the more polar solutes. On the other hand, retention factors for more hydrophobic solutes were considerably higher using the micellar solution with the longer chain alcohols, such as octanol, than for butanol. This was attributed to the incorporation of the long chain alcohols into the micelles, which could increase the micelle–water partition coefficient and the phase ratio due to an increase in hydrophobicity and in the volume of the micelle [215]. They also reported that selectivity was changed with the inclusion of alcohol modifiers for a group of alkyl phenol test solutes. This is a different behavior from that typically observed with polar modifiers like methanol or acetonitrile, where the retention factor is smaller in the presence of the modifier.

Several other reports [227–230] have focused on the effects of organic modifiers such as 1-hexanol, methanol, acetonitrile and dimethylformamide on SDS- and bile salt micelles. Katsuta et al. [227] demonstrated that selectivity can be altered by the inclusion of organic modifiers in MEKC systems. It was determined that this is primarily due to the saturation of the micellar palisade layer with the modifier and to the hydrogen bonding interaction between the modifier and analyte molecules.

Fig. 20 shows the MEKC separation of a group of ten estrogens. The top chromatogram shows no separation in a 50 m*M* SDS buffer solution. The

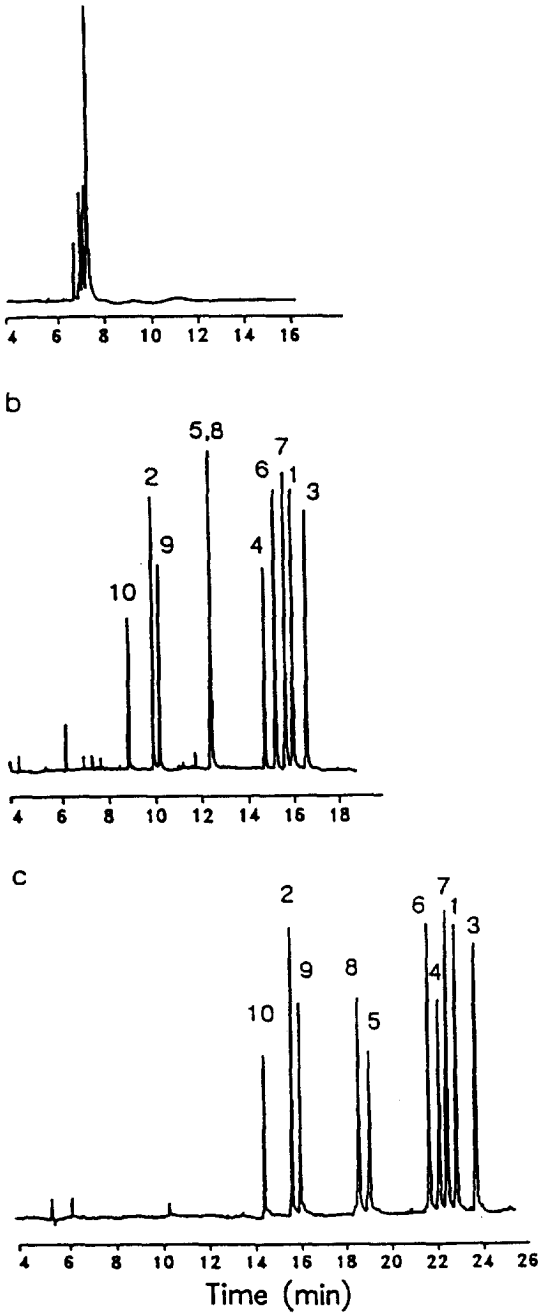


Fig. 20. Effect of an organic modifier in the MEKC separation of a group of estrogens: 50 m*M* SDS in phosphate buffer, pH 7.0. (Top) no organic solvent; (middle) 15% acetonitrile; (bottom) 20% methanol. Peak identification: 1 = 17β-estradiol; 2 = 16-keto-17β-estradiol; 3 = 2-methoxyestradiol; 4 = 2-hydroxyestradiol; 5 = 4-hydroxyestradiol; 6 = estrone; 7 = 2-methoxyestrone; 8 = 4-hydroxyestrone; 9 = 16α-hydroxyestrone and 10 = estriol. From Ref. [212] with permission.

addition of 15% acetonitrile improves the separation, with peaks 5 and 8 co-eluting. These peaks correspond to 4-hydroxyesteroidal (peak 5) and 4-hydroxyesterone (peak 8). Upon changing the type and concentration of the organic modifier to methanol, the two peaks are resolved and the elution order for peaks 4, 6 and 7 was changed [212].

