3

Advanced Sample Preparation

3.1 On-line sample preparation techniques in capillary electrophoresis - introduction

The importance of sample preparation is related to high demands on (i) the sensitivity of quantitative determination of trace analytes, (ii) the selectivity of monitoring of analytes in multicomponent sample matrix and (iii) the automatization and miniaturization of analysis [Mikuš & Maráková, 2010].

Sensitivity. One of the most pronounced limitations of capillary electromigration methods when compared to more traditional liquid-phase separation techniques, such as HPLC, is a poor concentration sensitivity of photometric detectors, which are the most popular among on-capillary CE detectors. The reached LODs are by two orders inferior in comparison to the HPLC technique because of a short optical pathlength and small sample injection volume. Two basic approaches can be distinguished among many efforts that have been made to improve the sensitivity of detection in CE. Either more sensitive detection schemes can displace the UV detection mode [Hernández et al., 2008, 2010; Hempel, 2000] or an increased analyte mass can be cumulated in its zone prior to detection utilizing a proper sample preparation, as it is reviewed in this section.

Selectivity. To achieve adequate separation selectivity, the analysis of real/complex samples in CE usually requires efficient sample treatment to remove interfering solutes, inorganic and organic salts, and particulate matter (as it is reviewed in this section). At the same time, the creation of a chiral environment is necessary for the separation of enantiomeric species by the electrokinetic chromatography (EKC) principle accomplished in zone electrophoretic mode simply by the addition of chiral selector molecules. In the same way, chiral selectivity can be implemented in isotachophoretic mode. Chiral separation principles in CE have been described in many review papers [Chankvetadze, 1997; Chankvetadze & Blaschke, 2001; Gübitz & Schmid, 2007, 2008; Preinerstorfer et al., 2009; Fanali, 2002; Fanali et al., 1998b; Ossicini & Fanali, 1997]. In addition to those, capillary electrochromatography (CEC) separation principles cannot be omitted for review, e.g., those by Schurig [Wistuba & Schurig, 2000a, 2000b]. On-line sample preparation and enantioseparation mechanisms should create a harmonized system, especially in chiral EKC and ITP where the chiral selector is not immobilized as it is in CEC, and all possible mutual negative interferences have to be carefully eliminated.

Automatization and miniaturization. As one moves toward small sample volumes, sample handling and preparation steps become more difficult and the concentration step is therefore preferably done on-line instead of off-line. The microfluidic devices, such as microchips, can provide several additional advantages over electromigration techniques performed in capillary format. The heat dissipation is much better in chip format compared

to that in a capillary and therefore higher electric fields can be applied across channels of the microchip. This fact enables, along with a considerably reduced length of channels, significant shortening of separation time. The sample and reagent consumption is markedly reduced in microchannels. Hence, the chiral MCE can provide a unique possibility of ultraspeed enantiomeric separations of microscale sample amounts. On the other hand, the efficiency is often weak due to the shape of the chips and the quality of the injection [Guihen et al., 2009]. Until now, electrophoresis, rather than chromatography, has been the primary principle applied in microchip separations for several reasons as follows: (i) the materials typically used for microchips can barely withstand the high pressures applied for chromatographic separations; (ii) the packing of the chromatographic particles into the channels without voids can be difficult, (iii) the performance of chromatography decreases with decreased column length [Preinerstorfer et al., 2009]. Nevertheless, both electrophoretic [Gong & Hauser, 2006; Piehl et al., 2004; Belder et al., 2006], as well as electrochromatographic, modes [Weng, et al. 2006] are applicable in microchip format. Nowadays, the on-line coupling of sample treatment systems to CE or MCE are of great interest because it allows the automatization of the analytical process (from sample preparation to data treatment), which is a current trend in analytical chemistry.

Thus, the importance of on-line sample preparation is pronounced when ultratrace analytes (ng/mL and less) are determined in minute amounts (μl, μg and less) of samples with complex matrices (variable in qualitative and quantitative composition). This is a common situation in the analyses of drugs, their metabolites and biomarkers in biological samples where preconcentration of analytes, elimination of matrix interferents, and minimized sample handling is necessary to obtain relevant analytical results. Mikuš and Maráková [Mikuš & Maráková, 2010] recently provided a review on the chiral capillary electrophoresis with on-line sample preparation. The latest panorama of sample preparation methods for animal/human and plant samples given by Chen et al. [Chen Y., 2008] has been composed from almost 500 references, highlighting some promising methods which have fast developed over recent years and giving a somewhat brief introduction on most of the well-developed methods, including on-line stacking methods in CE. Wu [Wu X.Z., 2003] illustrated new approaches to sample preparation for CE and Kataoka [Kataoka, 2003] highlighted their utilization in clinical and pharmaceutical analysis. In addition to these rather general reviews, specialized reviews are included at the beginning of each following subsection. These highlight the role of electromigration effects and interactions in on-line sample preparation, and summarize basic electrophoretic (stacking) and non electrophoretic (mostly chromatographic and extraction) on-line sample preparation techniques aimed at preconcentration and purification of complex samples in chiral pharmaceutical and biomedical research. Application examples are listed in Table 3.1 showing the chiral pharmaceutical and biomedical analyses supported by on-line sample preparation procedures described in this chapter. On the other hand, conventional off-line sample preparation techniques, such as solid-phase extraction, liquid-phase extraction, solid-liquid extraction and dialysis (included in the above mentioned reviews), are not discussed here.

3.2 On-line sample preparation techniques based on electrophoretic principles

Several reviews have been published recently, focusing on the on-line sample preconcentration CE techniques based on electrophoretic principles leading to the compression of a long sample plug into a narrow band with a high concentration of analytes, so called stacking techniques [Lin C.H. & Kaneta, 2004; Gebauer et al., 2009, 2011; Silva, 2009; Ryan et al., 2009; Malá et al., 2007, 2011; Urbánek et al., 2003]. Among the latest detailed review papers are those by Lin and Kaneta [Lin C.H. & Kaneta, 2004], Simpson et al. [Simpson et al., 2008], Breadmore [Breadmore, 2007], Breadmore et al. [Breadmore et al., 2009] and Malá et al. [Malá et al., 2011], including research articles gathered in the period 2000-2011. The papers provide fundamentals and applications of basic types of on-line sample preconcentration techniques. McKibbin and Terabe [Britz-McKibbin & Terabe, 2003] emphasized on-line preconcentration strategies for trace analysis of metabolites by CE. Ruiz and Marina [Ruiz & Marina, 2006] reviewed sensitive chiral analysis by CE.

The stacking procedures, described in section 3.2.1, are modifications of the basic zone electrophoretic and/or isotachophoretic and/or isoelectric focusing separation modes (Figure 2.2). The stacking procedures are based on increasing analyte mass in its zone during the electromigration process via electromigration effects, enhancing sensitivity in this way. In all the cases, the key requirements are that there is an electrophoretic component in the preconcentration mechanism and that the analytes concentrate on a boundary through a change in velocity. Then we can recognize (i) field-strength-induced changes in velocity (field-enhanced sample stacking [Kim J.B. & Terabe, 2003; Quirino & Terabe, 2000; Chien & Burgi, 1992; Weiss et al., 2001], isotachophoresis and transient isotachophoresis [Beckers & Boček, 2000; Schwer et al., 1993; Shihabi, 2002]), and (ii) chemically induced changes in velocity (dynamic pH junction [Britz-McKibbin & Chen, 2000; Aebersold & Morrison, 1990; Kim J.B. et al., 2003], sweeping [Kitagawa et al., 2006; Palmer et al., 2001; Quirino & Terabe, 1998; Quirino & Terabe, 1999; Quirino et al., 2000]), see Table 3.2. In addition to these techniques, the counter-flow gradient focusing [Shackman & Ross, 2007], electrocapture [Horáková et al., 2007] and many others can be considered as the techniques based on a combination of field-strength and chemically induced changes in velocity offering new and interesting possibilities in on-line sample preparation (mainly preconcentration).

Some of the stacking techniques (and their combinations) can provide, besides (i) the preconcentration, other benefits, such as (ii) an effective sample purification isolating solute (group of solutes) from undesired matrix constituents [Simpson et al., 2008] or they can be combined with (iii) the chemical reaction of the analyte(s) [Ptolemy et al., 2005, 2006], in this way simplifying the overall analytical procedure. On the other hand, great attention must be paid to the selection of the type of chiral selector as its charge can interfere with a sample preparation technique that employs electrophoretic principles.

Analyte	Sample	Chiral selector[a]	Sample preparation: type and main purpose[b]	Detection and LOD	Application	Ref.
Fenoprofen and amino acid derivates	River water	vancomycin	LVSEP-ASEI/on preconcentration (~10^3)	UV, 0.38-2.10 ng/mL	Spiked real samples	Wang Z.Y. et al., 2011
Cimaterol, clenbuterol, terbutaline	Human urine	β-CD	t-ITP and FESI/on preconcentration (250x)	1 ng/mL		Huang L. et al., 2011
Amlodipine	Plasma and urine	HP–α–CD	EME/ preconcentration (124x)	3 ng/mL		Nojavan & Fakhari, 2010
Primary amine drugs (amphetamine)	Human urine	(+)-(18-crown-6)-tetracarboxylic acid	SDME/in preconcentration (~10^3) + clean-up	UV, 0.5 ng/mL	Spiked real samples	Choi et al., 2009
Trihexyphenidyl	Serum	CM–β–CD	LLE/off clean-up, FESS/on preconcentration (490x)	DAD, 0.92 ng/mL	Spiked real samples	Li H. et al., 2008
Pheniramine and its metabolites, dimethindene, dioxopromethazine	Human urine	CE-β-CD	ITP-EKC; ITP/on preconcentration (~10^2) + clean-up	UV, 1.1-4.8 ng/mL	Spiked real samples and metabolic study	Mikuš et al., 2006a
Pheniramine and its metabolites	Human urine	CE-β-CD	ITP-EKC; ITP/on preconcentration (~10^2) + clean-up	DAD, 5.2-6.8 ng/mL	Metabolic study	Marák et al., 2007
Amlodipine	Human urine	HP-β-CD	ITP-EKC; ITP/on preconcentration (~10^2) + clean-up	DAD, 9.3-10.4 ng/mL	Pharmacokinetic study	Mikuš et al., 2008a
Metoxamine, carvedilol, terbutaline, metaproterenol	Serum	β-CD	PP/off clean-up, LVSS/on preconcentration (100-1000x)	DAD, <300 ng/mL	Spiked real samples	Denola et al., 2007
Gemifloxacin	Urine, saliva	CWE	EK clean-up, MCE with coupled channels/on clean-up	UV, LIF, 3-4x10^{-6} M	Spiked real samples	Cho et al., 2004

New adrenoreceptors antagonists	Plasma	HP-β-CD	SPE/off clean-up, FESI/on preconcentration (180x)	UV, MS, 3 ng/mL	Spiked real samples	Grard et al., 2002
Lorazepam and its metabolites	Urine	HP-β-CD, SDS micelles	EH/off, SPE/off clean-up, SWP/on preconcentration	UV, ESI-MS	Metabolic study	Baldacci & Thormann, 2006
S-timolol, 1R,2S-ephedrine	Infusion solution	Ketopinic acid and diisoproylidenek etogulonic acid (NACE)	EK + tITP/on preconcentration	DAD, 0.2 % S-timolol, 0.033% 1R,2S-ephedrine	Enantiomeric purity testing of pharmaceuticals	Hedeland et al., 2007
CBI-amino acids	Squirrel brain samples	S-β-CD	HD + LVSS-SWP/on preconcentration	LIF, 10^{-10} M	Biomedical study	Kirschner et al., 2007
Muramic acid and diaminopimelic acid	Bacterial cultures	OPA/NAC	HD + SPCD/on derivatization + preconcentration, DPJ/on preconcentration (100x)	UV, 2 and $0.2x10^{-6}$ M	Biomedical study	Ptolemy et al., 2005
Amino acids	Bacterial cultures	OPA/NAC, β-CD	EH, SPCD/on derivatization + preconcentration	UV, 0.4-$0.6x10^{-6}$ M	Biomedical study	Ptolemy et al., 2006a
DL-Glutamic acid, baclofen	urine	γ-CD	CFGF/on preconcentration (1200x)	DAD (glutamic acid), LIF (baclofen)	Spiked samples	Balss et al., 2004
Dexbrompheniramine	Pharmaceutical preparations	CE-β-CD	ITP-EKC; ITP/on preconcentration (~10^2) + clean-up	DAD, 2.5 ng/mL	Enantiomeric purity testing of pharmaceuticals	Marák et al., 2008
Terbutaline	Plasma, water matrices	DM-β-CD	EK + SPE/on preconcentration (7000x)	UV, $0.6x10^{-9}$ M	Spiked real and model samples	Petersson et al., 1999
Ephedrine derivatives	Urine	β-CD	SPME/on clean-up, FESI/on preconcentration	DAD, 3-5 ng/mL	Spiked real samples	Fang, H.F. et al., 2006b

(1*R*, 2*R*)-pseudoephedrine, (1*R*, 2*S*)-ephedrine, (1*S*, 2*S*)-pseudoephedrine, (*S*)-(+)-methamphetamine	Urine, serum	β-CD	LLE/on clean-up, FESI/on preconcentration (3800x)	DAD, 0.15 to 0.25 ng/mL	Spiked real samples	Fang, H.F. et al., 2006a
Aspartate	Tissue samples from rats	β–CD	Microdialysis/on clean-up, Derivatization/on	LIF, $9x10^{-7}$ M (D-aspartate)	Biomedical study	Thompson et al., 1999
D-serin + other neurotransmitters	Tissue homogenates	β–CD, HP–β-CD, HP-γ-CD	Microdialysis/on clean-up, Derivatization/on	LIF, $270x10^{-9}$ M; LIF (sheath flow detection cell), $21x10^{-9}$ M	Biomedical study	O'Brien et al., 2003
Isoproterenol	Intravenous dialysed samples	M-β-CD	Microdialysis/on clean-up, pH-mediated sample stacking/on preconcentration	Amperometric detection, 0.6 ng/mL	Pharmacokinetic study	Hadwiger et al., 1996
Bambuterol	Plasma		SLM/on + MLC/on clean-up, double stacking/on preconcentration (40 000x)	UV, $2.5x10^{-10}$ M		Pálmarsdóttir et al., 1996
Terbutaline, but also brompheniramine, propranolol, ephedrine	Plasma, synthetic samples	Alkyl- or hydroxyalkyl-β-CDs	SLM/on + MLC/on clean-up, double stacking/on preconcentration (400x)	UV, $5x10^{-9}$ M	Spiked real and model samples	Pálmarsdóttir et al., 1995

FMOC-carnitine	Synthetic samples	HDM-β–CD	FI/on derivatization	DAD, $5x10^{-6}$ M	Enantiomeric purity testing in model samples	Mardones et al., 1999
Fenoxy acid herbicides	Water matrices	Octyl-β-D-maltopyranoside	FESS/on preconcentration, derivatization/off	LIF, $0.5x10^{-9}$ M	Spiked model samples	Mechref & El Rassi, 1997
Ephedrine, amphetamine and related compounds	Hair	β–CD	LLE/off clean-up, FESS/on preconcentration	DAD, 25-75 ng/mL	Forensic analysis	Tagliaro et al., 1998
β–blockers	Serum	CM–β–CD	PP/off clean-up, FESI/on preconcentration (5-25x)	UV, 10-50 ng/mL	Spiked real samples	Huang L. et al., 2008
3-carboxyadipic acid	Minerals	Vancomycin	FESS/on preconcentration (1000x)	DAD, 10^{-7} M	Environmental analysis	Castro-Puyana et al., 2008
Sulindac and its metabolites	Plasma	DM-β-CD	LLE/off clean-up, FESI/on preconcentration (500x)	UV, $1\text{-}3x10^{-7}$ M	Pharmakokinetic study	Chen, Y.L. et al., 2006
Glufosinate	River water sample	γ-CD	SPE/off clean-up, LVSS/on preconcentration	LIF, $2x10^{-9}$ M	Environmental analysis	Asami & Imura, 2006
Flavins	Bacterial cell extracts, plasma and urine	β-CD, SDS	PP/off clean-up, DPJ-SWP/on preconcentration (60x)	LIF, $4x10^{-9}$ M	Spiked real samples, biomedical study	Britz-McKibbin et al., 2003b
Propiconazol	Grape	HP-γ-CD, SDS micelles	SPE/off clean-up, SWP/on preconcentration (100x)	DAD, 90-100 ng/mL	Spiked real samples	Ibrahim et al., 2007
Triadimenol	Methanol matrices	HS-β-CD, HP-γ-CD	SRMP/on preconcentration (10x)	DAD, 0.8-3.8 ng/mL	Spiked model samples	Otsuka et al., 2003

Tryptophan	Urine	α–CD	ITP-EKC; ITP/on preconcentration + clean-up (~99%)	UV, 1.5 ng/mL	Spiked real samples	Danková et al., 1999
Isoxyzolylpenicilines	Milk	HP–β–CD	LLE/off clean-up, LVSS/on preconcentration	DAD, 2 ng/mL	Food analysis	Zhu et al., 2003
Clenbuterol	Acetic acid matrices	DM–β–CD	tITP/on preconcentration	UV, 10^{-6} M	Spiked model samples	Toussaint et al., 2000

Table 3.1. Chiral CE determinations of biologically active compounds in various biological matrices employing advanced (on-line) sample preparation.

