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Review

Selectivity in capillary electrophoresis in the presence of micelles, chiral selectors and non-aqueous media

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Abstract

In addition to high efficiency, short analysis times and small sample volumes, a further attractive feature of capillary electrophoretic techniques is the possibility to achieve high selectivities. Usually, selectivity control also allows improvement in the resolution. A simple way to enhance the selectivity of capillary electrophoretic separations is to add one or more surfactants above their critical micelle concentration, or in the case of chiral separations to add a chiral selector to the background electrolyte. Because of the dynamic structure of micelles, the aggregation of monomers and size of the micelles can be easily adjusted. This review describes the various types of surfactants used in micellar electrokinetic capillary chromatography, and the chiral selectors employed in enantiomeric separations by capillary electrophoresis. Factors affecting the selectivity are noted. A brief discussion is included of the selectivity enhancement obtainable in non-aqueous media.
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1. Introduction

Selectivity and solvation in high-performance liquid chromatography (HPLC) are easily controlled through either the stationary phase or mobile phase interactions. High α values are thus usually obtainable in HPLC, but at the expense of long retention times. Capillary electromigration techniques offer the potential of high selectivities combined with short analysis times and high efficiencies, frequently to an extent not possible with other separation methods. The different modes of capillary electrophoresis (CE), with their different separation mechanisms, increase the flexibility of CE methods for selectivity control still further. Control of the selectivity usually also provides improvement in the resolution.

In micellar electrokinetic chromatography (MEKC) one or more surfactants are added to the electrolyte solution above their critical micelle concentration (CMC) to form micelles (pseudostationary phase). The same surfactants are also widely used as monomeric buffer additives in capillary zone electrophoresis (CZE). Separation in MEKC is based on electrophoretic mobilities of the analytes when partitioned into micelles. Partitioning of the analytes into a micellar pseudostationary phase can be utilised to improve the selectivity of the separation system. The mechanism of the analyte–micelle interaction involves a combination of hydrophobic–hydrophilic and dipolar interactions. The most attractive feature of MEKC is that neutral as well as charged analytes can be separated.

Several factors affect the selectivity in MEKC: the surfactant system, pH, temperature and addition of an organic solvent or other additives. Although manipulation of the electrolyte solution is an easy way to affect the selectivity in MEKC, it sometimes affects only the electroosmotic flow (EOF), the migration time and the resolution in CZE.

In the case of chiral separations, selectivity and high efficiency are much more easily achieved with CE than with chromatographic techniques utilising chiral phases. In the simplest case the selectivity can be manipulated simply by adding chiral selectors to the buffer solution. The number of chiral separations by CE has grown exponentially from the late 1980s to the present. Although inclusion complexation with cyclodextrins as buffer additives has clearly dominated in the applications, numerous new chiral selectors have been used in enantiomeric separation.

Both in MEKC and in chiral separations by CE, organic modifiers have frequently been employed to increase the selectivity and solubility of both analytes and additives. In chiral separations, the solubility of the chiral selectors is sometimes much better in organic solvents than in water. In non-aqueous media where the volume of organic modifier is 100%, the selectivity of capillary electrophoretic separations can be manipulated only by changing the organic solvent.

In this review article we describe the different means available for manipulating the selectivity in MEKC and in chiral separations by CE. All other capillary electrophoretic techniques are outside the scope of this paper. The effect of organic solvent on the selectivity in non-aqueous media will also be briefly described. The major aim of the paper is to demonstrate the ease and flexibility with which the relative order of analyte migration can be controlled by manipulating the composition of the running electrolyte.

2. Separation in MEKC

2.1. Theoretical background

When voltage is applied to an uncoated fused-silica capillary filled with a conductive electrolyte solution containing one or more surfactants, there will be an EOF (when the pH of the electrolyte solution is above ca. 2). In the case of anionic micelles their mobility in the solution will be towards the anode, i.e., in the opposite direction of EOF. However, at pH values greater than 5 the EOF will be stronger than the mobility of the micelle, leading to a net mobility of the negatively charged micelle towards the cathode [1]. The separation in MEKC is based on the partitioning of analytes between the micellar and aqueous phases in the capillary [2]. The difference in time it takes for an unretained component (with the velocity of EOF) and a component that is totally solubilised into a micelle to reach the detector is called the migration time window.

In MEKC the retention factor, k, is described as

$$k = n_{mc}/n_{aq} \tag{1}$$

where n_{mc} and n_{aq} are the numbers of the analytes in micellar and aqueous phases, respectively. In the case of an electrically neutral analyte, k can also be calculated as

$$k = (t_r - t_0)/\{t_0(1 - t_r/t_{mc})\} \tag{2}$$

where t_r is the migration time of the analyte, t_0 the migration time of an unretained solute (e.g., methanol, acetonitrile, mesityl oxide or formamide) and t_{mc} the migration time of an analyte that is believed to be fully solubilised into the micelle (e.g., Sudan III, Sudan IV, timepidium bromide, Orange OT, Yellow OB, anthracene). Owing to difficulties in predicting the interactions between analytes and micelles there will always be some uncertainty in the calculation of the retention factor. In particular, the determination of t_{mc} has proved difficult. To overcome this problem, the use of a homologous series of alkanes has been adopted for the determination of t_{mc} [3]. However, both methods (the use of one specific marker and the use of a homologous series) can be applied [4]. In addition, a procedure for the simultaneous estimation of t_0 and t_{mc}, based on the retention characteristics of a homologous series of molecules (alkyl benzenes and parabens), has been developed [5]. The calculated values of the migration times of EOF and the micelle were well in accordance with those obtained by empirical determinations.

In MEKC the selectivity (α) can be determined by the ratio of the retention factors of two solutes;

$$\alpha = k_2/k_1 \tag{3}$$

The effect of various parameters on the selectivity will be discussed below.

Eq. (2) for calculation of the retention factor is valid only for neutral compounds. A different equation is required for charged analytes, because now the analyte itself has a mobility and this will affect its net velocity [6]. The apparent electrophoretic velocity of the analyte ($v^*_{ep(s)}$) in the micellar solution is

$$v^*_{ep(s)} = v_{eo} - v_{m(s)} \tag{4}$$

where $v_{m(s)}$ is the velocity of the ionised analyte in the micellar solution and v_{eo} is the velocity of EOF

$$v_{eo} = -(\varepsilon\zeta/\eta)E \tag{5}$$

where ε, ζ and η are the permittivity, the zeta potential of the silica wall and the viscosity of the electrolyte solution, respectively. If we assume that the electrophoretic mobility of the micelle $v_{ep(mc)}$ stays constant even when analytes are incorporated or solubilised into the micelles, the apparent electrophoretic mobility of the analyte can be written as

$$v^*_{ep(s)} = (n_{aq}/(n_{mc} + n_{aq}))v_{ep(s)} + (n_{mc}/(n_{mc} + n_{aq}))v_{ep(mc)} \tag{6}$$

where $v_{ep(s)}$ is the electrophoretic velocity of an analyte in the buffer solution.

From the definition of the retention factor in Eq. (1) it follows that, in the case of charged analytes, k can be expressed as

$$k = (v^*_{ep(s)} - v_{ep(s)})/(v_{ep(mc)} - v^*_{ep(s)}) \quad (7)$$

Khaledi et al. [7] have presented a similar equation for the retention of anions

$$k = (t_r - t_{ion})/(t_{ion}(1 - t_r/t_{mc})) \quad (8)$$

where t_r and t_{ion} are the migration times of the solute in the presence and absence of the micelle, respectively, and t_{mc} is the migration time of the micelle [7].

2.2. Effect of type of surfactant

Probably the most common way to affect the selectivity in MEKC is to change the micellar solution. This can be done by choosing a different surfactant of the same or opposite charge or by adding another surfactant to the same electrolyte solution to form mixed micelles. Because of the dynamic structure of micelles (micelles in equilibrium with monomers) the aggregation of surfactants and size of the micelles are influenced by changes in micellar concentration, pH, temperature, ionic strength of the electrolyte solution and the addition of organic modifiers. Changes in the structures of the micelles will be discussed only briefly and emphasis placed instead on the effect of various types of surfactants on the selectivity of separations.

2.2.1. Anionic surfactants

The strength of the EOF is of significance when anionic surfactants are used for the separation of neutral compounds in uncoated fused-silica capillaries. Likewise, separations of mixtures of neutral and positively charged compounds will not succeed if the pH is too low because the EOF is then too slow to carry the micelles to the cathode. Most studies with anionic surfactants have been performed in neutral or basic electrolyte solutions.

The most frequently used anionic surfactant in MEKC separations is sodium dodecyl sulphate (SDS). This can be classified as a relatively hydrophobic micelle, which means that it reasonably well retains compounds with even medium logarithmic distribution coefficient (log K) or octanol–water partition coefficient (log P_{ow}) values. Examples of the critical micelle concentrations (CMCs) of SDS in different electrolyte solutions at 25°C are listed in Table 1. All these values are lower than the CMC in pure water, which is as expected since the electrostatic interactions between the charged hydrophilic headgroups are weakened when electrolyte is added, favouring micelle formation.

The influence of different organic solvents on the CMC values of SDS has been investigated by Jacquier and Desbene [14] by CE. They chose two amphiprotic solvents (methanol and ethanol) and two aprotic solvents (acetone and acetonitrile) and added these to a borax electrolyte solution at different volume percentages. All solvents had a marked effect on the CMC value (total variations between approximately 3.8 and 8 mM), causing both a decrease and an increase in CMC depending on the percentage of added solvent. In general the authors found that the aprotic solvents stabilised the micelles (decreased the CMC) at added volume percentages below 10%, and increased the CMC at higher concentrations (maximum volume percentages were 15% for acetonitrile and 20% for acetone). The amphiprotic solvents differed in their effect: methanol caused a small increase in CMC up to maximum volume percentage of 35%, whereas ethanol stabilised the micelles over a very broad range of volume percentages, with a minimum in CMC at 15%. There was no micellar aggregation with any of the organic solvents at higher organic solvent concentration (around 30–40%).