4.3.3.2. Glucose and urea

In addition to typical organic solvents, the use of other modifiers, like urea and glucose, has also been reported. Urea reduces the interaction of hydrophobic compounds with micelles by increasing their solubility in the aqueous solutions [231–235]. The retention factors of hydrophobic compounds in micellar solutions is therefore decreased dramatically. The size of the elution window has also been shown to increase with the addition of urea to MEKC systems.

Kaneta et al. [222] have reported the effects of adding glucose as a modifier to enhance the resolution in MEKC. In the separation of nine nucleosides, the inclusion of 1 *M* glucose resulted in variations in selectivity as well as in an increase in the electrophoretic mobility of SDS micelles and, consequently, to a wider elution window. For this mixture, glucose was reported to be even more effective than methanol in improving the separation [222].

4.3.3.3. Cyclodextrins

Another type of modifier that has been used in MEKC involves cyclodextrins (CDs) [236–242]. The first report of CD-modified MEKC (CD–MEKC) with SDS micelles was by Terabe et al. [236] for the separation of polycyclic aromatic hydrocarbons (PAHs). The hydrophobic cavity of CDs provides an alternative site of interaction to micelles for the hydrophobic solutes. Since uncharged CDs migrate at the EOF velocity and in the opposite direction to anionic SDS micelles, the net retention factor of solutes decreases in the presence of CDs. As a result, hydrophobic solutes that would otherwise co-elute with micelles can be better separated. In addition, CDs introduce a shape selectivity effect that is beneficial for the separation of structural isomers.

Fig. 21 shows an example of the use of two different types of CD for the separation of a group of

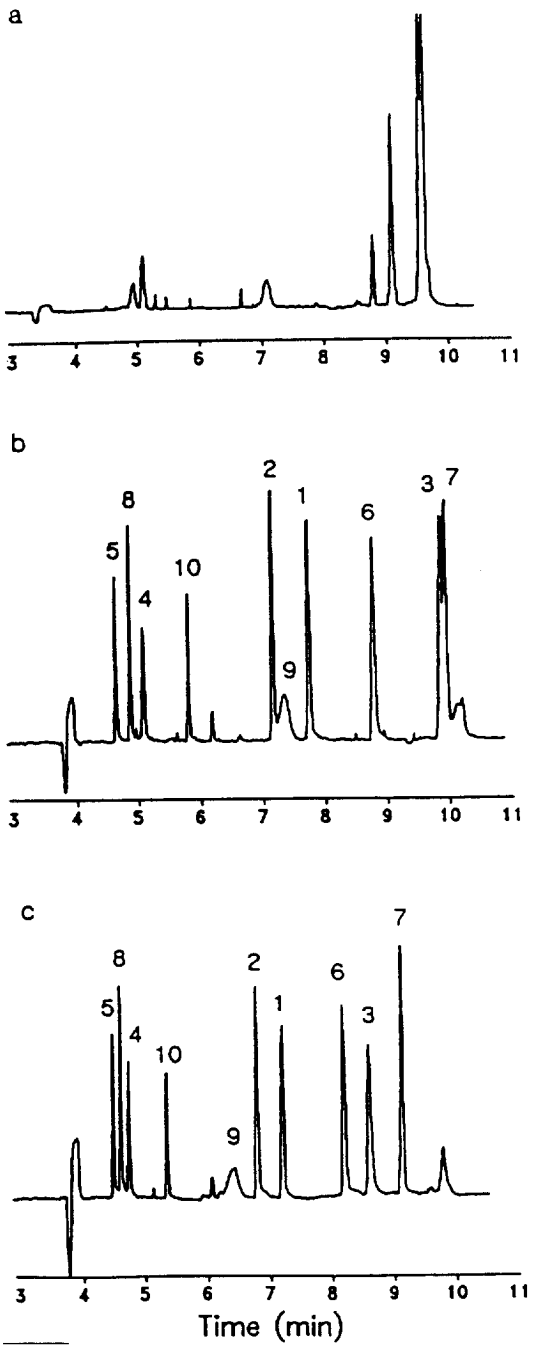


Fig. 21. Effect of cyclodextrin on the MEKC separation of a group of estrogens. 50 m*M* SDS in 10 m*M* sodium borate, pH 9.2. (a) No CD, (b) 20 m*M* β-CD; (c) 20 m*M* γ-CD. Peak identification see Figs. 3–14. From Ref. [212] with permission.