[a] Or derivatization agent creating diastereomeric products.

[b] An on- or off-line mode is given behind slash, preconcentration factor or amount of removed interfering compounds are given in brackets

ITP = isotachophoresis, tITP = transient isotachophoresis, EKC = electrokinetic chromatography, CFGF = counter-flow gradient focusing, CWE = crown ether, NACE = non-aqueous capillary electrophoresis, MCE = electrophoresis on microchip, SPCD = in-capillary sample preconcentration with chemical derivatization, OPA/NAC= *ortho*-phthalaldehyde/*N*-acetyl l-cysteine, S-β–CD = sulphated-β-CD, HS-γ-CD = highly sulphated-γ-CD, M-β-CD = methyl-β-CD, DM-β-CD = dimethyl-β-CD, CM-β-CD = carboxymethyl-β-CD, CE-β-CD = carboxyethyl-β-CD, HP-β-CD = hydroxypropyl-β-CD, HP-γ-CD = hydroxypropyl-γ-CD, HDM-β-CD = heptakisdimethyl-β-CD, EH = enzymatic hydrolysis, HD = hydrodynamic injection, EK = electrokinetic injection, FI= flow injection, DPJ= dynamic pH junction, SRMP=stacking with reverse migrating phase, FESS = field-enhanced sample stacking, LVSS = large volume sample stacking, FESI= field-enhanced sample injection, SPE = solid-phase extraction, LLE = liquid-liquid extraction, LPME = liquid-phase microextraction, LVSEP-ASEI=large volume sample stacking with EOF as a pump plus anion-selective exhaustive injection, PP = protein precipitation, CME = centrifuge microextraction, SDME = single drop microextraction, SLM = supported liquid membrane technique, DAD = diode array detection, UV-ultraviolet (absorbance detection), LIF = laser induced fluorescent detection, MS = mass spectrometry, LOD = limit of detection, SDS = sodium dodecylsulphate, FMOC = 9-fluorenylmethyl chloroformate, EME=electro membrane extraction.

Technique	Principle	Characteristic features
Electrophoretic (stacking)		
FESS: NSM, LVSS, FESI	Analyte from a low-conductivity sample solution zone is concentrated at the boundary of a high-conductivity separation solution zone. Concentration effect is related to conductivity ratio of these two zones ($c_{stacked} \sim G_{separation\ solution} / G_{sample\ solution}$). Water plug is injected prior to the injection of the analyte in FESI.	Advantages. Easy to perform by simply optimizing concentrations or electrical conductivities of the sample and separation solutions. Possibility to inject a selectively large amount of charged analyte from the sample with electrokinetical injection (FESI) with simultaneous preconcentration and purification aspect. Preconcentrations: NSM ~10-fold; LVSS ~100-fold; FESI >1000-fold Limitations. Limited applications {charged analytes, separations only cations or anions in one run, samples with a low-conductivity matrix (can be overcome by pH mediation)}. Requirements {removing of sample matrix (LVSS), EOF suppression}. Reproducibility with FESI (injecting amount is biased by electrophoretic mobility, time depletion of the analyte in the sample solution).
tITP	Temporary ITP action being replaced later by zone electrophoretic regime. Analyte migrates between the highest (leading ion) and lowest (terminating ion) electrophoretic mobility zones of electrolyte solution. The concentration of each separated zone is adjusted to that determined by the concentration of the ion in the foregoing neighbour zone ($c_{analyte\ ion} \sim c_{leading\ ion}$) resulting in the preconcentration of diluted sample zones.	Advantages. Flexible. Directly applicable to samples with a significant matrix ion (acting as a leader). Applicable for low-conductivity (diluted) samples with the aid of added leading ion (inserted in capillary or in sample). Direct analysis of water soluble supernatants of precipitated proteinic samples. Precipitation agent (e.g., acetonitrile) serves simultaneously as terminating ion (transient pseudoisotachophoresis). Sample purification aspect (neutral and oppositely charged compounds do not interfere). Preconcentration: more than three orders. Limitations. Preconcentration only cations or anions in one run. Analysis of neutral compounds is not possible.

DPJ	Based on significant changes in electrophoretic velocities of the analytes between different pH values (BGE solution vs. sample solution with a suppressor of analyte ionization). Long plug of sample zone is gradually titrated by the ion from the BGE solution and the analyte will be ionized in the neutralized zone. The analyte is focused at the neutralization boundary during the neutralization of the sample zone.	Advantages. Selective concentration of the analytes having a narrow range of p*K*a. Useful for the concentration of weakly acidic or basic analytes. Limitations. Electrokinetic dispersion plays an important role in this technique. Only for ionizable analytes (weak acids/bases). Analysis of neutral compounds is not possible.
SWP	Analytes are picked up and accumulated by the appropriate pseudostationary phase (e.g., micelles) that penetrates the micelle free sample zone. The length of the analyte zone after sweep is inversely proportional to the retention factor of the analyte (the ratio of the analyte amounts present in micelle and in solution), $L_{sweep} \sim 1 / k$.	Advantages. Electrokinetic dispersion is minimized by homogeneous electric field strength throughout the whole capillary. High concentration efficiency, possibly up to 5000-fold (according to the affinity analyte vs. pseudophase), or even up to several million-fold (when the length to the detection point is very short and very narrow detection window is employed, e.g., in MCE), or even more (with electrokinetic injection). Suitable for charged as well as neutral analytes. Sample purification aspect (migrate only analytes interacting with sweeping pseudophase), useful for complex matrices. Combinable with nearly every other stacking mechanism. Limitations. The narrow analyte zone created by sweeping tends to broaden quickly according to the diffusion along the capillary (separation efficiency significantly decreases with the length of capillary). Application is limited by availability of appropriate pseudophase.
CFGF	Counter-flow gradient focusing (CFGF) methods can focus the analyte into a concentrated plug via simultaneous acting an electrophoretic velocity and the opposite bulk solution flow so that the total velocity (the sum of both velocities) is equal to zero at a unique point (characteristic for the analyte).	Advantages. CFGF approaches are a potentially simple and versatile way in which to simultaneously concentrate and separate charged and neutral analytes. See also Pressure flow section in this table. Limitations. Specific electrolytes must be used in some of these techniques. See also Pressure flow section in this table.

EOF	Electroosmotic flow (EOF) is the motion of liquid induced by an applied potential across a porous material, capillary tube, membrane, microchannel, or any other fluid conduit. Electroosmotic velocity is dependent of the charge in the electrical double layer, solid surface vs. liquid.	Advantages. EOF enables movement of compounds regardless on their charge. Therefore analysis of neutral compounds is possible, and separation of neutral, positively and negatively charged compounds in one run is possible too. EOF enables an implementation of the countercurrent migration effects (EOF vs. analytes vs. selector) for an enhancement of the separability. A flat EOF profile is favourable for a high separation efficiency. Limitations. An EOF velocity depends of a solid surface area and charge, therefore a precise control of the EOF velocity can be difficult. Hence, the reproducibility of the EOF based systems is lower than the systems with suppressed EOF. It is very pronounced in e.g., CEC systems. EOF cannot be applied in the hydrodynamically closed electrophoretic systems.
Nonelectrophoretic		
Chromatography	The separation is based on a distribution of the analytes between the stationary and mobile phase according to the different interactions of these analytes with the stationary phase. This mechanism differentiates migration velocities of the analytes. Separations are based on chemical principles.	Advantages. An alternative separation mechanism to the electrophoretic one that enables to increase selectivity of the system. The stationary phase serves as an immobilized selector that eliminates any interferences of the selector with the detection. CEC represents a single column chromatographic implementation with EOF as a driving force. Limitations. The chromatographic systems do not provide a concentration of the analytes. Therefore, a chomatographically pretreated sample must be further treated before the electrophoretic separation. A coupling of the pressure driven chromatographic techniques with electrophoresis is difficult in terms of the instrumentation as well as the compactibility of the electrophoretic and chromatographic systems (mobile phase vs. electrolyte).

Extraction	The analytes are trapped on/in a suitable solid/liquid-phase according to the affinities. The distribution constant of the analytes in the sample vs. extraction phase system determines the extraction recovery. Separations are based on chemical principles. The concentration factor depends on the ratio of sample volume : extractor volume.	Advantages. An important sample purification (clean-up) and preconcentration method. The extraction selectivity can be easily modified by the type of extractor and a specific or more universal extraction can then be reached. Limitations. The whole analytical procedure is complex (extraction requires conditioning, loading/sorption, washing, labelling, if necessary, elution/desorption). In the single capillary systems, the entire sample must pass through the capillary, which can lead to fouling or clogging of the separation capillary and significant decreasing of reproducibility of the analyses when particularly problematic samples (like biological ones) are used. A hyphenation of the extraction techniques with electrophoresis is difficult in terms of the instrumentation, however, SPE can be implemented to the electrophoresis easier than LLE.
Membrane techniques	Separations are based on physical principles, i.e., size exclusion. In the microdialysis or filtration, small molecules are able to diffuse across the membrane while large molecules, such as proteins and cell fragments, are excluded. This is the principle of the sample cleanup. Moreover, large molecules are preconcentrated on the membrane in this way.	Advantages. Microdialysis is a widely accepted sampling and infusion technique frequently used to sample small molecules from complex, often biological, matrices. Filtration can be applied directly in electrophoresis avoiding a sample collection. Both techniques can concentrate large molecules, and purify large as well as small molecules. Limitations. Do not concentrate small molecules. In the microdialysis, the minimum volume required for analysis often determines the rate at which the dialysate can be sampled. The whole analytical procedure is complex (dialysis requires preconcentration of the analyte from dialysate). Single column filtration systems are relatively simple but less flexible and versatile. On the other hand, a hyphenation of the membrane techniques with electrophoresis is more difficult in terms of the instrumentation.

Pressure flow	Driving pressure or counter-pressure based flows are provided simply by the pumps and they are controlled electronically. This is important tool also in CFGF.	Advantages. One of the basic tools in the advanced systems enabling a controlled transfer of solutes in various segments of the hyphenated systems (e.g., electrophoretic and non electrophoretic). A counter-flow enables to increase virtually the effective length of the capillary or channel and, by that, increase the separability. Limitations. Fluctuations of these non selective flows decrease the reproducibility of the analysis. An additional instrumentation (pumps and auxiliaries) must be implemented into the analytical system. Dead volumes (tubing and connections) decrease the separation efficiency.

Table 3.2. The most important electrophoretic and nonelectrophoretic techniques and tools applicable in electrophoresis.
FESS = field-enhanced sample stacking; NSM = normal stacking mode; LVSS = large volume sample stacking; FESI = field-enhanced sample injection; tITP = transient isotachophoresis; DPJ = dynamic pH junction; SWP = sweeping; EOF = electroosmotic flow; CEC = capillary; SPE = solid-phase extraction; LLE = liquid-liquid extraction

3.2.1 Single column (in-capillary) electrophoretic techniques

3.2.1.1 Field-enhanced sample stacking

The field-enhanced sample stacking (FESS) is easy to perform in a zone electrophoretic mode (Figure 2.2a) by simply optimizing the sample solution and the separation solution, mainly in their concentrations or electrical conductivities, to constitute different electrical field strengths between the two solutions. To perform preconcentration, the discontinuous zones having different electrical conductivities (*G*) must be constructed along the capillary axis. The analyte from a low-conductivity sample solution zone is concentrated at the boundary of a high-conductivity separation solution zone. The concentration effect is related to the conductivity ratio of these two zones according to Equation 3.1 [Simpson et al., 2008]:

$$c_{stacked} = c_{injected} \cdot \gamma \tag{3.1}$$

where $c_{stacked}$ is the concentration of the analyte concentrated by FESS, $c_{injected}$ is the concentration of the analyte in the sample solution injected, γ is the ratio of the electrophoretic velocities of the ions between the two discontinuous zones (sample zone and BGS, *1* and *2*) having different conductivities (the ratios can be written for velocities, v_1/v_2, intensities of electric field, E_1/E_2, as well as resistivities, ρ_1/ρ_2). From this it is obvious that the sample solution should be prepared in a low-conductivity matrix (as in other stacking modes) that is limiting in terms of application, see Figure 3.1. This technique requires suppressing EOF as EOF velocity is also proportional to the field strength and mixing of the two solutions can occur at the boundary, causing broadening of the focused zone. In practice, we often stack in the presence of EOF, albeit at lesser enrichment. In fact, at high pH, stacking occurs for anions at the rear zone boundary as opposed to the front boundary for cations at low pH. The suppression (reduction) of EOF is more important when electrokinetic injection is employed. The FESS approach is useful for charged analytes, while neutral analytes cannot be directly concentrated (with the exception of their charged complexes) [Kim J.B. & Terabe, 2003]. The FESS can be carried out in the CZE or MEKC mode where the stacking action (see below) is the same while the final separation is based on the CZE or MEKC principles [Lin C.H., 2004; Kim J.B. & Terabe, 2003]. In fact, when a chiral separation is required, the final step following stacking procedure must be principally based on the EKC (chiral MEKC, CDEKC, etc.) mechanism, see chapter 2.

Several techniques have been developed by utilizing the FESS for sample preconcentration [Lin C.H. & Kaneta, 2004; Quirino & Terabe, 2000; Chien R.L. & Burgi, 1992].

(i) Normal stacking mode is the simplest mode, it requires a rather low amount of the injected sample, the EOF must be suppressed and ca. 10-fold concentration can be easily achieved. For schematic diagrams of the normal FESS model carried out in CZE or MEKC modes see Figure 3.2 and Figure 3.3a, respectively. The schemes of FESS with CZE or MEKC separation in Figure 3.2 and Figure 3.3 can be easily modified to the chiral EKC regime implementing chiral selector into the system. Then, the final step after stacking is a chiral EKC separation (i.e., FESS-EKC). For example, the scheme of FESS with MEKC separation in Figure 3.3 can be changed from the achiral (with achiral micelles) to the chiral one (with chiral micelles). Such chiral modification can also be

done for other stacking techniques with the zone electrophoresis separation step (see the techniques described below). Depending on the charge of chiral selector and polarity of electric field the normal or reversed stacking EKC models can be created that offer different separation selectivities. For example, depending on the charge of micelles and the polarity of the electric field, the normal (Figure 3.3a) or reversed stacking MEKC models (Figure 3.3b) can be created.

(ii) Large volume sample stacking (LVSS) requires removing the sample matrix. This can be done with polarity switching where the analysed anion has higher velocity in the opposite direction than the velocity of EOF, or without polarity switching where EOF has to be suppressed as the cations migrate oppositely to EOF. It is possible to separate only cations or anions in one run and more than 100-fold concentration can be achieved. For schematic diagrams of the LVSS model see Figure 3.4.