As mentioned above the most commonly used micelle has been SDS. Both its CMC and Krafft temperature are low enough to be useful in MEKC. The Krafft temperature is the temperature at which the solubility of the surfactant is increased by orders of magnitude in a relatively narrow temperature region (for SDS the Krafft temperature is 25°C); Above the CMC and Krafft temperature the change in CMC with increasing temperature is negligible. Other long-chain surfactants, such as SDS, have also been used in MEKC, but problems with poor reproducibilities of the migration times of the analytes have been reported [15]. Generally, with decreasing

Table 1
CMC values of SDS in selected electrolyte solutions at 25°C

Electrolyte solution	CMC (m*M*)	Method of determination	Ref.
Pure water	8.1	Several different	[8]
50 m*M* AMPSO (pH 9.0; adjusted with ammonia)	3.6	Conductometric titration	[9]
50 m*M* AMPSO (pH 9.0; adjusted with ammonia)	3.9	CE	[9]
100 m*M* borate, 50 m*M* phosphate (pH 7.0)	2.9	Conductometric titration	[10]
20 m*M* PIPES, 20 m*M* NaOH (pH 7.0)	3.8	Conductometric titration	[10]
100 m*M* BES, 100 m*M* NaOH (pH 7.0)	3.1	Conductometric titration	[10]
5 *M* urea, 100 m*M* borate, 50 m*M* phosphate (pH 7.0)	4.4	Conductometric titration	[10]
20% DMSO (v/v), 25 m*M* sodium tetraborate, 50 m*M* sodium dihydrogenphosphate (pH 7.0)	6.0	Conductometric titration	[11]
20% acetone (v/v), 25 m*M* sodium tetraborate, 50 m*M* sodium dihydrogenphosphate (pH 7.0)	6.3	Conductometric titration	[11]
5 m*M* sodium tetraborate (pH 9.2)	5.3	CE	[12]
100 m*M* sodium tetraborate, 100 m*M* sodium dihydrogenphosphate (pH 6.0[1])	2.0	Plot of *k* vs. SDS conc.	[13]
100 m*M* sodium tetraborate, 100 m*M* sodium dihydrogenphosphate (pH 6.5[1])	2.4	Plot of *k* vs. SDS conc.	[13]
100 m*M* sodium tetraborate, 100 m*M* sodium dihydrogenphosphate (pH 7.0[1])	3.1	Plot of *k* vs. SDS conc.	[13]
100 m*M* sodium tetraborate, 100 m*M* sodium dihydrogenphosphate (pH 7.7[1])	4.0	Plot of *k* vs. SDS conc.	[13]

[1] pH adjusted with 0.1 *M* HCl or 0.1 *M* NaOH.

surfactant chain-length, CMC increases rather dramatically, and the CMC of sodium decyl sulphate is as high as 33 m*M*. Such high surfactant concentration result in high currents and problems with Joule heating.

Also a longer-chain surfactant, sodium tetradecyl sulphate (CMC 2.1 m*M*, Krafft temperature 32°C), has been investigated [16]. The retention factors for the neutral aromatic compounds separated were different in SDS and sodium tetradecyl sulphate (with a larger time window in the latter system), but the migration order was the same. The retention factors for the analytes were further investigated with a micellar system containing sodium dodecyl sulphonate, which differs from SDS in the polar head group. Again a different selectivity was observed, but no changes in the migration order [16].

Another anionic surfactant, sodium N-lauroyl-N-methyltaurate (LMT), (CMC; 8.7, Krafft temperature; <0°C), has been used and compared with SDS for the separation of ionic compounds [17,18]. The polar headgroup of LMT is a sulphonate group. Not only the headgroup but also the tail differs from that of SDS; in LMT there are two carbons between the sulphonate group and a methylamide group. In the case of charged analytes, not only the hydrophobicity of the analytes is important but also their charge. For charged solutes the two micellar systems (SDS and LMT) have shown great differences in selectivity, and even the migration order was different for some compounds. No significant difference was seen in the selectivity for neutral analytes.

Another group of anionic surfactants that has been widely used for separations of both neutral and ionic analytes in MEKC is the bile salts. These biological surfactants, synthesised in the liver, have a steroidal structure. There has been considerable discussion about their micellisation. Perhaps the most popular theory about the aggregation suggests that the bile salts will form helical-structured micelles, with the monomers combined with each other at the hydrophobic face of the molecule. The two most widely used bile salts in MEKC are sodium cholate (SC) and sodium taurocholate (STC). Since only the ionic groups of the bile salts are different, migration times of some neutral solutes are about the same in SC and STC, [19]. Bile salts tend to have a lower solubilising effect on neutral hydrophobic compounds than does SDS, as has been demonstrated for some neutral compounds that could not be separated with

SDS but were successfully separated with bile salts [19]. In addition, some basic amino acids have been successfully separated in a MEKC system using SC [20]. The corresponding separation with an SDS system proved to result in very long migration times.

Yang and co-workers [21,22] have investigated the influence of surfactant type on migration behaviour and chemical selectivity in MEKC through linear solvation energy relationships (LSER) and functional group selectivities. In LSER models the influence of solute–solvent interactions is investigated by relating migration and structural descriptors of analytes. An anionic fluorocarbon, lithium perfluorooctane sulphonate (LiPFOS), was compared with two other anionic surfactants, SDS and SC, in an investigation of the retention behaviour of 60 aromatic compounds. The LSER results indicated that, for these compounds, the SDS micelles are slightly more apolar than the SC micelles, and the LiPFOS micelles are the most apolar of all. The authors attributed the differences in chemical selectivity of the surfactants mainly to differences in hydrogen bonding interactions. Furthermore, from the LSER studies they found the SC micelles to be the strongest hydrogen bond acceptor (HBA), and the LiPFOS micelles to be the strongest hydrogen bond donor (HBD). The SDS micelles were slightly weaker HBD micelles. The differences between the three anionic micelles in selectivity for a group of aromatic compounds are shown in Fig. 1.

The influence of the dodecyl sulphate counterion on selectivity has been investigated. In one case the sodium ion in SDS was changed to a divalent magnesium ion, resulting in magnesium dodecyl sulphate, with a much lower CMC than SDS, i.e., 1.2 m*M* versus 8.1 m*M* in water [23]. A low concentration of ethylenediaminetetraacetic acid tetrasodium salt (EDTA) had to be added to the buffer solution containing magnesium dodecyl sulphate to prevent the magnesium from adsorbing to the capillary surface, and the drifting of t_0. The retention factors were between 1.5 and 2.5 times as large with the magnesium dodecyl sulphate micelles as with the SDS micelles. Studies on the hydrophobic selectivities (i.e., methylene selectivity—measured by comparing the ratio of k values for members of a homologous series differing by one $-CH_2-$ group, or by plotting log k values versus carbon number) showed that the magnesium dodecyl sulphate micelles are much less polar than the SDS micelles. Also the functional group selectivity was tested, and revealed large differences between the micelles. The

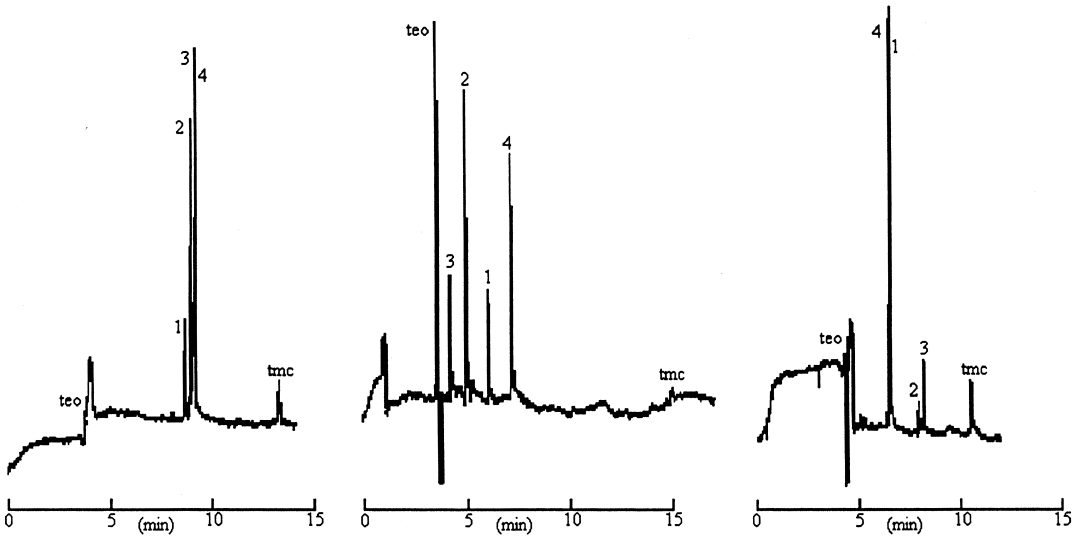

Fig. 1. Separation of aromatic compounds with (A) 40 m*M* SDS, (B) 40 m*M* LiPFOS, (C) 80 m*M* SC added to the electrolyte solution. Other conditions: 50 m*M* phosphate; pH 7.0; voltage 20 kV; wavelengths 210 nm (A), 214 nm (B), 254 nm (C). Numbering of compounds: 1 4-bromonitrobenzene, 2 bromobenzene, 3 4-iodophenol, 4 4-chloroacetophenone. t_{eo} and t_{mc} are the migration times of the EOF and micelle markers. Reprinted with permission from Ref. [21].

differences in selectivity were assumed to be due not only to dissimilar hydrophobicities but also to differences in the electric surface layer of the magnesium dodecyl sulphate micelle. Potassium and lithium dodecyl sulphate micelles were also investigated, and methylene selectivity studies showed the hydrophobicity of the dodecyl sulphate micellar systems to increase as the counterion was changed from Li^+ to Na^+ to K^+ [24]. Owing to the high Krafft temperature of KDS (40°C; CMC 7.8 m*M* in water), a minimum of 15% of acetonitrile had to be added to the buffer to solubilise KDS.

