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2

Advanced Chiral Separation

2.1 Chiral electromigration modes and enantioselective agents - introduction

Chiral separations by CE can be performed either indirectly, using a chiral derivatization agent forming irreversible diastereomeric pairs which can be resolved under achiral conditions, or directly, using chiral selectors as additives to the electrolyte, where reversible diastereomeric associates, enantiomer-chiral selector, are created that can be subsequently transformed into mobility differences of the individual stereoisomers [Chankvetadze & Blaschke, 2001; Rizzi, 2001]. In capillary electrochromatography (CEC), a hybrid CE / HPLC technique (i.e., CE with stationary phase), chiral stationary phases or chiral mobile phase additives are applied in enantioseparations [Huo & Kok, 2008].

Several disadvantages of the indirect enantioseparation approach, such as (i) the need of a functional group which can be derivatized, (ii) the derivatization reagent has to be of high enantiomeric purity, (iii) the derivatization represents an additional time consuming step with a risk of racemization under the reaction conditions, result in it being rarely used. Therefore, it is not surprising that only a few new chiral derivatization procedures, employing new chiral derivatization reagents, have been developed recently [Cheng J. & Kang J., 2006; Zhao S. et al., 2006a, 2006b].

More attractive and therefore much more frequently used are direct enantioseparations representing elegant and simple solutions in the majority of problems in chiral analysis. See, for instance, recent (2000-2011) chiral separations of drugs, their metabolites and biomarkers in various (mostly biological) samples listed in Tables 2.1 and 3.1 of this book (these tables are divided according to the manner of a sample preparation step, i.e., off- or on-line). In this chapter and Table 2.1 a chiral separation step is accompanied by a conventional off-line sample pretreatment and the chiral separation mechanism itself is highlighted. The latest fundamental reviews on chiral separations are given by Ward T.J. and Ward K.D. [Ward T.J. & Ward K.D., 2010] and Scriba [Scriba, 2011]. The papers by Gübitz and Schmid [Gübitz & Schmid, 2000a, 2007, 2008], Eeckhaut and Michotte [Van Eeckhaut & Michotte, 2006] and Preinerstorfer et al. [Preinerstorfer et al., 2009] provide detailed overviews on the different classes of chiral selectors, including newly introduced ones, that are used in common CE techniques, but also in MEEKC and MCE.

The following subsections summarize (i) basic electromigration modes and their possibilities in chiral separations, as well as (ii) basic characteristics of different groups of chiral selectors - giving a view on their complexing abilities (types of useful analytes) and advantages and limitations when introduced into CE. Recent applications in the enantioseparation of drugs in biological samples are discussed in the text and tabulated.

2.2 Electromigration techniques in chiral separations

Effective chiral separations can be performed by a wide range of electromigration techniques that provide a great variety of applicable separation mechanisms and, by that, a high application potential both analytically and preparatively. For the basic instrumental scheme of CE see Figure 2.1.

The latest review on advances of enantioseparations in CE is given by Lu and Chen [Lu H.A. & Chen G.N., 2011] and Scriba [Scriba, 2011]. Gübitz and Schmid [Gübitz & Schmid, 2000a, 2007, 2008] show recent progress in chiral separation principles in various CE techniques, namely capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), isotachophoresis (ITP), isoelectric focusing (IEF), capillary electrokinetic chromatography (EKC) and capillary electrochromatography (CEC). The authors included into their latest review [Gübitz & Schmid, 2008] microchip CE (MCE). Among the most recent reviews also belong refs. by Gebauer et al. [Gebauer et al., 2009, 2011], Silva [Silva, 2009] and Ryan et al. [Ryan et al., 2009] describing recent advances in the methodology, optimization and application of ITP, micellar EKC (MEKC) and microemulsion EKC (MEEKC), respectively. Preinerstorfer et al. [Preinerstorfer et al., 2009] included, besides common CE techniques, also MEEKC and MCE.
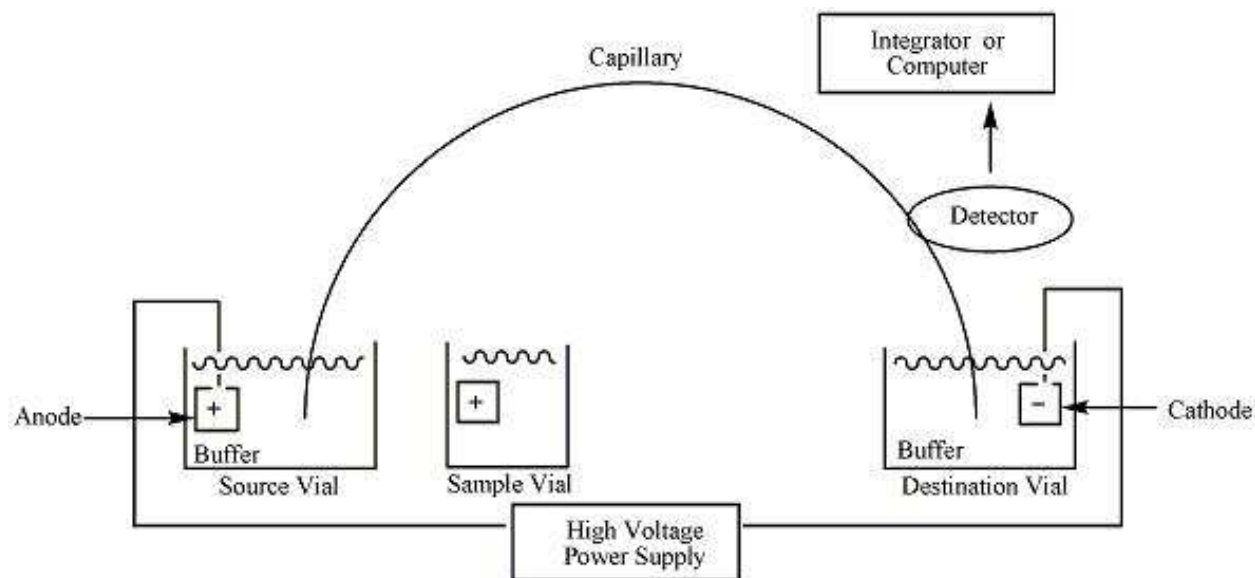

Figure 2.1. Instrumental scheme of capillary electrophoresis system.

Analyte	Separation method	Chiral selector[a]	pH	Biological sample	Sample preparation method[b]	Detection	LOQ	Application	Ref.
Amino acids, mexiletine	CEC	β-CD derivatives CSP	5.5-8.5	Human plasma	SPE	UV		Spiked samples	Li Y. et al., 2010
Catechin isomers	CDEKC	β-CD		Human plasma	PP	UV (high sensitivity detection cell)	4.1 and 1.5 ng/mL (LOD)	Real samples	El-Hady & El-Maali, 2008
Cetirizine and hydroxyzine	Polymeric EKC	maltodextrin	2.0	Human plasma	LLE	UV	10 ng/mL (LOD)	Spiked samples	Nojavan & Fakhari, 2011
Dioxopromethazine hydrochloride	CDEKC	β-CD	2.5	Human urine	LLE	ECL	4.0×10^{-6} M (LOD)	Spiked samples	Li X. et al., 2009
Ofloxacin	CDEKC	M-β-CD	2.8	Caco-cells	LLE, EK	UV	11.4-10.8 ng/mL	Spiked samples	Awadallah et al., 2003
Citalopram, desmethyl-citalopram	CDEKC	S-β-CD + acetonitrile	2.5	Plasma	LPME (19-31x)	UV	4.4-11.2 ng/mL	Clinical samples	Andersen et al., 2003
Primaquine, carboxyprimaquine	EKC	Maltodextrin	3.0	Mitochondrial fraction of liver of rats, plasma	LLE	DAD	40-100 ng/mL	Spiked samples	Bortocan & Bonato, 2004
Hydroxychloroquine and its metabolites	CDEKC	S-β-CD + HP-β-CD	9.0	Liver homogenate, plasma	LLE	DAD	129 ng/mL	Spiked samples	Cardoso et al., 2006
Propaphenone and its metabolites	CDEKC	S-β-CD + methanol	2.0	Serum	LLE	UV	10-12 ng/mL (LOD)	Spiked samples	Afshar & Thormann, 2006

Ketoprofen	CDEKC	HTM-β-CD	5.0	Serum	LLE	UV	500-1000 ng/mL	Pharmacokinetic study	Glowka & Karzniewicz, 2004a
Indobufen	CDEKC	HTM-β-CD	5.0	Serum	LLE	UV	200 ng/mL	Pharmacokinetic study	Glowka & Karzniewicz, 2004b
Apomorphine	CDEKC	HP-β-CD	3.0	Caco-cells	Direct injection	DAD	0.5×10^{-6} M	Spiked samples	Ha et al., 2004a
Lactic acid	CDEKC	HP-β-CD	7.0	Plasma	PP (10x)	DAD	$15\text{-}20 \times 10^{-6}$ M	Spiked samples	Tan et al., 2005
Serine	CDEKC	HP-γ-CD + D(+)-glucose	10.0	Neuronal cells (rat's brain)	Microdialysis, AD	LIF	0.3×10^{-6} M	Clinical samples	Quan et al., 2005
Methadone	CDEKC	S-β-CD	5.0	Serum	LLE, EK	UV	-	Clinical samples	Esteban et al., 2004
Mirtazapine and its metabolites	CDEKC	CM-β-CD	2.5	Plasma	SPE (37,5x)	DAD	5 ng/mL	Clinical samples	Mandrioli et al., 2004
Anisodamine	CDEKC	CM-γ-CD	2.5	Plasma (rabbits)	LLE, EK	UV	40-60 ng/mL	Pharmacokinetic study	Fan et al., 2004
Salbutamol	NACE (CDEKC)	HDAS-β-CD	6.0	Urine	SPE	DAD	375 ng/mL	Spiked samples	Servais et al., 2004
Metamphetamine and related compounds	CDEKC	HDAS-β-CD	1.7	Urine	LLE	MS	5 ng/mL	Spiked samples	Iio et al., 2005
t-tramadol, t-O-demethyltramadol	CDEKC	SBE-β-CD	2.5	Plasma, urine	LLE	UV	1.25 ng/mL	Pharmacokinetic study	Liu H.C. et al., 2004
Labetalol	CDEKC	ODAS-γ-CD, HDAS-β-CD	2.5	Plasma	SPE	DAD	-	Spiked samples	Goel et al., 2004
Deprenyl metabolites	CDEKC	DM-β-CD + CM-β-CD	2.7	Urine	SPE	UV	$0.1\text{-}0.5 \times 10^{-6}$ M	Metabolic study	Szökö et al., 2004
Mirtazapine and its metabolites	CDEKC	CE-β-CD	2.5	Urine	LPME, EH	DAD	62.5 ng/mL	Pharmacokinetic study	de Santana et al., 2008

Analyte	Technique	Chiral selector	pH	Matrix	Extraction	Detection	LOD / Concentration	Sample	Reference
Salbutamol	NACE	HDAS-β-CD (CDEKC)		Urine	SPE	MS	18-20 ng/mL	Spiked samples	Servais et al., 2006
Amphetamine derivates	CDEKC	S-γ-CD	2.5	Plasma	LLE	MS	100-400 ng/mL (LOD)	Spiked samples	Rudaz et al., 2005
Ketamine, norketamine	CDEKC	S-β-CD	2.5	Plasma (horse)	LLE	DAD	10 ng/mL (LOD)	Clinical samples	Theurillat et al., 2005
Warfarin	MEKC	Polysodium -N- undecenoyl- L,L-leucyl- valinate	6.0	Plasma	SPE	MS	100 ng/mL (LOD)	Clinical samples	Hou, J. et al., 2007
Cetirizine	CDEKC	S-β-CD	8.7	Plasma	LLE	UV	500 ng/mL (LOD)	Spiked samples	Chou et al., 2008
Ibuprofen and its metabolites	CDEKC	HTM-β-CD	5.0	Urine, plasma	SPE	UV	110 ng/mL (plasma), 1.0-1.1x10^3 ng/mL (urine)	Pharmacokinetic study	Glowka & Karazniewicz, 2007
Amlodipine	CDEKC	HP-β-CD	2.5	Serum	LLE	UV	700 ng/mL	Clinical samples	Wang R. et al., 2007
Disopyramide	CDEKC	S-β-CD	4.5	Plasma	LLE	ECL	8x10^{-8}-1.10^{-7} M (LOD)	Spiked samples	Fang L. et al., 2006
Baclofen	CDEKC	S-β-CD	9.5	Plasma	PP, AD	LIF	50x10^{-9} M (LOD)	Spiked samples	Kavran-Belin et al., 2005
Tioridazine 5-sulfoxide	CDEKC	HP-β-CD + S-β-CD	3.0	Plasma	LLE	DAD	250 ng/mL	Spiked samples	de Gaitani et al., 2003
Hydroxychloroquine and its metabolites	CDEKC	S-β-CD + HP-β-CD	9.0	Urine	LPME	DAD	10-21 ng/mL	Pharmacokinetic study	de Oliveira et al., 2007

Analyte	Technique	Chiral selector	pH	Matrix	Sample prep	Detection	LOD/Conc.	Sample type	Reference
Tramadol and its metabolites	CDEKC	SBE-β-CD	4.0	Plasma	LLE	MS	-	Metabolic study	Rudaz et al., 2000
Clenbuterol	CDEKC	DM-β-CD	2.5	Plasma	SPE	MS	740 ng/mL	Spiked samples	Toussaint et al., 2001
Baclofen	CDEKC	α-CD	9.5	Plasma	PP, AD	LIF	10 ng/mL (LOD)	Spiked samples	Chiang et al., 2001
Phenprocoumone	CDEKC	α-CD	5.4	Urine	Direct injection	LIF	200 ng/mL	Clinical samples	Chankvetadze et al., 2001b
Albendazole sulfoxide	CDEKC	S-β-CD	7.0	Plasma, saliva	LLE	UV, LIF	100 ng/mL (LOD)	Clinical samples	Prost et al., 2002
Mirtazapine and its metabolites	CEC	Vancomycin CSP	6.0	Urine	SPE	DAD	1000-2000 ng/mL	Spiked samples	Aturki et al., 2007
Methadone	CDEKC	HS-γ-CD	4.5	Oral fluids	LLE	DAD	7.6-8.1 ng/mL	Clinical samples	Martins et al., 2008
Methadone	CDEKC	S-γ-CD	2.5	Plasma	LLE-EK, PP-HD	MS	0.5 ng/mL (LLE-EK), 250 ng/mL (PP-HD)	Clinical samples	Schappler et al., 2008
Ketamine and its metabolites	CDEKC	S-β-CD (multiple isomer)	2.5	Plasma, urine	LLE	DAD	-	Metabolic study	Theurillat et al., 2007
Cinchona alkaloids	CDEKC	HDM-β-CD	5.0	Urine	SPE (10x)	DAD	100 ng/mL	Clinical samples	Tsimachidis et al., 2008
Hydroxy-mebendazole, hydroxyamino-mebendazole, aminomebendazole	CDEKC	S-β-CD	4.2	Plasma	LLE	UV	10-40 ng/mL (LOD)	Clinical samples	Theurillat & Thormann, 2008
3,4-metylendioxy-metamphetamine and its metabolites	CDMEKC	β-CD + sodium cholate	2.3	Urine	LLE	LIF	-	Spiked samples	Huang Y.S. et al., 2003

Ofloxacin and its metabolites	CDEKC	SB-β-CD	2.0	Urine	Direct injection	LIF	100-250 ng/mL	Metabolic study	Horstkötter & Blaschke, 2001
Methadone and its metabolites	CDEKC	CM-β-CD	4.0	Serum	LLE (10x)	MS	-	Clinical samples	Cherkaoui et al., 2001
Benzoporfyrine derivate mono and diacid	MEKC	Sodium cholate	9.2	Serum, microsomes	PP, SPE	LIF	2180-3500 ng/mL	Metabolic study	Penget al., 2002
Tramadol	CDEKC	CM-β-CD + M-β-CD	10.0	Urine	Direct injection	LIF	100 ng/mL	Pharmacoki netic study	Soetebeer et al., 2001
Carvedilol	CDEKC	succinyl-β-CD + M-α-CD	3.0	Plasma	LLE	LIF	1.56 ng/mL	Pharmacoki netic study	Behn et al., 2001
Chloroquine, deethylchloroquine	CDEKC	HP-γ-CD, CM-γ-CD, S-γ-CD	9.65	Plasma	LLE	LIF	0.5 ng/mL (LOD)	Clinical samples	Müller & Blaschke, 2000

Table 2.1. Chiral CE determinations of biologically active compounds in various biological matrices employing conventional (off-line) sample preparation.

a Mixed selector systems are indicated by a plus sign. Charge of ionizable chiral selectors is obvious from pH (next column).

b Preconcentration factor is given in brackets.

ITP = isotachophoresis, EKC = electrokinetic chromatography, MEKC = micellar electrokinetic chromatography, CDEKC = cyclodextrin mediated electrokinetic chromatography, CDMEKC = cyclodextrin mediated micellar electrokinetic chromatography, NACE = non-aqueous capillary electrophoresis, MCE = electrophoresis on microchip, CEC = capillary electrochromatography, S-β-CD = sulphated-β–CD, S-γ–CD = sulphated-γ-CD, HS-γ-CD = highly sulphated-γ-CD, M-α-CD = methyl-α-CD, M-β-CD = methyl-β-CD, DM-β–CD = dimethyl-β–CD, CM-β–CD = carboxymethyl-β-CD, CM-γ-CD = carboxymethyl-γ-CD, CE-β-CD = carboxyethyl-β-CD, HP-β-CD = hydroxypropyl-β-CD, HP-γ-CD = hydroxypropyl-γ-CD, HTM-β-CD = heptakistrimethyl-β-CD, SBE-β-CD = sulfobuthylether-β-CD, SB-β-CD = sulfobuthyl-β-CD, HDAS-β-CD = heptakisdiacethylsulfo-β-CD, ODAS-γ-CD = oktakisdiacethylsulfo-γ-CD, AD = analyte derivatization, EH = enzymatic hydrolysis, HD = hydrodynamic injection, EK = electrokinetic injection, FESS = field-enhanced sample stacking, LVSS = large volume sample stacking, SPE = solid-phase extraction, LLE = liquid-liquid extraction, LPME = liquid-phase microextraction, PP = protein precipitation, DAD = diode array detection, UV-ultraviolet (absorbance detection), ECL = electrochemiluminiscent detection, LIF = laser induced fluorescent detection, MS = mass spectrometry, LOQ = limit of quantification, LOD = limit of detection.

2.2.1 Capillary electrophoresis

The unique properties of CE in terms of enantioresolution, due to a combination of extremely high separation efficiency (N) and various electomigration effects, are comprehensively summarized by Chankvetadze [Chankvetadze, 2007] and generally described by the Equation 2.1 [Giddings, 1969]:

$$R_S = \frac{1}{4} \sqrt{N} \frac{\Delta\mu}{\mu_{av}}$$
2.1

where μ_{av} is the effective averaged mobility $\{\mu_{av}=1/2(\mu_1+\mu_2)\}$ and $\Delta\mu$ is the mobility difference $(\Delta\mu=\mu_1-\mu_2)$.

For enantioresolutions by CE the effective mobilities of the enantiomers have to be different $(\mu_1\neq\mu_2)$. This occurs due to (i) a difference in the complex formation constants of the enantiomer-chiral selector complexes $(K_1\neq K_2)$ and (ii) a difference in the mobility of the enantiomer-chiral selector complexes $(\mu_{c1}\neq\mu_{c2})$, as well as the mobility of the free enantiomer and the enantiomer-selector complex $(\mu_f\neq\mu_{c1}, \mu_f\neq\mu_{c2})$, as it can be seen from the mobility difference $(\Delta\mu)$ model (Equation 2.2) developed for two enantiomers (1, 2) and the concentration of selector (C) by Wren and Rowe [Wren & Rowe, 1992, 1993]:

$$\Delta\mu = \mu_1 - \mu_2 = \frac{\mu_f + \mu_{c1}K_1[C]}{1 + K_1[C]} - \frac{\mu_f + \mu_{c2}K_2[C]}{1 + K_2[C]}$$
2.2

It is apparent from this equation that CE offers many possibilities to manipulate enantioresolution via electromigration and complexing effects. This is also discussed in detail in the following subsections.

Besides electromigration and complexing effects, flow counterbalancing [Chankvetadze et al., 1999], as a combination of the bulk flow moving with the opposite migration of both a chiral selector and a chiral analyte, is another interesting possibility to effectively manipulate enantioresolution that will be briefly mentioned later.

The great advantages of CE in terms of the arrangement of the chiral separation system flexibly and simply are: (i) creation of continuous (CZE) and discontinuous / gradient (ITP, IEF) electrolyte systems providing a high variety of separation mechanisms. For basic CE modes see Figure 2.2. Here, interesting separation, as well as preseparation, possibilities are given by the differences in arrangement and diffusion properties of electrophoretic zones. (ii) Implementation of chiral selector(s) or, in other words, chiral pseudostationary phase(s), merely by their dissolving in such separation systems, creating a proper chiral separation environment. An extremely high resolution power of chiral CE can be amplified further by a large excess of a chiral selector dissolved in the electrolyte solution compared to the separation techniques with immobilized chiral selectors (CEC, HPLC) [Chankvetadze & Blaschke, 2001].