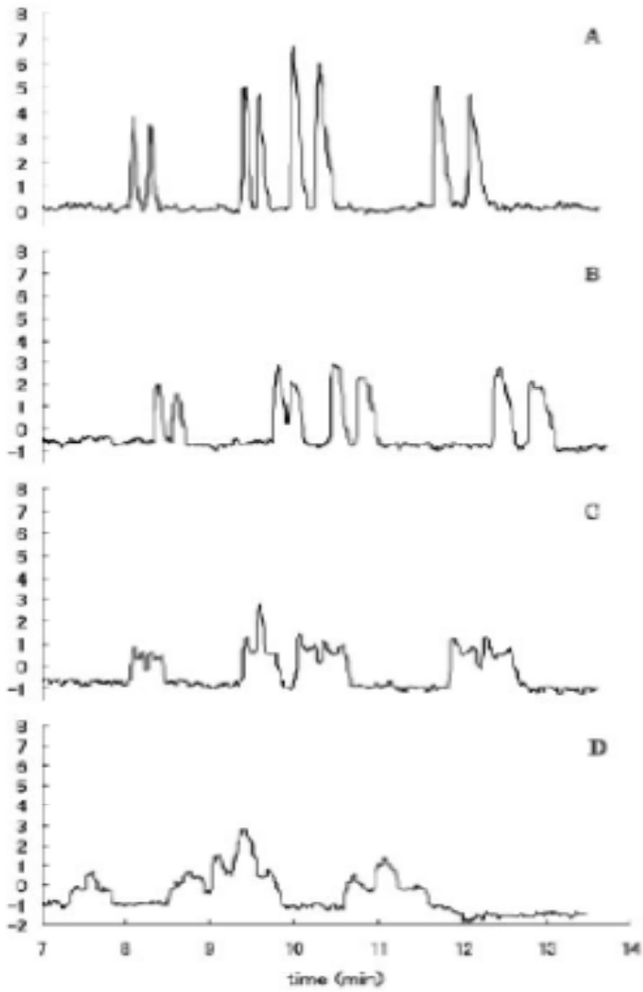

Figure 3.1. Effect of sample matrix on LVSS. Effect of NaCl on the stacking and enantioseparation of the analytes studied. Analytes 0.01 mg/mL in 20% ACN with [NaCl] in A = 0%, B = 0.1%, C = 0.2% and D = 0.4% w/v. Injection length: 20% capillary volume. Reprinted from ref. [Denola et al., 2007].

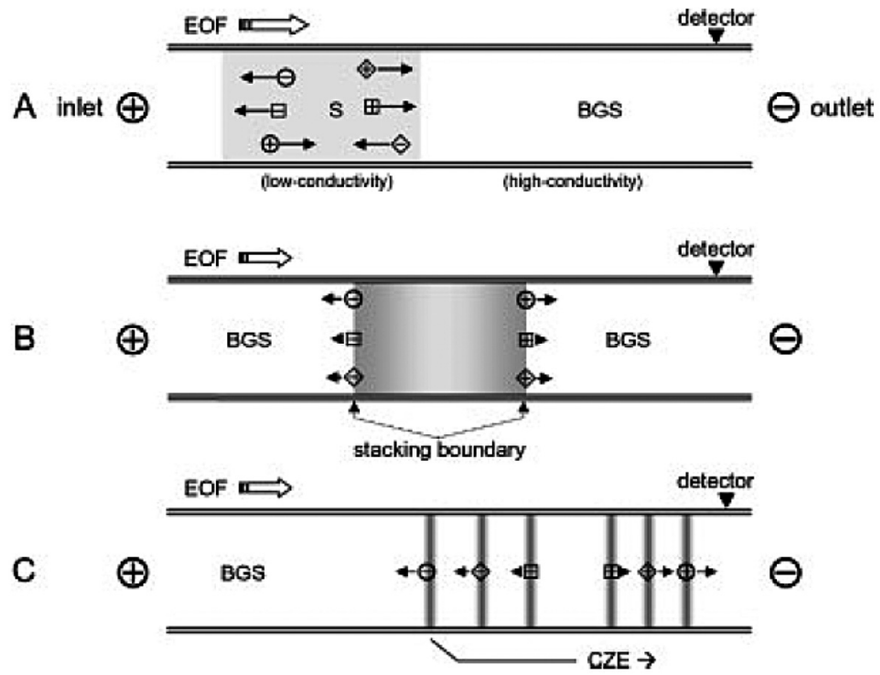

Figure 3.2. Schematic diagrams of the normal FESS model. (A) The capillary is conditioned with a BGS (a high-conductivity buffer), the sample, prepared in a low-conductivity matrix, is then injected to a certain length, and a high positive voltage is applied; (B) focusing of the analytes occurs near the boundaries between the sample zone and the BGS because of its mobility changes; (C) stacked analytes migrate and are separated by the CZE mode. Reprinted from ref. [Lin C.H. & Kaneta, 2004].

(iii) Field-enhanced sample injection (FESI) is based on the injection of a short (usually 2-3 mm, i.e., ca. 0.5% of the effective capillary length) water plug prior to the electrokinetical injection of the analyte. The EOF has to be reduced. Injection of a larger amount of the sample than in (ii) is possible. The injected amount is biased by the electrophoretic mobility. More than 1000-fold concentration is possible, however, injection reproducibility is influenced by depletion of the analyte in the sample solution. Selective injection is given by the charge of the sample constituents.

It should be highlighted that some of the above mentioned electrophoretic on-line preconcentration approaches can be utilized simultaneously also for on-line sample purification. For example, the use of the FESI technique can eliminate potential interfering compounds from the matrix via electrokinetic injection being selective to the sample constituents according to their charge. The selectivity of the electrokinetic injection can be easily influenced by the pH of the sample solution (adjusted by an appropriate buffer if necessary) where the solute of interest has unconditionally to be ionized while the ionization of potential interfering compounds should be suppressed. The FESI can be used for the exhaustive sample injection, e.g., almost all of the ions in a sample can be injected when the volume is small. This is a real advantage to the off-line solvent extraction, evaporation to dryness and redissolving of the analyte in a small amount of dilute buffer.

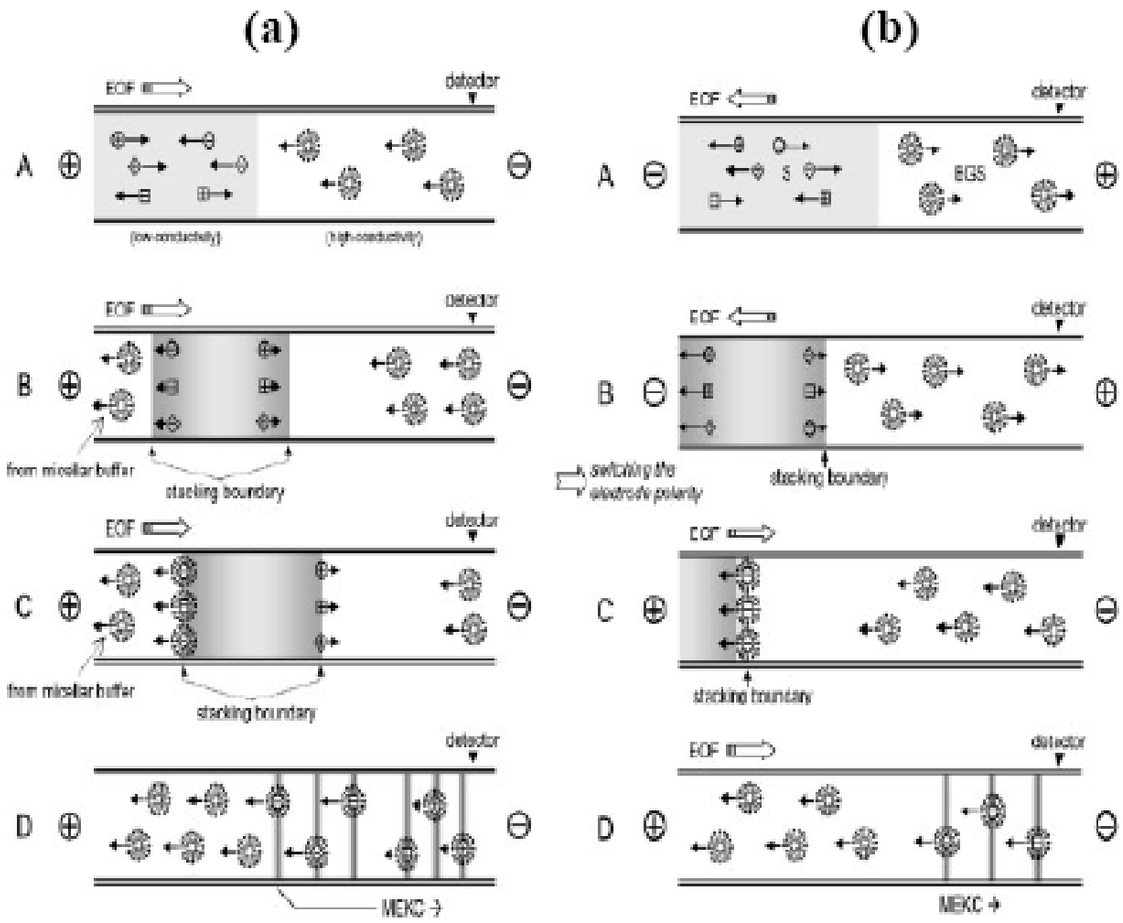

Figure 3.3. Schematic diagrams of stacking MEKC models. (a) A normal stacking MEKC model. (A) The sample is dissolved in a low-conductivity buffer, BGS, consisting of SDS to form the micelles; after the background and sample solution are injected, respectively, a positive voltage is applied; (B) the SDS micelles from the inlet enter the sample zone and then permit the analytes to migrate and become stacked; (C) then the SDS-analytes are separated by the MEKC mode. (b) A reversed stacking MEKC model. (A) The sample and BGS are prepared as described in Figure 3.3aA but a negative polarity is applied; (B) the EOF moves toward the inlet, the anionic analytes move toward the outlet and stack at one side of the boundary; (C) the electrophoretic current reaches approximately 95–99% of its original value, the polarity is quickly returned to positive, reversing the EOF; (D) then the SDS-analytes are separated by the MEKC mode. Reprinted from ref. [Lin C.H. & Kaneta, 2004].

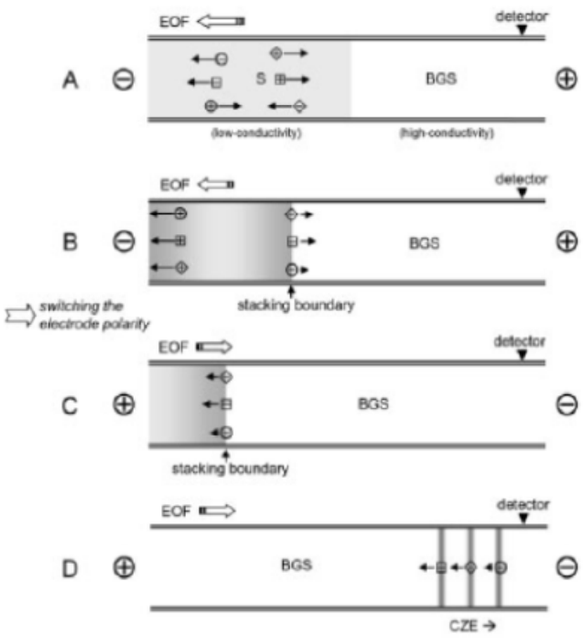

Figure 3.4. Schematic diagrams of the LVSS model. (A) The capillary is conditioned with a BGS (a high-conductivity buffer), the sample, prepared in a low-conductivity matrix, is then injected to a certain length, and then a high negative voltage is applied (EOF is toward the inlet); (B) the anionic analytes move toward the detection end (outlet) and stack at one side of the boundary, whereas the cations and neutral species move and exit the capillary at the injection end (inlet); (C) the electrophoretic current is carefully monitored until it reaches approximately 95–99% of its original value, and the polarity is then quickly returned to positive (EOF is reversed); (D) the following separation occurs by CZE mode. Reprinted from ref. [Lin C.H. & Kaneta, 2004].

However, the FESS methods suffer generally from poor applicability to real samples. (i) For optimal precision, hydrodynamic injection is preferred since we start with the correct number of ions in the capillary. On the other hand, sample depletion and injection reproducibility could be a problem when making more than one electrokinetic injection from a vial. In a practical sense, there is a balance between the degree of enrichment and the stability of the system. This is a significant problem when performing extreme enrichment from real samples. (ii) Poor applicability to real samples is primarily because the complicated (high-conductivity) matrices increase the conductivity of the sample and reduce the efficiency of stacking. This is typical for urine or blood samples which contain salts as matrix macroconstituents. Therefore, they are usually applied in conjunction with off-line sample pretreatment. A pH-mediated FESS method was introduced by the group of Lunte and is an indirect way of changing a high-conductivity sample into a low-conductivity sample to allow field-enhanced sample stacking [Weiss et al., 2001]. Schematic diagrams are shown in Figure 3.5. In the initial step, the sample is prepared in a high-ionic strength medium and is electrokinetically injected into the capillary. Then, a plug of strong acid is electrophoretically injected and a positive separation voltage is applied. The strong acid titrates the sample solution to create a neutral zone (a high-resistance zone). Thus, a proportionally greater field will develop across the neutral zone, causing the ions to migrate faster. As a result, the analytes are stacked at the boundary between the low-conductivity zone (prepared by on-line titration of the sample solution zone, e.g., by a strong acid plug) and the

high-conductivity BGS. A (chiral) separation by the zone electrophoresis mode then occurs. This is a simple and attractive approach for high-conductivity samples, which is growing in popularity. Another strategy introducing a sample with a higher ionic strength than the running buffer has been proposed by Landers et al. [Palmer et al., 1999]. For example, using sodium cholate as the pseudostationary phase and simply adding sodium chloride (or other ions) to the sample matrix, a reasonable enhancement in sensitivity has been achieved for a series of corticosteroids. In the first step the micelles, and not the analytes, are stacked at the boundary between the sample and buffer zone. Subsequently, the analytes migrating with the EOF are enriched in the zone with the high micelle concentration as the micelles are negatively charged and migrate in the opposite direction (Figure 3.6). Summarizing, the stacking effect is dependent on the affinity of the analytes to the micelles that were stacked before, at the boundary between the sample (with increased conductivity, e.g., by adding salt) and the buffer zone. This approach is attractive due to its robustness towards other sample constituents. Although these last two procedures have not been used for chiral analysis so far, their potential in this field is apparent. Other strategies for samples with high ionic strengths, also used in the chiral field, are based on appropriate combinations of different stacking techniques as briefly discussed, inter alia, in section 3.2.1.5.

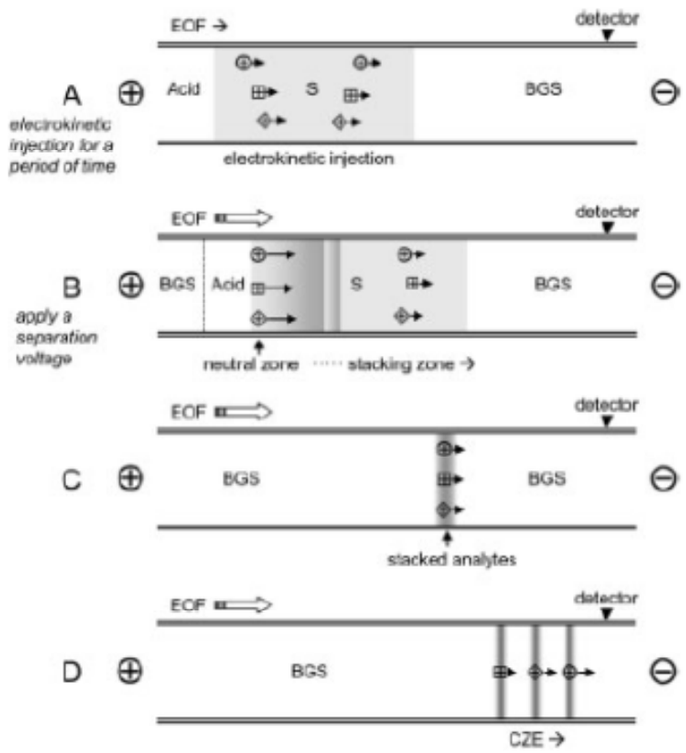

Figure 3.5. Schematic diagrams of a pH-mediated stacking model. (A) The capillary is conditioned with a high-conductivity BGS, the cationic analytes dissolved in a low-conductivity buffer are electrokinetic injected into the capillary, and then a plug of strong acid is also electrokinetically injected; (B) a positive separation voltage is applied; (C) the strong acid titrates the sample solution to create a neutral zone causing the ions to migrate faster and become stacked; (D) the subsequent separation occurs by the CZE mode. Reprinted from ref. [Lin C.H. & Kaneta, 2004].