ten estrogens [212]. Note that the same mixture was also separated using organic modifiers (see Fig. 20). γ-Cyclodextrin has a larger cavity that provides a better selectivity for the mixture than β-CD. Most of the reported applications of CD–MEKC have involved the use of γ-CD, which apparently provides better results than found with other types of CD. Note that charged CDs can also be used either in conjunction with the micelles or alone for the separation of uncharged solutes.

4.3.4. pH

In addition to the primary partitioning mechanism into micelles, one can incorporate secondary chemical equilibria (SCE), such as prototropic, ion-pairing or metal–ligand complexation, in the bulk aqueous solution. The use of acid–base equilibria is particularly important as it involves the separation of ionizable compounds. A large majority of small molecules with pharmaceutical and clinical significance have acidic or basic functional groups. For the separation of compounds with very similar electrophoretic mobilities, incorporation of micelles can result in better selectivity. For mixtures of charged and uncharged compounds, MEKC is the method of choice. The retention behavior of ionizable compounds is much more complicated than that of uncharged solutes. Both charged and uncharged forms of the solutes can interact and migrate with the micelles. The charged fraction of the solutes would also migrate in the bulk aqueous media at its own electrophoretic mobility. For these groups of compounds, controlling the pH is of great significance, as the pH determines the position of the acid–base equilibrium and, consequently, the net charge on the molecule. The migration behavior of ionizable compounds in MEKC has been quantitatively described through simple mathematical models that were developed on the basis of the acid–base equilibrium and micelle–water partitioning equilibria for the charged and uncharged forms of solute. The equations describe the retention factor or net mobility as a function of two experimental variables, pH and surfactant concentration, as well as the dissociation constant and the micelle–water partition coefficients [115,116,243,244]. If the values of the equilibrium constants are known for a given compound, one can

easily predict the corresponding migration behavior over a wide range of pH values and micelle concentrations. In a majority of cases, however, constant values are unknown. One can achieve an estimate of the values by measuring retention at a few initial pH values and micelle concentrations and by fitting the experimental values to the models through non-linear regression. The retention behavior of a group of seventeen amines was successfully predicted on the basis of only five initial experiments at different pH values and SDS concentrations. A high correlation was observed between the predicted and observed migration parameters. Fig. 22 shows an example of the predicted chromatograms at various pH values for the group of amines. Large variations in retention and selectivity, due to changes in the pH value,