In fact, enantiomeric separations performed by CE may be included in an EKC mode because the discrimination of the enantiomers of a chiral compound is due to their different interactions with a chiral selector, that is, enantiomers are distributed in a different way between the bulk solution and the chiral selector according to a chromatographic (interaction) mechanism. So the electrophoretic and chromatographic principles are acting simultaneously in EKC (notice that is principally true not only for chiral but also achiral separations modified by a selector). Therefore, in this monograph we consider all the enantiomeric separations performed in the zone electrophoretic mode to be EKC separations with the exception of chiral ITP and chiral IEF separations (no alternative terms are introduced in the literature).
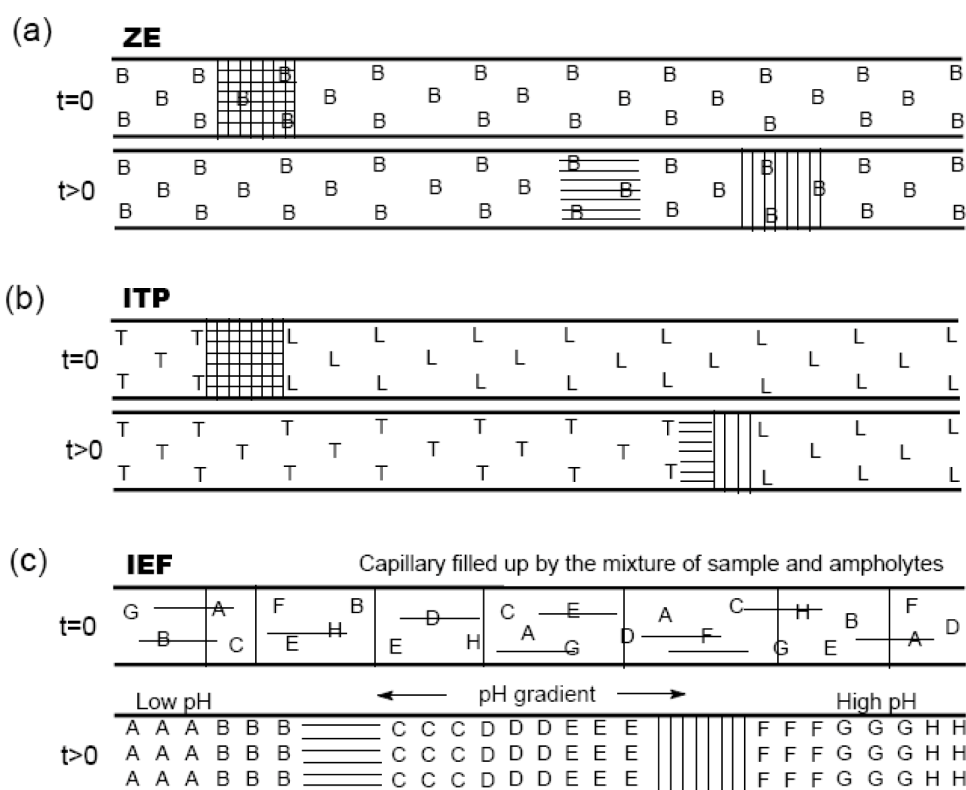
Figure 2.2. Separation principles in capillary electrophoresis: (a) zone electrophoresis (ZE), where B is background electrolyte, (b) isotachophoresis (ITP), where L is leading electrolyte and T is terminating electrolyte, with different electrophoretic mobilities of these electrolytes, (c) isoelectric focusing (IEF), where A-H are ampholytic electrolytes, with different pI values of these electrolytes. Reprinted from ref. [Boček, 1987].

2.2.1.1 Interactions in enantioseparations and their manipulation

Thanks to a great variety of applicable chiral selectors with different physico chemical properties and complexing abilities (see section 2.3), chiral CE separation systems with high performance variability can be created. Here, several basic enantioresolution mechanisms can be recognized that are based on:

- Inclusion (host-guest) complexation {cyclodextrins (CDs), crown ethers (CWEs)},
- Ligand-exchange (metal complexes),

- Affinity interactions (proteinic biopolymers, macrocyclic antibiotics),
- Polymeric complexation (saccharidic biopolymers),
- Micelle / microemulsion solubilization (micelles, micelle polymers, oils),
- Ion-pairing (ionic compounds in non-aqueous media).

Thus, the separations of enantiomeric couples with a wide range of polarities, charges and sizes can be easily accomplished [Gübitz & Schmid, 2000a, 2007, 2008; Preinerstorfer et al., 2009; Gebauer et al., 2009, 2011; Silva, 2009; Ryan et al., 2009], see examples in section 2.4, Table 2.1 and Table 3.1.

On the other hand, very subtle differences/modifications of the structure within the same group of chiral selectors also can provide significant differences in (enantio)selectivity, see Table 2.2 (notice differences in CE enantioresolutions under the same conditions, but different chiral selector – differing in one methyl group in their molecules). This demonstrates another powerful tool to manipulate (enantio)selectivity from the complex forming point of view in CE enantioseparations.
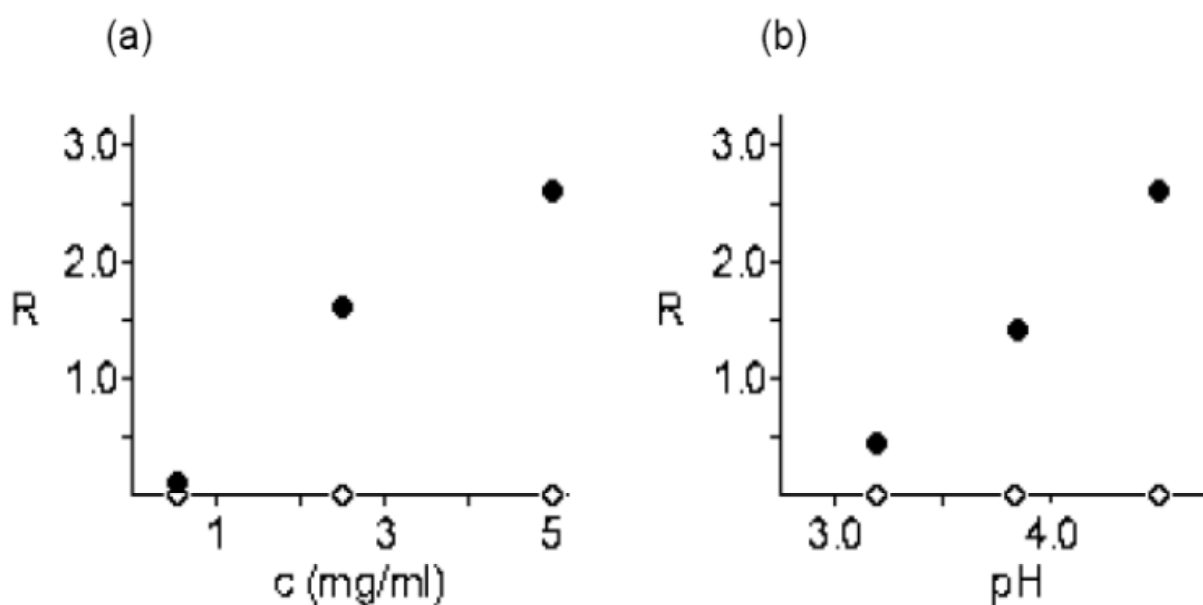
Figure 2.3. Influence of pH and concentration of chiral selector on the resolution of pheniramine enantiomers demonstrating the effectivity of charged chiral selector and countercurrent separation mechanism in EKC enantioseparation. (a) The concentration dependences at 0.5, 2.5 and 5.0 mg/mL concentrations of CE-β-CD (●) and native β-CD (○) were obtained at pH 4.5 (20 mM ε-aminocaproic acid - acetic acid BGE); (b) the pH dependences were obtained at 5 mg/mL concentrations of the CDs and the glycine- or ε-aminocaproic acid – acetic acid BGEs with pH 3.2-3.8 or 4.5, respectively. 0.2% (w/v) methyl-hydroxyethylcellulose served as an EOF suppressor in BGE. The driving current was stabilized at 100-120 μA. CE-β-CD = carboxyethyl-β-cyclodextrin. Reprinted from ref. [Mikuš et al., 2005a].

Analyte	Electrolyte system				
DNP-D,L-amino acid	R [ES2]	R [ES3]	R [ES4]	R [ES6]	R [ES7]
DNP-D,L-glutamic acid	2.6	1.0	1.6	0.4	1.7
DNP-D,L-methioninesulfone	0.9	0.0	1.6	0.0	1.3
DNP-D,L-methionine sulfoxide	0.9	0.3	1.1	0.0	0.9
DNP-D,L-α-amino-n-butyric acid	2.4	0.0	1.4	0.5	1.2
DNP-D,L-norvaline	2.7	1.7	2.4	0.8	2.2
DNP-D,L-citrulline	1.3	0.0	1.3	0.0	1.0
DNP-D,L-methionine	2.1	3.2	4.3	0.9	3.3
DNP-D,L-norleucine	5.1	6.5	6.0	1.3	4.5
DNP-D,L-ethionine	3.1	5.2	5.9	0.8	4.2
DNP-D,L-isoleucine	7.2	6.0	8.6	1.7	5.3
DNP-D,L-leucine	11.8	9.9	12.1	2.2	7.6

Table 2.2. Enantioresolutions of 2,4-dinitrophenyl (DNP) labelled amino acids under different complexing and acid-base conditions[a]

[a]Electrolyte systems (ESs) were prepared at two different pH values: (i) 100 mM morpholinoethanesulfonic acid + 10 mM histidine + 0.2% methylhydroxyethylcellulose (w/v) + 20 mM cyclodextrin derivative, pH 5.2. (ii) 50 mM H$_3$BO$_3$ + 100 mM 1,3-bis(tris(hydroxymethyl)-methylamino)propane + 0.2% methylhydroxyethylcellulose (w/v) + 20 mM cyclodextrin (CD) derivative, pH 9.6. ES1: 6^L-deoxy-6^L-monomethylamino-β-CD positively charged at pH 5.2, ES2: 6^L-deoxy-6^L-dimethylamino-β-CD positively charged at pH 5.2, ES3: 6^L-deoxy-6^L-trimethylammonium-β-CD positively charged at pH 5.2, ES4: 6^L-deoxy-6^L-monomethylamino-β-CD uncharged at pH 9.6, ES5: 6^L-deoxy-6^L-trimethylammonium-β-CD positively charged at pH 9.6.
R – enantioresolution (for a given ES).
Reprinted from ref. [Mikuš & Kaniansky, 2007].

Aqueous media. In CE, the improved separation enantioselectivity of charged solutes can be observed, in many cases, with oppositely charged chiral selectors compared to neutral ones (**Figure 2.3**). Higher stability of the formed complexes is one of the factors responsible for this enhanced enantioselectivity, as it was demonstrated by CE [Vespalec & Boček, 2000; Wenz et al., 2008] as well as nuclear magnetic resonance (NMR) measurements [Kitae et al., 1998], and as it is described in terms of complex formation mechanisms with particular chiral selectors in section 2.3. In aqueous media the complexing ability of ionizable compounds can be tuned by the pH of the buffer (changing the size of the effective charge) in this way creating optimal CE separating conditions (**Figure 2.3b**). Due to an enhanced enantioresolution power of such systems, very low amounts of charged chiral selectors are often sufficient for the successful CE enantioseparations (**Figure 2.3a**), and in some cases even micromolar concentrations are sufficient [Gübitz & Schmid, 2000a; Blanco & Valverde, 2003].
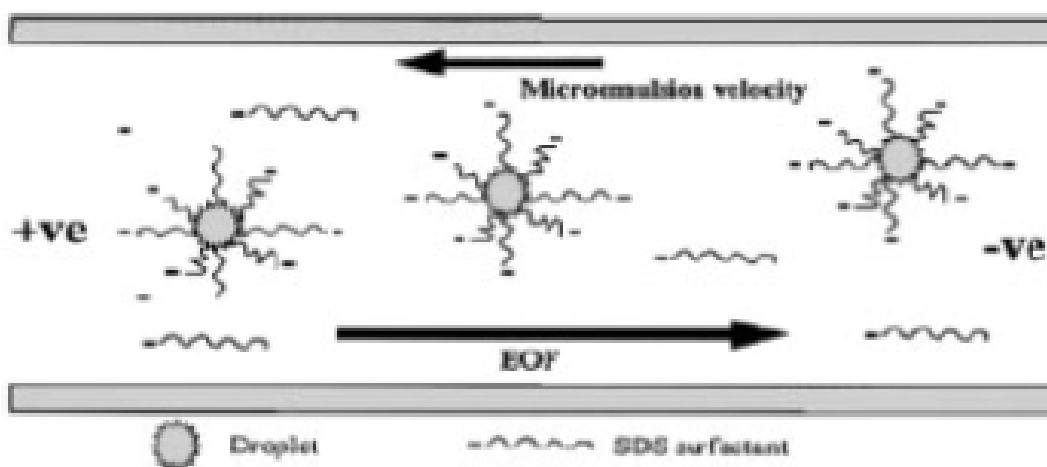
Figure 2.4. Schematic representation of MEEKC separation. MEKC separation has principally the same experimental arrangement but no oil droplets are present in micelle cores. Hydrophobic analytes are distributed preferably into droplet (MEEKC) or micelle core (MEKC). Reproduced from [Altria K.D. et al., 2003].

Amphiphilic media. CE is usually carried out in aqueous background electrolytes (BGEs) and therefore it is useful for the separation of hydrophilic solutes and samples of aqueous nature. On the other hand, the formation of stable complexes (associates) of hydrophobic analytes (that are many of natural biologically active compounds) can be accomplished in aqueous solutions using amphiphilic pseudostationary phases with proper hydrophobic bounding sites. Thus, typically, chiral micelles or chiral mixed micelles (in MEKC) and microemulsions (in MEEKC) help solving additional problems in chiral CE, such as enantioseparation of hydrophobic analytes in aqueous buffers [Preinerstorfer et al., 2009; Silva, 2009; Ryan et al., 2009; Kahle & Foley, 2007a], see **Figure 2.4**. In this field, chiral micelle polymers appeared recently as a very attractive alternative to the conventional micelle systems offering significant benefits not only in separation (fast complexing kinetics), but also detection (especially MS) schemes (see 2.3.8). Such amphiphilic systems are beneficial for the analyses of water-based samples, such as body fluids, creating a powerful alternative to HPLC-MS.

Non-aqueous media. The elimination of the aqueous media in non-aqueous CE (NACE) can provide additional selectivity with respect to that obtained in aqueous CE, and favours the analysis of solutes with poor water solubility [Karbaum & Jira, 1999; Valkó et al., 1996; Wang F. & Khaledi, 1996]. In the same manner, non-aqueous solvents show several advantages regarding solubility of chiral selectors and reduce unwanted interactions with the capillary wall. Different forms of chemical equilibria in aqueous and non-aqueous systems can lead to different selectivities as a result of the fact that weak interactions which are disrupted by water can become effective in non-aqueous systems (see e.g., ion-pair formation, 2.3.7). Moreover, in non-aqueous solvents, less Joule heating is produced and since higher voltage can be applied, retention times are shorter.
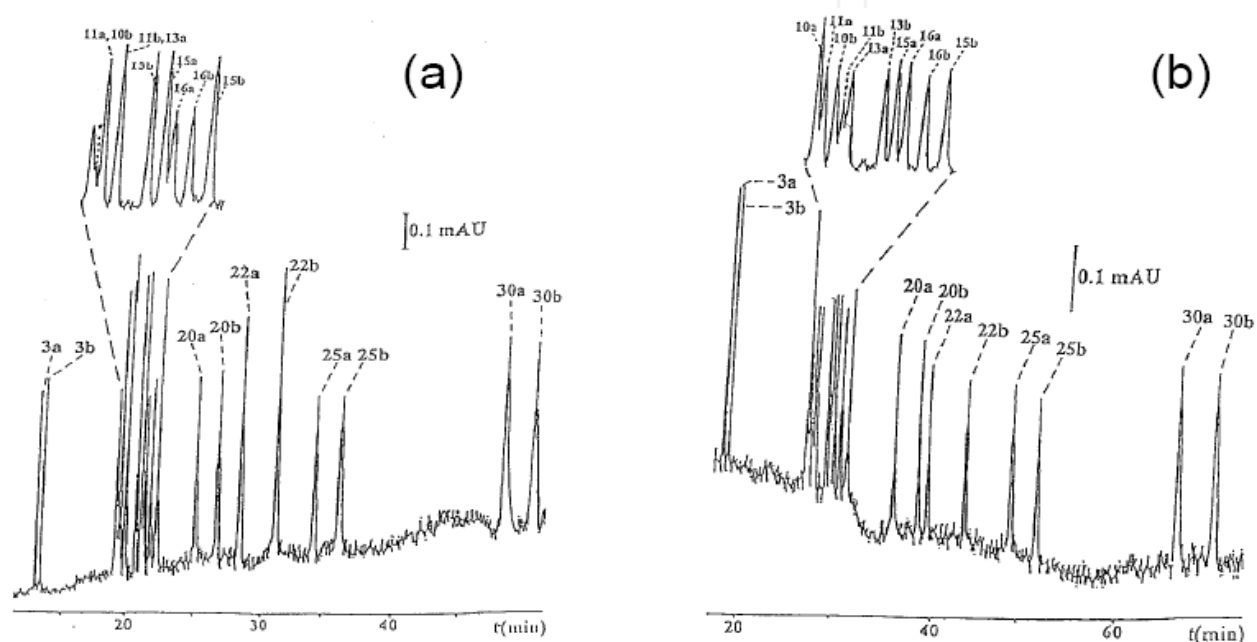
Figure 2.5. Influence of complementary complexing agents on the CE separation of a mixture of DNP-amino acid racemates. Electrolyte system with pH 5.2 and 20 mM 6ᴵ-deoxy-6ᴵ-monomethylamino-β-CD (as in Table 2.2) without any other coselector (a), with addition of 2 mM γ-CD (b). Peak labelling: 3 = DNP-DL-glutamic acid, 10 = DNP-DL-methioninesulfone, 11 = DNP-DL-methionine sulfoxide, 13 = DNP-DL-α-amino-n-butyric acid, 15 = DNP-DL-norvaline, 16 = DNP-DL-citruline, 20 = DNP-DL-methionine, 22 = DNP-DL-norleucine, 25 = DNP-DL-ethionine, 30 = DNP-DL-α-amino-caprylic acid. Reproduced from [Mikuš et al., 2001].
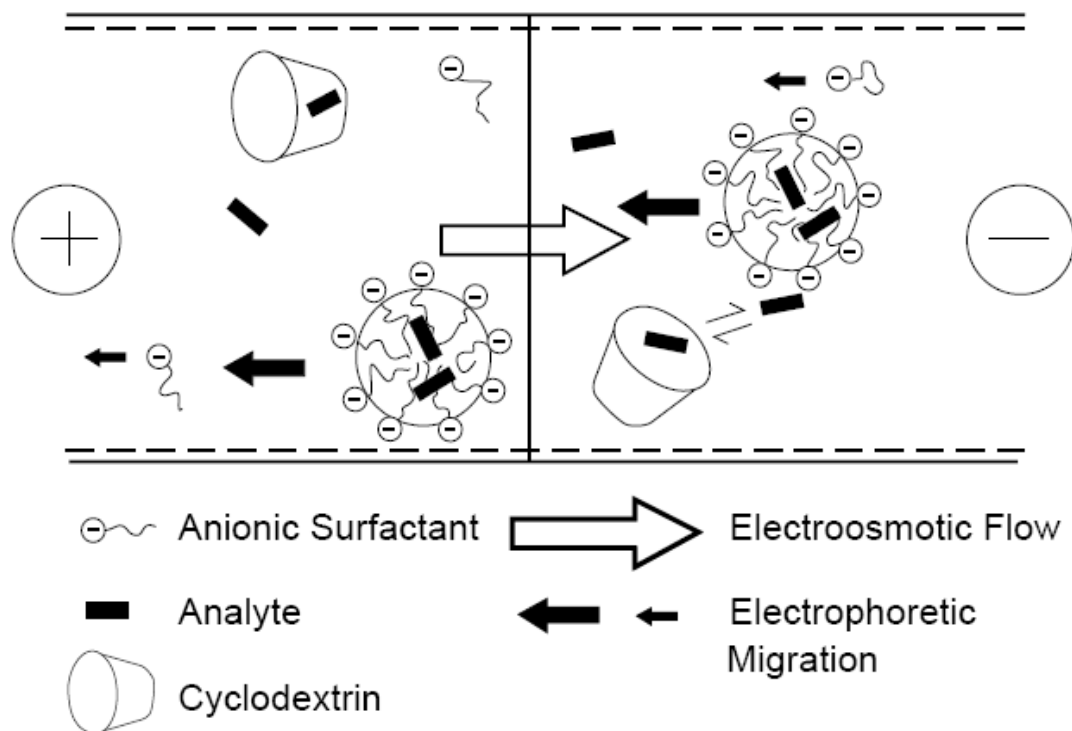
Figure 2.6. Schematic of the separation principle of CDMEKC showing multiple complexing equilibria. The detector window is assumed to be positioned near the negative electrode. Reproduced from [Terabe, 1992].