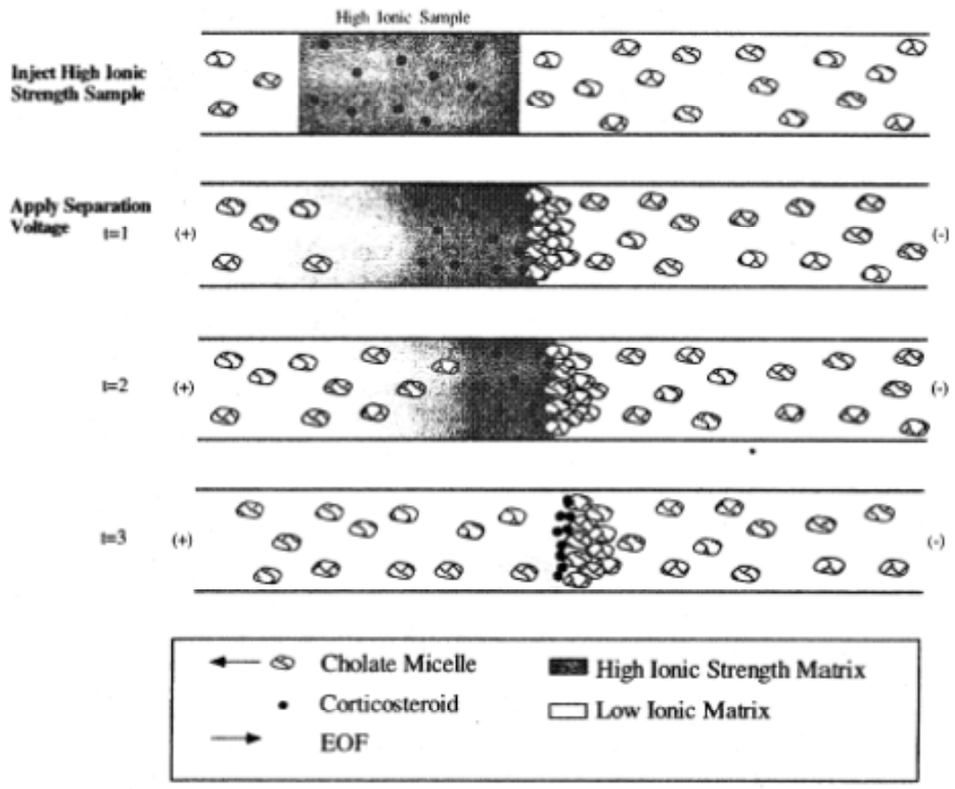

Figure 3.6. Mechanism for stacking in MEKC with a high ionic strength matrix. Reprinted from ref. [Palmer et al., 1999].

3.2.1.2 Isotachophoresis and transient isotachophoresis

Analytes are separated in isotachophoresis (ITP) as adjoining successive zones in the order of decreasing electrophoretic mobilities, migrating between the leading (the highest electrophoretic mobility) and terminating (the lowest electrophoretic mobility) electrolyte solution zones (Figure 2.2b). In the presence of voltage applied, the concentration of each separated zone is automatically adjusted to that determined by the concentration of the ion in the foregoing neighbour zone according to Equation 3.2 [Simpson et al., 2008]:

$$c_A = \frac{c_L \mu_A \left(\mu_L + \mu_Q\right)}{\mu_L \left(\mu_A + \mu_Q\right)} \qquad 3.2$$

resulting in the preconcentration of diluted sample zones. In this equation, $\mu_L > \mu_A$ and c_A, c_L are concentrations of analyte ion, A, in the adjoining zone to the leading ion, L, zone and μ_A, μ_L, μ_Q electrophoretic mobility of A, that of L, and that of the counter ion Q (assumed the same ion for A and L), respectively. ITP is a flexible and powerful method for on-line concentration. It is directly applicable to samples with a significant matrix ion and can also provide very high enrichment factors for low-conductivity samples.

The principle of ITP can be applied to preconcentration in zone electrophoresis techniques (CZE, EKC), which is termed as transient ITP (tITP) [Beckers & Boček, 2000; Schwer et al., 1993]. Schematic diagrams of tITP are shown in Figure 3.7. There are several modifications in tITP to perform sample preconcentration, such as the following configurations (i) a low electrophoretic mobility background solution (e.g., borate) is inside the capillary, a leading electrolyte (e.g., chloride ion) solution is injected as the first plug, the sample solution is injected as the second plug, sample solution zones are preconcentrated by the tITP mechanism and further migrate as zones in zone electrophoresis, (ii) as in (i) but the sample and leading solutions are mixed and then injected as one plug. It is particularly suited to the analysis of trace components in samples with a significant matrix ion in which that matrix ion functions as a leader. This is called 'sample self-stacking' in the literature. (iii) Transient pseudoisotachophoresis is a modification of previous tITP modes, based on the addition of a water miscible organic solvent (e.g., acetonitrile, acetone, methanol, 2-propanol) serving as a pseudoterminating ion [Shihabi, 2002]. Of course, it cannot be a terminating ion in the true sense. The organic solvent serves as a zero mobility low-conductivity zone. This approach is attractive for the analysis of biological fluids because acetonitrile added 2:1 to the sample (making 66% acetonitrile) is used for protein precipitation and it is therefore possible to directly inject the supernatant and achieve a 20-30 fold improvement in sensitivity.

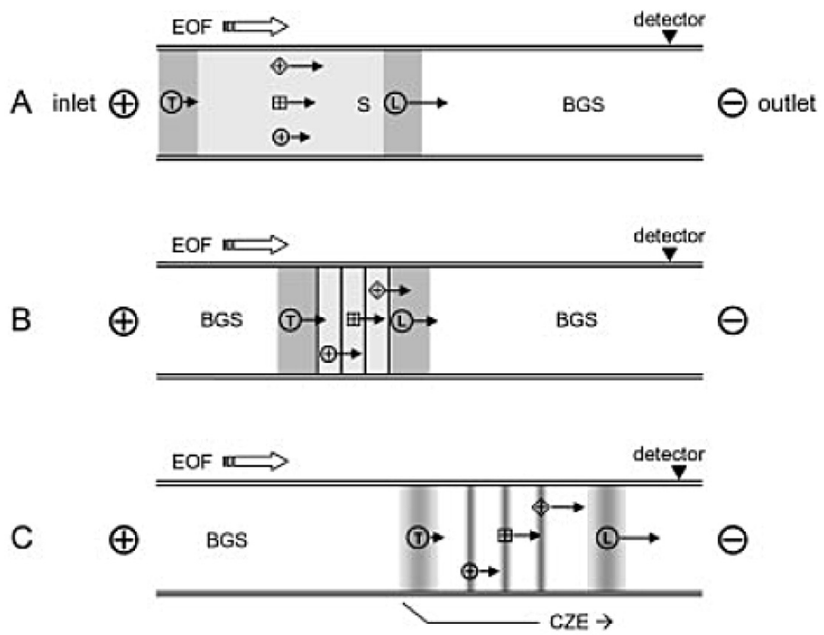

Figure 3.7. Schematic diagrams of a tITP model for cations. (A) The capillary is conditioned with a BGS, the leading electrolyte and sample solution, and terminating electrolyte are then injected in turn - a high positive voltage is also applied; (B) concentration of the analytes occurs between the leading and the terminating ions during tITP migration; (C) the concentrated analyte zones are separated by the CZE mode. Reprinted from ref. [Lin C.H. & Kaneta, 2004].

Like FESI, tITP and ITP is adversely affected by changes in sample conductivity. The ITP and tITP techniques are applicable to charged analytes only and simultaneous analysis of

oppositely charged analytes is not possible. On the other hand, this limitation can be understood as an advantage in terms of selective removal of neutral or oppositely charged sample matrix constituents.

3.2.1.3 Dynamic pH junction

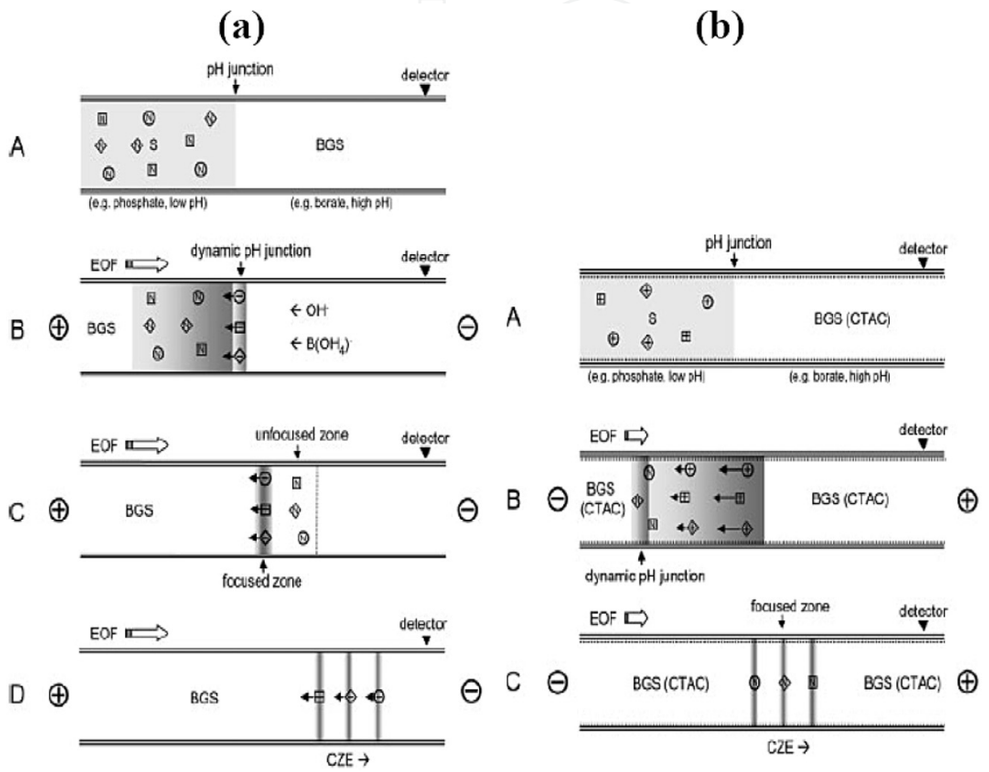

Figure 3.8. Schematic diagrams of dynamic pH junction models. (a) The normal and (b) reversed dynamic pH junction models. (a) (A) The capillary is filled with a high pH-BGS and a section of sample solution (prepared in a lower-pH buffer); (B) a high positive voltage is applied, resulting in a discontinuous electrolyte zone; (C) the anionic analytes are focused on the boundary of the pH junction; (D) separation of the analytes occurs by the CZE mode. (b) (A) The capillary is filled with BGS (prepared in a higher-pH buffer containing CTAC) and sample solution (prepared in a lower-pH matrix); (B) a negative voltage is applied because of the addition of CTAC the EOF moves toward the outlet; the cationic analytes move toward the inlet and change to neutral at the rear boundary due to the change in pH; (C) separation of the analytes occurs by the CZE mode. Reprinted from ref. [Lin C.H. & Kaneta, 2004].

The dynamic pH junction (DPJ) technique utilizes significant changes in ionization states of the analytes or electrophoretic velocities between different pH values [Britz-McKibbin & Chen, 2000; Aebersold & Morrison, 1990]. Possible configuration is as follows: the capillary is filled with an alkaline background solution (high concentration, low electrophoretic mobility), a long plug of a weakly acidic analyte dissolved in an acidic matrix (high concentration, high mobility) is injected, a positive voltage is applied at the injection end, the acidic sample zone is gradually titrated by the hydroxide ion in the alkaline background

solution from the cathodic side and the analyte will be ionized in the neutralized zone. The negatively ionized analyte will migrate toward the anode, but if it enters into the acidic sample zone it will be protonated again to neutral and stop the electrophoretic migration. Thus, the weakly acidic analyte can be focused at the neutralization boundary during the on-line neutralization of the sample zone. Two modes of the DPJ can be used for the analyte preconcentration, namely the normal (Figure 3.8a) and reversed (Figure 3.8b) DPJ model, providing conditions for preconcentration of anionic or cationic analytes, respectively. This focusing technique is different from sample stacking since the conductivity of the sample matrix is not of great importance; it can be less than or greater than that of the BGS. The electrokinetic dispersion plays an important role in this technique [Kim J.B. et al., 2003]. The DPJ technique can selectively concentrate analytes having a narrow range of p*K*a and it is useful for the concentration of weakly acidic or basic analytes, as well as zwitterionic analytes. This technique has been used in the chiral field in appropriate combinations with other on-line preconcentration techniques, see section 3.2.1.5.

3.2.1.4 Sweeping

Sweeping (SWP) can be defined as a phenomenon whereby analytes are picked up and accumulated by the appropriate pseudostationary phase (micelles, microemulsions, charged cyclodextrins) that penetrates the sample zone. The homogeneous electric field strength is given by the similar conductivity of the nonmicelle sample zone (provided by the addition of salt if needed) and the running micelle solution. Homogeneous electric field strength is assumed throughout the whole capillary under SWP conditions different from field-enhanced stacking techniques. The schematic diagrams of SWP preconcentration are shown in Figure 3.9. The length of the analyte zone after sweep, L_{sweep}, is inversely proportional to the retention factor of the analyte, k (the ratio of the analyte amounts present in micelle and in solution), according to Equation 3.3. [Simpson et al., 2008]:

$$L_{sweep} = L_{inj}\left[\frac{1}{(1+k)}\right] \tag{3.3}$$

where L_{inj} is the length of the sample solution injected. It is apparent that the analyte having a higher k value is more efficiently concentrated. According to the affinity of the analyte to the pseudostationary phase the concentration efficiency can be very high, possibly up to 5000-fold. The narrow analyte zone created by SWP, however, tends to broaden quickly according to the diffusion along the capillary (CE) or channel (MCE). Therefore, when the length to the detection point is very short and a very narrow detection window is employed, very high concentration efficiency, up to several million-fold, can be observed [Kitagawa et al., 2006]. Electrokinetic injection in combination with SWP has made it possible to inject a large volume of samples, and, by that, additionally increase sensitivity [Palmer et al., 2001]. However, commonly used SWP pseudophases do not provide specific interactions with the analyte and many interfering compounds from the sample matrices with affinity to the pseudophase can be swept (enriched) together with the analyte. Therefore, a sample clean-up technique along with the SWP technique can be required to introduce into analytical protocol too, especially when analysing biological samples [Hempel, 2000; Baldacci & Thormann, 2006].