Various tetraalkylammonium ions, such as tetramethylammonium bromide, tetraethylammonium bromide, tetrapropylammonium bromide, tetrabutylammonium bromide, cetyltrimethylammonium bromide and tetrapentylammonium bromide, can be added to an SDS micellar solution to alter the selectivities in MEKC [25,26]. Interestingly, mixed micellar systems of SDS and CTAB (50 m*M* and 15 m*M*, respectively) have not resulted in a reversal of the EOF [25]. The possible combination of a tetraalkylammonium ion as counterion with SDS, instead of sodium, has been suggested.

2.2.2. Cationic surfactants

Use of cationic surfactants in MEKC causes a reversal of the EOF due to electrostatic interactions between the negatively charged fused-silica wall and the positively charged surfactant monomers [27]. The reversal of EOF occurs at surfactant concentrations even below the CMC. This has been shown for cetyltrimethylammonium bromide (CTAB), for which the reversal of EOF was observed between CTAB concentrations of 0.035 m*M* and 0.1 m*M* (CMC of CTAB in water is 0.92 m*M*) [28]. A theory has been proposed for the formation of hemimicelles or two-dimensional aggregates of the hydrophobic chains [29]. The critical concentration at which the association of the cationic ions was observed was referred to as the hemimicelle concentration. The effect of CTAB concentration on the EOF was investigated, with methanol as the marker, at pH 6.0–7.8. It was shown that, after the reversal of the EOF, there was a steady increase in EOF with increasing CTAB concentration (at surfactant concentrations of 1 m*M* to 50 m*M*). In the case of SDS the EOF remained virtually constant (pH 7.0) with increasing SDS concentration. SDS and CTAB were compared for the separation of some PTH-amino acids at pH 7.0 and completely different selectivities were obtained with the two micellar systems [27]. The similar comparison of selectivities was made for the separation of some positively charged peptides [30], but here the ionic interactions between the peptides and the negatively charged micelles were too strong, resulting in poor separation. When CTAB was used as the micellar phase, a full separation of all the peptides was achieved.

In the separation of some inorganic anions, an increase of cetyltrimethylammonium chloride (CTAC) concentration resulted in marked selectivity differences and even altered the migration order of the compounds [31].

2.2.3. Neutral and zwitterionic surfactants

Although the zero electrophoretic mobilities of neutral surfactants cannot be exploited in MEKC separation of non-ionic compounds, they can successfully be used in the separation of ionic compounds. The problems with Joule heating encountered with ionic surfactants at increasing concentration are avoided in the case of non-ionic surfactants, which means that these surfactants can be added to the buffer at high concentrations and high voltages still be used. The neutral surfactant Tween 20 has been used under acidic conditions for the separation of eleven peptides [32]. Remarkable improvement in the selectivity was obtained when 200 m*M* Tween 20 was added to the electrolyte solution. Decreasing the pH from 6 to 3 enhanced the separation of the peptides.

Like the neutral surfactants the zwitterionic surfactants do not contribute to the net conductivity of the electrolyte solution. The zwitterionic surfactant 3-(N,N-dimethylhexadecylammonium) propanesulphonate (PAPS) has been used in MEKC both as a micelle and as a dynamically coating reagent [33,34]. The CMC of PAPS in a 0.2 *M* sodium phosphate pH 2.5 buffer was found to be 25 μ*M*. Above this concentration PAPS was adsorbed to the silica walls of the capillary. PAPS was added to the low-pH buffer for the separation of some polypeptides. Comparison was made between CZE and MEKC and big improvements in the separation were observed with the PAPS buffer solution. The authors suggested

that the zwitterions not only prevent the silica walls from peptide adsorption but also help to break up peptide–peptide interactions. The use of a zwitterionic surfactant for the separation of a mixture of charged and neutral solutes in acidic media has recently been described [35]. The neutral solutes in the mixture could not, however, be separated without the addition of sulphonic acids or SDS to the buffer.

2.2.4. Mixed micelles

Various mixed micellar solutions have been tested in MEKC and shown to improve the selectivity. These include mixed anionic–non-ionic surfactants [e.g., SDS and polyoxyethylene-23-dodecanol (Brij-35] [36–39], SDS and Tween 20 [40], bile salts and polyoxyethylene-4-dodecyl ether) [41], anionic–anionic surfactants [e.g., SDS and SC [42], SDS and sodium octyl sulphate [43], two different bile salts [44–46], fluorocarbon (LiPFOS) and hydrocarbon surfactant (LiDS) [47]]; anionic–cationic surfactants [e.g., mixtures of anionic (FC 128) and cationic (FC 134) fluorosurfactants [48]]; and anionic–zwitterionic surfactants [e.g., N-dodecyl-N,N-dimethylammonium-3-propane-1-sulphonic acid (SB-12) and SDS [49]]. The formation of mixed micelles with different selectivity than the corresponding single micelles has been demonstrated by several authors. One major advantage of the mixed anionic–non-ionic and anionic–zwitterionic systems is that there is no increase in the currents with increasing concentration of non-ionic or zwitterionic surfactant. Mixed fluorocarbon and hydrocarbon surfactants form two types of micelles: one fluorocarbon-rich and the other hydrocarbon-rich. Mixtures of LiPFOS and LiDS, at different mole fractions, have been investigated for the separation of peptides and large differences in selectivity were achieved by changing the micellar composition (the total micelle concentration was kept constant) [47]. A large variety of micelles can be formed with other kinds of mixed micelles. In the case of mixed SDS and SC we have observed that the addition of SC to an SDS solution (15 m*M*) decreases the fraction of monomeric SDS, meaning that the first molecules of SC to be added are solubilised in the SDS micelles [9]. Further, the addition of cholate has a minor effect on the micellar size. Mixing of SDS and SC gives mixed micelles with a wide range of SDS to SC ratios. We compared SDS and SC with the mixed micelles of SDS and SC for the separation of some neutral compounds. Big differences in the selectivities were obtained. In the case of mixed micelles of SDS and SC used in MEKC for the separation of some corticosteroids, a mathematical optimisation of the selectivity showed the concentrations of SDS and buffer to be the most critical parameters (the parameters optimised were pH and the concentrations of SDS, SC and buffer) [50]. Fig. 2 shows the separation of fifteen corticosteroids with a mixed SDS–SC system.

To assist the separation of some proteins, Hult et al. [48] added a mixture of cationic and anionic fluorosurfactants (FC 128 and FC 134) to the buffer. A fluorosurfactant admicellar bilayer was formed on the silica walls and with this system both negatively and positively charged proteins were successfully separated at pH 7 in a 10 m*M* phosphate buffer. Changes in the FC 128/FC 134 ratio affected the selectivity of the separation. Worth mentioning, too, is the separation of some porphyrins with the use of bovine serum albumin (BSA) and SDS or bile salts as buffer additives [51]. The SDS–BSA complex was assumed to provide selectivity for the porphyrins. Without SDS in the solution the separation was unsatisfactory, probably due to the adsorption of BSA and/or porphyrins onto the bare silica wall. As with SDS, bile salts are also known to form complexes with proteins like BSA.

2.2.5. In situ charged micelles

A very different type of surfactant is the in situ charged micelles which are based on the complexation between an alkylglucoside surfactant and alkaline borate [52–54]. Since the complexation occurs in the electrolyte solution the micelles are extremely dependent on pH and the concentration of borate in the solution. Hence, with these micelles, with adjustable surface charge densities, the retention window can easily be manipulated through t_{mc} without drastically affecting t_0 [52]. This holds at alkaline pH because the surface silanols are then fully ionised, i.e., the overall surface charge density of the wall remains constant. The in situ charged micelles exhibit different selectivities.