Combinations of selectors. The possibility of various chiral selectors being easily combined with one another, as well as with achiral additive(s) (introducing multiple complexing equilibria), increases the chance of successfully separating not only particular enantiomeric pairs (via enhanced chiral recognition), but also multicomponent mixtures of enantiomeric pairs (via enhanced molecular recognition) [Mikuš & Kaniansky, 2007; Mikuš et al., 2001; Carlavilla et al., 2006], see Figure 2.5. MEKC systems based on mixed micelles (micelle plus another selector, e.g., CD), introduced by Terabe et al. [Nishi, H. et al., 1991], can provide new and interesting possibilities in (enantio)recognition in comparison with single type micelle systems. For the scheme of the separation principle of CDMEKC showing multiple complexing equilibria see Figure 2.6. Rundlett and Armstrong [Rundlett & Armstrong, 1995] proposed another chiral system based on mixed micelles with vancomycin where the authors illustrated the presence of the mixed micelle as a qualitatively new chiral selector (Figure 2.7). As a special case, dual selector systems can be presented, composed from two different chiral selectors being inactive in enantiorecognition when used alone, acting via synergistic effect and providing unique enantioseparation possibilities in CE [Gübitz & Schmid, 2008; Lurie, 1997; Fillet et al., 2000].

The possibility of combining different chiral systems in on-line coupled CE techniques (i.e., different chiral selectors in different CE techniques) can be utilized for a further significant enhancing of enantioresolution in comparison to single column application [Fanali et al., 2000].
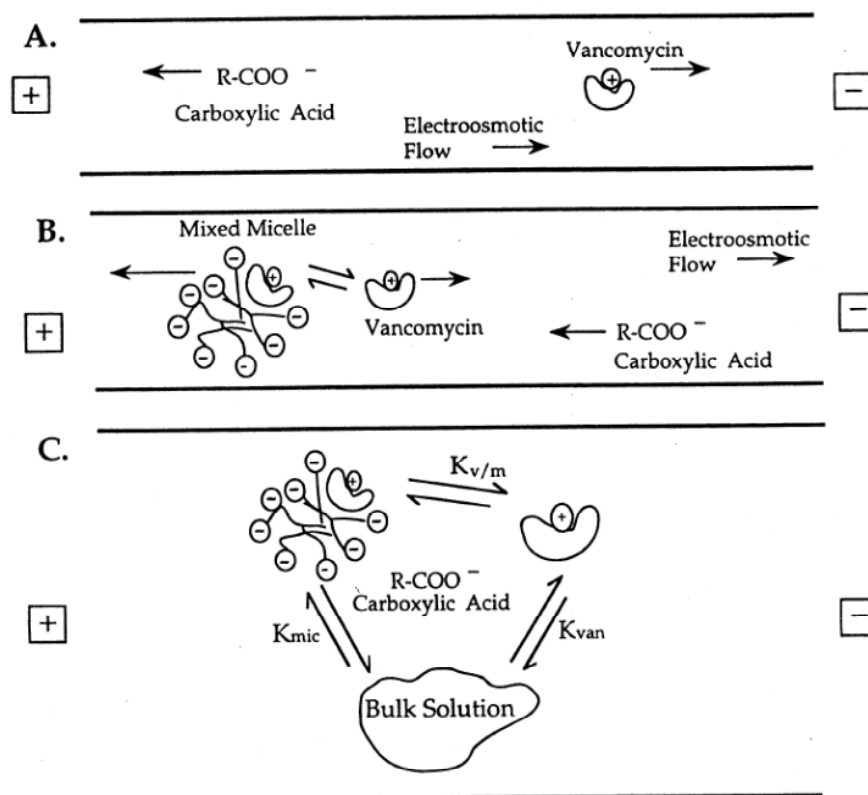
Figure 2.7. Representation of the electrophoretic mobilities of the analytes, chiral selector and mixed micelles in (A) buffer containing vancomycin (relative migration times: $t_{vancomycin} < t_{eof} < t_{acid}$) and (B) buffer containing vancomycin and SDS (relative migration times: $t_{eof} < t_{acid} < t_{vancomycin} < t_{SDS}$). (C) Shows the equilibria of acid analytes (between the bulk solution and the free vancomycin or mixed micelle). Reproduced from ref. [Rundlett & Armstrong, 1995].

2.2.1.2 Electromigration effects in enantioseparations and their manipulation

In chromatographic techniques the selectivity of enantioseparations is entirely defined by the chiral recognition, i.e., by the difference between the affinities of the enantiomers towards the chiral selector. Therefore, the selectivity of enantioseparations in common chromatographic techniques may, in the best case, approach the thermodynamic selectivity of the chiral recognition, but will never exceed it. One major consequence of the mobility contribution in separations in CE is that the apparent separation selectivity may exceed the thermodynamic selectivity of the recognition [Chankvetadze, 2007]. This belongs among unique features of electromigration methods, being not present in chromatographic methods, which can be advantageously utilized in enantiomeric separations. See the reviews on fundamental aspects of chiral electromigration techniques discussing the general aspects of migration models and the enantiomer migration order [Scriba, 2003; Chankvetadze, 1997; Chankvetadze & Blaschke, 2001]. A high enantioresolution power of CE, given by an extremely high separation (peak) efficiency, can be therefore further enhanced by electromigration effects based on increasing mobility difference between free and complexed forms of the enantiomer, as proposed by Wren and Rowe [Wren & Rowe, 1992, 1993], see Equation 2.2 in section 2.2.1. A contribution of intrinsic mobility of the chiral

selector to changes of effective mobility of the charged as well as electroneutral compounds in CE is illustrated in simplified form in Figure 2.8. Moreover, the EOF mobility can additionally influence overall mobility of analytes according to the principles of additivity of particular mobility terms. From these facts the enormous potential of CE to manipulate the separability is apparent, including chiral compounds.
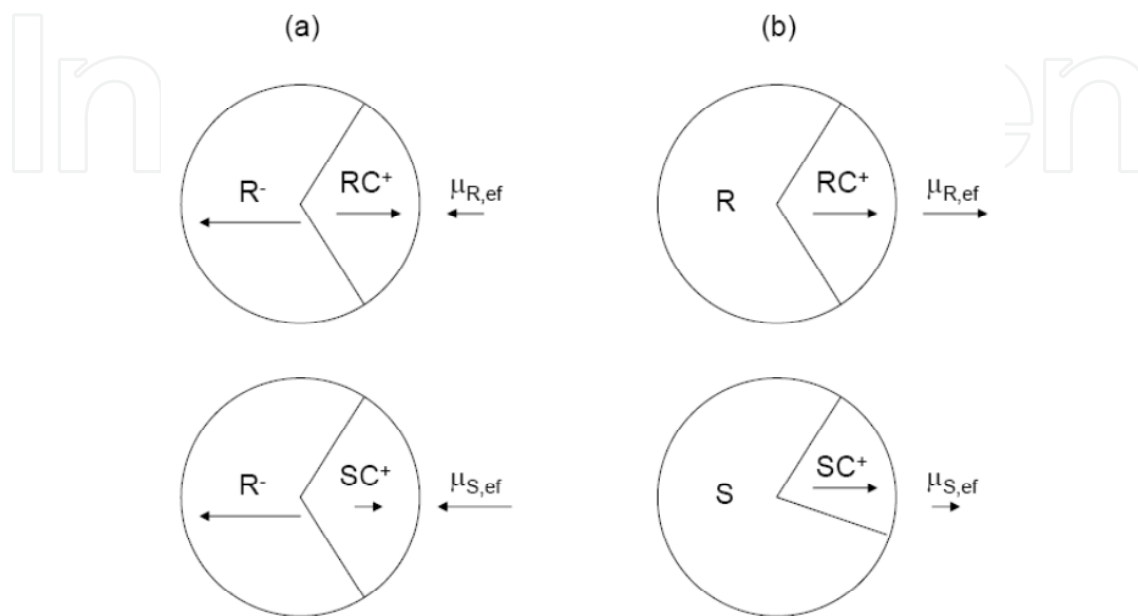
Figure 2.8. Influencing of the effective mobility of: (a) ionic enantiomers R, S, (b) neutral enantiomers R, S, by a charged chiral selector C^+. Inside the diagrams, the arrows indicate mobility contributions while the cut-outs indicate complex stability contributions. As some of the possible examples, diagrams (a) illustrate the main role of mobility differences between complexes while diagrams (b) illustrate the main role of complex stability differences between complexes for obtaining differences in effective mobilities of R and S enantiomers, $\mu_{R,ef}$, $\mu_{S,ef}$.
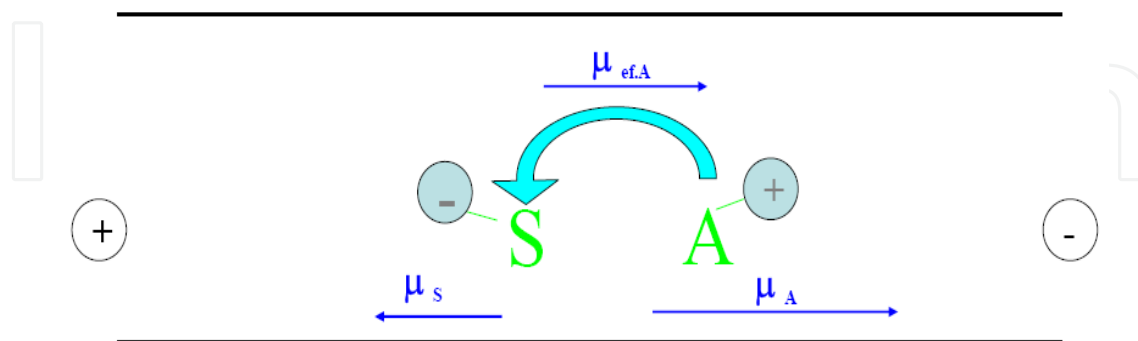
Figure 2.9. Scheme of a countercurrent migration CE system. A = analyte, S = selector.

Countercurrent migration systems. The electromigration effects enhancing enantioresolution can be implemented into CE via countercurrent migration of charged chiral selector and oppositely charged analyte enantiomers, see Figure 2.8a and Figure 2.9. Electrophoretic

mobility of ionizable chiral selectors can be effectively tuned by the pH of the buffer creating very efficient chiral countercurrent migration CE systems, see the results from the relevant CE measurements in Figure 2.3b and Table 2.2. Enhanced effectivity of such systems is reflected in considerably decreased amounts of charged chiral selectors necessary for the successful CE enantioseparations (Figure 2.3a). Thanks to the many new charged chiral selectors (especially CDs and micelles) the possibilities to create new, effective countercurrent separation systems increase, see examples in section 2.4, Table 2.1 and Table 3.1. Besides enhanced enantioresolution, this migration mode is extremely useful also for hyphenated detection systems, eliminating detection interferences of chiral selectors due to their migration from the detector site (see chapter 4).
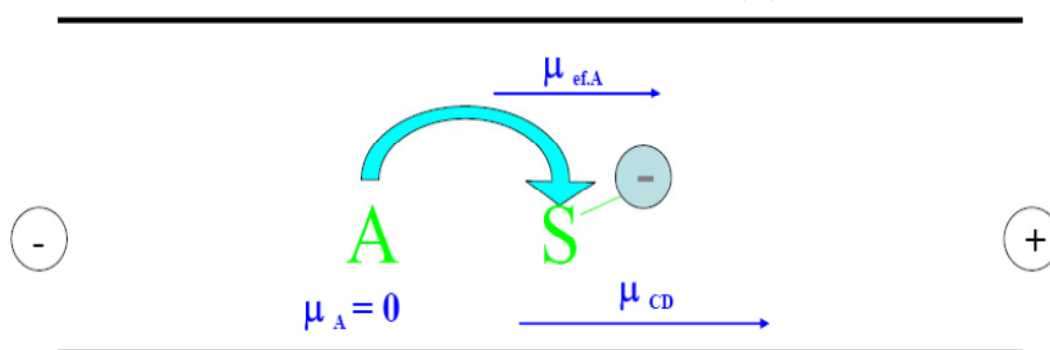
Figure 2.10. Scheme of a carrier molecule-based CE system. A = analyte, S = selector.

Carrier molecule-based migration systems. In addition, charged chiral selectors (or charged chiral pseudostationary phases) spread the application range of CE separating uncharged enantiomers according to their distribution between moving selector and solution phase, making CE (e.g., MEKC, CD-EKC) a universal separation technique like HPLC [Mikuš et al., 2005b; Zandkarimi et al., 2009], see Figure 2.8b and Figure 2.10 and examples in section 2.4.

EOF supported migration systems. Great variability of direct, countercurrent and carrier migration modes in CE, producing electrophoretic systems of different separation selectivities, is given not only by possible combinations of one or more charged and uncharged, as well as chiral and achiral additives, but also by the EOF modifying velocity and direction of movement of the species (analytes, selectors) present in the separation system, and by combinations of both electrophoretic and electroosmotic migration effects (Figures 2.4, 2.6, 2.7). These effects can also be utilized, besides enhancing enantioresolution and/or speeding analysis, for the manipulation of the enantiomer migration order [Scriba, 2003].

The benefits of the advanced migration modes can be pronounced not only in chiral resolution, but they can also simultaneously take effect in achiral resolution [Mikuš et al., 2001, 2006a; Marák et al., 2007; Mikuš & Kaniansky, 2007]. In biomedical analyses, they can be useful in simultaneous separation of structurally related analytes, e.g., chiral drugs and their metabolites, chiral drugs in multicomponent matrices, etc., see examples in section 2.4, Table 2.1 and Table 3.1. In this way, enhanced achiral resolution can also minimize requirements on sample preparation (purification) isolating the enantiomers of the interest from the matrix constituents during electrophoretic run [Mikuš et al., 2006a].

2.2.1.3 Counter-flow in enantioseparations and its manipulation

Another promising mode of chiral CE separations is flow counterbalanced capillary electrophoresis (FCCE). The difference between countercurrent [Chankvetadze et al., 1994] and flow counterbalancing CE [Chankvetadze et al., 1999] techniques is that in the latter case a chiral selector and a chiral analyte do not migrate in the opposite directions to each other, but the bulk flow moves with a defined velocity in the opposite direction to the effective mobility of the analyte zone. The principle of this technique is schematically shown in Figure 2.11 [Chankvetadze et al., 1999].
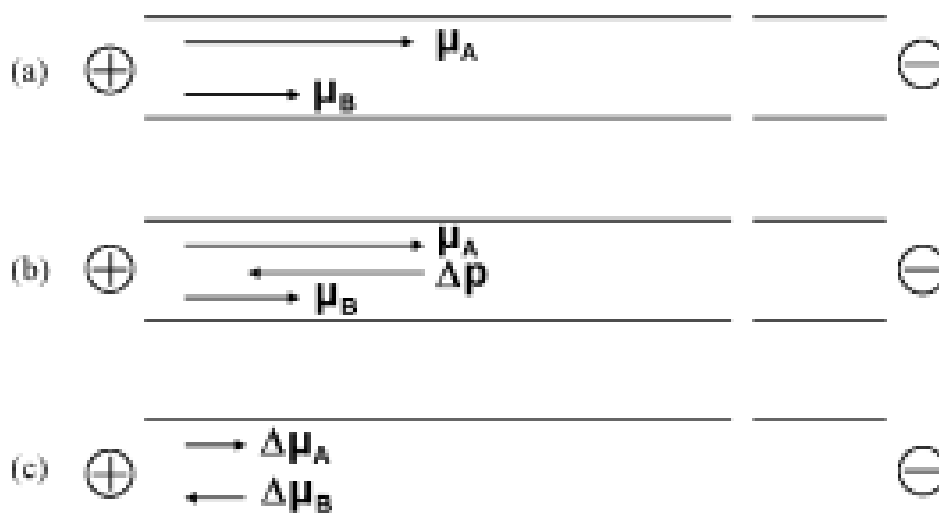
Figure 2.11. A schematic representation of the flow counterbalanced separation principle in CE: (a) without counterbalanced flow; (b) with counterbalanced flow; (c) resulting mobilities. Reproduced from ref. [Chankvetadze et al., 1999].

In FCCE, the sample is driven forward by electromigration and then backward by a pressure-induced flow. The pressure/vacuum, the EOF, hydrodynamic pressure (levelling of the inlet and outlet vials), etc., may be used as a driving force for countermobilities in this technique [Chankvetadze et al., 1999]. The samples travel back and forth in the capillary until sufficient separation is obtained [Zhao J. et al., 1999]. In the mode of FCCE as proposed by Culbertson and Jorgenson [Culbertson & Jorgenson, 1994], the electric field and the pressure are applied alternatively, but not simultaneously as the driving forces. In another mode of FCCE, the counterbalancing driving force, such as pressure, may be applied to the separation chamber continuously during the entire time of electrokinetic separation [Chankvetadze et al., 1999]. An enormous increase of apparent separation factor in chiral and achiral CE separations may be achieved using this technique.

The potential advantage of FCCE can be seen from the Equation 2.3 [Zhao J. et al., 1999]:

$$R_S = \left(\mu_1 - \mu_2\right)\frac{E\sqrt{t}}{4\sqrt{2D}} \qquad\qquad 2.3$$

where *E* is the electric field strength, *D* is the average effective diffusion coefficient of two analytes (*1*, *2*), and *t* is the electrophoretic migration time. The advantages of the flow counterbalancing technique include the following: (i) enormous, in principle unlimited, enhancement of the apparent separation factor may be achieved in zonal discontinuous separations as shown in section 2.4. (ii) This technique allows easy discontinuous zonal separation of a binary mixture into a continuous separation with stepwise migration of the sample components from the inlet towards the outlet vial. (iii) FCCE may be used for micropreparative purposes and offers significantly higher sample capacity compared to discontinuous separations. Other potential advantages of mobility counterbalancing techniques are discussed in ref. [Chankvetadze et al., 1999].

The mobility counterbalancing technique is certainly not limited to binary mixtures and it can easily be applied in a stepwise mode for the separation of multicomponent samples. Counterbalancing of analyte electrophoretic mobility by pressure has been tried by Culbertson and Jorgenson [Culbertson & Jorgenson, 1994] for the enhancement of the detection sensitivity in achiral CE. Later, the same technique was used for the separation of isotopomers of phenylalanine [Culbertson & Jorgenson, 1999]. Several FCCE modes have been developed and applied for enantioseparations so far, see examples in section 2.4.

2.2.2 Capillary electrochromatography

Capillary electrochromatography (CEC) combines electrophoretic and chromatographic separation mechanisms that can be beneficial in highly effective enantioresolutions, for the recent advances in this field see the review by Lu and Chen [Lu H.A. & Chen G.N., 2011]. For the CEC separation principle see Figure 2.12. Chiral stationary phases (CSPs) known from HPLC may be used in CEC. CSPs are based on immobilization of chiral selector onto/into appropriate polymeric matrix (e.g., polysiloxanes, modified silica structures, methacrylates). CDs, proteins, polysaccharides, macrocyclic antibiotics are the most often used chiral molecules for the chiral stationary phases, offering in this rigid state modified enantioselectivity in comparison with their free (mobile) forms. In addition to those, new CSPs, such as ionic liquids functionalized β-cyclodextrin-and carbosilane dendrimer-bonded chiral stationary phases, are synthesized for producing modified enantioselectivity, [see e.g., Zhou Z. et al., 2010; Shou et al., 2008]. Moreover, the polymeric structure of these stationary phases can additionally influence CEC enantioresolution. An overview of previous developments in chiral CEC is given in former review articles [Gübitz & Schmid, 2000b; 2008; Lämmerhofer et al., 2000; Fanali et al., 2001; Kang et al., 2002].