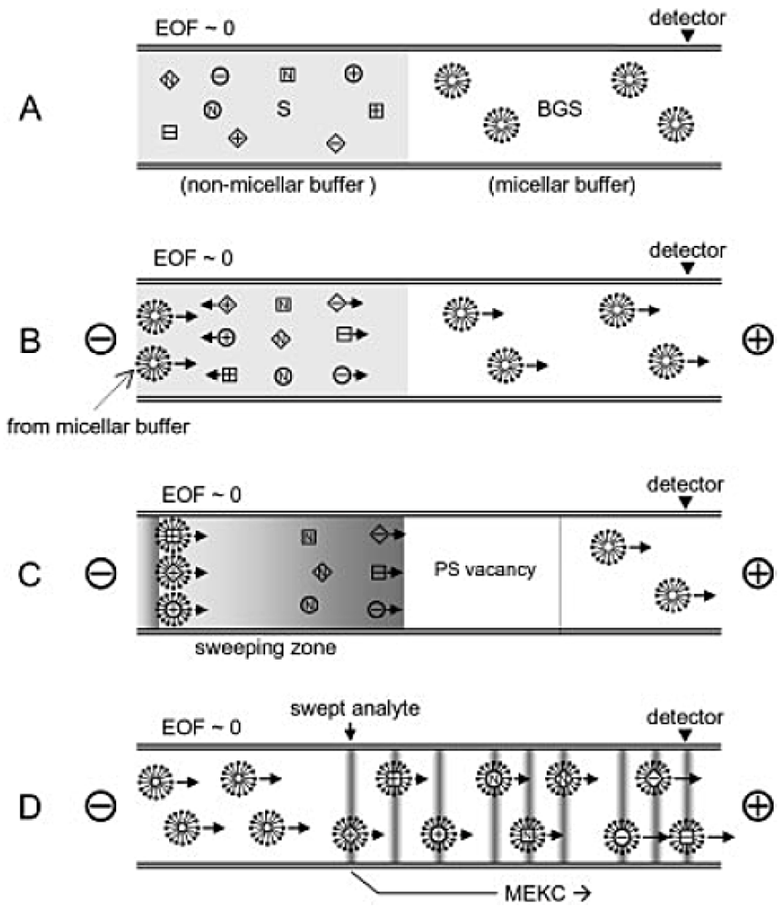

Figure 3.9. Schematic diagrams of a reversed sweeping MEKC model. (A) The BGS consists of a surfactant (for example, SDS, a negatively charged surfactant) and electrolytes to form a micelle buffer, but the samples are dissolved in a nonmicelle buffer; (B) after the injection of the BGS and the sample solution, a negative polarity is applied to power the CE separation; (C) the cations and anions move toward the inlet and outlet , respectively, and anionic SDS micelles enter the capillary sweeping the analytes; (D) the analytes are completely swept by SDS, the subsequent separation occurs by the MEKC mode. Reprinted from ref. [Lin C.H. & Kaneta, 2004].

SWP was originally developed for the on-line concentration of neutral analytes for MEKC separation [Quirino & Terabe, 1998]. The SWP approach is suitable also for charged analytes, regardless of the charge of the analyte and the direction of EOF. Strong Coulomb interactions between oppositely charged analytes and the pseudophase can be reflected in a higher k value and, subsequently, more efficient preconcentration [Quirino & Terabe, 1999; Quirino et al., 2000]. SWP is a perfect combination for nearly every other stacking mechanism. Its complementarity so far has really only been used for sensitivity enhancement, but it could also be used to selectively enrich specific analytes. This will become increasingly important as more integrated methods are developed for the analysis of more complex samples with less off-line sample pretreatment (clean-up).

3.2.1.5 Single column combinations of sample preparation techniques based on electrophoretic principles

A combination of two of the electrophoretic on-line sample preconcentration techniques described in sections 3.2.1.1-3.2.1.4 can be more efficient in increasing detection sensitivity, preconcentration selectivity and spreading application capabilities (i.e., analysis of a wider range of analytes, differing by their charge, polarity, etc., in one experiment), according to particular demands. It can also overcome some limitations of these methods when used separately.

Most of these combinations have been used only in achiral analyses so far, however, their potential in the chiral field is apparent. As it is believed that they will appear in the chiral field in the near future, it is useful to list them (corresponding schemes of these techniques can be found in the cited references or in ref. [Lin, C.H. & Kaneta, 2004]):

(i) FESI + tITP (i.e., electrokinetic supercharging, EKS): 3000-fold concentration, analysis of ionic analytes in diluted samples [Fang L. et al., 2006]. Compared with conventional electrokinetic injection, the enhancement factors can be greatly improved, e.g., to be 250-fold [Huang, L. et al., 2011].

(ii) pH-mediated FESS + DPJ: only for ionisable analytes [Chou Y.W. et al., 2008]. This technique has been developed to enhance analyte focusing for CE for the analysis of physiological samples. The process results in ultra-narrow peak widths and no dilution of the sample to lower ionic strength is necessary. In comparison with normal base stacking and electrokinetic injection, mass loading capacity can be increased with this technique without degradation in peak shape and resolution is dramatically improved.

(iii) tITP + DPJ: 50-fold increase in sensitivity, nM LODs of cationic metabolites [Hou J. et al., 2007]. The authors depict three major transitions experimentally observed in the process: (a) initial tryptophan (Trp) focusing at the back end of the sample-BGE boundary, (b) partial focusing of Trp with residual peak fronting and (c) complete focusing of Trp within the original sample plug. The authors demonstrated that CE could serve as an effective preconcentrator, desalter and separator prior to ESI-MS, while providing additional qualitative information for unambiguous identification among isobaric and isomeric metabolites. The proposed strategy is particularly relevant for characterizing yet unknown biologically relevant metabolites that are not readily synthesized or commercially available.

(iv) FESI + SWP: up to million-fold increase in sensitivity [Rudaz et al., 2005; Servais et al., 2006]. This technique has two modes. (a) The cation selective exhaustive injection (CSEI) + SWP. The CSEI-SWP-MEKC method provides for a more sensitive detection than sweeping and is sufficiently flexible to offer the potential for achieving an increase in the detection limit of more than 100 000-fold, for positively chargeable analytes [Rudaz et al., 2005]. (b) Alternatively, anion selective exhaustive injection (ASEI)-SWP-MEKC, using a cationic surfactant, offers under optimized conditions an approximately 1000 to 6000-fold improvement in LODs of negatively chargeable analytes [Servais et al., 2006]. Applications of the CSEI-SWP-MEKC method: (methamphetamine, ketamine, morphine and codeine in hair, LODs of 50–200 pg/mg hair) [Huang Y.S. et al., 2003], (amphetamine, methamphetamine and hydroxymethamphetamine in urine, LODs of 15–20 ppm) [Theurillat & Thormann, 2008], (amphetamine, methamphetamine and methylenedioxymethamphetamine, 6–8 ppt LODs, several 1000-fold improvement in detection sensitivity compared with typical injection) [Tsimachidis et al., 2008; Theurillat et al., 2007].

(v) Electrocapture. Horáková et al. [Horáková et al., 2007] presented a novel approach which they called 'electrocapture'. This technique makes possible the determination of nanomolar concentrations of weak acidic analytes in CE. The method consists of long-running electrokinetic sample injection and stacking (electrokinetic immobilization) of the analytes at a boundary of two electrolytes with different pH values (pH 9.5 and 2.5), and consequent mobilization of the stacked uncharged analytes in a micelle system (containing SDS micelles). The micelle system can provide for further concentrating the analytes by sweeping. An approximate 4600-fold increase of the sample concentration (in comparison with the standard CZE) can be achieved during the preconcentration step.

Some of the techniques based on combined electrophoretic principles in a single column arrangement have also been used for chiral analyses, or, at least, a chiral separation environment has been utilized in these techniques. These are the following methods:

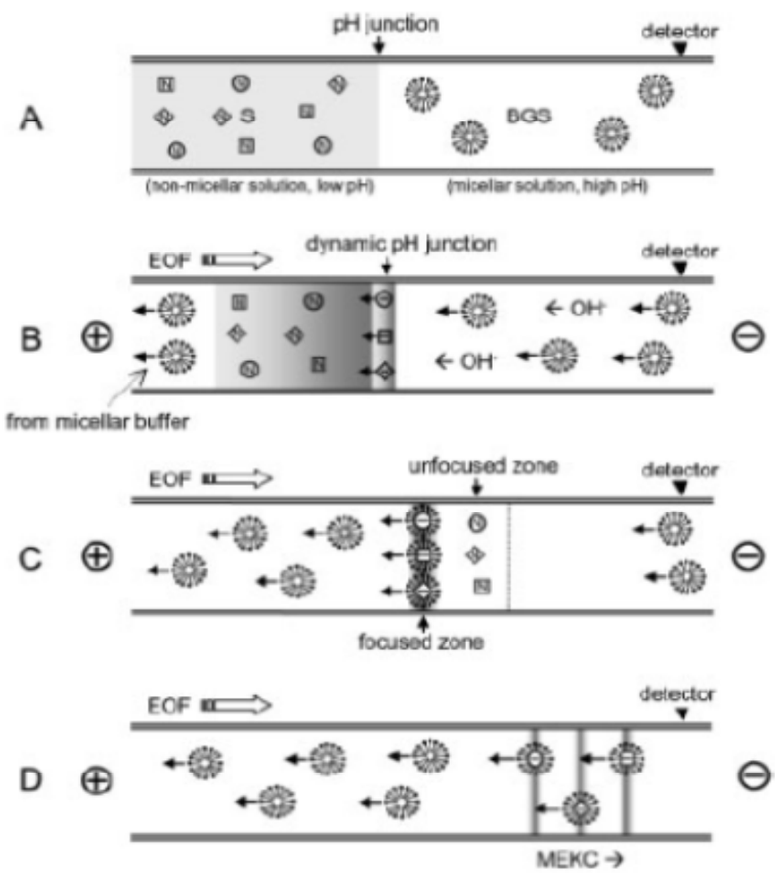

Figure 3.10. Schematic diagrams of the DPJ-SWP model. (A) The micelle (such as SDS) BGS and the sample solution (a nonmicelle buffer) are injected into the capillary, respectively; (B) when the injection is complete, a positive polarity is applied (if a negatively charged SDS surfactant is used) to power the CE separation; (C) the neutral analytes are converted to anions and are swept by the SDS micelles; (D) separation occurs by the MEKC mode. Reprinted from ref. [Lin C.H. & Kaneta, 2004].

(i) SWP + DPJ: suitable for a mixture of neutral and weakly acidic/basic analytes [Britz-McKibbin et al., 2002], for illustrative scheme see Figure 3.10. This method is very effective for overcoming the often poor band-narrowing efficiency of conventional SWP (using anionic micelles) and the DPJ for hydrophilic and neutral analytes, respectively, even if the migration time needed for separation is much longer than that of a conventional DPJ or SWP. This method was applied for plasma and urine samples. A picomolar detectability can be achieved by CE-LIF detection without the need for laborious off-line preconcentration and clean-up [Britz-McKibbin et al., 2003].
(ii) LVSS (a combination of field-enhanced stacking and pH-mediated stacking) + SWP: LODs up to 10^{-10} M with LIF detection, applied for animal tissues [Kirschner et al., 2007], for illustrative scheme see Figure 3.11.
(iii) FESI + tITP: applied for the simultaneous on-line preconcentration and enantioseparation of drugs in urine samples. The detection limits in ng/mL levels can be easily obtained [Huang, L. et al., 2011].

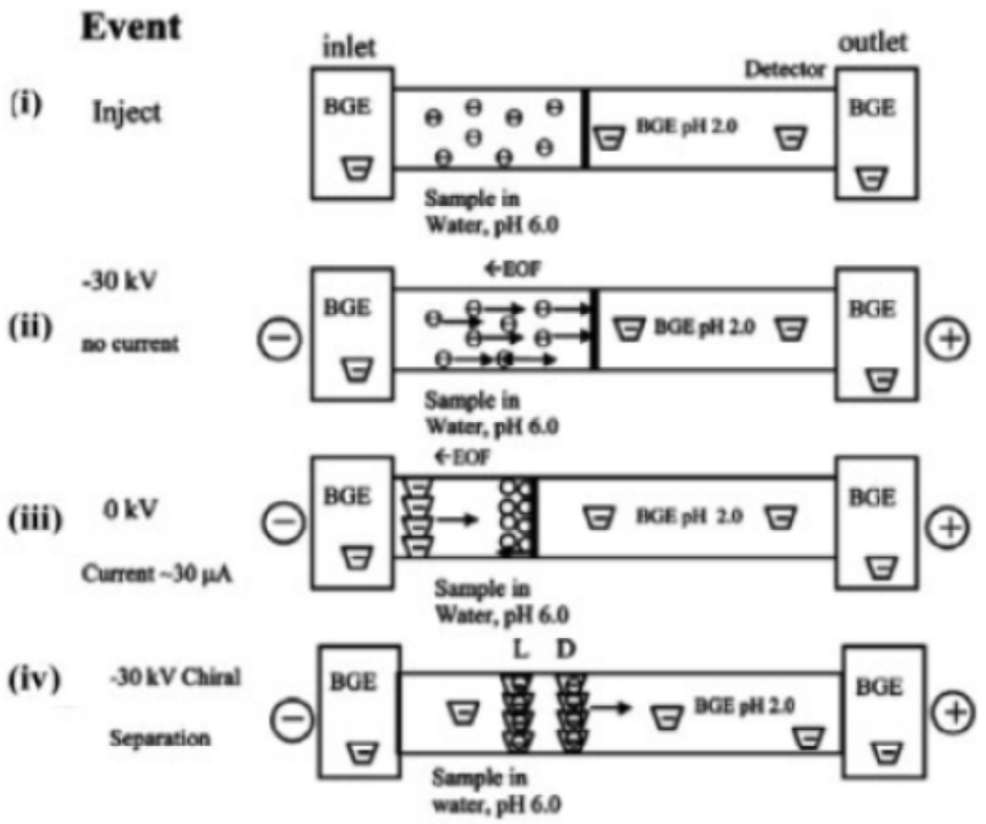

Figure 3.11. Schematic diagram of the stacking/sweeping: (i) hydrodynamic injection of large volume (1/3 of the capillary) of the CBI–amino acids in water at pH 6.0; (ii) migration toward the pH junction at the outlet side of the injection plug of the anionic CBI–amino acids; (iii) pumping water out of the capillary and movement of the stacked analyte band toward the inlet by the EOF; (iv) sweeping of the HS-β-CD through the stacked band of analyte. Adapted from ref. [Kirschner et al., 2007].