2.2.6. Surfactants and cyclodextrins

Chiral separations have most frequently been

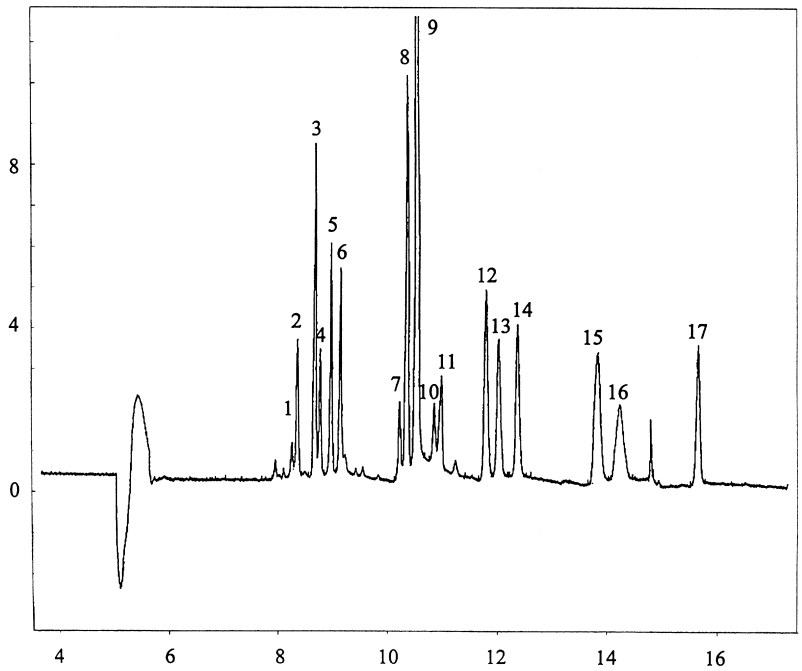

Fig. 2. Separation of corticosteroids by mixed micellar electrokinetic chromatography (MMEKC). Electrolyte solution: 49 m*M* AMSPO, 18 m*M* SDS, 55 m*M* SC, pH 9.0. Running conditions: capillary 60 cm (l_{det}), 68.5 cm (l_{tot}), 25 kV, 254 nm, injection 3 s 50 mbar, 25°C. Analytes separated: 1 1-dehydroaldosterone, 2 17-isoaldosterone, 3 probenecid*, 4 cortisone, 5 *d*-aldosterone, 6 4-androstene-11β-ol-3,17-dione, 7 cortisol, 8 fludrocortisone acetate, 9 dexamethasone, 10 21-deoxycortisol, 11 benzoic acid*, 12 4-androstene-3,17-dione, 13 corticosterone, 14 11-deoxycortisol, 15 17α-hydroxyprogesterone, 16 11-deoxycorticosterone, 17 progesterone. * Marker compounds (Ref. [137]).

achieved through the addition of cyclodextrins (CDs) to buffers. These separations are discussed in the following section of this article. However, there are several examples of how the addition of various CDs to buffers containing SDS improves the separation of structural homologues of alkylphenols [55] and C_2–C_{14} fatty acids [56], estrogens [57], corticosteroids [58] and polyaromatic hydrocarbons [59]. The separation mechanism in CD-MEKC is based on differential partitioning of compounds between the micellar and the CD-aqueous phase. Selectivity improvements through the addition of various CDs (α, β, γ-CD) together with SDS and mixtures of neutral and ionic CDs (without SDS) [60] have also been investigated. In addition, SC–CD mixtures have been used [61].

2.2.7. Double- and triple-chain surfactants

Since the choice of surfactant significantly affects the selectivity in MEKC, the introduction of new types of surfactants as pseudostationary phase is of great interest. Double-chain surfactants with two sulphonate groups have been used for the separation of naphthalene derivatives [62,63]. The structures of the synthesised double-chain surfactants are shown in Fig. 3. All three surfactants have extremely low CMCs: 0.014 m*M* for DBTHX, 0.017 m*M* for DBTHP and 0.043 m*M* for DBTDMHP, in water. The Krafft temperatures are below 0°C. Comparison of the migration behaviour of the naphthalenes in DBTHX and SDS revealed very different selectivity for the two systems. Also, wider migration time windows were seen for the double-chain surfactants than for SDS at the same concentration. Much higher concentrations of SDS were needed for the separation of the naphthalenes, and baseline separation was not achieved even at 60 m*M* SDS concentration. Migration times of the naphtalene derivatives were longer with a triple-chain surfactant, DDBTN, having two sulphonate groups than for the double-chain

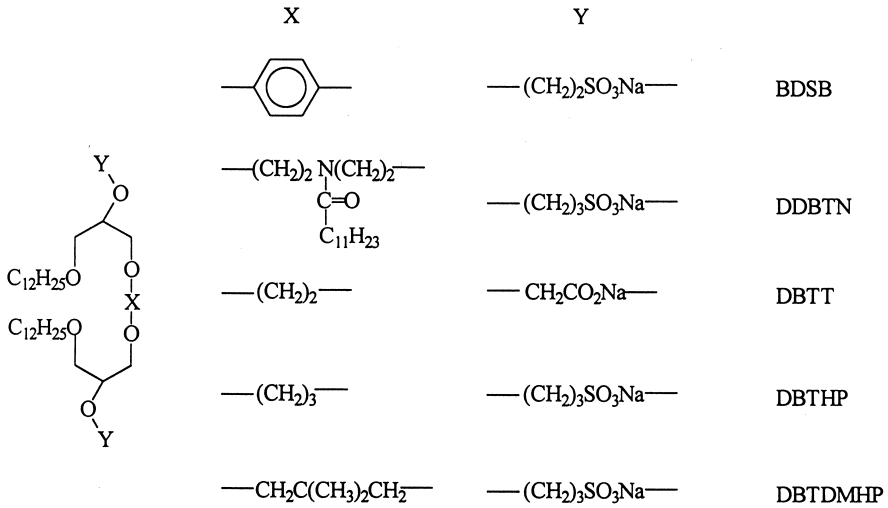

Fig. 3. Structures of double- and triple-chain surfactants.

surfactants, and there were some differences in the migration order [64]. The selectivity was completely different with SDS and the triple-chain surfactant.

2.2.8. High-molecular-mass surfactants

The high-molecular-mass surfactants used in MEKC are either oligomers of monomeric surfactants or block copolymers with surface-active properties. High-molecular-mass surfactants are considered to form the micelle from a single molecule, which has been termed a molecular micelle. Since their CMCs are close to zero the molecular micelles are considered to be highly stable irrespective of the experimental conditions. The oligomers of sodium 10-undecylenate (SUA) have been compared with micelles of SDS and SUA in a study of the migration times and order of some aromatic compounds [65,66]. The migration order with the SUA oligomer was different from the order with the SDS and SUA micelles. The interior of the oligomer is more polar than the interior of the micelles of SUA. Noteworthy are the high concentrations of methanol (60%) and acetonitrile (55%) that can be used in the case of the oligomers. The block copolymers used as buffer additives in MEKC are illustrated in Fig. 4. These have been shown effectively to alter the selectivity in MEKC [67–70,22]. Both anionic and cationic block copolymers have been investigated—the EOF being reversed with the latter.

2.3. MEKC separations of compounds as their metal complexes

An alternative way to chemically increase the selectivity control of MEKC is to add metals to the MEKC buffer solution [71–73]. The method has been shown to be useful for compounds that form complexes with metals, e.g., porphyrins, oligonucleotides and polyaminopolycarboxylic acids. Results have shown that less metal ions were adsorbed to the silica walls when SDS is present, demonstrating the capability of the metal ions to adsorb on the micellar surface. However, even with micelles in the solution there is a small increase in t_0 with added metals, i.e., a decrease in the ζ-potential of the silica wall due to metal ions adsorbed on the wall. Since the complexation constants of compounds formed with the micelle–metal surfaces differ with the metal ion added, the selectivity can easily be increased by changing the metal. Both anionic and cationic surfactants can be utilised.

2.4. pH

When compounds are charged, pH variations in the buffer may lead to changes in the dissociation of the compounds, affecting their charge, and thereby the solute–micelle ionic interactions and electrophoretic mobilities [6]. The rather small EOF at

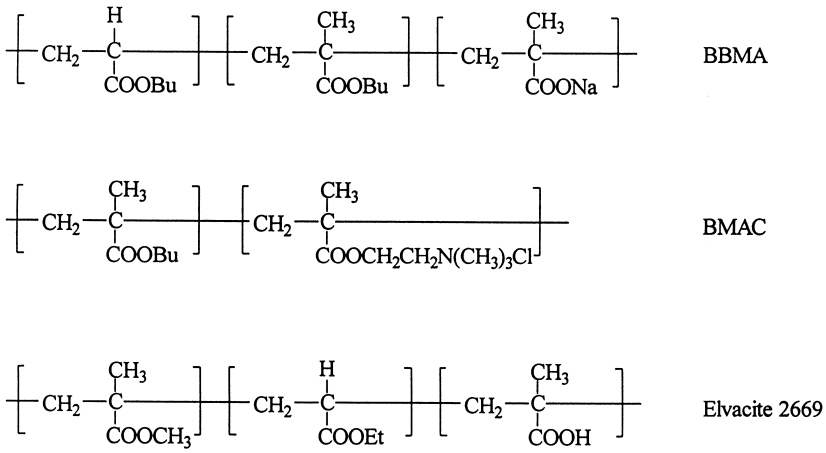

Fig. 4. Structures of block copolymers.

acidic pH values makes it difficult to use anionic micelles with high electrophoretic mobilities in low-pH buffers. Thus in the case of SDS, its direction at pH values below 5 is towards the cathode. An investigation of the micelle-induced pK_a shifts for ionic compounds has shown to affect the selectivity significantly [7]. Compounds with similar pK_a values in aqueous solutions may have different pK_a values in micellar solutions. However, the reverse situation may cause problems; compounds with different pK_a values in aqueous solutions may have similar values in micellar solutions. A consequence of this phenomenon is that variations in selectivity with pH may be larger with MEKC than with CZE.

The selectivity of analytes that are electrically neutral over a wide pH range, which will not dissociate at the pH values used with fused-silica capillaries, is not affected by changes in pH. However, if the pH is adjusted there will be changes in the ionic strength of the electrolyte solution and this may affect the selectivity. Such variations in the ionic strength may lead to changes in the partition coefficients of the analytes between the aqueous and the micellar phase [74].

2.5. Temperature

The effect of temperature on the solubility of micelles has been discussed above (Krafft temperature). In measurements of the CMC of SDS at 20°C to 50°C there was only a negligible increase in the values with temperature [10]. The distribution coefficients, in turn, are heavily influenced by temperature [10]. Increase in the temperature caused the distribution coefficients of some aromatic compounds between the SDS micelle and the aqueous phase to decrease. Since the dependence of the distribution coefficient on temperature varies with the analyte, there will also be selectivity differences due to temperature. In the case of ionic analytes, temperature variations can lead to changes in the pK_a values, and to different micelle–solute interactions [10].