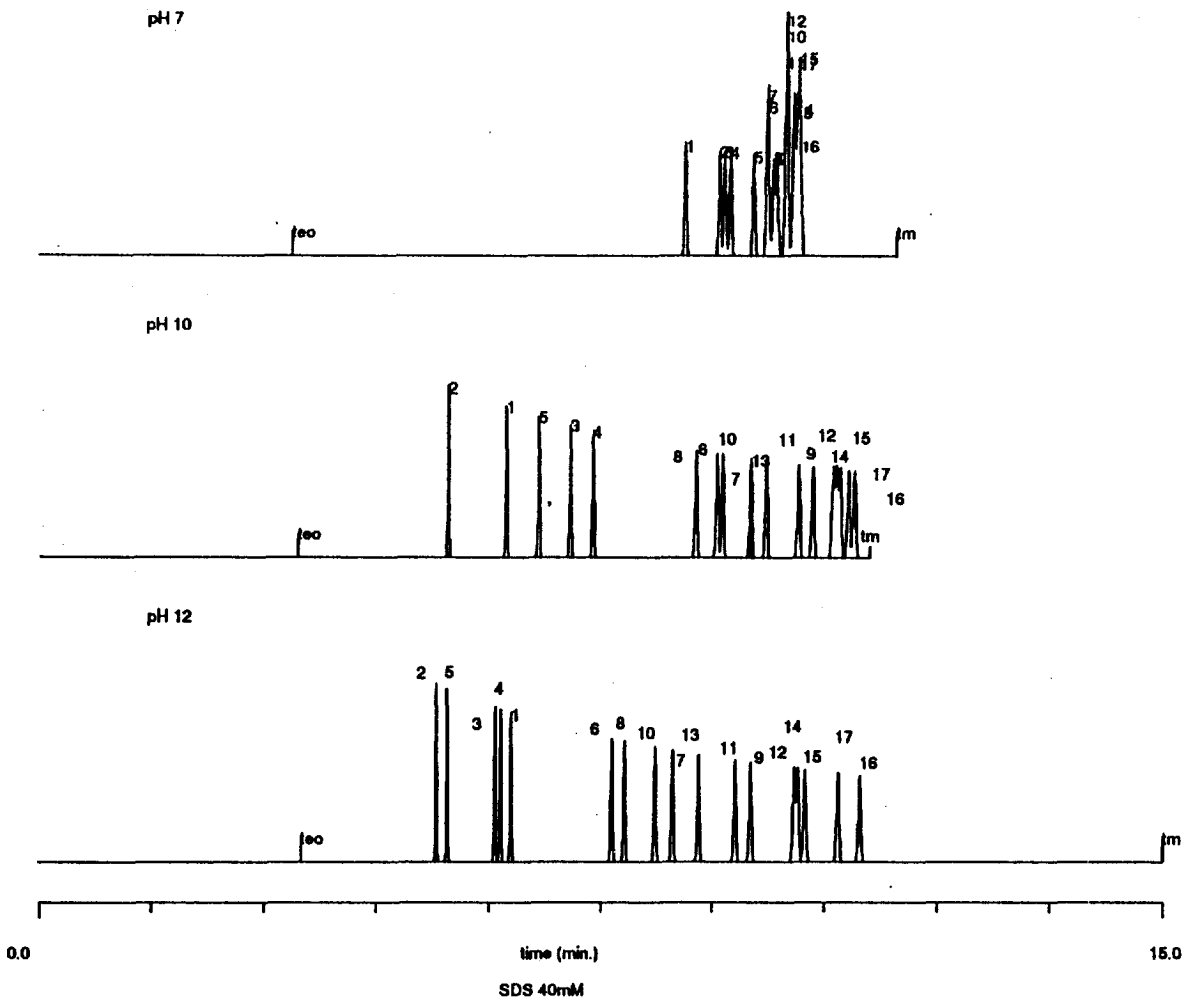


Fig. 22. Computer assisted optimization in MEKC. Predicted retention behavior of seventeen aromatic amines as a function of pH. Peak identification: 1=nicotine; 2=4-nitrobenzylamine; 3=benzylamine; 4=3-methyldopamine; 5=norephedrine; 6 ephedrine; 7 phenethylamine; 8=4-nitrophenethylamine; 9=N-methylphenethylamine; 10=4-chlorobenzylamine; 11=2-methylphenethylamine; 12=phenylpropylamine; 13=4-bromobenzylamine; 14=2-tolylethylamine; 15=4-chlorophenethylamine; 16=phenyl-*n*-butylamine; 17=1-(methylphenyl)propylamine. From Ref. [243] with permission.

indicate the significance of this parameter. These quantitative models are quite useful for predicting and optimizing the parameters with a minimum number of initial experiments. Fig. 23 illustrates the successful separation of a mixture of seventeen amines in less than 15 min.

Other optimization methodologies have also been reported in MEKC. The usefulness of an iterative regression strategy to optimize selectivity in CZE and MEKC has been reported by Corstjens et al. [245,246]. Pyell and Butehorn [247] have examined the role of temperature for minimizing the analysis time and optimizing resolution. Temperature is not a widely used parameter as its effects on selectivity are not pronounced. Temperature has a great effect on viscosity (and therefore EOF) and this can be significant, as shown in Fig. 24, where the separation of a group of amino acids at two different temperatures is compared [133].