3.2.1.6 Single column combination of sample preparation techniques based on electrophoretic principles with chemical reaction

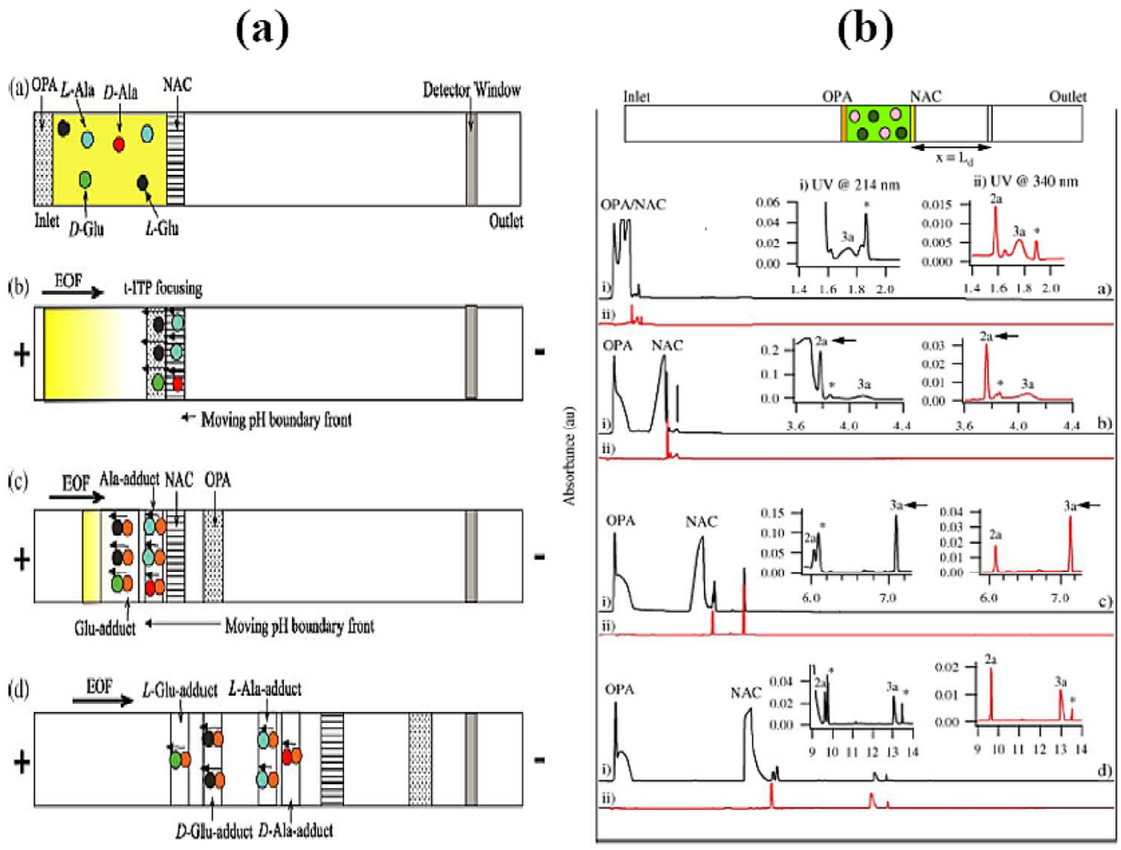

Figure 3.12. SPCD-CE method. (a) Proposed mechanism and kinetics of analyte electrokinetic focusing with in-capillary derivatization of amino acid enantiomers by SPCD-CE using OPA/NAC based on time-resolved electrophoretic experiments depicted in Figure 3.12b. Note the rapid analyte focusing with labelling of the long sample plug via tITP with subsequent zone passing of reagents, as well as the distinct time-dependent electrokinetic focusing of labelled amino acid-adducts at later stages by the moving pH boundary. (b) Series of electropherograms showing the distinct time-dependent processes of electrokinetic focusing and in-capillary OPA/NAC derivatization by SPCD-CE. Electropherograms were monitored with UV absorbance: (i) 214 nm and (ii) 340 nm. Arrows note time-delayed analyte electrokinetic focusing mediated by a dynamic pH junction. All samples contained 20 μM D-Ala and D-Glu in 40mM phosphate, pH 6.0. A long sample plug (6.1 cm, using a sample injection of 100 s) was placed at different positions from the capillary window using a low pressure rinse (0.5 psi or 3.5 kPa) in order to change the effective capillary length (Ld) from (a) 0 cm, (b) 11.4 cm, (c) 22.8 cm and (d) 30.5 cm. Other conditions: 140mM borate buffer, pH 9.5; voltage 25 kV; capillary length 67 cm; internal diameter; 50μm; UV 340 nm. Analyte peak number corresponds amino acid-isoindole adducts, where 2a: D-Ala, 3a: D-Glu. Reprinted from ref. [Ptolemy & Britz-McKibbin, 2006].

In-capillary sample preconcentration with chemical derivatization (SPCD) is included as an innovative strategy [Ptolemy et al., 2005, 2006; Ptolemy & Britz-McKibbin, 2006]. The general principles of SPCD–CE for single-step enantioselective analysis of submicromolar levels of analytes are: (i) multiple hydrodynamic injection sequence (appropriate arrangement of sample and derivatization reagent(s) zones); (ii) on-line sample preconcentration (electrokinetic focusing); (iii) in-capillary chemical labelling by zone passing of derivatization reagent(s); (iv) chiral separation of diastereomeric analyte adducts formed, see illustrative schemes in Figure 3.12a and corresponding electropherograms in Figure 3.12b.

3.2.1.7 Single column combination of electrophoretic migration with counter-flow

Counter-flow gradient focusing (CFGF) approaches [Shackman & Ross, 2007] are a potentially simple and versatile way in which to simultaneously concentrate and separate charged and neutral analytes. These types of techniques have the potential to concentrate each sample component in a uniquely different place in the separation space (similarly to the IEF where each component is concentrated at its pI region, see Figure 2.2c). Over the last decade a number of new approaches that can focus analytes at positions according to their electrophoretic mobility (electric field gradient focusing, temperature gradient focusing) and interaction with a pseudophase (micellar affinity gradient focusing) have been developed. It is worthwhile noting that counter-flow gradient focusing methods are exceptionally flexible: they can be used as an analytical method in itself or as an electrophoretic equivalent to solid-phase extraction (SPE), whereby analytes can be selectively captured and/or released in a well-defined and controlled manner.

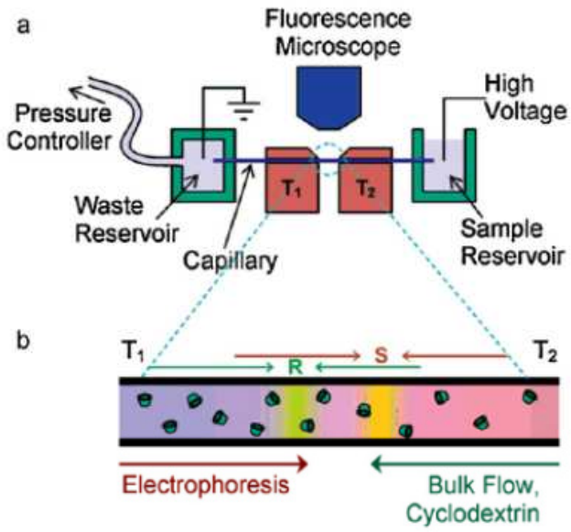

Figure 3.13. TGF method. (a) Schematic illustration of the TGF apparatus. A linear temperature gradient is formed along the capillary in the 2-mm space between the copper blocks regulated at temperatures T1 and T2. (b) Schematic of chiral TGF separations. T1, 13°C (left side in image); T2, 40°C; +1000 V/cm; BGS,10 mM γ-CD in 1 M Tris–borate (pH 8.3). The D-enantiomer peak is to the left in the figure; the L-enantiomer is to the right. Reprinted from ref. [Balss et al., 2004].

The method termed temperature gradient focusing (TGF) [Ross & Locascio, 2002; Balss, 2004; Danger & Ross, 2008; Kitagawa & Otsuka, 2011], applied also in the chiral field, relied upon the use of a buffer with a temperature-dependent ionic strength (such as tris-borate) so that application of a temperature gradient would result in an electrophoretic velocity gradient. For the scheme of the TGF apparatus and separation mechanism see Figure 3.13. For real-time chiral TGF separation see an application example in section 3.5. The advantages of the TGF method included easier implementation than electric-field gradient focusing (EFGF), as well as the ability to focus wider classes of analytes, see review [Shackman & Ross, 2007]. The maximum reported concentration enhancement was 10 000-fold in 100 min. A disadvantage of TGF can be the limited variety of buffers having the necessary temperature-dependent ionic strength.

3.2.2 Column-coupled (hyphenated) electrophoretic techniques

The theory and analytical potentialities of the column coupled electrophoretic techniques carried out in the capillary, as well as channel (microchip), formats and applied in the field of advanced pharmaceutical and biomedical analysis are comprehensively presented in the latest monograph chapter of the authors [Mikuš & Maráková, 2011]. On the other hand, in the present book/section the theory and potentialities of the column-coupled electrophoresis with chiral aspect are given.

The CE performed in a hydrodynamically closed separation system (hydrodynamic flow is eliminated by semipermeable membranes at the ends of the separation compartment) can be easily implemented into advanced CE systems, e.g., into those operating with coupled columns [Kaniansky & Marák, 1990; Kaniansky et al., 1993, 1994a, 1994b]. In addition to the single column (in-capillary) sample preconcentration and purification approaches (section 3.2.1), an on-line column combination of two CE methods can effectively solve problems of sample preparation and final analysis in one run in a well-defined way, i.e., producing high reproducibility of analyses. The Kaniansky group has been carrying out detailed research on the basic aspects, instrumentation and utilization of on-line coupled CE techniques for many years with interesting results. The proposed and commercially available CE-CE systems have a modular composition that provides a high flexibility in arranging particular modules in the separation unit, creating desirable CE-CE combinations (e.g., ITP-ITP, ITP-EKC, EKC-EKC, small or large volume injection) capable of solving a wide range of advanced analytical problems.

One pioneering work [Kaniansky & Marák, 1990] demonstrates the analytical potential of on-line coupling of ITP with CZE in the trace analysis of multicomponent ionic mixtures, see the instrumental scheme in Figure 3.14 for a general point of view. The ITP stage serves for the sample preparation and CZE stage for the final separation and detection of the analyte, and the following benefits can be recognized: (i) the ITP technique is useful for the separation of only cations or only anions in one run and this can be considered as an in-column ITP sample clean-up. (ii) ITP provides preconcentration of the sample constituents along with the analyte (see 3.2.1.2). (iii) In the column coupling separation system the post-column ITP sample clean-up (removing of undesired zones migrating in ITP) is performed via a proper switching of the direction of the driving current to the counter-electrodes. This enables transfer of an ITP zone of the concentrated analyte with only minimum interfering

compounds (up to 99% or even more interfering compounds can be isolated [Danková et al., 1999]) into the second CE stage for the final separation and detection. It should also be mentioned that the use of tubes with larger internal diameter in CE analysis (300-800μm, typical in hydrodynamically closed separation systems) is favourable for their enhanced sample load capacities (30μl sample injection volumes are then common) linked with lower LODs [Kaniansky et al., 1997]. Using an ITP-CZE method, it is possible to analyse directly ultratrace charged analytes (lower ng/mL regions even with conventional detectors, e.g., UV absorbance detector) in complex ionic matrices (e.g., biological fluids) with minimum sample handling (e.g., only dilution) and to improve the LOD commonly more than 100-fold (depending on ionic matrix composition/concentration) in comparison with single column CE.

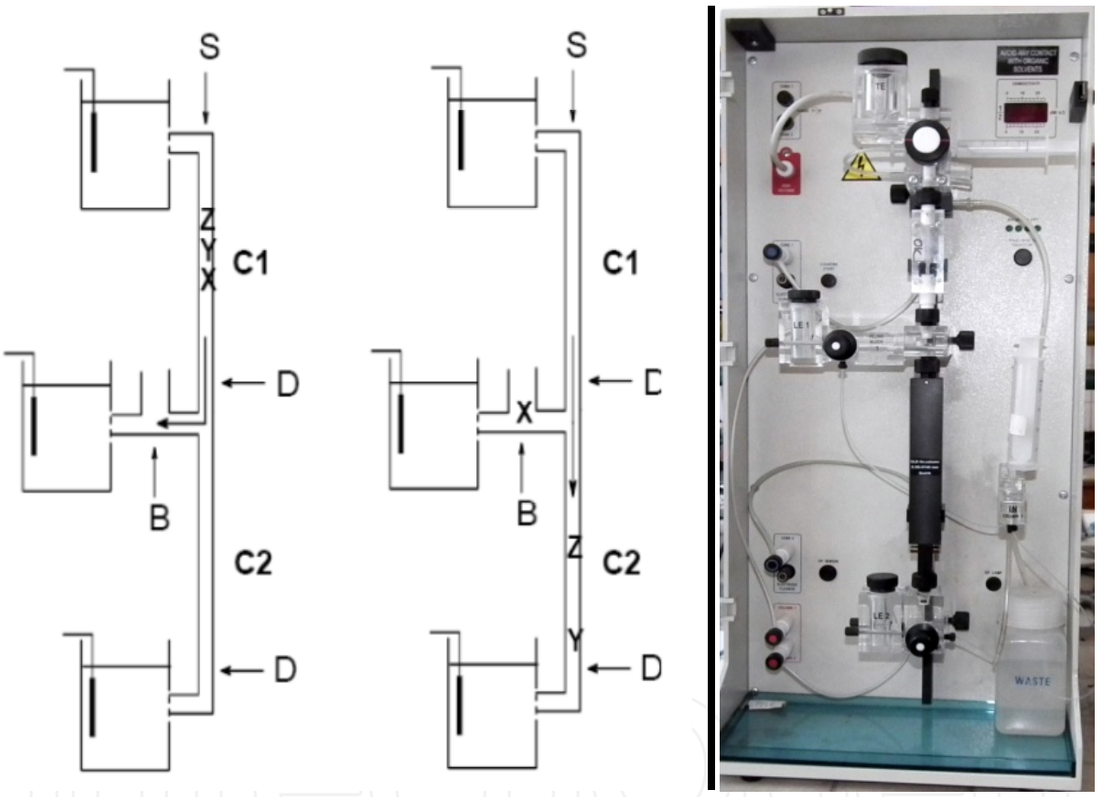

Figure 3.14. ITP-EKC technique in the capillary format and column coupling configuration of the separation units for the direct analysis of samples with the unpretreated complex matrices. The instrumental schemes (left and middle) and the photo of the corresponding commercial equipment, capillary electrophoresis analyser EA-102 (Villa-Labeco, Spišská Nová Ves, Slovakia), (right). On-line sample preparation: removing matrices X, preconcentration of enantiomers Y, Z in the first ITP stage (column C1). Final separation: enantioseparation of Y and Z in the second EKC stage (column C2). C1 – ITP column, C2 – EKC column, B – bifurcation block for coupling C1 and C2, D – positions of detectors. The instrumental schemes are adapted from ref. [Tekeľ & Mikuš, 2005].

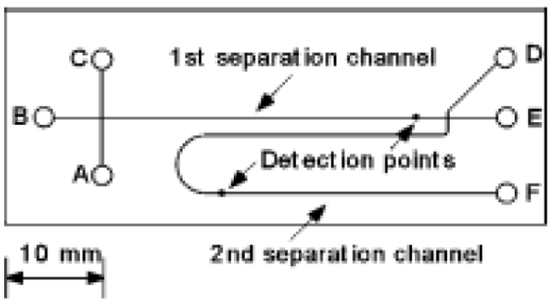

Figure 3.15. Schematic layout and dimensions of the microchip with on-line coupled separation channels. Reservoirs are labelled as: (A) run buffer; (B) sample; (C) sample waste; (D) buffer waste; (E) run buffer containing a chiral selector; and (F) buffer waste containing the chiral selector. The channel depth was 35 μm and width at a half-depth was 60 μm. The dots represent the detection points, which are located at 32 and 38 mm from the first and second injection crosses, respectively. Reprinted from ref. [Cho et al., 2004].

The CE-CE methodology is easily adaptable to chiral analysis. ITP-EKC is the most popular combination for the ultrasensitive determinations of chiral analytes present in complex matrices, see the schematic separation of electrophoretic zones in Figure 3.14 and several application examples in section 3.5. Neutral, as well as charged, chiral selectors are usually implemented in the EKC stage of the ITP-EKC combination [Danková et al., 1999; Fanali et al., 2000; Mikuš et al., 2006a, 2008a, 2008b; Marák et al., 2007]. Nevertheless, an ITP-ITP combination is also applicable for chiral analyses as the implementation of both neutral and charged chiral selectors in the ITP is possible [Ölvecká et al., 2001, Mikuš et al., 2006b; Kubačák et al., 2006a, 2006b, 2007].

On-line combinations of the electrophoretic techniques performed on chip (coupled channels), see Figure 3.15, offer additional advantages of microscale analysis as they are described in the introduction part of chapter 3 and section 2.2.3 (and references given therein) and also provide good possibilities for speed chiral analyses of drugs in minute amounts of sample with the model [Ölvecká et al., 2001], as well as complex matrices [Cho et al., 2004], as illustrated by an application example in section 3.5.

3.3 On-line sample preparation techniques based on nonelectrophoretic principles

The on-line sample preparation can be carried out advantageously also combining CE with other than electrophoretic techniques. Most of these approaches are based on extraction or chromatographic principles, but also other techniques, such as membrane filtration or microdialysis (separations based on physical principles), can be used. Electrophoresis and nonelectrophoretic on-line sample preparation techniques can be properly combined to achieve a desired effect. Lately several such approaches have been introduced, as illustrated by application examples in section 3.5.