2.6. Organic modifiers

Several papers have been published on the use of organic modifiers to improve selectivity in MEKC. As a rule of thumb, adding organic modifiers to the buffer leads to extended migration time windows. The addition of organic solvents to the buffer improves the solubility of hydrophobic compounds in the aqueous buffer or, in other words, reduces the distribution of compounds into the micelles. As mentioned earlier, however, high concentrations of organic solvents may prevent aggregation. Organic solvents have also been suggested to hinder the interaction between large peptides and the micelle [75]. The changes in EOF with the addition of organic modifiers are due to changes in the viscosity of the buffer or in the ξ-potential of the silica wall. Examples of organic modifiers used together with

Table 2
Organic solvents used with SDS in MEKC

Modifier	% or concentration of added modifier	SDS conc. (m*M*)	Buffer solution	Compounds separated	Ref.
Acetone	30%	25 m*M*	Borate–phosphate (pH 7.0)	13 PAHs	[11]
Acetone	15%	50 m*M*	100 m*M* borate (pH 8.1)	7 drugs	[78]
Acetonitrile	5%	60 m*M*	25 m*M* borate (pH 8.85)	3 microcystins, nodularin	[80]
Acetonitrile	20%	75 m*M*[1]	5 m*M* borate, 5 m*M* phosphate (pH 8.6)	4 estrogens	[76]
Dimethylformamide	17%	40 m*M*	20 m*M* CAPS (pH 11)	porphyrins	[72]
DMSO	50%	25 m*M*	Phosphate (pH 7.0)	9 PAHs	[11]
1-Hexanol	100 m*M*	75 m*M*	10 m*M* sodium phosphate–2 m*M* borax (pH 6.8)	5 aromatic compounds	[77]
Methanol	10%	60 m*M*	25 m*M* borate (pH 8.85)	3 microcystins, nodularin	[80]
Methanol	20%	70 m*M*	5 m*M* disodium phosphate, 2.5 m*M* sodium borate	6 aromatic compounds	[79]
2-Propanol	10%	50 m*M*	100 m*M* borate (pH 8.1)	7 drugs	[78]
Methyl ethyl ketone	15%	50 m*M*	100 m*M* borate (pH 8.1)	7 drugs	[78]
Urea	6 *M*	50 m*M*	20 m*M* borate–20 m*M* phosphate (pH 9.0)	8 corticosteroids	[81]
Urea	4.3 *M*	100 m*M*	100 m*M* borate–50 m*M* phosphate	23 PTH-amino acids	[81]

[1] 75 m*M* sodium cholate.

SDS are listed in Table 2. The conditions given are optimum by means of selectivity. In addition to the organic modifiers mentioned above, 1-butanol, 2-butanol, cyclohexanol, ethanol, ethylene glycol, phenol, 1-propanol and tetrahydrofuran have been used as modifiers in MEKC.

2.7. Buffer

The choice of buffer is of major importance in MEKC separation. The buffer influences the CMC of the surfactant and is critical in regard to the working pH values since the buffering capacity will be decreased outside the pH range of the buffer. The buffer can also be used to directly affect the selectivity of a MEKC separation. This has been shown, for example, for some amino acids in a boric acid–SDS electrolyte solution [20]. Increasing the boric acid concentration (50–130 m*M*) while keeping SDS constant (150 m*M*) leads to increased migration times of the analytes, and even changes the migration order. The effect of changing the buffer on the selectivity has also been studied. Five different (inorganic and organic) buffers with pK_a values between 6.49 and 8.09 have been investigated for the separation of positively charged *bis*(amidinohydrazones) at pH 7.0 with 1 m*M* CTAB in the electrolyte solution [82]. Marked differences in the migration times were observed, without any reversals in the migration order.

2.8. Microemulsions

Microemulsion electrokinetic chromatography (MEEKC) employs a microemulsion as the pseudostationary phase. Microemulsions are microheterogeneous liquids that are optically transparent, thermodynamically stable and have high solubilising power. Microemulsions consist of water, oil, a surfactant and a co-surfactant. The microemulsion components can be chosen according to the following rule: the carbon number of the surfactant is equal to the carbon number of the co-surfactant plus the carbon number of the oil. To our knowledge, only oil-in-water (o/w) microemulsions have been used in MEEKC so far. The surfactants have been SDS [83–89] or trimethyltetradecylammonium bromide (TTAB) [88]. A medium-length alkyl-chain alcohol

has been used as the co-surfactant, i.e., 1-butanol. As has been shown in several papers [88,89], different selectivities are easily obtained by changing the oil, or core phase, in the microemulsion. The time window is easily decreased in MEEKC by increasing the volume fraction of the organic components [83]; moreover the time window can be manipulated by altering the SDS fraction of the microemulsion [86]. Comparisons between MEKC and MEEKC have been made for the separation of some aromatic compounds and cold medicine ingredients [86], and for some steroids [84]. In addition, MEEKC, MEKC and mixed micellar electrokinetic chromatography (MMEKC) have been compared for the separation of some herbicides (Fig. 5) [85]. The separation efficiencies were higher in MMEKC and MEEKC than in MEKC, but the migration time window was wider in MEEKC than in MMEKC or MEKC. In general, there are a large number of parameters in MEEKC that can be optimised to improve the selectivity.

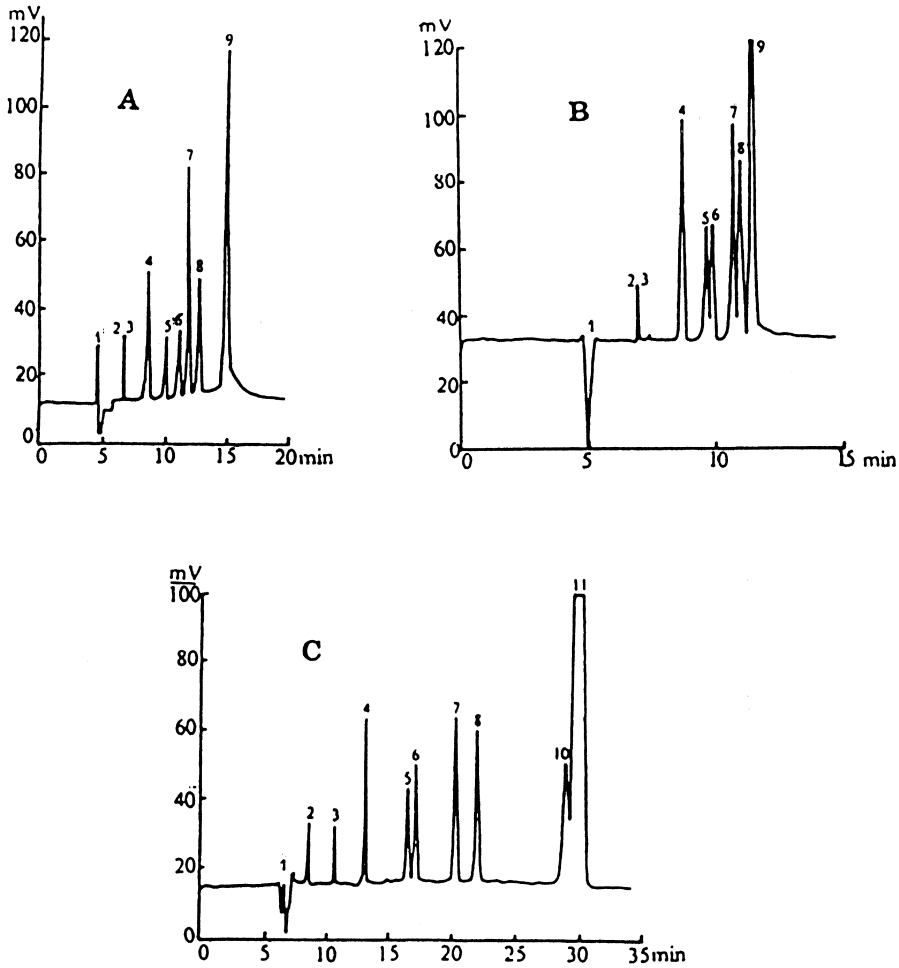

Fig. 5. Separation of herbicides by MEKC, MMEKC and MEEKC. Applied voltage 24 kV. (A) MEKC: 50 m*M* SDS, 12.4 m*M* KH_2PO_4, 3.8 m*M* $Na_2B_4O_7$ (pH 7.0). (B) MMEKC: 50 m*M* SDS, 2% (v/v) polyethylene glycol 400 monolaurate, 12.4 m*M* KH_2PO_4, 3.8 m*M* $Na_2B_4O_7$ (pH 7.0). (C) MEEKC: 50 m*M* SDS, 800 m*M* *n*-butanol, 70 m*M* *n*-octane, 12.4 m*M* KH_2PO_4, 3.8 m*M* $Na_2B_4O_7$ (pH 7.0). Peaks: 1 dimethylformamide; 2 chlorsulfuron; 3 fenuron; 4 monuron; 5 fluometuron; 6 chloroturon; 7 dinuron; 8 linuron; 9 Sudan III; 10 impurity in *o*-diphenylbenzene; 11 *o*-diphenylbenzene. Reprinted with permission from Ref. [85].