Chiral stationary phase
+ Effective charge of bulk solution
● Effective charge of capillary surface
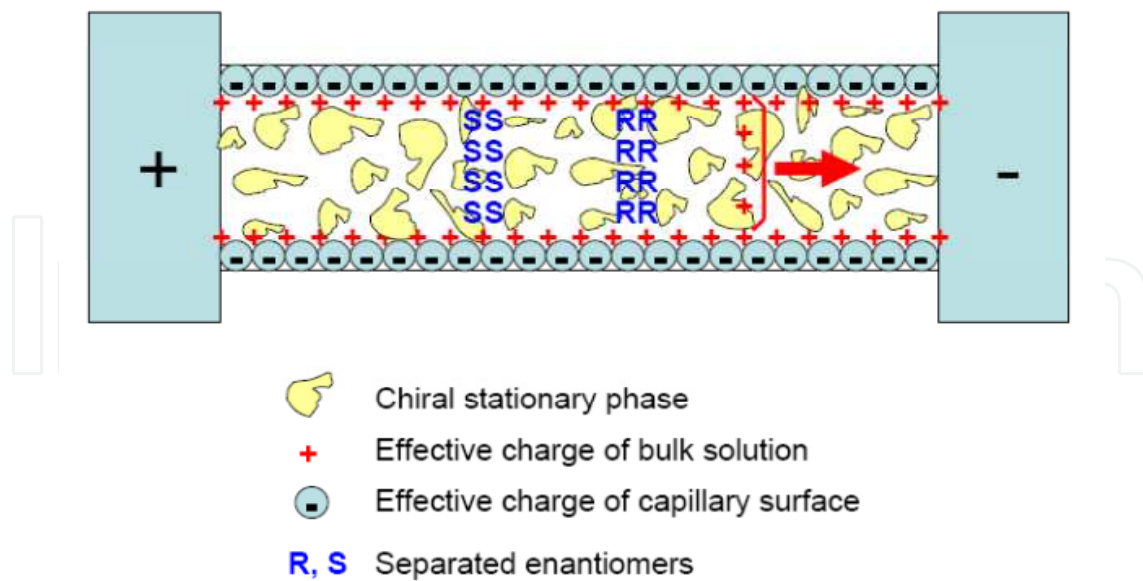R, S Separated enantiomers

Figure 2.12. Separation principle of capillary electrochromatography.

Chiral CEC stationary phases included in capillary wall coatings, particle packings or monolytes (as an example see Figure 2.13) are beneficial in situations with special requirements on separation buffer (aqueous, non-aqueous), stability and solubility of compounds (analytes, selectors, additives, etc.) in separation system, and some on-line detection modes (e.g., avoiding a contamination of detector by selector, often in the case of UV absorbance and mass spectrometry) [Huo Y. & Kok, 2008]. Depending on a chiral selector embedded into the monolith, the resulting chiral monolith can provide significant differences in the chiral selectivity, as illustrated in Figure 2.14. Imprinted chiral phases can offer an enhanced specificity of chiral CEC analyses [Nilsson et al., 2004; Turiel & Martin-Esteban, 2004].
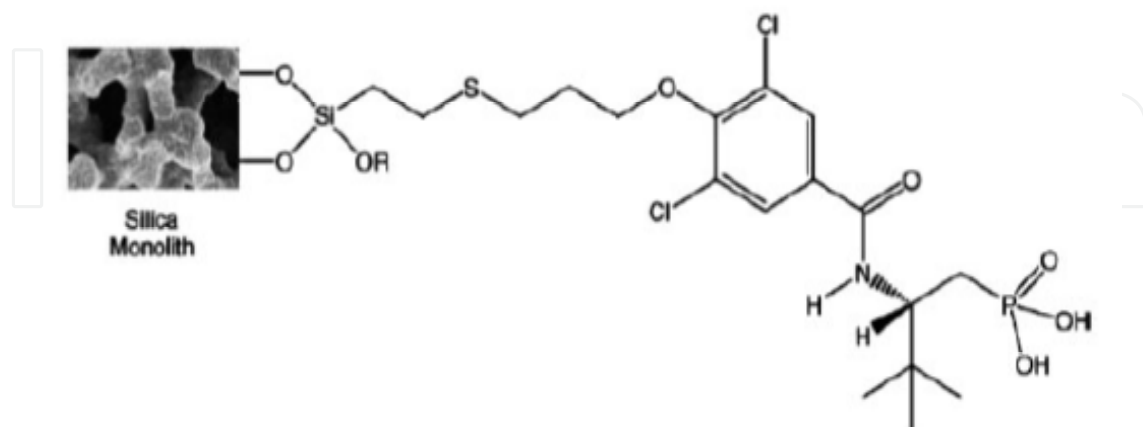
Figure 2.13. Surface-structure of enantioselective silica-based monolithic cation-exchange capillary column with aminophosphonic acid-derived chiral selector. Reproduced from ref. [Preinerstorfer et al., 2006].
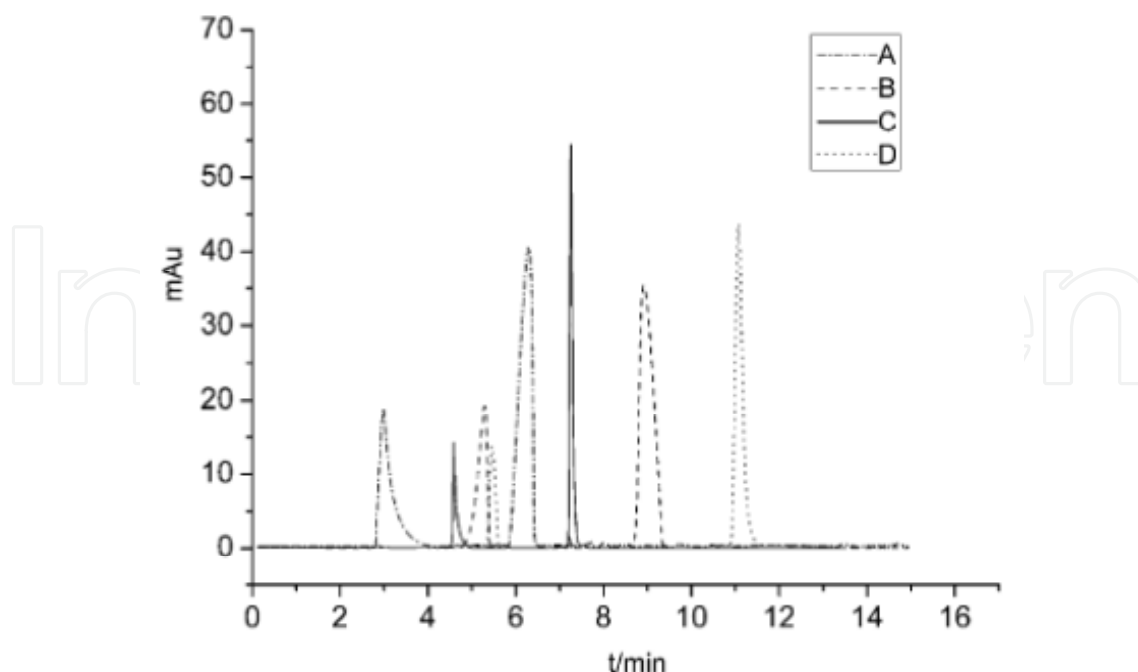
Figure 2.14. Separation of D,L-phenylalanine by four chiral monolithic CSPs. (A) Fabricated with NH$_2$-β-CD; (B) fabricated with β-CD; (C) fabricated with Asp-β-CD; (D) fabricated with HP-β-CD. Temperature: 20°C; voltage: −10 kV; injection:−2 kV, 2 s; mobile phase: phosphate, 5mM, pH= 6.5. Reprinted from ref. [Li Y. et al., 2010].

Compared to HPLC, where a conical flow profile caused by hydrodynamic flow leads to band broadening, in CEC a rather plug-like profile generated by the EOF (see Figure 2.12) results in higher peak efficiency. However, there are also several disadvantages in CEC such as the complicated packing procedures, formation of air bubbles in the case of packed capillaries due to Joule heating, lower reproducibility of migration times due to fluctuation of EOF with different packings and sample matrices [Gübitz & Schmid, 2000, 2008; Lämmerhofer et al., 2000; Fanali et al., 2001; Kang et al., 2002]. Pretreatment of samples with complex matrices is necessary before CEC analysis to maintain separation reproducibility (cleaning of CEC columns is much more difficult and less efficient than CE columns), see examples in section 2.4 and Table 2.1.

2.2.3 Microchip capillary electrophoresis

Microfluidic devices, such as microchips (Figure 2.15), can provide several additional advantages over electromigration techniques performed in capillary format [Li O.L. et al., 2008]. The heat dissipation is much better in chip format compared with that in a capillary and therefore, higher electric fields can be applied across microchip channels. This fact enables, along with a considerably reduced length of channels, significant shortening of separation time, see an example in Figure 2.16. Sample and reagent consumption is markedly reduced in microchannels, hence, the chiral MCE can provide the unique possibility of ultraspeed enantiomeric separations of microscale sample amounts. Both electrophoretic [Gong & Hauser, 2006; Piehl et al., 2004; Belder et al., 2006; Belder, 2006] and electrochromatographic modes are applicable [Weng X. et al., 2006].
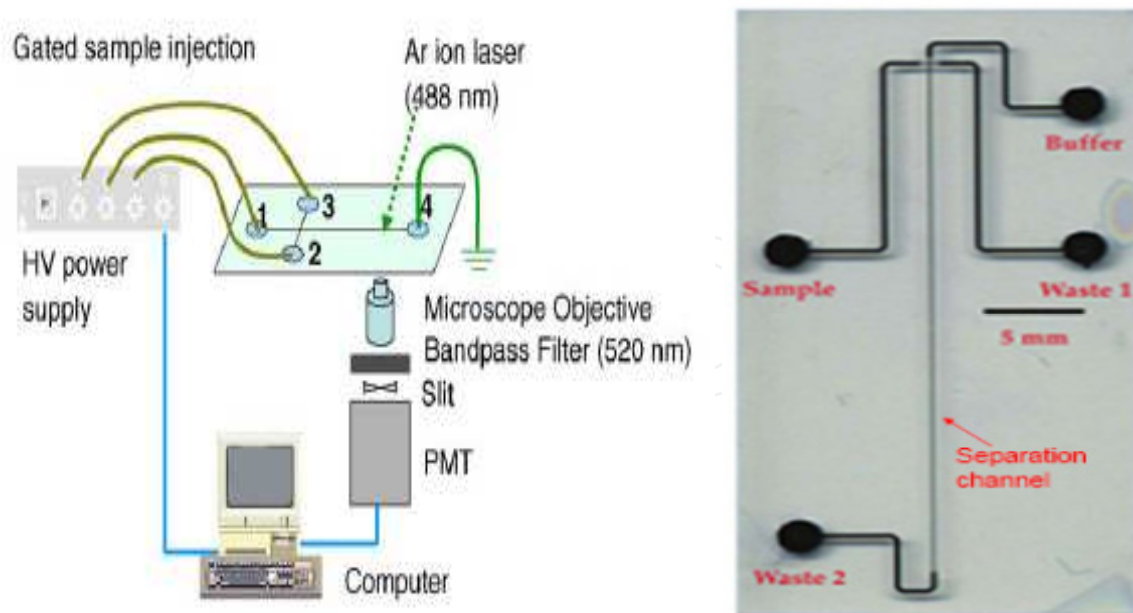
Figure 2.15. MCE. Experimental arrangement of microchip electrophoresis (left). Arrangement in the left single-channel chip: (1) sample, (2) run buffer, (3) sample waste and (4) buffer waste. The electrophoretic microchip – real detail (right). Arrangement in the right single-channel chip: reservoirs (sample, buffer, waste) and separation channel. For a chiral MCE, separation channel can be filled with chiral electrolyte (CE mode), or chiral/achiral electrolyte with chiral/achiral stationary phase (CEC mode). Reproduced (left) from [Kim M.S. et al., 2005].
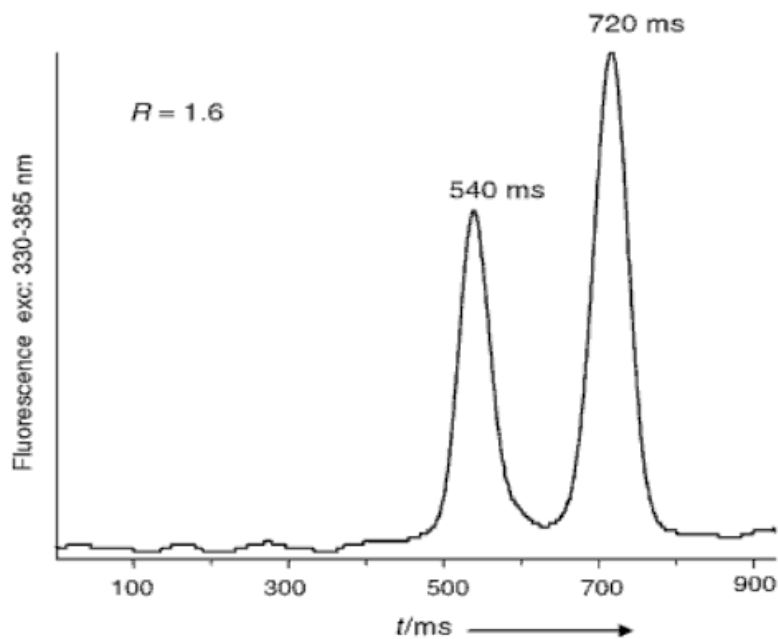
Figure 2.16. Subsecond chiral separation of DNS-tryptophan. Electrolyte: 2% HS-γ–CD, 25 mM triethylammonium phosphate buffer pH 2.5. A high field strength up to 2600 V/cm and short separation length of several millimetres were employed. Reproduced from ref. [Belder, 2006].
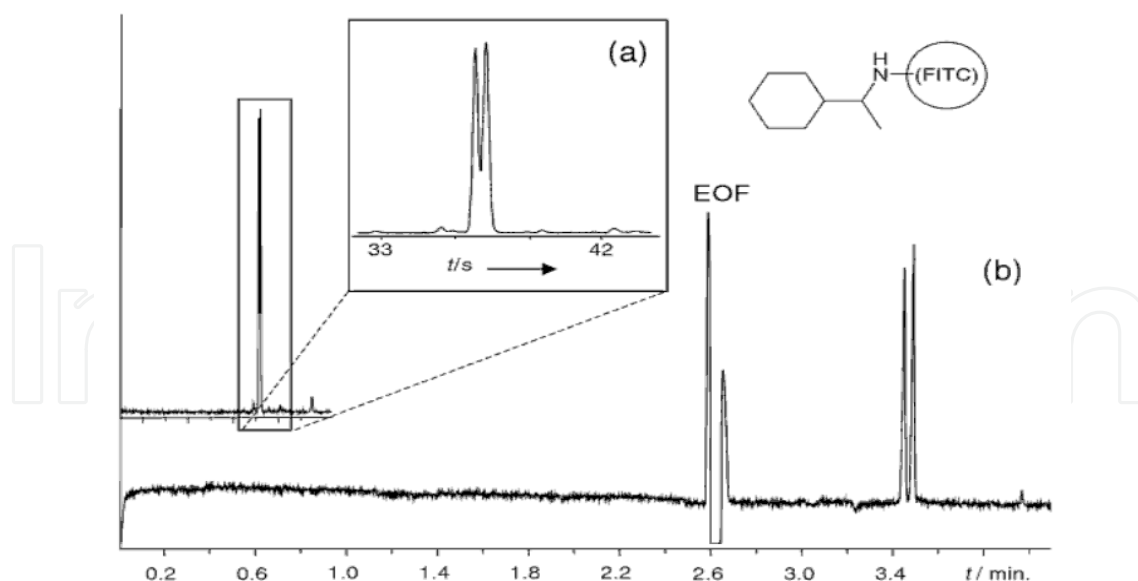
Figure 2.17. Comparison of chiral separations obtained in microchip electrophoresis (MCE) (a) and in classical capillary electrophoresis (CE) (b). For both the experiments the same electrolyte was used, while the column length was limited to 7 cm in MCE, a column of 40 cm effective length was used in CE. Reproduced from ref. [Belder, 2006].

In practice, however, the resolution achievable in MCE devices is often lower compared to that obtainable in classical CE utilizing considerably longer separation capillaries. This is shown in Figure 2.17, where the chiral separation of an FITC-labelled amine obtained at typical conditions in MCE and in classical CE is compared. In order to obtain sufficient resolution in chiral MCE, different strategies have been used [Belder, 2006], such as (i) enhancing the enantioselectivity of the system as much as possible (changing the type and amount of chiral selector, adding coselector, etc.), (ii) using folded separation channels, the column length can be extended without enlarging the compact footprint of the device, as shown in Figure 2.18, (iii) using coated channels, internal coatings improve separation performance by the suppression of both analyte wall interaction and electroosmosis; the impact of channel coating with poly(vinyl alcohol) on a chiral separation in MCE is shown in Figure 2.19 for the separation of an FITC-labelled amine. The use/combination of the above-mentioned tools applicable in MCE gives a good chance for real-time process control and for multidimensional separations, and makes MCE a powerful tool in real chiral applications (pharmaceutical, biomedical, etc.).
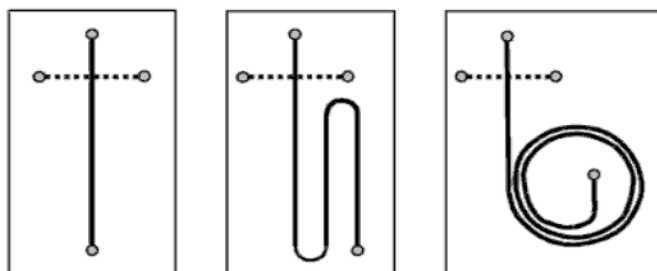
Figure 2.18. Channel layouts enabling long separation channels on a small device. Reproduced from ref. [Belder, 2006].
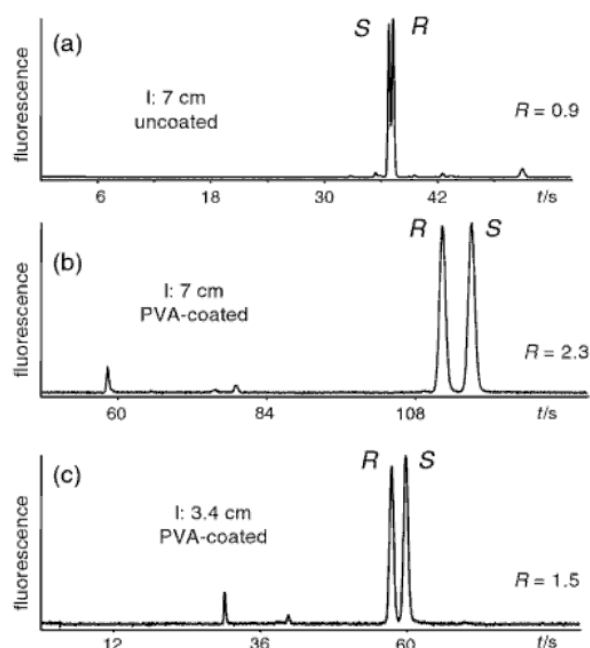
Figure 2.19. Influence of PVA-channel coating on chiral resolution of FITC-labelled (R)- (-) and (S)-(+)-1-cyclohexylethylamine in MCE. The effective separation lengths were 7 cm for the uncoated channel (a) and 7 cm (b) and 3.4 cm (c) for the PVA-coated microchip. Buffer: 40 mM CHES, 6.25 mM HP-γ-CD, pH 9.2. Reproduced from ref. [Belder, 2006].

For examples of practical applications of the chiral MCE, see section 2.4 and Table 3.1. A brief comparison of CE and MCE, and clinical applications of MCE are reviewed in ref. [Li, S.F.Y. & Kricka L.J., 2006]. Chiral separations in microfluidic devices are nicely reviewed by Belder [2006].

2.3 Chiral selectors as complexing agents, advantages and limitations

Conventional as well as new chiral selectors suitable for CE are described in the following subsections. From this description, showing complexing properties (i.e., mechanism of chiral discrimination), advantages and limitations of various classes of chiral selectors, the high flexibility of chiral CE for the separation of a wide range of structurally different compounds is apparent.