On the other hand, attention must be paid when on-line sample preparation based on sorption-desorption or distribution mechanism is combined with a chiral system, as chiral

molecules can influence these mechanisms through competitive complexing equilibria. For example, a partial filling of the separation capillary with chiral electrolyte can be employed to avoid the elution of the enriched solutes during flushing of the sorbent with chiral electrolyte [Petersson et al., 1999].

3.3.1 Chromatographic techniques

A microcolumn liquid chromatography (MLC) can be used in an on-line arrangement with the CE for sample purification and concentration allowing the injection of microlitre volumes into the electrophoresis capillary [Bushey & Jorgenson, 1990; Pálmarsdóttir et al., 1995]. For the instrumental scheme of the MLC-CE see Figure 3.16. The combined system has a much greater resolving power and peak capacity than either of the two systems used independently of each other. The selectivity and sensitivity gain of combining MLC with CE for determination of low concentrations of chiral drugs in biosamples is exemplified in section 3.5. However, the MLC-CE coupling is technically much more difficult than the CE-CE because it has to be accompanied by collection, evaporation and reconstitution of fraction isolated by MLC.

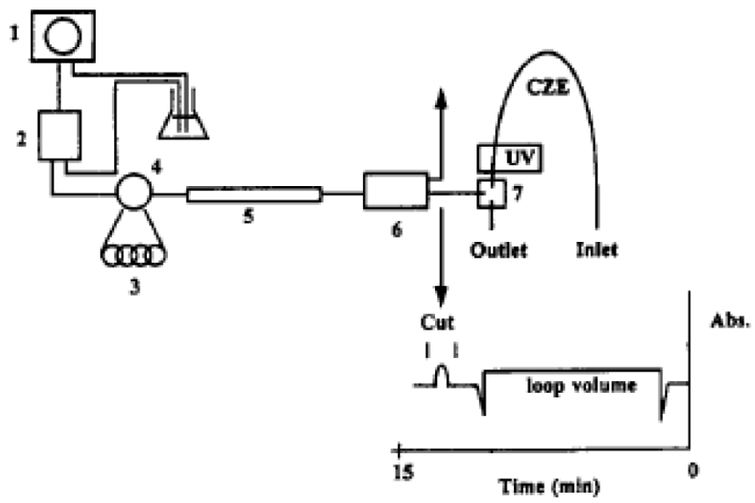

Figure 3.16. Experimental set-up of MLC coupled on line with CE. (1) Pump; (2) flow processor; (3) loop; (4) valve; (5) analytical column; (6) p.-dumper interface; (7) Tee adapter. Reprinted from ref. [Pálmarsdóttir et al., 1995].

3.3.2 Extraction techniques

Extraction techniques now play a major role in sample preparation in CE. These techniques can be used not only for reconstitution of the sample from small volumes, but also for sample clean-up in complex matrices and desalting for very saline samples that would interfere with the electrophoretic process. Considerable progress has been made towards the coupling of solid-phase extraction (SPE) with subsequent electrophoresis, while coupling of liquid-phase extraction (LLE) with electrophoresis is less used. The review by Breadmore et al. [Breadmore et al., 2009] gives attention to on-line or in-line extraction methods that have been used for electrophoresis.

3.3.2.1 Solid-phase extraction / microextraction

Solid-phase extraction (SPE) is the most attractive way of coupling extraction with CE and, especially, MCE. This is in particular because it can provide significant improvements in sensitivity without the use of electrokinetic injection [Puig et al., 2007a, 2008; Bertoncini & Hennion, 2004]. Depending on the nature of the adsorbent chemistry, this can be specific for certain analytes, such as through the use of a biopolymeric phase (see a generalized mechanism in Figure 3.17), or more generic for the extraction of a range or classes of compounds, such as a C18 reverse phase material, ion-exchanger resins, etc. These solid phases can be present in various formats, such as particles, parallel open channels and monoliths.

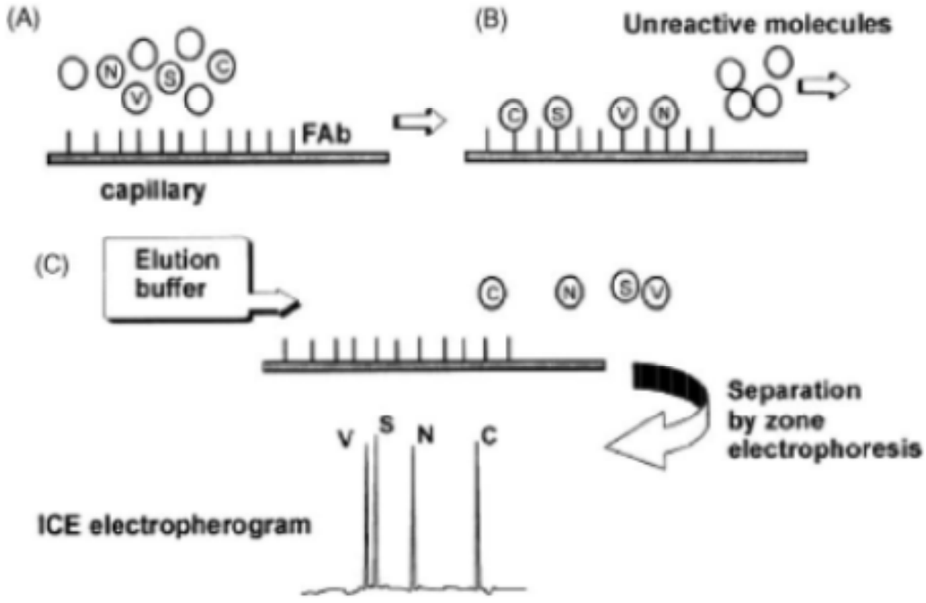

Figure 3.17. Diagrammatic representation of the 3-D biospecific extraction employing biopolymer coupled to CE technique. (A) Analyte percolation and capture phase. (B) Washing of non-retained compounds. (C) Acid elution of the analytes and separation by CE. V, S, N, C = chiral analytes. Adapted from ref. [Phillips, 1998].

In-line systems are created inserting solid-phase column into capillary (see Figure 3.18a, b) and they are attractive thanks to their low cost and easy construction. The whole analytical procedure includes conditioning, loading/sorption, washing, (labelling, if necessary), filling (by electrolyte), elution/desorption, separation and detection, see an example in Figure 3.18c. One of the main limitations of performing in-line SPE is that the entire sample, washing and elution solvents must pass through the capillary, which can lead to fouling of the separation capillary, particularly when problematic samples are used.

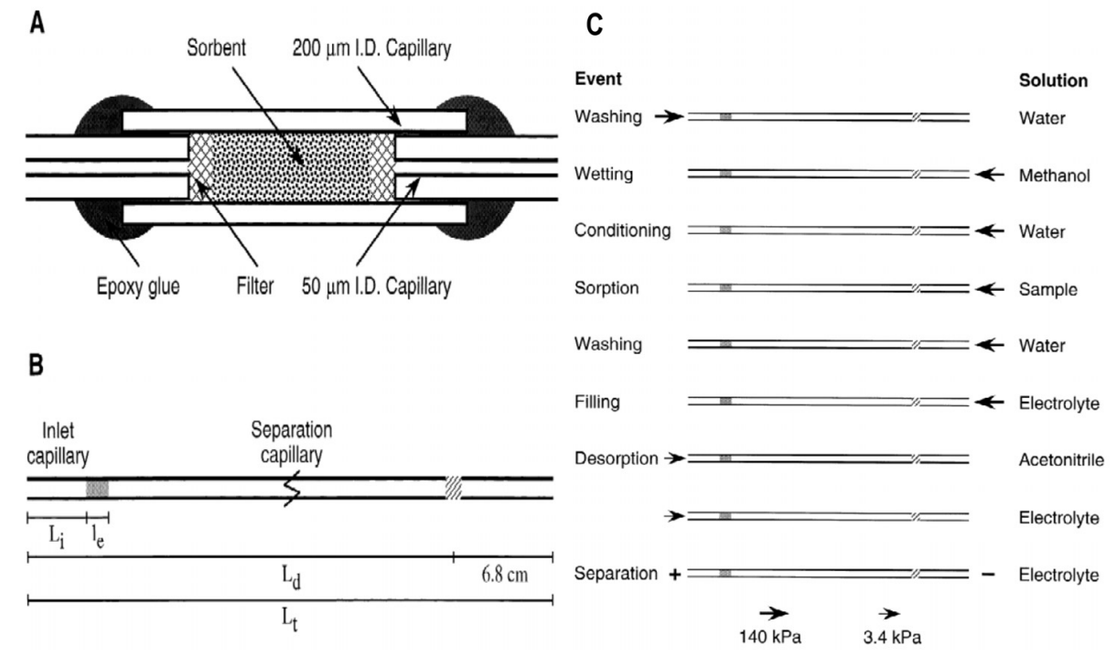

Figure 3.18. Miniaturised on-line SPE for enhancement of concentration sensitivity in CE. Cross-section of (A) the extractor and (B) the enrichment capillary where L_t (28–58 cm) is the enrichment capillary total length, L_d (21.2–51.2 cm) is the length to the detector, L_i (5.4 cm) is the length of the inlet capillary and l_e (1–3 mm) is the extractor length. (C) Sample enrichment procedure for terbutaline dissolved in water. Arrows indicate flow directions. The post-sorption washing with water is optional, as the electrolyte filling, in which non-retained solutes are flushed out of the capillary, usually is enough for rather clean samples. Reprinted from ref. [Petersson et al., 1999].

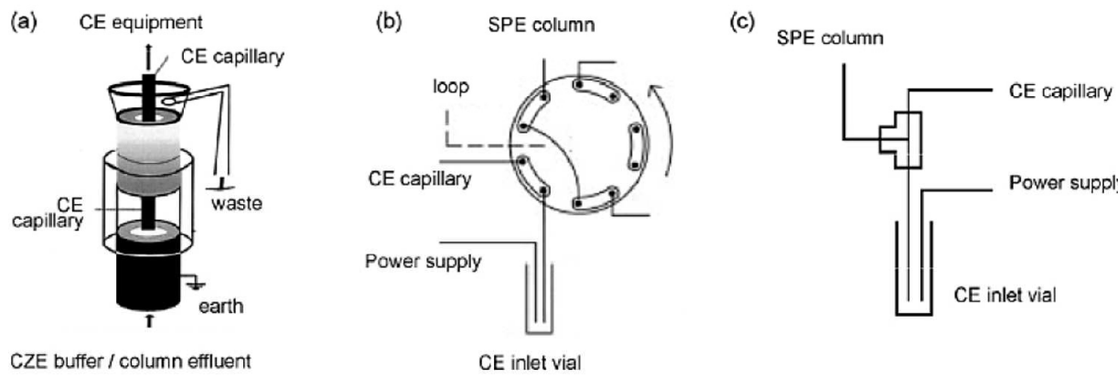

Figure 3.19. Schematic diagram of the three types of interfaces for on-line SPE–CE coupling: (a) vial interface; (b) valve interface; (c) T-split interface. Reproduced from refs. (a) [Stroink et al., 2003], (b) [Tempels et al., 2007] and (c) [Puig et al., 2007b].

In order to overcome this issue, on-line methods may be used, although care must be taken to ensure that no efficiency is lost in the transferral (e.g., dead volume must be minimized). Nowadays, the most used on-line SPE interfaces are the vial-type, the valve-type and the T-

split-type [Bonneil & Waldron, 2000; Tempels et al., 2006; Puig et al., 2007b; Jiménez & de Castro, 2008]. Figure 3.19 shows a schematic representation of these interfaces. On-line SPE interface ensures that during the extraction waste solvents from the wash step are redirected, providing a cleaner extract for analysis. However, one of the major limitations of this approach is that because of the dead volume of the system, the entire eluate is not injected for separation, hence, some of the sensitivity gain is lost. Zhang and Wu [Zhang L.H. & Wu X. Z., 2007] presented a novel and conceptually simple approach to overcome this by creating a small hole in the capillary just after the SPE phase. The hole in the capillary allowed sample and wash solutions to be redirected to waste away from the separation capillary, while ensuring that the entire volume of solution used to elute the analytes was used for electrophoretic separation. While the integration level is impressive and the improvement in the LOD is sufficient (more than 10 000-fold is not unusual), practical analyses can be limited by the loading times in some cases (injection time can even be several hours). With respect to shortening analysis time, microchips offer a more attractive way of integrating SPE with electrophoresis, see examples in the review paper by Breadmore et al. [Breadmore et al., 2009]. However, unlike capillary format (SPE-CE), on-line coupled SPE-MCE has not been applied in chiral analysis so far.

Solid-phase microextraction (SPME) is an increasingly used technique because it is simple, can be used to extract analytes from very small samples and provides a rapid extraction and transfer to the analytical instrument. Moreover, it can be easily combined with other extraction and/or analytical procedures, improving to a large extent the sensitivity and selectivity of the whole method [Pawliszyn, 1997; Lord & Pawliszyn, 2000; Ouyang & Pawliszyn, 2006; Saito & Jinno, 2003]. As an example, an interface for SPME–CE–MS coupling is given in Figure 3.20. The on-line coupling of microextraction with the chiral CE has been described in the literature. For example, a direct chiral analysis of primary amine drugs in human urine by single drop microextraction in-line coupled to CE was demonstrated by Choi et al. [Choi K et al., 2009]. Examples on sensitive chiral analyses by means of microextraction-CE are stated in section 3.5. However, such coupling has not been widely used in practice because of its inherent drawbacks regarding the low injection volumes typically required in the CE (which are crucial to obtaining a good separation efficiency) and also because the different sizes of the separation capillaries usually used for CE and the SPME fibres [Liu Z. & Pawliszyn, 2006].

A general problem with the SPE/SPME-CE is the poisoning of the concentrator by matrix components and also their adsorption on the capillary wall. The use of coated capillaries, like poly(vinyl alcohol) (PVA), can decrease problems involved with protein adsorption. However, PVA capillaries are not available in the dimensions often required. Washing with sodium hydroxide is excluded due to incompatibility with the silica-based sorbent. Alternatives, then, are the use of polymer-based sorbents [Knudsen & Beattie, 1997] or a detergent such as SDS [Lloyd & Wätzig, 1995].

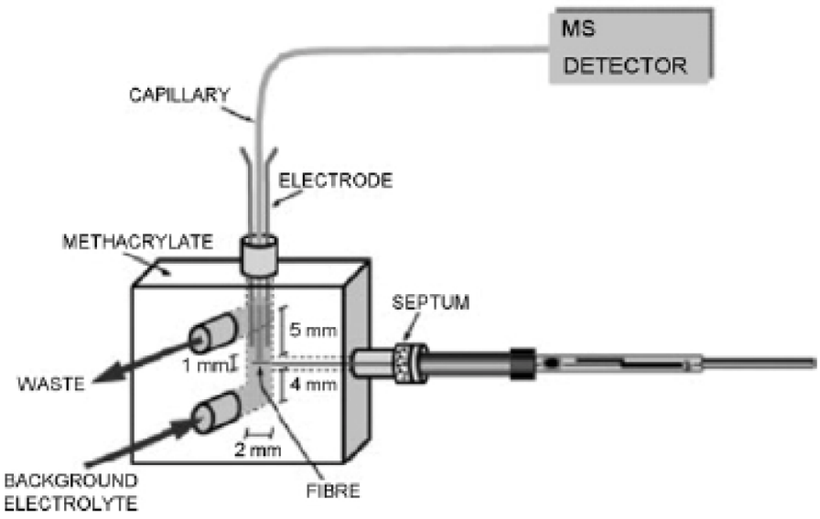

Figure 3.20. Interface for SPME–CE–MS coupling. Reproduced from Santos et al. [Santos et al., 2007].