3. Chiral separations

3.1. Theoretical considerations

Enantiomers exist in two forms that are non-superimposable mirror images. Enantiomers of the same compound are difficult to separate because they have the same physicochemical properties in an achiral environment. Their dissociation constants, diffusion coefficients and electrophoretic mobilities are identical, for example, which means that they cannot be distinguished by CE or any other achiral separation method. However, they may behave differently in a chiral environment. One feasible strategy for the separation of enantiomers is to exploit their differential interactions with chiral selectors i.e., optically pure compounds that have at least one chirality element (usually chiral centre represented by asymmetric carbon atom) and functional groups with such configuration that allows spatially dependent interactions with the chiral analyte. In the interaction between enantiomers and the chiral selector, diastereomeric complexes with different physicochemical properties are formed in a dynamic equilibrium process. Diastereomeric molecules may be formed in chemical reactions between the analytes and the chiral selector, and after the reaction the diastereomers can be separated in an achiral environment. Here we shall focus on separations based on dynamic equilibria between the analyte enantiomers and a chiral selector dissolved in the background electrolyte, which is by far the most widely used approach in CE. In other applications the chiral selector may be incorporated in a gel [90], bonded to the capillary wall [91] or molecularly imprinted to a polymer [92].

It is important to know what factors affect the selectivity of chiral separations. Wren and Rowe [93] developed and later slightly modified [94] a model that accounts for the effect of the concentration of a neutral chiral selector on the separation of fully charged analytes. They predicted that the apparent mobility difference of the analytes passes through a maximum value as the concentration of the chiral selector is increased. According to the model the optimum chiral selector concentration (c_{opt}) is determined by the association constants of the diastereomeric complexes (K_R and K_S). The stronger the binding the lower c_{opt} is. The apparent mobility difference is higher if the relative difference in K_R and K_S is high.

Very often in practice the predicted maximum in the chiral selectivity as a function of the chiral selector concentration cannot be observed. Rawjee and co-workers [95,96] have developed a model that accounts for the effect of both the pH and the concentration of a neutral chiral selector. The protonation of weak acid and weak base analytes will change as a function of the pH. The charged and uncharged analytes interact with the chiral selector separately. The proposed model leads to a rather complex but nevertheless straightforward equation for the chiral selectivity of the separation of weak acids and bases. Here we show the equation for the separation of weak acids [95]:

$$\alpha_{R/S} = \frac{1 + \frac{\mu_{RC^-}}{\mu^-} K_{RC^-}[\mathrm{C}]}{1 + \frac{\mu_{SC^-}}{\mu^-} K_{SC^-}[\mathrm{C}]} \cdot \frac{1 + K_{SC^-}[\mathrm{C}] + \frac{[\mathrm{H_3O^+}]}{K_a}(1 + K_{\mathrm{HSC}}[\mathrm{C}])}{1 + K_{RC^-}[\mathrm{C}] + \frac{[\mathrm{H_3O^+}]}{K_a}(1 + K_{\mathrm{HRC}}[\mathrm{C}])} \quad (9)$$

where $\alpha_{R/S}$ is the selectivity, μ_{RC-} and μ_{SC-} are the mobilities of the diastereomeric complexes of the charged analytes, μ^- is the mobility of the dissociated acid (equal for enantiomers R and S), K_{RC-} and K_{SC-} are the association constants of the charged analytes with the chiral selector, [C] is the chiral selector concentration, K_a is the acid dissociation constant (equal for R and S) and K_{HRC} and K_{HSC} are the association constants of the non-dissociated enantiomer–chiral selector complex. A similar equation can be formulated for weak bases [96]. A more complex equation has been presented by the same authors for the resolution as a function of [C] and pH [97].

On the basis of the equations three distinctly different separation types can be distinguished: the desionoselective, where only the neutral form (Fig. 6), the ionoselective, where only the charged form interacts and the duoselective, where both the charged and neutral form of the two enantiomers interacts selectively with the chiral selector (Fig. 7).

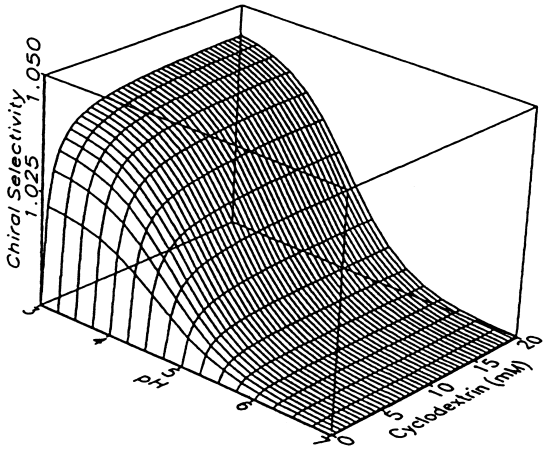

Fig. 6. Theoretical selectivity surface for ibuprofen, a desionoselective separation as a function of pH and the cyclodextrin (CD) concentration. Reprinted with permission from Ref. [95].

Desionoselective separations can be achieved in a relatively narrow pH range close to the pK_a of the analyte. The chiral selector concentration has little effect on the resolution if it exceeds a minimum value. In ionoselective and duoselective separations selectivity can be achieved in a wider pH range. Although the selectivity passes through a maximum as a function of the chiral selector concentration, this maximum cannot always be seen in practice because of the limited solubility of some resolving agents. In contrast to the desionoselective mode, in both iono- and duoselective separations it is theoretically possible to change the migration order of the enantiomers by choosing the right set of pH and [C] values.

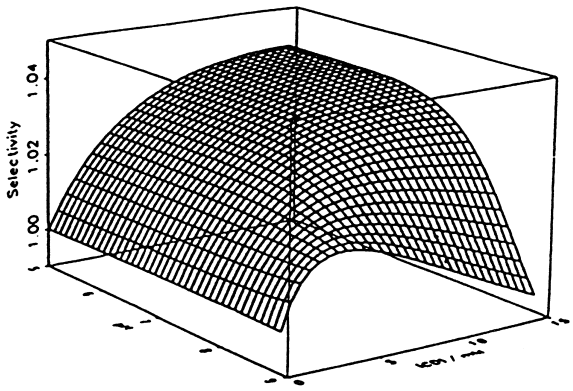

Fig. 7. Theoretical selectivity surface for homatropine, a duoselective separation as a function of pH and the cyclodextrin (CD) concentration. Reprinted with permission from Ref. [96].

Surapaneni et al. [98] have proposed a model for the separation of neutral enantiomers with a combination of charged and neutral cyclodextrins in the absence of EOF. The selectivity is described as follows:

$$\alpha_{R/S} = \frac{\mu_{RC^-}K_{RC^-}}{\mu_{SC^-}K_{SC^-}} \cdot \frac{1 + K_{SC^-}[C^-] + K_{SC}[C]}{1 + K_{RC^-}[C^-] + K_{RC}[C]} \qquad (10)$$

where μ_{RC-} and μ_{SC-} are the mobilities of the complexes formed by enantiomers and the charged chiral selectors; K_{RC-}, K_{SC-} and K_{RC}, K_{SC} are the association constants of the enantiomers with the charged and the neutral chiral selector, respectively; and [C^-] and [C] are the concentration of the charged and neutral chiral selector, respectively.

One may call pH and the chiral selector concentration the operator dependent parameters. However, the operator can also alter the solute dependent parameters (the association constants of the charged and neutral analytes with the resolving agent, the pK_a and mobility of the analytes) by changing the temperature, adding organic modifiers, or replacing the aqueous background electrolyte with organic solvents. Although the effect will be identical on the electrophoretic mobility and the pK_a of the R and S enantiomers, for the association constants the effects may differ resulting in changes in selectivity. By using appropriate optimisation schemes one can maximise the selectivity of the separation [99].

It is not easy, if possible at all, to predict which chiral selector will allow selective interaction for a certain pair of enantiomers. Molecular modelling can provide some guidance, but the calculations are laborious and the predictions not always justified. In practice, analysts follow the trial-and-error approach in combination with (or without) the use of published separation data from CE and other separation methods. This approach seems to work well, as the ever growing literature on chiral separations by CE shows. The success may be partly explained by the availability of numerous chiral selectors and partly by the relative ease, time- and cost-effective nature of testing them. More than 100 chiral selectors have been used so far in CE. From the vast literature it is not easy to identify a solute type that constitutes a

particularly difficult chiral separation problem. Perhaps very small chiral molecules, such as 2-butanol would be examples of a real challenge.

Here we briefly discuss only some of the most important types of chiral selectors and make no attempt to list all of them. Figs. 8 and 9 shows the structure of some resolving agents. A detailed overview of chiral separations by CE can be found in a recent review by Fanali [100].

3.2. Types of chiral selectors

3.2.1. Cyclodextrins (CDs)

CDs [101] are by far the most useful chiral selectors. CDs are cyclic oligosaccharides built up from D-(+)-glucopyranose units linked by α(1,4) bonds. The native α- β- and γ-CDs consist of 6, 7 and 8 glucose units, respectively. Their structure is similar to a truncated cone, the secondary 2- and 3-hydroxyl groups being on the wider rim of the

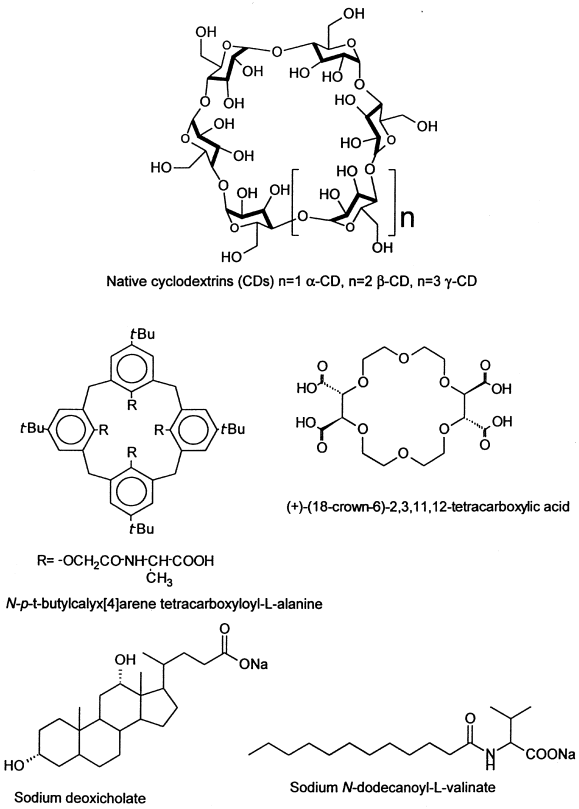

Fig. 8. Structural formulae of selected chiral selectors.