Pyell and Butehorn [248] discussed strategies for the rapid, one parameter optimization of concentrations of SDS and modifiers, like urea and glucose. They also developed a computer-aided method for the simultaneous optimization of the concentrations of SDS and urea for the separation of various mixtures, like nitroaromatic compounds, urea pesticides and amines [249]. Bretnall and Clarke [250] also investigated the optimum modifier composition for the separation of several cardiovascular drugs. They examined different modifiers, such as various alcohols and ketones as well as acetonitrile. Ng et al.

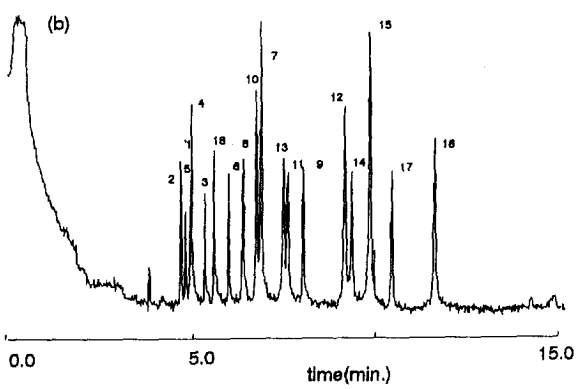


Fig. 23. MEKC separation of seventeen aromatic amines using 40 m*M* SDS, pH 11.0, and 10% acetonitrile. Peak identification is the same as in Fig. 22. From Ref. [243] with permission.

[251] used an interpretive optimization scheme based on overlapping resolution mapping for the separation of flavonoids. Beattie and Richards [252] investigated the optimization of separation conditions, such as different electrolyte compositions and capillary wall modifications, for the separation of proteins. Wan et al. [253] used a full factorial design to optimize the SDS concentration and pH for the separation of diastereomeric amino acids.

In addition to optimizing MEKC separations, several reports have focused on the enhancement of the detectability and efficiency of uncharged [188,233], as well as charged [137], compounds in MEKC through zone sharpening. Nielsen and Foley [188] reported that zone sharpening of neutral solutes by sharpening the zones of the charged micelles that serve as carriers for neutral (and charged) molecules. Using electrokinetic injection, zone sharpening can be accomplished only when the effective electrophoretic mobility of the solute and the electroosmotic velocity migrate in the same direction during the injection process. A cationic–zwitterionic mixed micellar system had to be used in the sample buffer, with the running buffer containing SDS micelles, in order to achieve zone sharpening for a homologous series of alkyl phenyl ketones. As a result, limits of detection for the solutes were reduced by as much as an order of magnitude. Efficiencies for heptanophenone exceeding 1 000 000 theoretical plates were generated in under 10 min on a 50-cm capillary.

Liu et al. [233] also reported the on-column concentration of neutral molecules by field-amplified sample stacking. The neutral analytes were dissolved in a low concentration micellar solution (above the CMC) with a lower ionic strength than that of the running micellar buffer. The negatively charged micelles migrate rapidly into the boundary between the sample and the running buffer, where they stack up. A 75–80 fold increase in sensitivity was observed for dioxins.

4.4. Band dispersion

In MEKC, plate counts exceeding 100 000 are typically observed. As mentioned earlier, MEKC offers superior efficiency over HPLC and CEC. As a CE technique, factors such as longitudinal diffusion,