2.3.1 Cyclodextrins

Cyclodextrins (CDs), cyclic oligosaccharides with (typically) 6-8 D-glucose units in the macrocycle (α-, β-, γ-CDs, see Figure 2.20, although recently δ-CD was also introduced into CE [Wistuba et al., 2006]), are the most often employed chiral additives for the CE enantiomeric separations of low molecular organic compounds due to their outstanding broad selectivity spectra and other beneficial properties, such as UV transparency, availability, wide application range (polar, nonpolar, charged, uncharged analytes), and reasonable solubility in water [Chankvetadze, 2008; Scriba, 2008; Juvancz et al., 2008; Cserhati, 2008; Fanali, 2009]. Moreover, a fast complex forming kinetics with CDs is beneficial for highly efficient CE enantioseparations. From these reasons (and other facts

discussed below) it is not surprising that CDs are dominating chiral selectors also in enantioselective analysis of many drugs in biological samples, as it is apparent from Table 2.1 and Table 3.1.
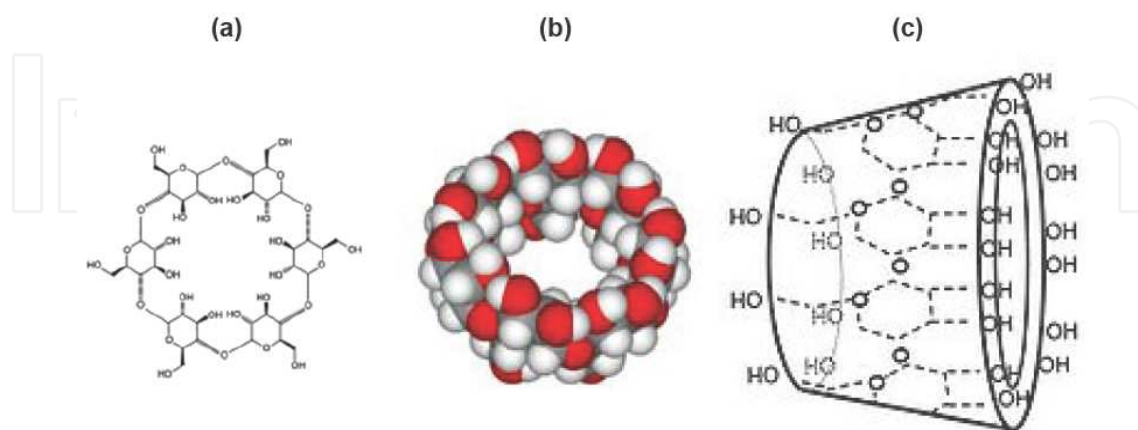

Figure 2.20. Cyclodextrins. (a) Chemical structure of α-CD. (b) Space filling model of β-CD. (c) γ-CD toroid structure showing spatial arrangement.
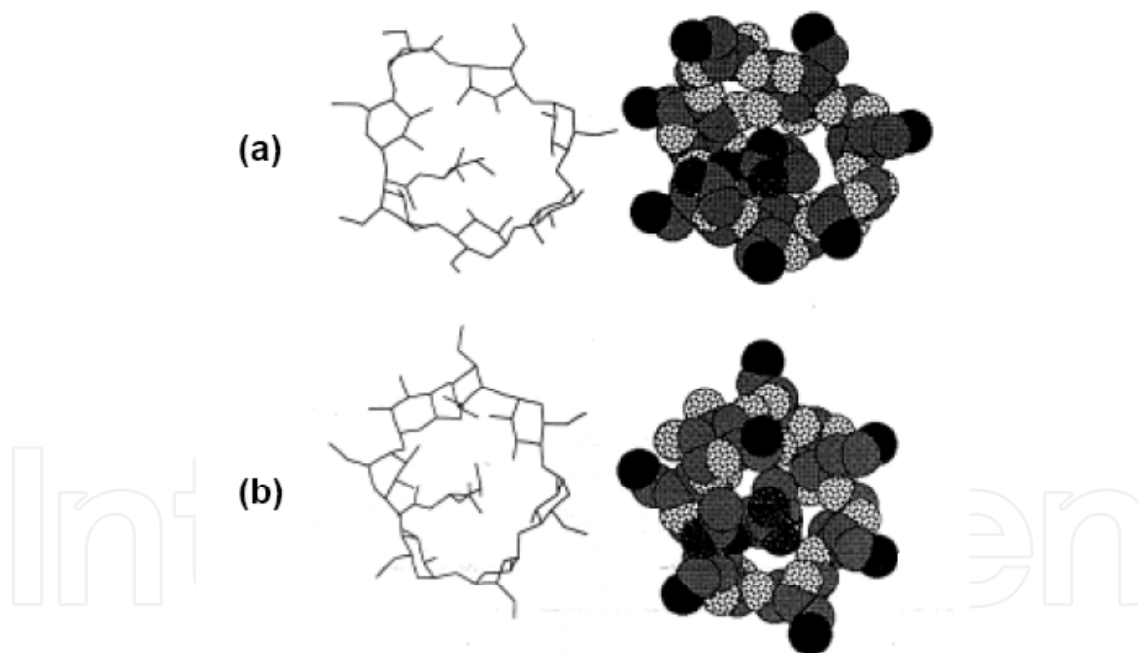

Figure 2.21. Structures of inclusion CD complexes. (a) per-NH_3^+-β-CD-(S)-AcLeu complex, (b) per-NH_3^+-β-CD-(R)-AcLeu complex. The complexes were derived from the molecular mechanics – molecular dynamics (MM-MD) calculations. Reproduced from ref. [Kitae et al., 1998].

The basic mechanism of chiral discrimination using CDs is based on the inclusion of the analyte (guest), or at least its hydrophobic part, into the relatively hydrophobic cavity of CD (host), see an example in Figure 2.21. Here different sterical arrangements of chiral compounds in chiral CD cavity results in differences in stability of the formed complexes.

Compounds containing an aromatic system in their molecules, including many drugs, are usually well-suited for inclusion into the CD cavity and hence, a good enantiorecognition between enantiomers is often easily achieved [Vespalec & Boček, 2000; Mikuš et al., 2002; Thiele et al., 2009; Denmark, 2011; Palcut & Rabara, 2009].

The hydroxyl groups present on the rim of the CD can be easily modified by chemical reactions with various functional groups creating a great amount of derivatized CDs with the desired properties, especially (i) complex forming ability, (ii) solubility, (iii) migration and (iv) detection capabilities. Therefore, it is not surprising that even in the field of newly developed chiral selectors the novel CD derivatives prevail significantly [Preinerstorfer et al., 2009]. The preparation of selectively substituted derivatives [Gübitz & Schmid, 2000a; Nzeadibe & Vigh, 2007; Cucinotta et al., 2010] is preferred as this eliminates the creation of mixtures of CDs having different substitution patterns, and, by that, different complexing / electromigration properties, see Figure 2.22, that can cause difficulties with separation reproducibility in CE [Vespalec & Boček, 1999; Mikuš et al., 1999; Mikuš & Kaniansky, 2007]. Several different groups of CD derivatives can be distinguished, namely (i) neutral CDs, (ii) negatively and positively charged CDs, (iii) amphoteric CDs and (iv) polymerized CDs.
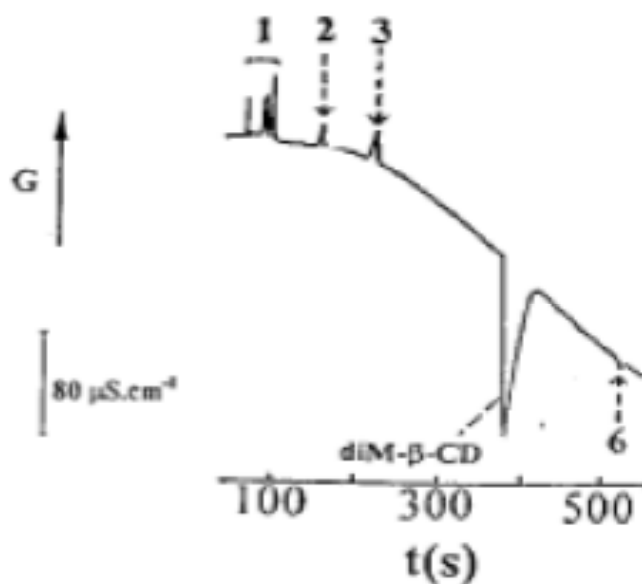
Figure 2.22. Electropherograms showing different electromigration properties of the β-CD aminoderivates. The sample constituents were separated according to differences in their actual ionic mobilities. There are clearly visible differences in migration velocities of the CD derivatives with different substitution degree (peaks 2, 3, and diM-β-CD) in the electropherogram. The concentration of the monoaminoderivative, 6^I-deoxy-6^I-dimethylamino-β-CD, the major constituent in the analysed preparative, was ca. 1.7 mM. Tentative peak assignments: 1 = a migration region of the alkali and alkaline earth metal cations and alkyl- and arylamines; 2 = triamino-β-CD derivatives; 3 = diamino-β-CD derivatives; diM-β-CD = 6^I-deoxy-6^I-dimethylamino-β-CD; 6 = unidentified constituents. Contactless conductivity detection was used in this CE experiment to monitor the non-absorbing analytes. Reproduced from ref. [Mikuš et al., 1999].

Neutral CDs (e.g., replacing hydroxyl groups with alkyl or hydroxyalkyl groups) can offer modified depth and flexibility of the cavity, as well as the free cross-section of its smaller opening, leading to better accommodation of the guest and increased stability of the resulting inclusion complex [Vespalec & Boček, 2000; Blanco & Valverde, 2003], as an example see Figure 2.23. This can improve solubility of CDs and their complexes, as well as enantiomeric recognition in comparison with their native forms as illustrated on many biological samples (urine, plasma, rat brain), such as 2-hydroxypropyl-β-CD vs. lorazepam and its chiral 3O-glucuronides, methyl-O-β-CD vs. isoproterenol [Hadviger et al., 1996], hydroxypropyl-β-CD vs. amlodipine [Mikuš et al., 2008a], 2-hydroxypropyl-β-CD and 2-hydroxypropyl-γ-CD vs. 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) or cyanobenz[*f*]isoindole (CBI) derivatives of serine [Quan et al., 2005; Zhao S. L. et al., 2005a], for details see Table 2.1 and Table 3.1.
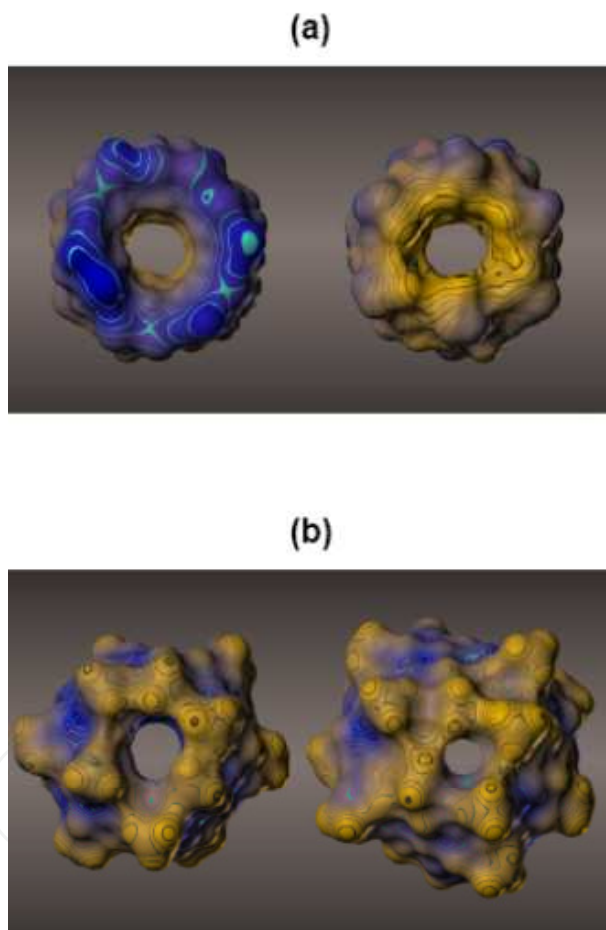
Figure 2.23. Modification of the structure and character of α-CD after its derivatization. (a) native α-CD, (b) per-O-methyl-α-CD. In the MOLCAD (molecular computer-aided design) structures the hydrophilic parts are coloured with blue while the hydrophobic parts are coloured with yellow. Reprinted from [csi.chemie.tu-darmstadt.de].

The improved chiral recognition was observed many times with charged CDs compared to neutral ones, see Figure 2.24 {compare NMR traces (b) and (d)}, as a result of the enhanced stability of host-guest complexes due to the additional strong electrostatic (Coulombic)

interactions of oppositely charged functional groups of complexing partners [Vespalec & Boček, 2000; Wenz et al., 2008; Kitae et al., 1998]. For an example of the CD complex stabilized by the Coulombic interaction see Figure 2.25. The complex stability depends on the size of charge that is important especially for the effective use of ionizable CD derivatives in CE (having carboxyl or amino groups in macrocycle), as it was demonstrated (mainly from the electromigration point of view) in section 2.2. Substitution pattern is another tool for a quite significant tuning of the enantiorecognition ability of charged CDs [Mikuš & Kaniansky, 2007; Kitae et al., 1998], as illustrated in Figure 2.24 {compare NMR traces (b) and (c)}. The ionic character of CD derivatives is also responsible for their excellent water solubility. Enhanced enantiomeric recognition as well as solubility of these CDs and their complexes in comparison with their native forms was illustrated on many biological samples (urine, squirrel brain, human plasma), including carboxyethyl-β-CD vs. H_1-antihistaminic drugs (dioxopromethazine, dimethindene, pheniramine) [Mikuš et al., 2006a, 2008b; Marák et al., 2007], a highly sulfated-β-CD vs. CBI derivatives of serine, glutamate and aspartate [Kirschner et al., 2007], a highly sulfated-β-CD vs. CBI derivatized baclofen [Kavran-Belin et al., 2005], a highly sulfated-β-CD vs. a racemic antiarrhythmic drug (disopyramide) [Fang, L. et al., 2006], for details see Table 2.1 and Table 3.1.
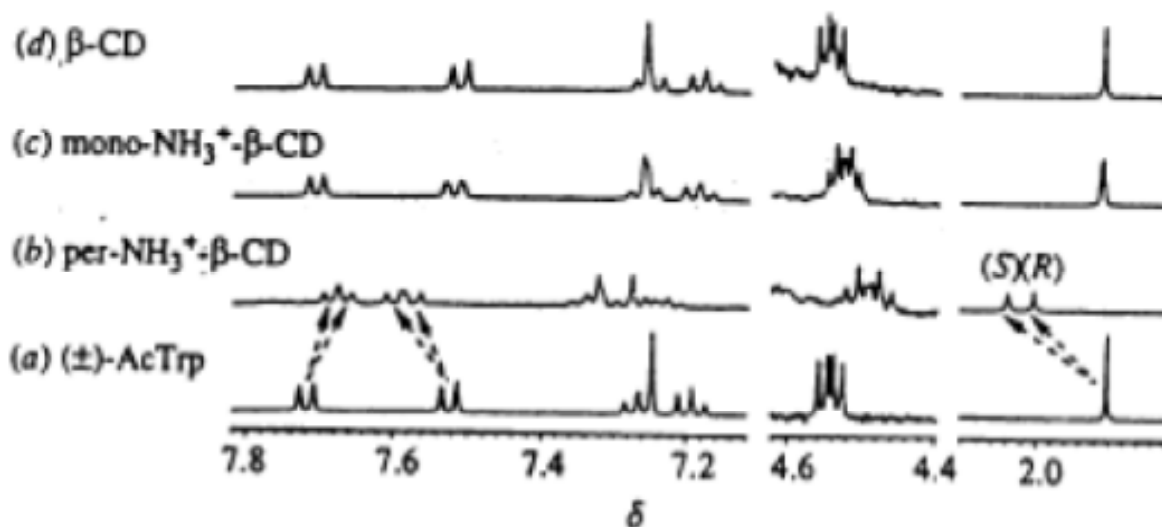

Figure 2.24. ^{1}H NMR spectra of (±)-AcTrp (2×10^{-3} M) in D_2O at pD 6.0 in the absence and the presence of native β-CD, and selectively substituted β-CD derivatives (8×10^{-3} M). Here, only per-NH$_3^+$-β-CD exhibited enantiorecognition capability for (±)-AcTrp (split NMR signals). Reproduced from ref. [Kitae et al., 1998].

Polymerized CDs are mostly prepared by cross-linking the CDs with bifunctional reagents like diepoxydes and diisocyanates [Fenyvesi, 1988]. Associated analytes have more reduced mobility (close to zero) that can lead, for some analytes, to their better CE enantioseparation in comparison with the use of the native monomeric CDs. Moreover, improved solubility and rigid structure of CD cavites in polymer can additionally influence (enantio)recognition and utilization [Fenyvesi, 1988; Ingelse et al., 1995; Ševčík et al., 1996]. For example, polymeric β-CD exhibited excellent molecular recognition for multicomponent mixtures of amino acid enantiomers [Mikuš et al., 2001].
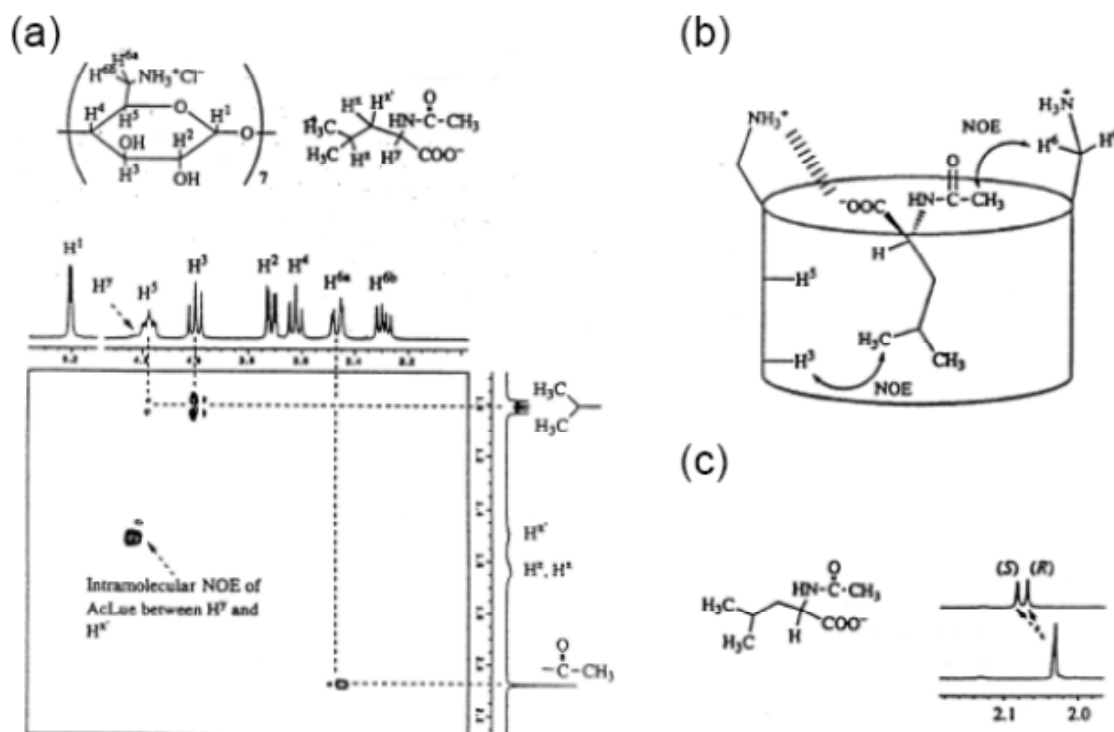
Figure 2.25. Illustration of the enhanced chiral recognition due to Coulomb interaction as an attractive force. (a) ROESY spectrum of the per-NH_3^+-β-CD-(S)-AcLeu system in D_2O at pD 6.0 and 25°C. The spectrum was measured for the solution of a mixture of per-NH_3^+-β-CD (8×10^{-3} M) and (S)-AcLeu (4×10^{-3} M) in N_2-saturated D_2O. The mixing time for the ROESY measurement was 250 ms. (b) A plausible structure of the per-NH_3^+-β-CD-(S)-AcLeu complex deduced from ^{1}H NMR spectroscopy. (c) ^{1}H NMR signals of the CH_3 protons at the acetyl group of *N*-acetylated leucine, AcLeu, (2×10^{-3} M) in the absence (lower) and the presence of protonated heptakis(6-amino-6-deoxy)-β-cyclodextrin, per-NH_3^+-β-CD, (8×10^{-3} M) (upper) in D_2O at pD 6.0 and 25°C. Reproduced from ref. [Kitae et al., 1998].

2.3.2 Crown ethers

Chiral crown ethers (CWEs) are cyclic polyethers that form stereoselectively inclusion complexes with primary amines (**Figure 2.26**). The CWE complexation differs from CD complexation by the inclusion of the hydrophilic part of the analyte (e.g., protonated amino group) in the cavity [Kuhn, 1999].
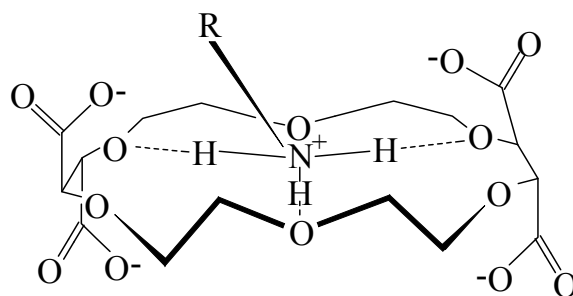
Figure 2.26. Chemical structure of the complex of crown ether with primary amine.

18-Crown-6-tetracarboxylic acid ($18C_6H_{14}$) found widespread application as a chiral selector in CE for the chiral separation of amino acids, dipeptides, sympathomimetics and various drugs containing primary amino groups [Kuhn, 1999]. Wang et al. [Wang C.Y. et al., 2003] demonstrated applicability of a new CWE, (S,S)-1,7-bis(4-benzyl-5-hydroxy-2-oxo-3-azapentyl)-1,7-diaza-12-crown-4, for the chiral CE separations. However, there are several limitations with CWEs. The separation electrolyte may not contain cations, such as potassium or ammonium ions, because they compete with the enantiomers for the CWE cavity. This increases claims on the purity and composition of electrolyte systems, and limits some applications. Therefore, according to our best knowledge, there is only one application of CWE to the chiral analysis of biological sample from 2000 [Cho et al., 2004]. $18C_6H_{14}$ was applied for enantioseparation of gemifloxacin in urine (for detail see Table 3.1).