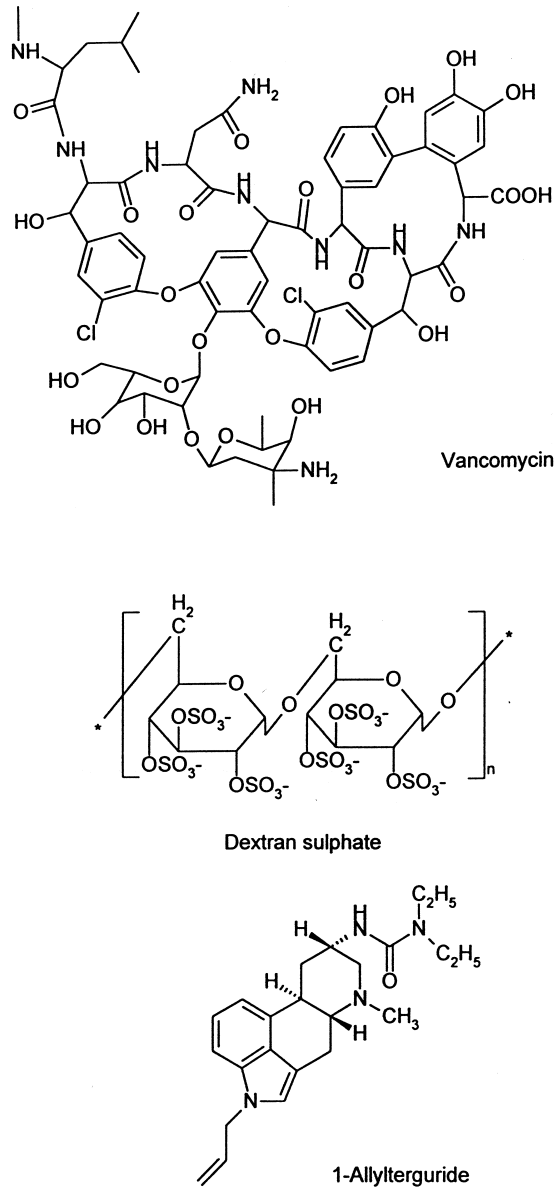

Fig. 9. Structural formulae of selected chiral selectors.

torus, and the primary hydroxyls at carbon atoms 6 on the narrower opening. Since the secondary hydroxyls are attached to asymmetric carbons and cannot rotate, they are perfect sites for sterically selective interactions. The solute–CD interactions very often involve the penetration of the analyte to the relatively hydrophobic cavity of the CD. The inner diameter of the α- β- and γ-CD cavity is 0.57,

0.78 and 0.95 nm, respectively. The different cavity sizes provide the CDs with different selectivity. CDs are generally considered to form inclusion complexes, but separation may sometimes be achieved without the analyte deeply penetrating the CD cavity. For example, in NMR studies Lipkowitz et al. [102] found the aromatic ring of tryptophan to be tilted and near the top of the cavity of α-CD rather than deeply embedded in it. Nevertheless, baseline separation of tryptophan enantiomers is possible with α-CD [103].

Substitution of the hydroxyl groups of the CDs results in new chiral selectors, which usually have improved solubility in water and different selectivity for many solutes. Many substituted CDs are commercially available. Some of them such as 2,6-di-O-methyl-β-CD and 2,3,6-tri-O-methyl-β-CD are of well defined structure, others (e.g., hydroxypropyl-β-CD) are mixtures of homologues with different degrees and patterns of substitution. This raises the question of repeatability, but on the other hand differences in the degree of substitution can be exploited for optimisation of the selectivity [104]. Introducing charged (chargeable) groups to the CD structure allows the separation of neutral compounds. Carboxymethyl-β-CD, a weak acid, can be used in either charged or neutral form, resulting in reversal of the migration order of the enantiomers [105]. Fig. 10 shows the reversal of the migration order of ephedrine enantiomers, with carboxymethyl-β-CD used in neutral form at pH 2.7 and in anionic form at pH 7.2. Recently, sulphobutyl-β-CD has gained popularity because it selectively interacts with a wide range of chiral solutes. The resolution and selectivity with sulphobutyl-β-CD are sometimes very high. Without optimisation an R_s value as high as 26 has been achieved [106] for the chiral separation of 5-cyclobutyl-5-phenylhydantoin, and the same high value for hydrobenzoin at pH 3.8 where the analytes are neutral but the sulphobutyl-β-CD is negatively charged. To our knowledge these are the highest R_s values ever reported for a chiral separation by CE.

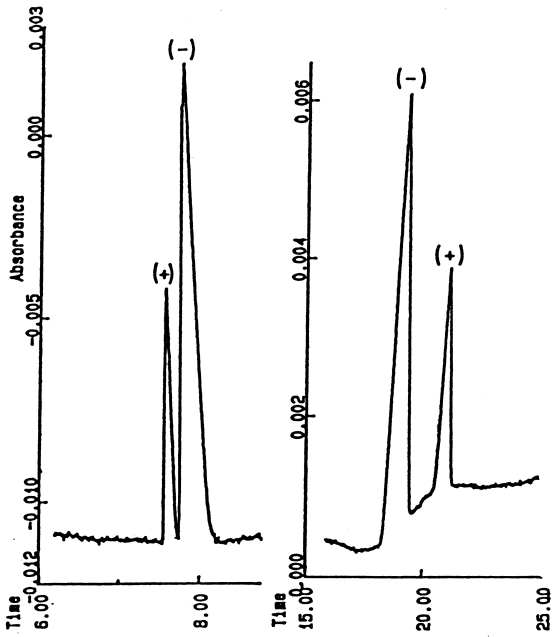

Fig. 10. Reversal of migration order of ephedrine enantiomers by changing pH. Coated capillary 37 cm (30 cm effective length)×75 μm; detection 214 nm, sample: (±)-ephedrine spiked with (−)-ephedrine; (A) 2% carboxymethyl-β-CD in 20 m*M* citric acid, pH 2.7, E=400 V/cm; (B) 1.5% carboxymethyl-β-CD, pH 7.2, E=270 V/cm. Reprinted with permission from Ref. [105].

3.2.2. Macrocyclic antibiotics

Macrocyclic antibiotics such as vancomycin [107], rifamycin B [108] and ristocetin A [109] readily form host–guest complexes with a wide range of analytes. The presence of several asymmetric carbon atoms and many different functional groups in these large molecules makes possible selective interaction with an impressive number of analytes. The structure of vancomycin is shown in Fig. 9. The resolution is often very high [110] with this class of chiral selectors (up to R_s=20). Macrocyclic antibiotics strongly absorb UV light. Fortunately, they can be used for chiral separations at relatively low concentrations, typically 1–5 m*M*, therefore direct UV detection is possible at 254 nm with vancomycin and ristocetin A. Indirect UV detection has been used for rifamycin B [108]. Although, macrocyclic antibiotics have been introduced to CE as chiral selectors only relatively recently, this group may be considered one of the most successful types of resolving agents.

3.2.3. Chiral crown ethers

18-Crown-6-ether has a ring size that is suitable for host–guest complex formation with potassium, ammonium and protonated primary amines. Although, this molecule is not itself chiral, a chiral derivative, (+)-18-crown-6-tetracarboxylic acid

(Fig. 8), has been found to be a useful resolving agent for many primary amines, including amino acids [111].

3.2.4. Chiral calixarenes

Calixarenes are a new group of macrocycles with good ability for host–guest complex formation. The first chiral separation in CE with this type of resolving agents was published just recently: N-*p*-*tert*.-butylcalyx[4]arene tetracarboxyloyl-L-alanine (Fig. 8) was successfully used for the separation of 1,1′-binaphthyl-2,2′-diyl hydrogenphosphate enantiomers [112]. One can expect more separations to be conducted with chiral calixarenes in the future.

3.2.5. Ligand–exchange complexes

The very first chiral separation by CE was made in 1985 by Gassmann et al. [113] using ligand–exchange complex formation. In the ligand–exchange mechanism the chiral selector is the complex of a metal cation and optically pure chiral ligands. The analyte enantiomers can displace one ligand in the complex. If the association constants of the resultant complexes are different the separation of the two solute enantiomers is possible.

3.2.6. Proteins

Proteins are widely used as chiral stationary phases in HPLC [114]. A number of proteins (e.g., bovine and human serum albumin) and glycoproteins (e.g., ovomucoid, α_1-acid glycoprotein, avidine) have been introduced to CE as resolving agents dissolved in the buffer [115–117]. Unfortunately, there are some practical problems with the use of proteins in CE: they tend to adsorb to the capillary wall, they have considerable UV absorption, the efficiency of the separation is typically low and the peak symmetry may be very distorted. Some ways have been found to reduce these shortcomings, however. Adsorption to the wall can be minimised by using coated capillaries, the protein can be kept away from the detection window by using partial capillary filling technique [118] and the peak shape can be improved by the addition of organic solvents. The main application of protein chiral selectors continues to be in the binding studies of enantiomeric drugs. For practical separation problems selective and easy-to-use resolving agents are likely to be found in other chiral selector groups.