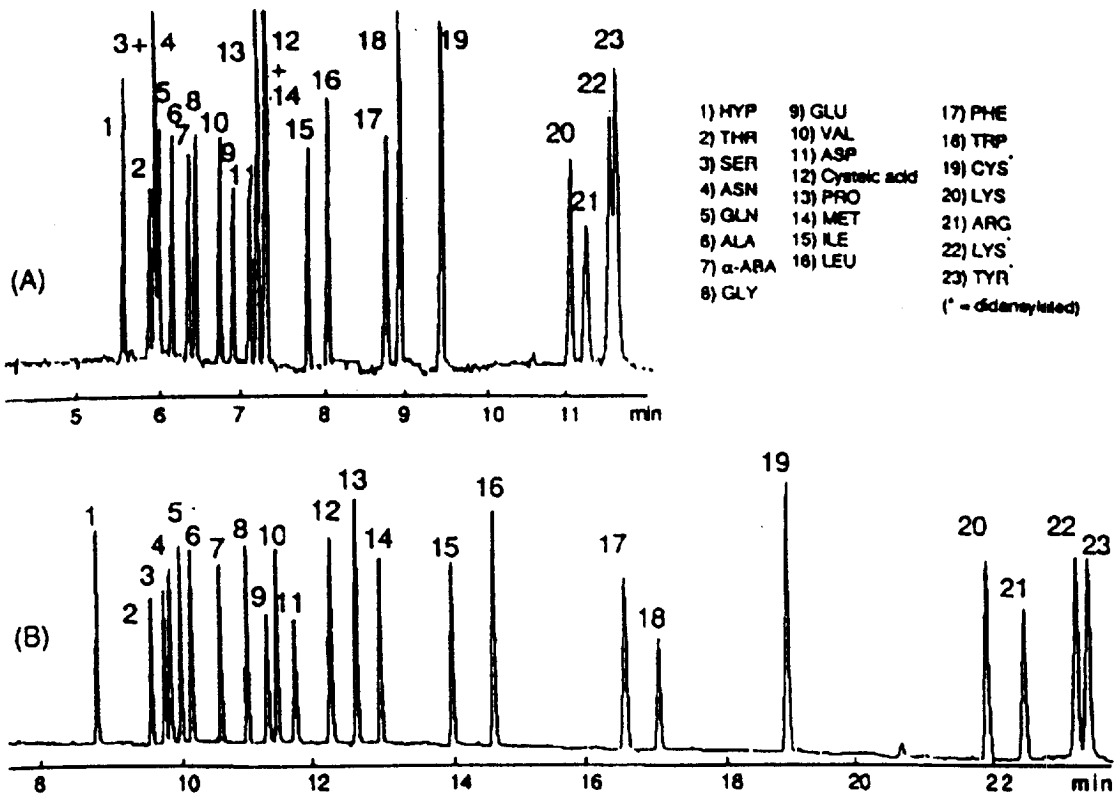


Fig. 24. Influence of temperature on the MEKC separation of dansylated amino acids (A) 20°C, 100 m*M* SDS and (B) 10°C, 102.5 m*M* SDS. Buffer: 20 m*M* borax, pH 9.2. From Ref. [133].

sample plug size, detection window size and time constant, joule heating and wall adsorption can contribute to band broadening in MEKC. However, the exact mechanisms for band dispersion due to the presence of micelles are not yet known. Several mechanisms, such as micellar overload, mass transfer kinetics, intermicellar diffusion and radial variation in the partition coefficient due to a temperature gradient, have been proposed [254–256]. An excellent review of band dispersion in MEKC is reported by Davis [257]. Clearly, a need exists for a more extensive study of band broadening mechanisms and the optimization of parameters for approaching the upper limit of efficiency.

4.5. Scope of MEKC applications

The applicability of this HPCE-based technique that was primarily developed for the separation of uncharged solutes has grown far beyond its initial intent. The range of applications cover wide groups of organic, inorganic and biochemical compounds that are of interest in various disciplines such as pharmaceutical, clinical, biotechnology, biochemical, food and environmental interests. Some of the recently reported applications of MEKC with different types of pseudo-stationary phases or modifiers are listed in the following paragraphs.

Anionic alkyl chain surfactants, especially SDS micelles, have been used in a variety of applications, such as the MEKC separation of phenols and other acidic solutes [115,137], pharmaceutical amines [116], organic solvents [117], keto compounds and carbohydrate derivatives [119,122,140], fungicide and phytotoxin [120], caffeine metabolites in human urine [121], DNA adducts [123], pharmaceuticals, such as antibiotics, vitamins, sulfonamides and xanthines [124,125,131,139], peptides and proteins

[126–130], organometallic solutes and ligands [118,132], amino acids [133,136,146,147], sulfur and nitro compounds in environmental applications [134,142], flavonoids in foods [135], nucleic acids constituents in body fluids [138], etc. The resolving power of the SDS–MEKC systems, however, have been somewhat limited for certain groups of solutes, such as PAHs and steroids [143–145].

Bile salt micelles have been effective in the MEKC separation of steroids [145], amino acids [146,147], benzodiazepines [149], synthetic colors [150], bioactive compounds [151], environmental analysis [152,155,157], proteins and peptides [153,164], bilirubin [154], organic acids [155,156,160], hydrophobic compounds including PAHs [158,161,163], chiral and diastereoisomers [159] and anti-HIV agents [162].