2.3.3 Polysaccharides/oligosacharides

A variety of linear neutral and charged carbohydrates were found to be applicable as selectors for chiral CE separations. The low UV absorption and often high attainable separation efficiency make these selectors attractive for CE [Riekkola et al., 1997]. The enantiorecognition capability of various neutral oligo- polysacharides (maltodextrins, dextrins, dextrans) is significantly influenced by their higher molecular structures, like helical hydrophobic cavities or pores of polymeric networks [Nishi, H. et al., 1996a]. Two applications to biological samples from 2000 [Bortocan & Bonato, 2004; Nojavan & Fakhari, 2011] show enantioselective separation and determination of primaquine and its metabolite, carboxyprimaquine, in rat liver mitochondrial fraction and plasma samples, and cetirizine and hydroxyzine in human plasma, by maltodextrin as chiral selector (for detail see Table 2.1).
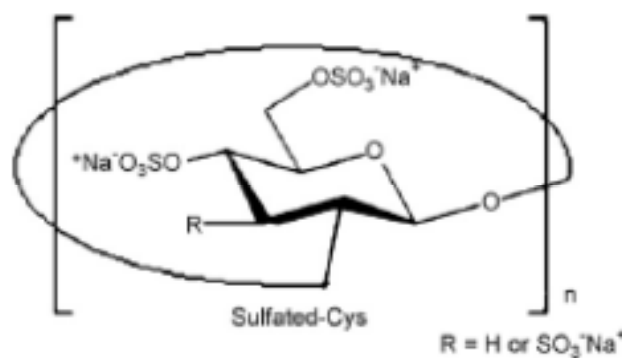

Figure 2.27. Chemical structure of HS-Cys. Reprinted from ref. [Park et al., 2004].

Electrostatic interactions provide additional stereoselectivity effects in the case of ionic polysacharides [Nishi H., 1997] (heparin, dextran sulphate, chondroitin sulphate, hyaluronate, chitosans, aminoglycosides, colominic acid, highly sulfated cyclosophoraoses) suitable for a variety of basic drugs such as β-blockers and sympathomimetics [Nishi H., 1997; Nishi, H.S. et al., 1996b; Du et al., 2002; Park et al., 2004]. For example, recently Park et al. [Park et al., 2004] synthesized highly sulfated cyclosophoraoses (HS-Cys) (Figure 2.27) from a family of neutral cyclosophoraoses isolated from *Rhizobium leguminosarum* and applied them as chiral selectors for the resolution of β-blocker and sympathomimetic enantiomers. Contrary to the sulfated compounds, the neutral cyclosophoraoses themselves showed no chiral recognition ability for these drugs investigated.

2.3.4 Proteins

Proteins provide strong and highly selective interactions, so called affinity interactions, with the analytes. Stereoselectivity is further influenced by the tertiary structure of proteins. Different analytes can selectively bind to different binding sites of proteins. Proteins can be used, depending on their ionization state, for neutral, basic or acidic analytes. However, there are several limitations associated with the use of proteins in CE enantioseparations, mainly low obtainable separation efficiency, adsorption of proteins to the capillary wall and high UV absorption. To eliminate these disturbing effects, capillary coating and/or partial filling techniques and/or indirect detection have to be used [Vespalec & Boček, 2000; Tanaka & Terabe, 1995; Armstrong et al., 1994a].
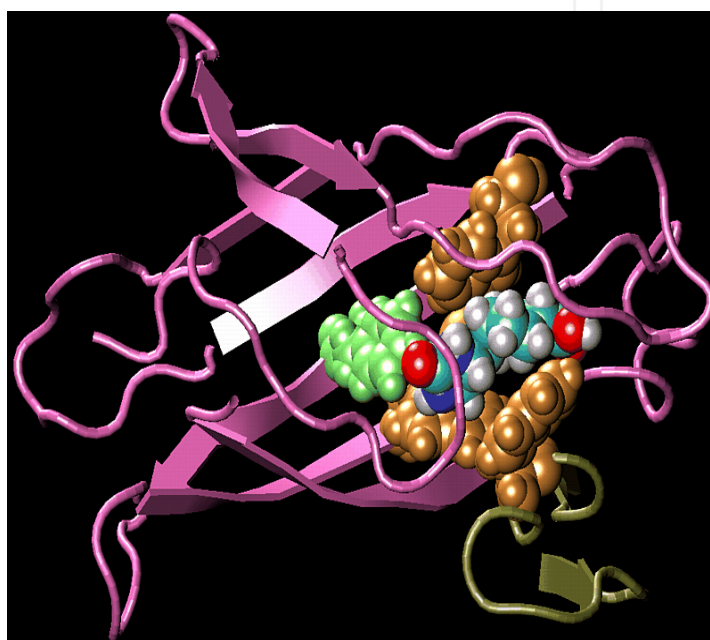
Figure 2.28. 3D structure of avidin. A typical arrangement of avidin complex (avidin-biotin). Reprinted from [ks.uiuc.edu].

Various proteins, e.g., bovine serum albumin, α1-acid glycoprotein, ovomucoid, avidin (Figure 2.28), transferring, pepsin, penicillin G acylase, have been successfully employed in CE enantioseparations of amino acids and drugs. [Haginaka, 2000; Hödl et al., 2006; Gotti et al., 2006; Martínez-Gómez et al., 2007]. Although this group of selectors is frequently used in model CE enantioseparations, it is less often applied in pharmaceutical analysis, see e.g., ref. by Martinez-Gomez et al. [Martínez-Gómez et al., 2007] devoted to the **e**nantiomeric quality control of antihistamines in pharmaceuticals by affinity electrokinetic chromatography with human serum albumin as chiral selector. However, no chiral application to biological samples has been referred from 2000, probably because of achieving the goal with less problematic selectors such as CDs and their derivatives.

2.3.5 Macrocyclic antibiotics

Macrocyclic antibiotics, introduced by Armstrong et al. [Armstrong et al., 1994b], possess several asymmetric centres and many functional groups and structures (like hydrophobic pocket) allowing multiple interactions (affinity, inclusion, etc.) with the analytes and

providing extremely high enantiorecognition capability. Since these compounds have strong UV absorption, partial filling methods [Desiderio et al., 1997] and countercurrent approaches [Oswald & Ward, 1999] have been applied to overcome detection problems. Moreover, capillaries have to be coated for the elimination of adsorption of antibiotics at the capillary wall [Wang Z. et al., 2007].
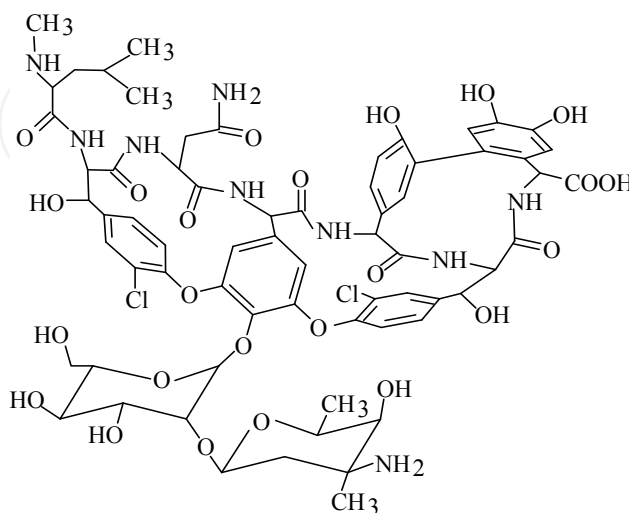

Figure 2.29. Chemical structure of vancomycin.

Several classes of antibiotics have been introduced as chiral selectors showing different enantiorecognition capabilities towards amino acids, drugs, etc.: ansamycins (rifamycin B, rifamycin SV), glycopeptides (vancomycin, ristocetin, teicoplanin, avoparcin), aminoglycosides (streptomycin sulfate, kanamycin sulfate, fradiomycin sulfate), macrolides (erythromycin), polypeptides, [see e.g., Wang Z. et al., 2007; Hou J.G. et al., 2003; Ha P.T.T. et al., 2004b]. For the chemical structure of vancomycin see Figure 2.29.

Lack of recent CE applications to biological samples can be explained by the same arguments used for proteins. On the other hand, applications in this field were accomplished by antibiotics immobilized in CEC stationary phases (see section 2.2.2 and Table 2.1).

2.3.6 Ligand-exchange selectors

Ligand-exchange selectors {usually chelate complexes (hemicomplexes) of amino acids with metal cations, e.g., histidine-copper(II)} can form ternary complexes with appropriate analytes (compounds containing aminocarboxyl or hydroxycarboxyl groups like amino acids and hydroxyl acids) having different stability for the two enantiomers [Gassmann et al., 1985]. Rizkov et al. [Rizkov et al., 2010] demonstrated beta-Amino alcohol selectors for enantioselective separation of amino acids by ligand-exchange capillary zone electrophoresis in a low molecular weight organogel. An approach related to the ligand-exchange of metal complexes is the formation of mixed borate–diol ternary complexes [Kodama et al., 2006]. For example, recently the use of (5S)-pinandiol (SPD) as the chiral selector in the presence of borate as a central ion and SDS has been described (**Figure 2.30**) [Kodama et al., 2005]. Three diols (1-phenyl-1,2-ethanediol, 3-phenoxy-1,2-propanediol and 3-benzyloxy-1,2-propanediol) were successfully resolved by this technique. Di-n-amyl L-

tartrate-boric acid complex chiral selector in situ synthesis and its application in chiral non-aqueous capillary electrophoresis is presented by Wang et al. [Wang L.J. et al., 2011].
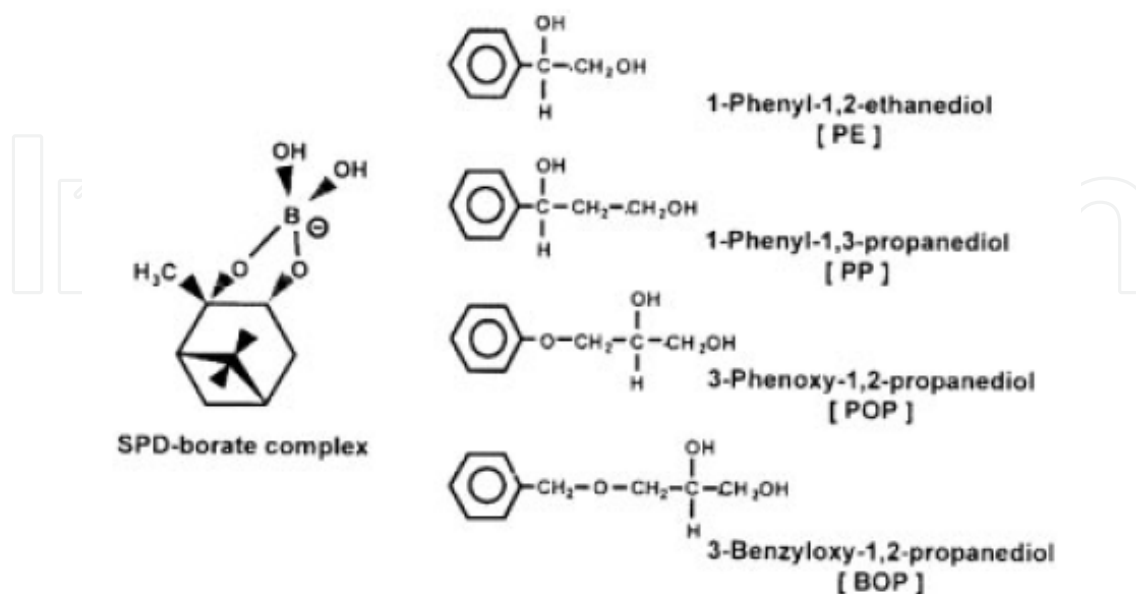

Figure 2.30. Chemical structures of SPD–borate complex and analytes. Reprinted from ref. from [Kodama et al., 2005].

The limited stability of these selectors, their UV absorption (detection difficulties) and rather slow ligand-exchange kinetics (poor enantioresolution) [Blanco & Valverde, 2003] could be the main reasons for a rare utilization of these selectors in chiral CE. Recently, tartaric acid–Cu(II) complex has been used as a selector for the chiral separation of drugs with amino alcohol structure [Hödl et al., 2007], however, it has not been employed in real bioanalyses so far.

2.3.7 Ion-pairing reagents

Ion-pairing reagents {e.g., (+)-S-Camphor-10-sulfonic acid; 2R,3S,4R,5S (−) 2,3,4,6-di-O-isopropylidene-2-keto-l-gulonic acid; 1S,4R(+) ketopinic acid, see examples in Figure 2.31}, are applicable in CE usually in non-aqueous medium as the essential interactions (hydrogen bondings and dipole–dipole interactions) are less effective in aqueous medium [Bjornsdottir et al., 1996; Carlsson et al., 2001; Hedeland et al., 2007]. Exceptions are the use of chiral counterions as supporting agents for separations with coselectors (e.g., CDs) in aqueous organic mixtures [Kodama et al., 2003].
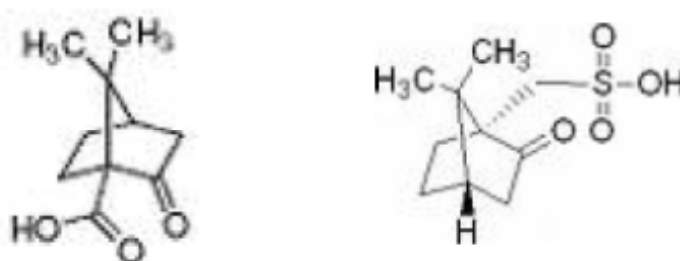

Figure 2.31. Chemical structures of ion-pairing reagents. (S)-(+)-ketopinic acid (left), (+)-(S)-camphor-10-sulfonic acid (right). Reprinted from [sigmaaldrich.com].

The ion-pairing mechanism under non-aqueous conditions took part in the enantioseparation of many drugs such as β-blockers [Bjornsdottir et al., 1996; Carlsson et al., 2001; Hedeland et al., 2007]. It cooperated also in bioanalytical applications separating enantiomers of salbutamol with heptakis-(2,3-di-O-acetyl-6-O-sulfo)-β-CD in urine samples under non-aqueous EKC conditions [Servais et al., 2004, 2006] (for detail see Table 2.1).

2.3.8 Amphiphilic molecules, micelles, micelle polymers and microemulsions

Amphiphilic molecules, composed of a polar head group and a hydrophobic tail, can form micelles above their critical micelle concentration (Figure 2.32). Micelles are aggregates (usually spherical) with a surface created by heads and core by tails. A typical recognition of analytes is based on the formation of associates with the micelles (less polar parts of analytes tend to enter the micelle core) having different stability for the two analytes of different hydrophobicity, or in other words, differences in their partition coefficients between the micelle phase and the electrolyte bulk phase. Chiral recognition is given by modifying partition coefficients of chiral analytes due to different steric effects between chiral analytes and chiral core or chiral surface of micelles. Micelle pseudostationary phases were introduced into CE by Terabe et al. [Terabe et al., 1984]. Different classes of charged as well as neutral amphiphilic molecules, namely bile salts, saponines, long-chain N-alkyl-lamino acids, N-alkanoyl-L-amino acids, N-dodecoxycarbonyl amino acids, alkylglycosides, alkylglucosides, were applied as chiral selectors in aqueous solutions for a wide range of substances of a hydrophilic or hydrophobic nature, see the specialized review article [Palmer & McCarney, 2004a]. For example, sodium cholate was used for the enantiomeric separation of mono and diacid benzoporfyrine derivates in serum and microsome samples [Peng et al., 2002] (for details see Table 2.1).
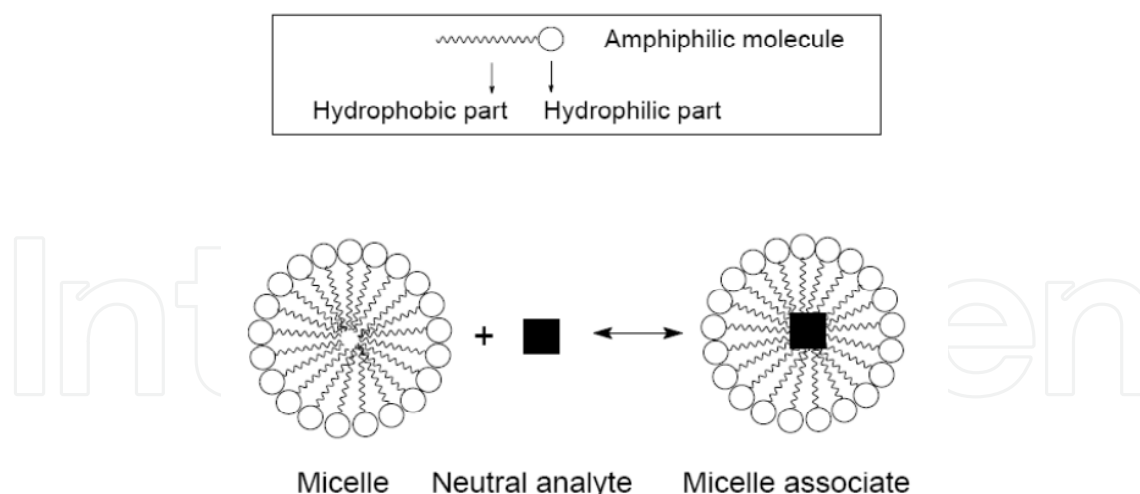

Figure 2.32. Mechanism of formation of micelle associate with neutral molecule. Reprinted from ref. [Terabe et al., 1984].

Micelle polymers {e.g., poly- sodium N-undecenoxy carbonyl-L-leucinate/isoleucinate (Figure 2.33), 3-[(3-dehydroabietamidopropyl)dimethyl-ammonio]-1-propane-sulfonate, undecyl-L-valine} can offer additional benefits in comparison to conventional micelles, such as elimination of intrinsic micelle equilibrium (enhanced chiral recognition), wide

concentration range applicable (no critical micelle concentration) and therefore, higher obtainable signal to noise ratio (S/N). Further, the covalent bonds between surfactant monomers are stable (less background noise from ionized surfactant monomers of low molecular weights in mass spectrometer) and there are no disruptions in micelle formation (wider range of buffer additives applicable, e.g., organic modifiers). Micelle polymers have lower surface activity, association kinetics is fast due to a shallower and faster penetration of the solute into the compact polymeric structure (enhanced efficiency) [Riekkola et al., 1997; Palmer & McCarney, 2004b; Palmer, 2007; Zhao, S. et al., 2007; Akbay et al., 2005; Hou, J. G. et al., 2006; Rizvi et al., 2007]. Many of these properties are beneficial for enhancing chiral, as well as achiral, recognition {a broad range of structurally diverse racemic compounds such as phenylethylamines, β-blockers, 2-(2-chlorophenoxy) propionic acid, benzoin derivatives, derivatized amino acids and benzodiazepines} and for improving detection (very pronounced in MS) when compared with traditional micelle systems [Rizvi et al., 2007]. Therefore, the utilization of these selectors tends to rise up as it is apparent from the following recent applications in biological samples (human urine, plasma), such as poly(sodium *N*-undecenoxy carbonyl-L-leucine) sulfate vs. pseudoephedrine [Rizvi et al., 2007], polysodium-*N*-undecenoyl-L,L-leucyl-valinate vs. warfarin [Hou, J. et al., 2007], for details see Table 2.1.
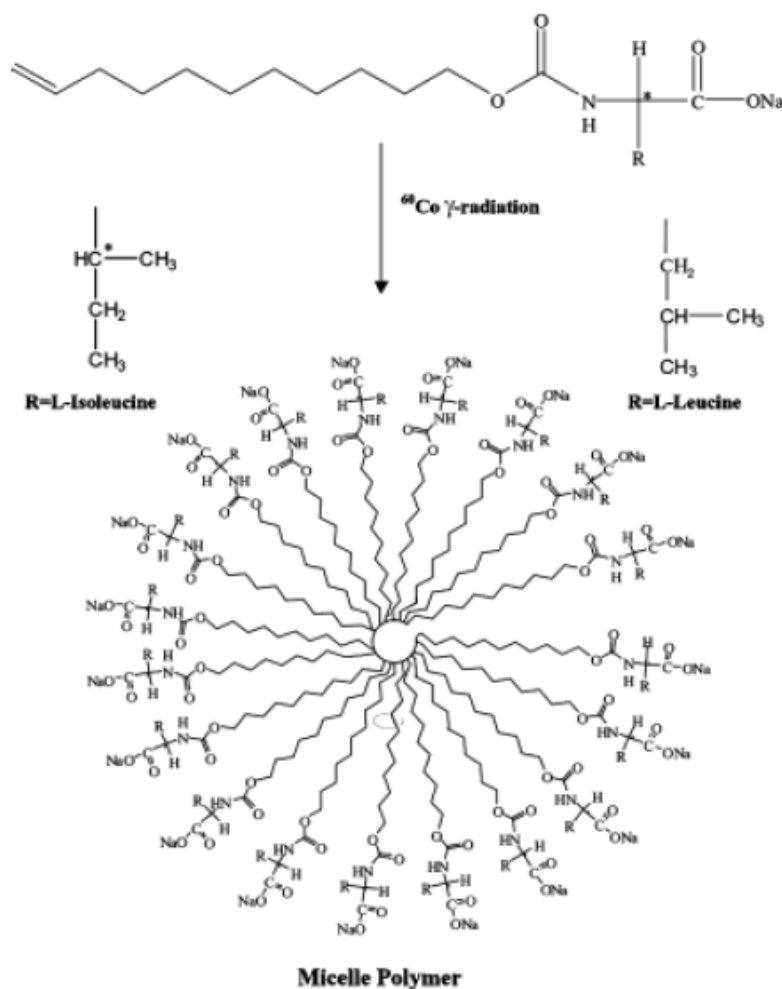

Figure 2.33. Structure of monomer and micelle polymer of alkenoxy surfactants. Reprinted from ref. [Rizvi et al., 2004].
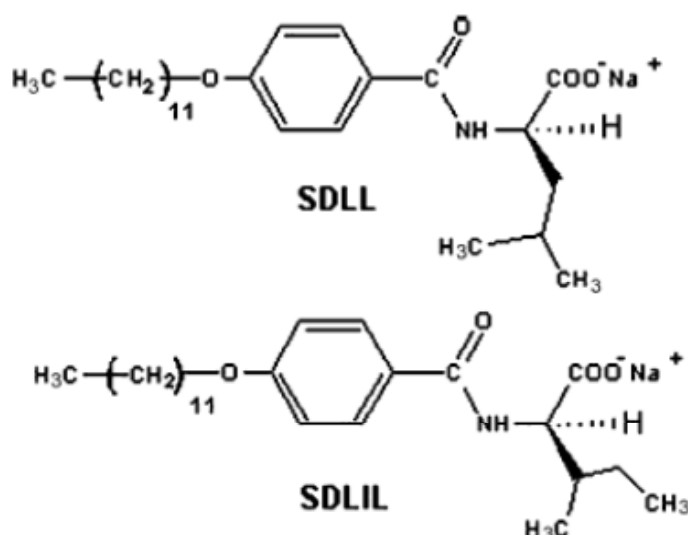
Figure 2.34. Molecular structures of the vesicle-forming amphiphilic molecules. Sodium *N*-[4-*n*-dodecyloxybenzoyl]-L-leucinate (SDLL) and sodium *N*-[4-*n*-dodecyloxybenzoyl]-L-isoleucinate (SDLIL). Reprinted from ref. [Mohanty & Dey, 2006].