3.2.7. Oligo- and polysaccharides

Besides CDs, which are cyclic oligosaccharides, there are other sugar derivatives that can be applied in chiral separations. Neutral polysaccharides such as cellulose are not soluble in water. Neutral oligosaccharides (e.g., maltooligosaccharides) and polyionic polysaccharides (e.g., heparin, dextran sulphate) are however, water soluble and have been successfully used as chiral selectors [119,120]. The low UV absorption of oligo- and polysaccharides and the often high efficiency of the achieved separations makes these resolving agents highly attractive. Fig. 9 shows the structure of the dextran sulfate monomer.

3.2.8. Chiral micelles

Means of influencing selectivity in MEKC have been discussed in Section 2 of this paper. Here we focus only on those factors with a bearing on chiral separations. Partitioning of the solute enantiomers between the free solution and the micelles allows separation if the distribution constants of the enantiomers are different and either the micelles or the analytes (or both of them) are charged. Surfactants differ from other chiral selectors in that they do not act alone but in molecular aggregates. To our knowledge, no enantioseparation with chiral surfactants under their CMC has been reported. Very little is known about the chiral recognition mechanism on the molecular level in MEKC.

Many different chiral surfactants can be used for enantioseparation. Bile salts [121] such as sodium deoxycholate, and long alkyl-chain amino acids [122] such as sodium N-dodecanoyl-L-valinate, are the most widely used chiral surfactant types (Fig. 8).

The chiral surfactants may be used in combination with non-chiral ones. For example, digitonin, a neutral chiral surfactant, and SDS form mixed micelles [123]. SDS does not participate in the chiral recognition, but by introducing charge to the micelle it influences the mobility and the resolution. Chiral MEKC is often combined with CDs [124].

An inherent disadvantage of MEKC that leads to peak broadening is the slow mass transfer between the bulk solvent and the micellar phase. An interesting approach to overcome this problem is the use of polymerised micelles. Undecyl-L-valine has been polymerised by ^{60}Co γ-irradiation [125]. The polymerised micelle is believed to have a more compact structure than the normal micelle, and the penetration

of the solutes is therefore less deep and faster although the selectivity of the separation remains good. In addition to the improved efficiency, it may be advantageous that polymerised micelles have no CMC.

3.2.9. Microemulsions

An interesting way of achieving enantioselectivity is described by Aiken and Huie [126] who used a microemulsion for the chiral separation of ephedrine. The chiral selector was the oil (2*R*,3*R*)-di-*n*-butyl tartrate.

3.2.10. Ergot alkaloids

Recently, ergot alkaloids such as 1-allylterguride (Fig. 9) have been used for the separation of organic acids [127]. The asymmetric carbon atoms, the presence of a basic nitrogen and the π-acceptor indole ring make these molecules suitable chiral selectors. The low solubility of the ergot alkaloids can be overcome by the addition of methanol to the buffer.

4. Non-aqueous media

Water in the background electrolyte can be completely replaced by organic solvents. The EOF is intense and the solubility of the buffer ions is sufficient in many organic solvents. The advantages and disadvantages of non-aqueous media in CE have been discussed in detail in a recent review [128]. Here we shall focus on the effect of organic solvents on the selectivity.

Possibly the most attractive feature of organic solvents is that their physical and chemical properties are very different from each other and from water, allowing the important characteristics of separations to be controlled on a wider scale than with water alone. Table 3 summarises some characteristic properties of solvents used in CE. Not all the listed properties are directly relevant to the selectivity. For example, the viscosity of the solvent affects the mobility of all analytes in the same way. An organic solvent affects the acid–base properties of the analytes, allowing the separation of analytes difficult to resolve in aqueous buffers [129]. Depending on the solvent, the pK_a values of chargeable compounds can be many orders of magnitude different. Amphiprotic solvents undergo autoprotolysis. Some amphiprotic solvents, such as water and alcohols, are about equally good proton donors and acceptors, while others are characterised by stronger acidic (e.g., acetic acid) or basic character (e.g., formamide). Amphiprotic solvents with acidic character enhance the basicity of solutes and reduce their acidity. Amphiprotic basic solvents (e.g., formamide, N-methylformamide, N,N-dimethylformamide) have the opposite effect [129]. Aprotic solvents are not capable of autoprotolysis but they can accept protons. Important examples of this solvent type are acetonitrile and dimethyl sulphoxide. Inert solvents such as hexane are neither capable of autoprotolysis nor of donating or accepting protons to any considerable extent.

Fig. 11 shows the separation of drugs in two organic solvent mixtures [130]. The selectivity improved and even the migration order of some negatively charged analytes changed when methanol in a background electrolyte consisting of methanol–ace-

Table 3
Physicochemical parameters of selected organic solvents and water [136]

Solvent	η (cP)	ε	Solvent type	pK_{auto}	Polarity	T_{boil} (°C)
Water	0.89	80	Amphiprotic	14.0	10.2	100
Formamide	3.30	111	Basic amphiprotic	16.8	9.6	210
N-Methylformamide	1.65	182	Basic amphiprotic	10.7	6.0	182
N,N-Dimethylformamide	0.80	36.7	Basic amphiprotic	29.4	6.4	153
N,N-Dimethylacetamide	0.78	37.8	Basic amphiprotic	24.0		166
Dimethyl sulphoxide	2.00	46.7	Aprotic	33.3	7.2	189
Acetonitrile	0.34	37.5	Aprotic	[1]	5.8	82
Methanol	0.54	32.7	Amphiprotic	17.2	5.1	65
Ethanol	1.07	24.6	Amphiprotic	18.9	4.3	78

[1] No detectable autoprotolysis.

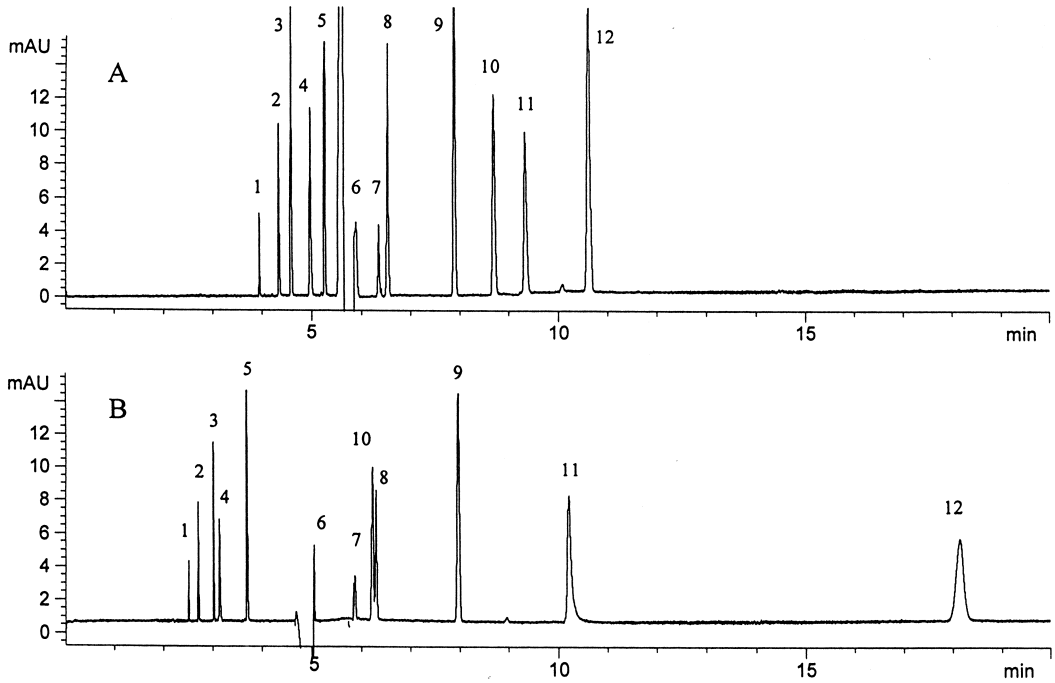

Fig. 11. Separation of a drug mixture in (A) ethanol–acetonitrile–acetic acid (50:49:1) containing 20 m*M* ammonium acetate and in (B) methanol–acetonitrile–acetic acid (50:49:1) containing 20 m*M* ammonium acetate. Capillary: 58.5 cm (50 cm effective length)×50 μm fused-silica, applied voltage: 30 kV, injection: 50 mbar×3 s, temperature: 22°C, detection 214 nm. Analytes: 1 amphetamine, 2 ephedrine, 3 levorphanol, 4 dextromoramide, 5 morphine, 6 hydrochlorothiazide, 7 benzoic acid, 8 meso-2,3-diphenylsuccinic acid, 9 probenecid, 10 chlorothiazide, 11 1,2-phenylenediacetic acid, 12 ethacrynic acid. From Ref. [130].

tonitrile–acetic acid (50:49:1) and containing 20 m*M* ammonium acetate was replaced by ethanol.

The solubility of quinine is much better in methanol than in water. The dielectric constant of methanol is lower than that of water and therefore ion pairing more easily takes place in methanol. The improved solubility and the ion pairing interactions with the analytes have allowed the use of quinine as a chiral selector for N-3,5-dinitrobenzoyl substituted amino acids in methanol [131].

The limited aqueous solubility [101] of β-cyclodextrin (18 m*M*) is a disadvantage because sometimes not enough chiral selector can be dissolved to achieve appropriate selectivity. β-Cyclodextrin is not a very good chiral selector for dansyl-amino acids in aqueous buffers, partly due to its poor aqueous solubility. However, in N-methylformamide more than 700 m*M* β-cyclodextrin can be dissolved [132]. We have obtained fast and efficient chiral separation of dansyl-amino acids with high resolution in N-methylformamide with β-cyclodextrin as a chiral selector [132,133].

The association constants of host–guest complexes differ with the organic solvent and organic solvent mixtures and this difference can be exploited for the optimisation of the selectivity of chiral separations in non-aqueous media [134,135].

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