The usefulness of cationic micelles have been explored in a number of MEKC applications. A few examples are the separation of phenolic carboxylic acids [166], charged molecules with nearly identical electrophoretic mobilities, like bis(amidinohydrazones) [167], nucleic acid constituents [169,175], cocaine and illicit drugs [170], adrenergic blocking agents [171], glycosylated compounds [168,172,173] and aromatic hydrocarbons [174].

The popularity of mixed micellar pseudo-stationary phases has dramatically increased over the past few years [186–198]. Some examples of the groups of compounds that have been separated by mixed micelles in MEKC include nitroaromatic explosive compounds [142], corticosteroids [145,186], amino acids [188,191], organic anionic species [190], herbicides [191], fatty acids [192], analgesic compounds in pharmaceutical samples [193], tetracycline antibiotics [195] and drugs in body fluids [197].

Organic modifiers have been used to improve the MEKC separation of cardiovascular drugs [208], herbicides [209], amino acids [210], chlorophylls [211,219], taxol and related antitumor compounds [212], benzodiazepines [213], vitamins [214], illicit drugs [216,221], porphyrin compounds [217], coumarines [218], natural compounds with anticancer activity [220], nucleosides [222], hydrophobic compounds including PAHs, alkyl benzenes, alkyl phenyl ketones, alkyl parabenes and fullerenes [199–202,205–207,224] and phthalate esters [225].

Cyclodextrin-modified MEKC has been quite effective in the separation of various classes of compounds, such as PAHs [236,239,241], chlorinated benzenes and trichlorobiphenyl isomers [236], enantiomeric mixtures [237,240], steroids [238] and mycotoxins [242].

5. Applications of MLC and MEKC in quantitative structure–activity relationships (QSAR) studies

The main applications of MLC and MEKC in QSAR studies have been for the estimation of log P_{ow}, although there have been a few reports where retention in these two techniques has been directly related to biological activity.

Both methods offer similar advantages for the determination of physico-chemical parameters such as partition coefficients; for example, small sample size requirement, speed, high sample throughput, reproducibility, suitability for substances containing impurities and mixtures, wide dynamic range and feasibility for automation.

The relationship between retention in MLC and log P_{ow} has been reported for various groups of aromatic compounds [258]. In many cases, linear relationships were observed between the retention factor, k', and log P_{ow}. Usually, log k' is linearly related to log P_{ow}. As in conventional RPLC, the nature of the relationship depends on the composition of the mobile and the stationary phases. For example, much better correlations were observed using a cationic surfactant of CTAB compared to anionic SDS.

Breyer et al. [259] also reported the first successful application of MLC to quantitative retention–activity relationships (QRAR). They observed high correlations between the bioactivity of a group of 26 substituted phenols and the corresponding MLC retention factors. A multiparameter QSAR model was needed to achieve the same correlation between the bioactivity of these compounds and conventional molecular descriptors (such as log P_{ow}, pK_a, etc.). The single QRAR model in MLC was attributed to the fact that the information on hydrophobic and electrostatic interactions is already incorporated in MLC retention data.

In MEKC, the relationship between retention and

log P_{ow} depends on the type of pseudo-stationary phase (Fig. 14). As mentioned above, bile salts seem to provide the most appropriate system for the estimation of log P_{ow}. Yang et al. [102] reported direct relationships between the retention of a group of corticosteroids and two types of bioactivities; adsorption in the small intestine of rat and binding to human serum protein. Various micellar systems were investigated and correlations as high as 0.96 were observed in QRAR studies.