The use of vesicle-forming amphiphilic molecules (e.g., sodium N-[4-n-dodecyloxybenzoyl]-L-leucinate) [Mohanty & Dey, 2006] and ionic liquids (i.e., salts in the liquid state, organic salts with low melting points soluble in both polar and nonpolar solvents, e.g., undecenoxycarbonyl-L-pyrrolidinol bromide) [Rizvi & Shamsi, 2006] can be counted among the recent trends in chiral CE with new potentialities for biologically active chiral compounds. For example, two vesicle-forming single-tailed amino acid derivatized surfactants sodium *N*-[4-*n*-dodecyloxybenzoyl]-L-leucinate (SDLL) and sodium *N*-[4-*n*-dodecyloxybenzoyl]-L-isoleucinate (SDLIL) (Figure 2.34) have been synthesized and used as a pseudo-stationary phase in MEKC for various model chiral analytes, namely atropisomers (±)-1,1′-bi-2-naphthol, (±)-1,1′-binaphthyl-2,2′-diamine, (±)-1,1′-binaphthyl-2,2′-diylhydrogen phosphate and Tröger's base and chiral compound benzoin [Mohanty & Dey, 2006]. Results of these studies have suggested formation of vesicles in aqueous solutions. Microenvironment of the vesicle determined the depth of penetration of the analytes into vesicle, and, in this way, it influenced the separation selectivity. The application of novel ionic liquid-like surfactants and their polymers for chiral separation of acidic analytes in MEKC was reported first by Rizvi and Shamsi in 2006 [Rizvi & Shamsi, 2006]. The two model acidic analytes, (*rac*)-α-bromophenylacetic acid and (*rac*)-2-(2-chlorophenoxy)propanoic acid, were successfully separated with the amino alcohol-derived chiral ionic liquids (given in Figure 2.35) and their polymers at 25 mM surfactant concentration.
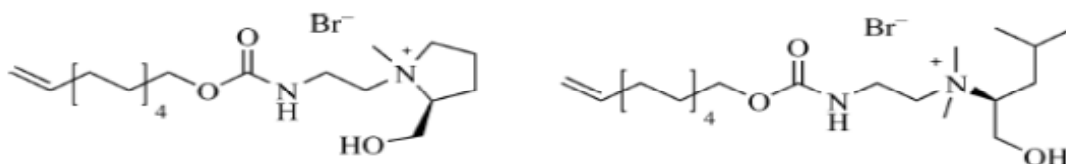

Figure 2.35. Amino alcohol-derived chiral ionic liquids for capillary electrophoresis. Reprinted from ref. [Rizvi & Shamsi, 2006].

Microemulsions, spherical aggregates of nanometre size consisting of an oil core covered/stabilized by amphiphilic molecules in water solution (Figure 2.36), are optically transparent and thermodynamically stable phases that offer a new alternative to micelles for chiral separations aimed at hydrophobic molecules. Chiral microemulsions can utilize one chiral entity (either surfactant, cosurfactant or oil are chiral) or more than one chiral entity (e.g., both surfactant and cosurfactant are chiral) [Ryan et al., 2009; Kahle & Foley, 2006]. Only a few applications to chiral separations have been described (none yet in biological samples), including chiral oils, chiral amphiphilic molecules, as well as CDs as chiral additives [Marsh et al., 2004; McEvoy et al., 2007]. Kahle and Foley [Kahle & Foley, 2007a; 2007b, 2007c] investigated one-, two- and three-chiral-component microemulsions, composed of the chiral surfactant dodecoxycarbonylvaline, the cosurfactant 2-hexanol and one of the chiral oils dibutyl or diethyl tartrate for MEEKC. Six basic drugs, viz. four ephedrine derivatives and two β-blockers, were used as test solutes. For most analytes, enantioselectivity, as well as resolution, were higher with the best dual-chirality system compared with the best one-chiral-component microemulsion due to synergistic effects.
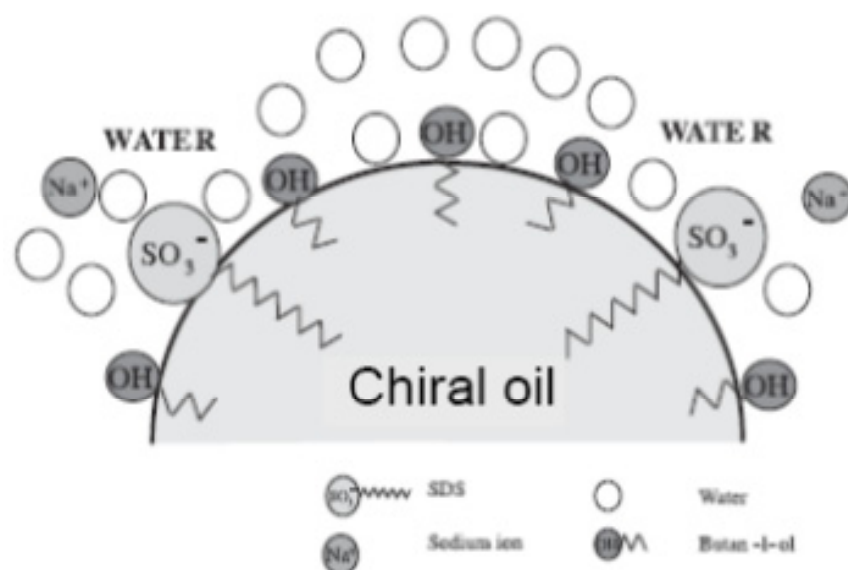

Figure 2.36. Schematic representation of an oil-in-water microemulsion droplet. Adapted from ref. [Ryan et al., 2009].

2.3.9 Alternative chiral selectors

Several alternative chiral selectors, compared to those described in sections 2.3.1-2.3.8, have been employed in chiral CE, including calixarenes (macrocyclic compounds consisting of benzene rings linked by methylene groups and chiral substituent, see Figure 2.37), cyclosphoraoses (unbranched cyclic β-D-glucans), hemispherodextrins (capped CDs), tergurides (ergot alkaloids), amphiphilic aminosaccharides, cyclopeptides, guanosine gels, aptamers (single-stranded RNA or DNA oligonucleotides 15 to 60 base in length that bind with high affinity to specific molecular targets), e.g., anti-arginine 1-RNA aptamer, dendrimers (repetitively branched cascade molecules, see Figure 2.38), etc. [Van Eeckhaut & Michotte, 2006; Gübitz & Schmid, 2004, 2008; Riekkola et al., 1997; Mokhtari et al., 2011, Chen Y.M. et al. 1998]. These selectors can solve particular/specific problems in chiral

separations, however, their use is less universal in most cases as they can be expensive and their availability is limited. This could also be the reason why no application in chiral bioanalysis has been demonstrated so far.
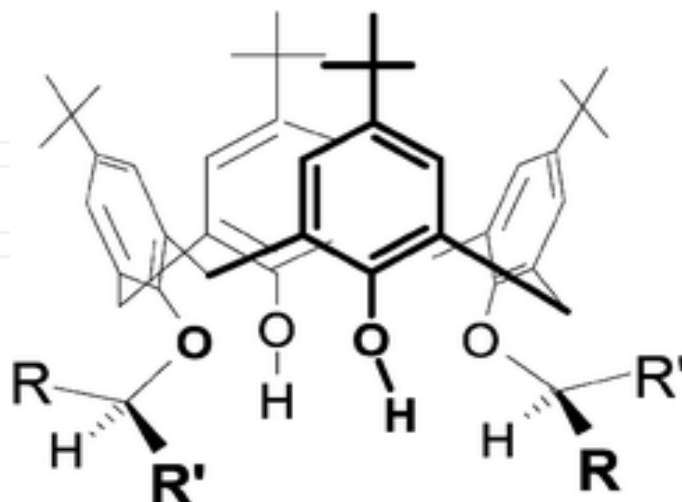

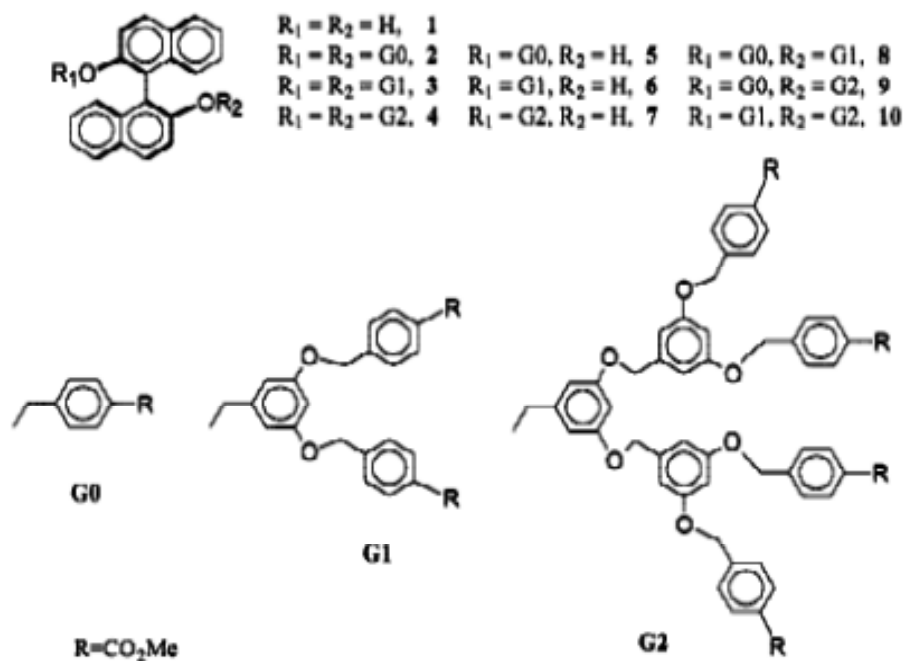Figure 2.37. Chiral calixarene. Reprinted from [rsc.org].

Figure 2.38. The chemical structures of the chiral dendrimers with axial chirality. Reprinted from ref. [Chen, Y.M. et al., 1998].

2.4 Applications of chiral separation systems in bioanalysis

The following examples illustrate successful EKC enantioseparations of drugs in biological samples utilizing some of the progressive chiral principles mentioned in section 2.2. and/or new chiral selectors mentioned in section 2.3. CEC and MCE applications in this field are also included.

New chiral selectors. A sensitive MEKC-MS method using poly(sodium *N*-undecenoxy carbonyl-L-leucine) sulfate was developed for enantioselective analysis of pseudoephedrine in human urine [Rizvi et al., 2007]. The permanent charge of this micelle chiral selector allows for setting separation conditions (electrolyte pH) suitable for obtaining the highest detection sensitivity maintaining sufficient enantioresolution. Other new chiral selectors and new chiral stationary phases can be also found in some applications given for the advanced electromigration modes and mechanisms below.

Countercurrent migration. Rudaz et al. [Rudaz et al., 2005] used the countercurrent migration of the negatively charged highly sulfated-γ-CD to achieve a highly effective enantioseparation of amphetamine derivatives in plasma. This strategy allowed using a low concentration (0.15%) of the chiral selector for the complete enantioseparation of seven amphetamine derivatives in analysis times of less than 6 min.

Anionic CD, the heptakis(2,6-diacethyl-6-sulfato)-β-CD, migrating in the opposite direction to the analytes, was highly effective in low concentrations (0.85 mM) for the simultaneous chiral CD-EKC-MS separation of methamphetamine, 3,4-methylenedioxyamphetamine and amphetamine in clinical human urine samples [Iio et al., 2005].
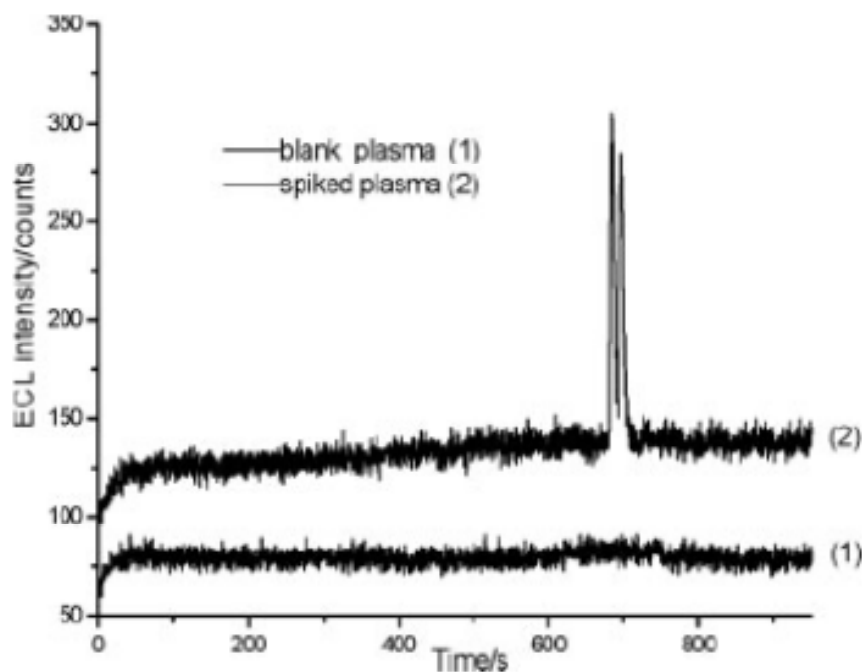

Figure 2.39. Electropherograms of extracts of blank plasma and plasma of spiked concentration of 4 μM racemic drug. Conditions: detection buffer, 5 mM Ru(bpy)$_3$ $^{2+}$ in 100 mM (pH 6.5) phosphate buffer; separation buffer, 3 mg/mL of S-β-CD in 40 mM (pH 4.5) acetate buffer; applied voltage, 22 kV; injection time, 5 s. Reprinted from ref. [Fang, L. et al., 2006].

The CD-EKC method based on a combination of discontinuous buffer (separation buffer differed from detection solution, essential for chemiluminiscence detection) with low concentration (3 mg/mL) of anionic highly sulfated-β-CD was used for the baseline

enantioseparation of an oppositely charged racemic antiarrhythmic drug, disopyramide, in spiked plasma samples, see Figure 2.39 [Fang, L. et al., 2006].

Naphthalene-2,3-dicarboxaldehyde (NDA) derivatized baclofen was separated in human plasma using the CD-EKC-LIF method based on a highly sulfated-β-CD [Kavran-Belin et al., 2005]. The anionic CD migrated in the opposite direction toward negatively charged baclofen (pH 9.5) transported by EOF toward the cathode.

The NACE-MS with countercurrent migration of heptakis(2,3-di-O-acetyl-6-O-sulfo)-β-CD present in acidified methanol medium was used for the separation and determination of low levels of the enantiomers of a basic chiral drug (salbutamol) in biological samples (human urine) [Servais et al., 2006].
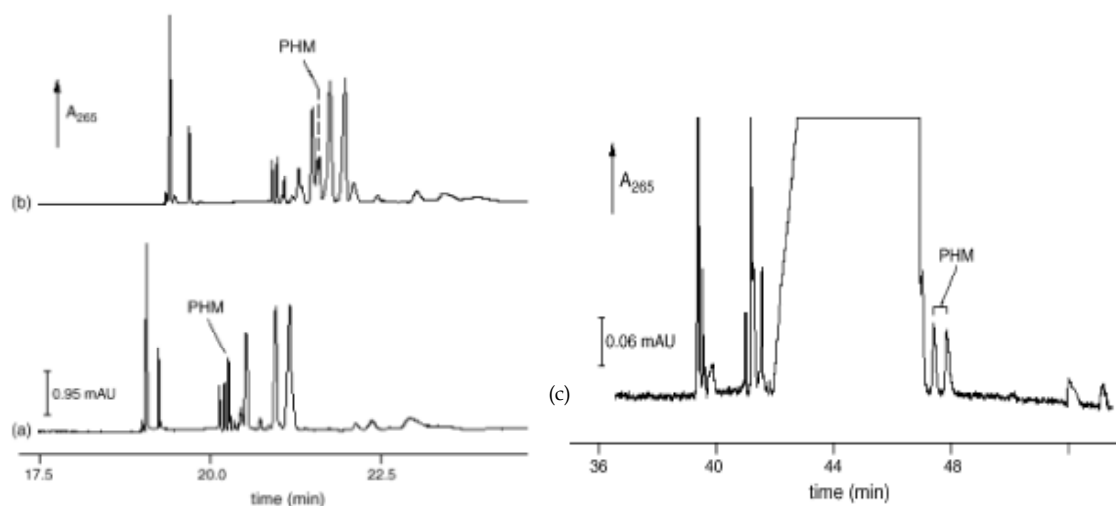

Figure 2.40. Electropherograms showing the effect of the charge of ionizable chiral selector, CE-β-CD, on the EKC separation of PHM enantiomers in a partially pretreated urine matrix. The separations were carried out with the uncharged CE-β-CD (a, b) and with the negatively charged CE-β-CD (c). Separating conditions: (a, b) EKC electrolyte system consisting of a 25mM glycine-acetic acid buffer, pH 3.2, 0.2 methylhydroxyethylcellulose as an EOF suppressor, with a 0 (a) and 15 (b) mg/mL concentration of CE-β-CD, (c) EKC electrolyte system consisting of a 25mM ε–aminocaproic acid-acetic acid buffer, pH 4.5, 0.2 methylhydroxyethylcellulose as an EOF suppressor, with a 2.5 mg/mL concentration of CE-β-CD. Sample and analyte: (a, b) PHM was present in the sample (20-times diluted urine) at a 150 ng/mL concentration. (c) PHM was present in the sample (4-times diluted urine) at a 30 ng/mL concentration. Reprinted from ref. [Mikuš et al., 2006a].

Negatively charged carboxyethyl-β-CD considerably improved enantioresolution of several oppositely charged H$_1$-antihistamines (pheniramine and its metabolite, dimethindene, dioxopromethazine) in comparison with native CDs [Mikuš et al., 2006a]. Countercurrent migration and enhanced complexation were responsible not only for improved chiral separation of the drugs and the metabolite, but also achiral separation allowing resolution of the drug enantiomers from their metabolic products, as well as from the sample matrix constituents when metabolic study of pheniramine in urine samples was carried out [Marák et al., 2007]. A high effectivity of the separation was demonstrated by a low concentration of

the chiral selector enabling optimum separating conditions for the trace chiral analytes in concentrated biological matrices [Mikuš et al., 2006a], see Figure 2.40.

Charged chiral pseudophases/carrier systems. The enantioseparations of cyanobenz[*f*]isoindole serine (CBI) derivatives of glutamate and aspartate in squirrel brain samples were accomplished with a highly sulfated-β-CD as chiral selector at low pH and reverse polarity [Kirschner et al., 2007].

Mixed selector systems. Aspartate enantiomers (NDA derivatives) were separated in the samples prepared from tissue of the central nervous system of *A. californica* using mixed micelles composed from β-CD and SDS [Miao et al., 2005].
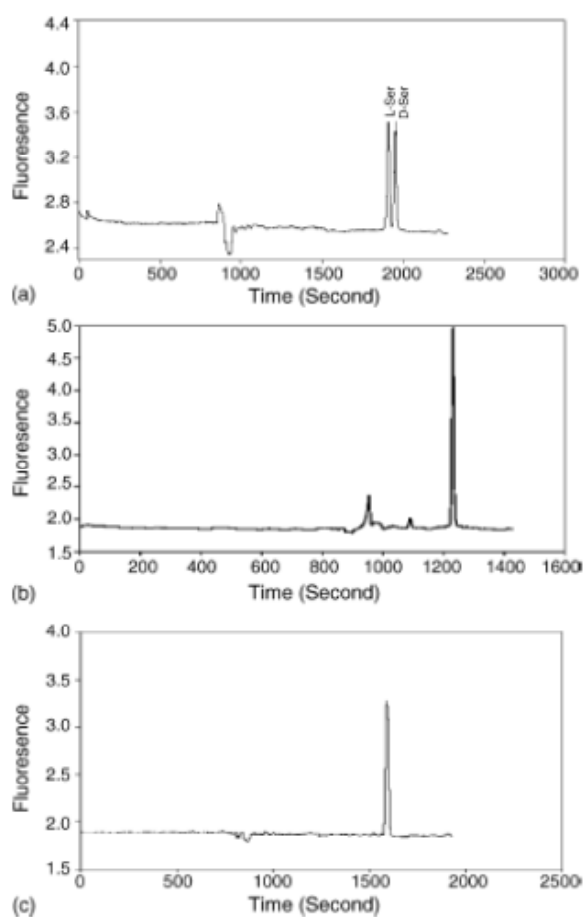

Figure 2.41. Separation of CBI-Ser enantiomers using different running buffers: (a) 100mM borate (pH 9.5), 30mM β–CD and 60mM SDC; (b) 100mM borate (pH 9.5) and 30mM β-CD; (c) 100mM borate (pH 10.0), 60mM SDC. Capillary was 50 μm i.d.×50 cm effective length. Voltage applied was 15 kV. Ser enantiomer concentration was 2.0 μM. Reprinted from ref. [Zhao, S.L. et al., 2005b].

The CD-MEKC separation of CBI-D/L-serine enantiomers was achieved by using a dual chiral selector system consisting of β-CD and chiral micelles formed by deoxycholic acid (SCD) [Zhao, S.L. et al., 2005b]. The essential role of the mixed chiral selector system for the

enantioresolution in comparison with the capability of individual chiral selectors is demonstrated in Figure 2.41, compare electropherograms (a-c). In addition, an effectivity of the mixed chiral selector system in the enantioseparation of CBI-D/L-serine enantiomers in real biological matrices was demonstrated in this work. In this way, for the first time, peaks corresponding to L-serine and D-serine were completely separated in *Aplysia* ganglian (a sea mollusc widely used as a neuronal model) homogenates.

The evaluation of the enantioselectivity of glycogen-based dual chiral selector systems towards basic drugs in capillary electrophoresis is presented by Chen et al. [Chen J. et al., 2010].

Flow counterbalanced systems. Several FCCE modes have been developed and applied for enantioseparations so far, like microfluidic temperature gradient focusing [Balss et al., 2004], electric field gradient focusing [Koegler & Ivory, 1996a, 1996b, Ivory, 2000, Huang, Z. & Ivory, 1999]. Notice that these techniques have a great potential not only in enantioseparations, but also in a sample pretreatment (focusing, see chapter 3).
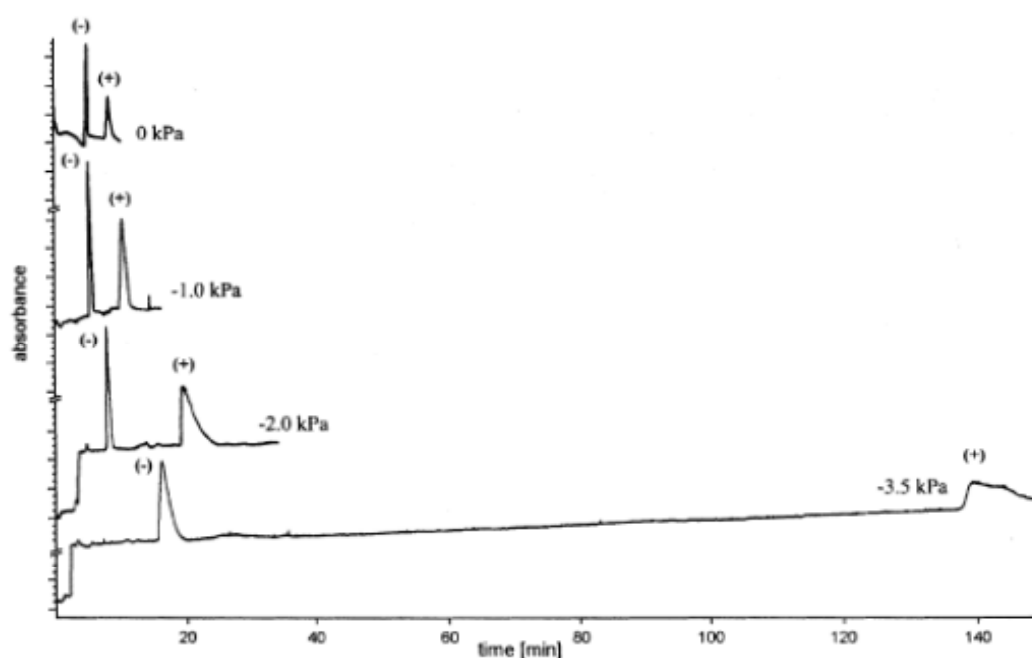

Figure 2.42. Effect of increasing counterpressure on the separation of (±)-chlorpheniramine in the presence of 2 mg/mL CM-β–CD). Reproduced from ref. [Chankvetadze et al., 1999].

The effect of increasing counterpressure on the separation of (±)-chlorpheniramine in the presence of 2 mg/mL CM-β-CD) was studied by Chankvetadze et al. [Chankvetadze et al., 1999] and remarkable results are illustrated in Figure 2.42. Applications of counterpressure techniques for the enantioseparations in complex ionic matrices, however, have not been demonstrated.

Synchronous cyclic capillary electrophoresis (SCCE) was proposed by Jorgenson's group [Zhao J. et al., 1999] as a technique which allows overcoming the dispersion problems in FCCE caused by the parabolic counterflow profile. This technique was applied for isotopic and chiral separations. In the third cycle, the chiral compound (α-hydroxybenzyl)

methyltrimethylammonium with a selectivity of 1.0078 was almost baseline separated in 3.5 h [Zhao J. & Jorgenson, 1999].

CEC. CEC separations are carried out mainly in packed capillary columns. Immobilization provides an effective solution especially for the chiral selectors with problematic detection properties, such as proteins, macrocyclic antibiotics, etc., that was demonstrated on many model examples, such as vancomycin CSP vs. warfarin and various β-blockers (atenolol, metoprolol, pindolol, oxprenolol, alprenolol, propranolol, carteolol, talinolol) vs. MS detection [Zheng J. & Shamsi, 2006; Zheng J. et al., 2006].
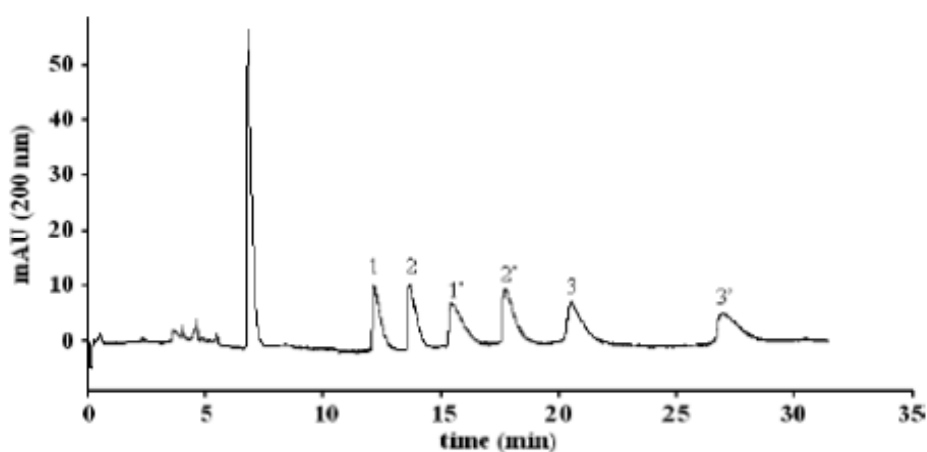

Figure 2.43. Electrochromatogram of blank urine sample spiked with 10 µg/mL of racemic MRT and 8-OH-M and 20 µg/mL of racemic DMR. Both samples were subjected to the SPE procedure. Electrochromatographic conditions: stationary phase composition, vancomycin-CSP, mobile phase, 100 mM ammonium acetate buffer (pH 6)/H$_2$O/MeOH/ACN (5:15:30:50, by vol.). Other CEC conditions: capillary column, 75 µm i.d., 33 cm total length, 23 cm packed length, 24.5 cm effective length; applied voltage, 25 kV; capillary temperature, 20°C; pressurized column at both ends with 10 bar; injection by pressure at 10 bar, 0.5 min, followed by a plug of mobile phase at 10 bar, 0.2 min. (1) MRT, (2) 8-OH-M and (3) DMR. Reprinted from ref. [Aturki et al., 2007].

Various new CSPs are also employed in model analyses. A new chiral capillary electrophoresis column coated by carbosilane dendrimers with peripheral Si-Cl groups and beta-cyclodextrin was prepared by Shou et al. [Shou et al., 2008]. The separation characteristics of stability and longevity of coated capillaries modified by carbosilane dendrimers were excellent. The varying of separation efficiency was less than 5%, after running for one month. The optimum conditions contained the running voltage of 12 kV, the UV detector wavelength of 214 nm, the sample injected time of 7 s and the phosphate buffer solution concentration of 40 mM. Chlortrimeton, hydrochloric promethazine and benzedrine were selected as the separation model targets. Especially, G2P columns could be used as separate chlortrimeton enantiomers effectively. Under these conditions, the column efficiency was 2.5 x 105 plates/m with a resolution of 1.43 and baseline separation.

Other examples of the CEC application are illustrated on biological samples. For example, CEC based on vancomycin CSP offered an effective solution not only for the enantioseparation of

mirtazapine (MRT) and its metabolites {8-hydroxymirtazapine (8-OH-M) and *N*-desmethylmirtazapine (DMR)} in urine samples (pretreated by off-line solid-phase extraction), but also for their reliable UV absorbance detection [Aturki et al., 2007], see Figure 2.43.
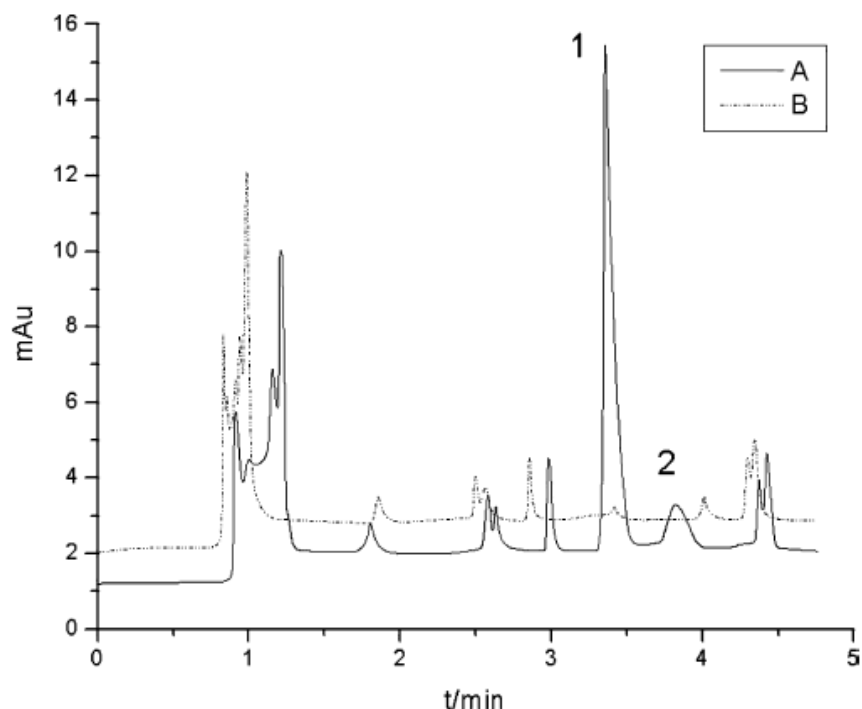

Figure 2.44. Separation of mexiletine hydrochloride in human plasma sample by Asp-β–CD fabricated monolithic column. (A) Plasma sample; (B) free plasma. 1 and 2, the enantiomers' peaks of mexiletine hydrochloride. Mobile phase: phosphate/trolamine buffer, 5mM, pH= 4.5; temperature: 20°C; voltage: −10 kV; injection: −2 kV, 2 s. Reprinted from ref. [Li Y. et al., 2010].

Very attractive applications due to rapid separations and good enantioresolutions are represented by the chiral monolithic based CEC. A monolithic column fabricated with Asp-β–CD was applied to the separation of mexiletine hydrochloride enantiomers in a human plasma sample. Because of complex matrices, as little as possible trolamine was added to the phosphate buffer to decrease absorption between the sample and the solid-phase. Figure 2.44 shows the electropherogram of mexiletine hydrochloride extracted from the plasma sample. From Figure 2.44, two enantiomers were separated by baseline, the migration times were 3.46 and 3.84 min, respectively. Some components in plasma were also determined, but they did not interfere with the determination of the enantiomers. Thus, the method was applied to the complex matrix sample, and the column had good selectivity.

MCE. MCE with (+)-18-crown-6-tetracarboxylic acid as a chiral selector was applied for the countercurrent chiral separation of gemifloxacin in urine samples after an appropriate sample pretreatment (see in 3.2.2) [Cho et al., 2004]. A successful chiral separation was achieved in ca. 3 min, with an extremely small amount of the chiral selector (50 μM), and minute amounts of both sample as well as BGE. This demonstrated significant potentialities of electromigration methods in highly effective ultraspeed microscale chiral analyses of drugs in complex matrices.
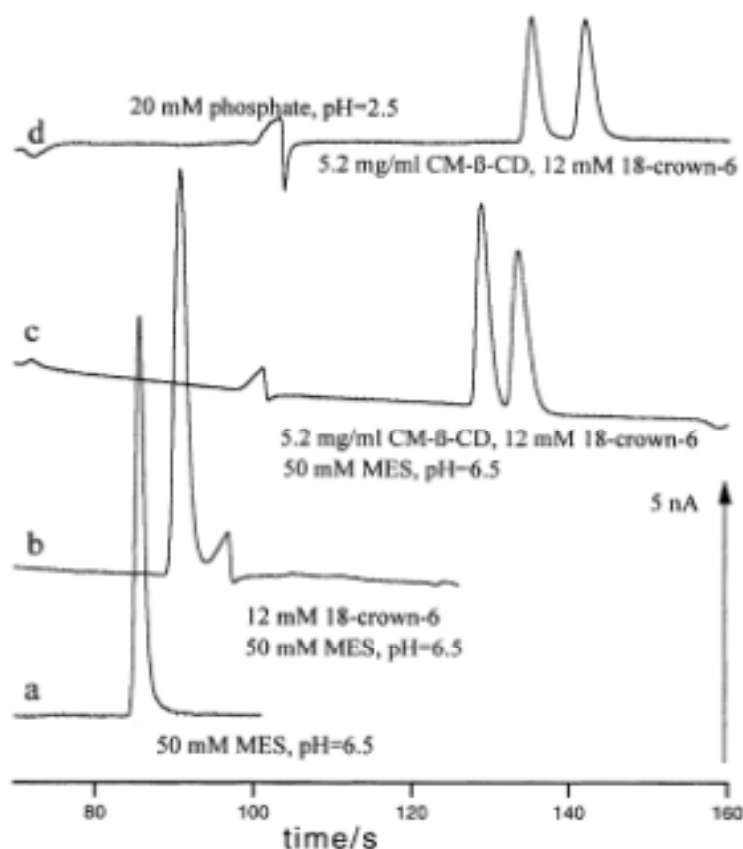
Figure 2.45. Enantiomeric separation of noradrenaline with CM-β-CD by formation of sandwich complexes with 18-crown-6 at pH 6.5 and 2.5. Separation voltage, 3 kV; detection potential, 1500 mV; injection, 1 kV (3 s); concentration of standard, 10^{-4} M. Reprinted from ref. [Schwarz & Hauser, 2001].

A combination of an advanced enantioseparation mechanism (dual chiral selector system) with advanced CE format (MCE) was demonstrated by Schwarz and Hauser [Schwarz & Hauser, 2001]. The separation of the enantiomers of noradrenaline in the presence of 18-crown-6 is illustrated in Figure 2.45. The crown ether on its own has a small effect on the migration, but cannot lead to a chiral separation (Figure 2.45b). In combination with the carboxymethylated β-cyclodextrin (Figure 2.45c), a chiral separation which is better than the one achieved by only carboxymethylated β-cyclodextrin is obtained. The resolution factors (*R*) were determined as 1.30 and 1.35 (5.2 mg/mL CM-β-CD, pH=6.7) with and without the addition of 12 m*M* 18-crown-6, respectively. Moreover, such an advanced system is capable of also separating multicomponent mixtures of enantiomers, as presented in Figure 2.46.
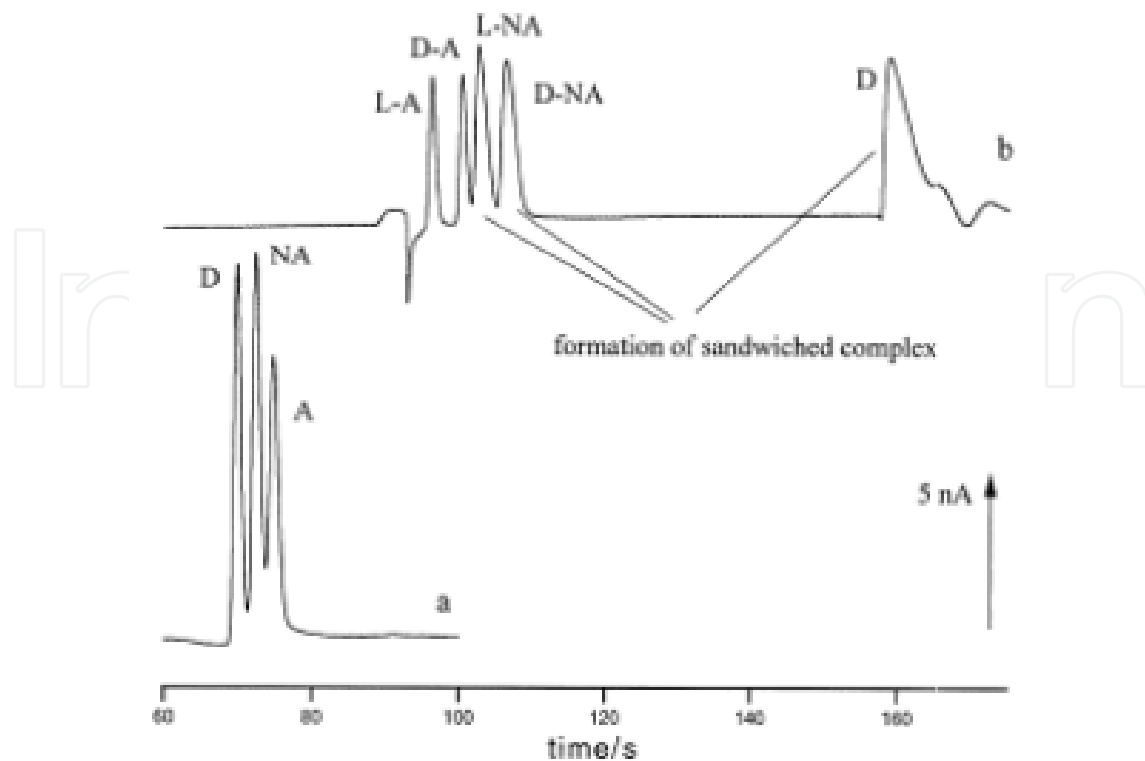
Figure 2.46. Rapid enantiomer MCE separation and amperometric detection of a mixture of adrenaline, noradrenaline and dopamine employing sandwich complexes. Buffer: (a) 50 mM MES, pH=6.7; (b) 20 mM phosphate, pH=3.0, 5.2 mg/mL CM-β-CD, 12 mM 18-crown-6. Separation voltage, 3 kV; detection potential, (a) 1300 mV, (b) 1500 mV; injection, 1 kV (3 s); concentration of standards, 10^{-4} M. Peak marks: dopamine hydrochloride, D, adrenaline, A, noradrenaline, NA. Reprinted from ref. [Schwarz & Hauser, 2001].