In addition to the advantages mentioned earlier, MEKC has certain unique features that make it even more attractive for QSAR studies. One is the feasibility of adjusting the composition of the micellar pseudo-stationary phase by simply changing the type of surfactant(s) in the system in order to provide better chemical models for the interactions in biological systems, or for facilitating log P_{ow} determination. The composition of the pseudo-stationary phase in MEKC can be easily changed by rinsing the capillary with the new micellar solution. Also, micelles are amphiphilic aggregates with an anisotropic microenvironment that provides both hydrophobic and electrostatic sites of interaction. In this respect, they are more structurally similar to biomembranes than to *n*-octanol or RPLC stationary phases (the latter is anisotropic but not amphiphilic). As a result, micelles have long been known as simple chemical models for biomembranes. In MEKC, solute partitioning is between the bulk aqueous phase and the micelles, which resembles the hydrophobic partitioning into biomembranes. In chromatographic systems such as RPLC and MLC, one has to essentially eliminate mobile phase selectivity in order for compounds to elute according to their hydrophobicity. In RPLC, solutes partition from a mixed hydro-organic solvent into an alkyl-bonded phase that is also enriched with the organic modifier from the mobile phase. In MLC, the three-phase equilibrium complicates the retention behavior and has a great influence on selectivity. Thus, retention order might not be according to hydrophobicity. Finally, a great potential in MEKC is the possibility of standardization of retention. An advantage of log P_{ow} is that it is a single and continuous scale, at least in theory. In practice, large variations exist in the log P_{ow} values (by as much as one order of magnitude), which reflects the difficulties involved in measurements. In MEKC, the retention factor is directly related to the partition coefficient into micelles, which, in principle, is also a single and continuous scale for a given surfactant system. One can then expect an increasing trend in the number of applications of MEKC in QSAR-related studies.

6. Conclusions

Since the 1980s, both MLC and MEKC have been used in hundreds of applications. These micellar-mediated LC and CE techniques provide unique capabilities for solving separation problems. In spite of many great advantages, poor efficiency is the greatest disadvantage of MLC that has hampered its widespread use in routine analytical laboratories.

A primary reason for the exploding interest in MEKC separations is a combination of high efficiency, versatility, feasibility of manipulating selectivity, through changes in the chemical composition of micellar solutions, and incorporation of secondary equilibria, speed and ease of use.

Comparing the two, MEKC has a greater resolving power and is easier to use. In fact, this statement is true when MEKC is compared to other conventional chromatographic techniques, such as RPLC, IPC, and IEC, as well as CEC. MEKC provides higher efficiencies than all of these techniques and is more versatile and more flexible. The limited elution window is a disadvantage, however, the problem can be improved through the use of mixed micelles, polymeric phases, and incorporation of organic modifiers. Another area of limitation is the on-line coupling of MEKC with mass spectrometry (MEKC–MS). The major obstacle with MEKC–MS is the contamination of the ion source by micelle-forming surfactants. Recently, successful attempts to on-line coupling MEKC with electrospray MS have been reported [260,261]. Lamoree et al. [260] made some modifications to the interface to minimize contamination of the ion source, while Ozaki et al. [261] demonstrated the possibility of using a high-molecular-mass surfactant in MEKC with on-line MS detection. Yang et al. [262] reported the use of partial-filling MEKC with MS detection.

With regard to future trends, the area of on-line coupling with MS deserves more attention. Also,

more MEKC applications on microfabricated devices are likely [263]. As mentioned earlier, the chemical composition of the pseudo-stationary phase in MEKC plays an important role in the separation process. An area of research that has attracted interest and deserves even more attention is the use of pseudo-stationary phases with new and different chemistries. This would greatly enhance control over chemical selectivity, extend the range of applications, and should lead to better separations. Various groups of pseudo-stationary phases, such as micelle-forming synthetic and biological surfactants, polymerized micelles, ionic polymers, dendrimers and other types of organized media, have already been examined. In addition, through incorporation of various types of modifiers, especially organic solvents and mixed pseudo-stationary phases, one can induce dramatic effects in MEKC separations. This combination of various types of organized assemblies and modifiers provides an unprecedented range of choices for the manipulation of selectivity and that can easily be incorporated into the separation process. In order to fully take advantage of this great flexibility, however, better characterization of these pseudo-stationary phases and of the roles of various modifiers is necessary. The use of LSER methodology provided promising preliminary results in achieving a better understanding of the separation mechanism in MEKC. Through a better understanding of the nature of solute interactions with individual and mixed pseudo-stationary phases as well as of the effects of modifiers, one can select the composition of the MEKC buffer on a rational basis. Such a capability will result in the better separation of mixtures of increasing complexity and also in a broadening of the scope of MEKC applications to wider groups of compounds.

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