3.3.2.2 Liquid-phase extraction

The miniaturization of liquid-phase (or liquid-liquid) extraction (LLE) has benefits in minimizing organic solvent consumption and sample amount requirements. Moreover, it simplifies and (partially) automates the extraction process. However, there are a number of technical issues that must be overcome for the development of an on-line integrated system. Recent progress covering the whole field of liquid-phase microextraction can be found in reviews on the subject by Bjergaard and Rasmussen [Bjergaard & Rasmussen, 2008], Lee et al. [Lee et al., 2008] and Xu et al. [Xu L. et al.; 2007]. The authors summarize miniaturized and highly flexible formats for LLE combinable with separation techniques, including CE, and they also gives views on environmental and bioanalytical applications of this coupled technique.

Integration of LLE with CE is based on an on-line back extraction system with FESI [Fang H.F. et al., 2006a]. In this approach the weak bases are first extracted into an organic solvent following a conventional off-line LLE protocol. The organic solvent containing the analytes is then placed in the sample vial and a small amount of water is placed on top. The analytes distribute between the organic phase (donor solution) and aqueous phase (acceptor solution), where they are partially protonated. Electrokinetic injection of the charged analytes depletes the analytes from the aqueous layer, disrupting the equilibrium, and more analytes are transferred from the organic phase into the water plug. Upon entering the capillary, analytes stack by normal FESI principles. Using this approach, a several thousand-fold increase in sensitivity can be obtained accompanied with sample purification. Other modifications of this approach are based on the use of a Teflon micromembrane [Almeda et al., 2007] or propylene hollow fibre [Nozal et al., 2007] filled with an acceptor solution and placed between the capillary and sample vial, see a scheme of LLE-CE equipment in Figure 3.21. For a detailed illustration, a schematic description of the construction of the liquid-phase microextraction unit can be seen in Figure 3.22.

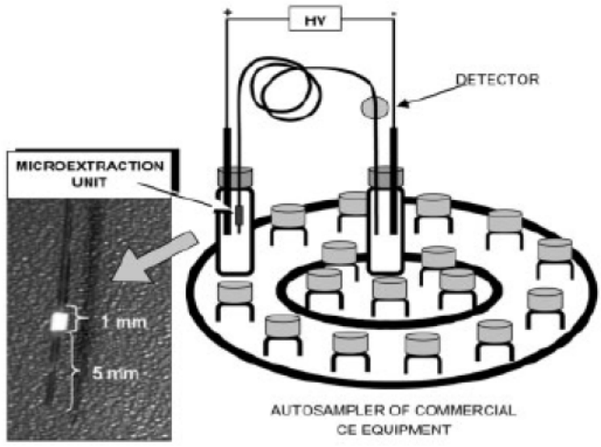

Figure 3.21. In-line liquid-phase microextraction – capillary electrophoresis (LLE-CE) arrangement for the determination of nonsteroidal antiinflammatory drugs in urine. Reprinted from ref. [Nozal et al., 2007].

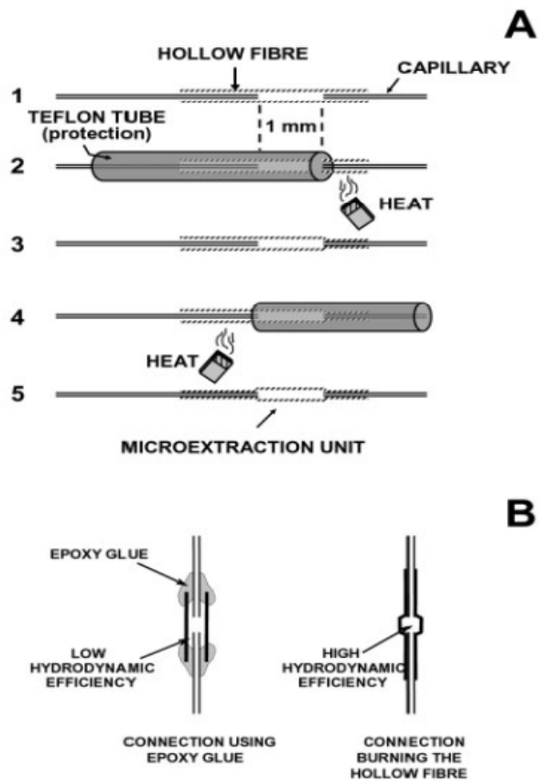

Figure 3.22. (A) Description of the construction of the liquid-phase microextraction unit. (1) Capillaries lining up and insertion in the hollow fibre; (2) protection of the hollow fibre with a Teflon tube and burning of the free part for capillary-hollow fibre connexion; (3) system with one connexion; (4) performance of the second connexion and (5) microextraction unit integrated into the capillary. (B) Comparison of connexions performed with epoxy glue and by burning the hollow fibre. Reprinted from ref. [Nozal et al., 2007].

3.3.3 Membrane filtration, microdialysis

Analytes can also be concentrated by inducing a velocity change due to their size by physically restricting their movement, so called concentration by physically induced changes in velocity. This has traditionally been most easily performed with large molecules, such as proteins and DNA [Yu C.J. et al., 2008]. Implementation of nanoporous media (nafion membrane, anionic hydrogel plug, etc.) in microchips (MCE is dominant in this field) has led to a number of interesting developments where the concentration of much smaller molecules is possible [de Jong et al., 2006; Holtzel & Tallarek, 2007; Dhopeshwarkar et al., 2008; Long et al., 2006]. Sensitivity enhancements of 4–6 orders of magnitude make this method powerful for sensitivity enhancement. A small piece of membrane (e.g., with 10 nm pores) can be integrated into a microchip also for the isolation of small molecules from crude samples. The potential of this approach was demonstrated with the analysis of biomarkers in blood without any off-line protein removal [Long et al., 2006]. A scheme of membrane – preconcentration/purification device implemented on-line into the CE is given as an example in Figure 3.23, where the detail of membrane insertion can be clearly seen.

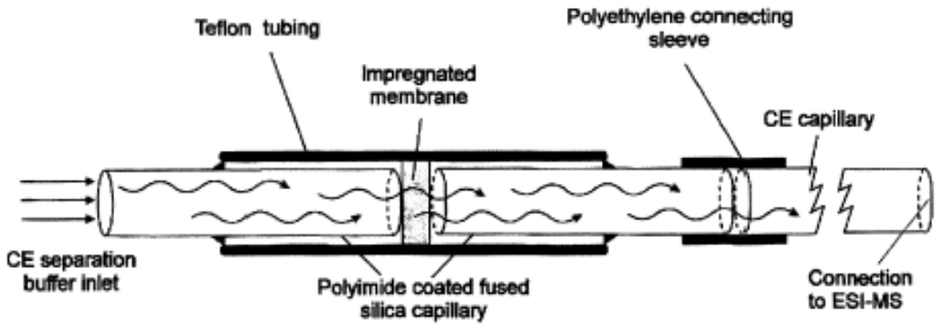

Figure 3.23. Membrane – preconcentration device with styrene-divinyl-benzene membrane to concentrate samples on-line in CE. Reprinted from ref. [Barroso & de Jong, 1998].

A microdialysis is frequently used to sample small molecules from complex, often biological, matrices [Adell & Artigas, 1998; Robinson & Justice, 1991; Chaurasia, 1999]. For example, some amino acid neurotransmitters are heterogeneously distributed in the brain and colocalized with *N*-methyl-D-aspartate (NMDA) receptors, suggesting a role in neurotransmission. In this analytical field, initial tissue assays for D-serine and D-aspartate biomarkers were based on a somewhat labour-intensive clean-up procedure followed by, e.g., a 70 min HPLC separation and LIF detection [Hashimoto et al., 1992, 1995]. On the other hand, in microdialysis, small molecules are able to diffuse across the dialysis membrane into the probe, while large molecules, such as proteins and cell fragments, are excluded. The clean-up provided by the microdialysis can allow analysing for neurotransmitters in tissue (e.g., brain) homogenates directly [Thompson, J.E. et al., 1999]. In the microdialysis, the minimum volume required for analysis often determines the rate at which the dialysate can be sampled. On-line microdialysis-CE-LIF assays (for the instrumental scheme see Figure 3.24) eliminate fraction collection. This elimination of fraction collection, combined with the high mass sensitivity of LIF or electrochemical detectors, makes sampling rates on the order of seconds possible [Thompson, J.E. et al., 1999; Hogan et al., 1994; Zhou S.Y. et al., 1995, 1999; Lada & Kennedy, 1995, 1997; Lada et al., 1998]. On-line microdialysis-CE assays for neurotransmitters to date have been most

successful for easily resolved analytes such as glutamate and aspartate [Thompson, J.E. et al., 1999; Zhou S.Y. et al., 1995; Lada et al., 1997, 1998; Lada & Kennedy, 1996]. Efficiency and peak capacity of high-speed CE separations are often not high enough to resolve complex mixtures. Recently, improvements in injection technique and detection limits have improved separation efficiency, e.g., allowing singly charged amine derivatives, such as γ–amino-*n*butyric acid (GABA), to be analysed [Bowser & Kennedy, 2001]. Recently, on-line microdialysis-CE has been adapted to chiral determinations of neurotransmitters in multicomponent amino acid mixtures, as well as biological matrices [O'Brien et al., 2003], as shown in section 3.5.

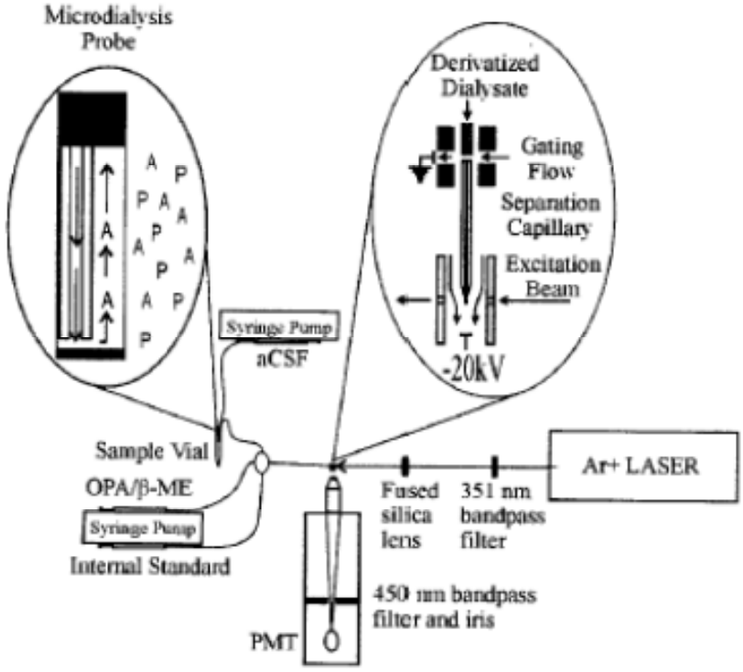

Figure 3.24. Diagram of the microdialysis-CE-LIF instrument. The whole automated procedure consists of following steps/modules performed on-line: (i) Microdialysis, (ii) derivatization, (iii) Flow-gated injection interface, (iv) High-speed CE with LIF detection. Reprinted from ref. [O'Brien et al., 2003].

3.3.4 Combination of electrophoretic stacking with nonelectrophoretic techniques

The electrophoretic stacking and nonelectrophoretic on-line sample preparation principles can be properly combined with each other to achieve the desired effect. Lately several such hybrid on-line sample preparation techniques have been introduced into the CE that offer excellent solutions, especially, for the sample clean-up (often accomplished by nonelectrophoretic principles) and analyte preconcentration (often accomplished by electrophoretic principles) in one experiment. Some of them, namely (i) extraction + stacking [Fang H.F. et al., 2006a, 2006b], (ii) dialysis + stacking [Hadwiger et al., 1996], (iii) chromatography + stacking [Pálmarsdóttir & Edholm, 1995; Pálmarsdóttir et al., 1996, 1997], were successfully applied also in chiral analyses of biologically active compounds in biological samples as presented in section 3.5.

The great potential with the hybrid on-line sample preparation techniques lies in their complementarity that enables the accumulation of positive effects and/or overcoming the weak points of the individual sample preparation techniques (discussed in sections 3.2 and 3.3). For example, the extraction used as the first step of the sample preparation can simplify the sample matrix (e.g., removing major interfering ionic constituents from the sample matrix) that is essential for some of the stacking modes (e.g., FESS requires a low-conductivity sample) used as the second step.

3.4 Flow injection

A combination of on-line sample preparation techniques based on electrophoretic and nonelectrophoretic principles can be performed in a single column or column coupling arrangement. Another interesting possibility with how to implement various sample preparation procedures on-line is a combination of the flow injection (FI) with electrophoresis. The combination of FI with electrophoresis using capillaries and chips is reviewed by Lü et al. [Lü W.J. et al., 2009]. Here, the basic principles, instrumental developments (including newly designed interfaces for FI-CE) and applications of FI-CE system from 2006 to 2008 are reviewed. The technique of combined flow injection CE (FI-CE) integrates the essential favourable merits of FI and CE. It utilizes the various excellent on-line sample pretreatments and preconcentration (such as cloud point extraction, SPE, ion-exchange, DPJ and head-column FESS technique, analyte derivatization) of FI, which has the advantages of high-speed, accuracy, precision and avoiding manual handling of sample and reagents. Therefore, the coupling of FI-CE is an attractive technique; it can significantly expand the application of CE and has achieved many publications since its first appearance. The significant potential of the FI-CE method in the automatization of sample derivatization and chiral separation was demonstrated by Mardones et al. [Mardones et al., 1999], and the proposed FI-CE scheme is shown in Figure 3.25.

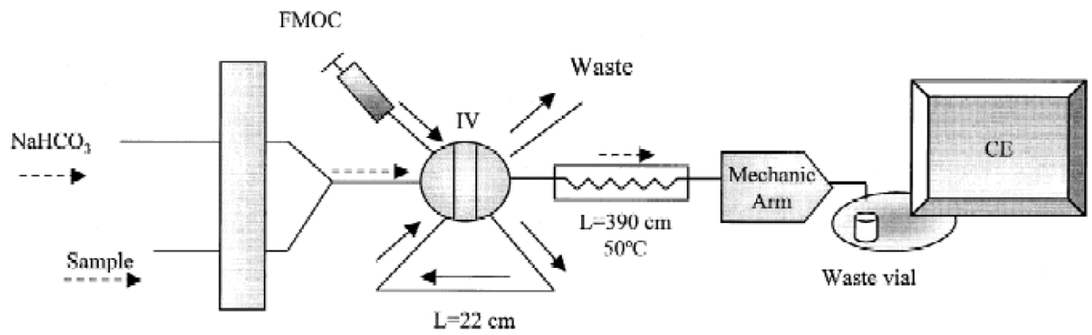

Figure 3.25. FI manifold used for the derivatization of the carnitine enantiomers and their on-line introduction into the CE system. Reprinted from ref. [Mardones et al., 1999].

3.5 Applications

The sample preparation techniques on-line combined with the CE, described in sections 3.2-3.4 (see for the theory and schemes), have been applied in many models, as well as real situations. In a lesser extent, given by (i) the higher complexity of experimental arrangement with chiral additive(s), as well as (ii) the natural proportion between currently solved chiral and achiral analytical problems, these techniques have been on-line combined with chiral CE systems.

Chiral CE determinations of biologically active compounds in various real matrices, e.g., environmental, food, beverage and mainly biological (clinical, forensic), as well as pharmaceutical samples, employing an on-line sample preparation, are listed in Table 3.1.

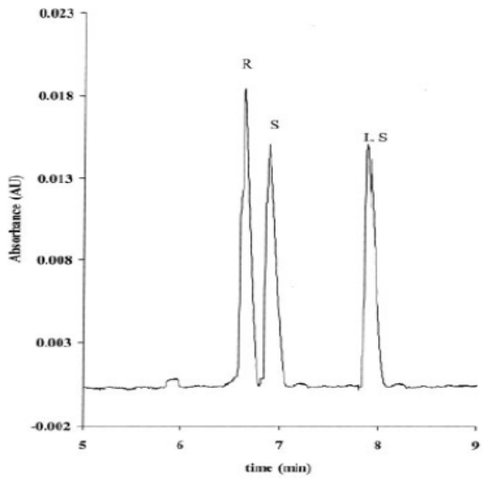

Figure 3.26. Analysis of plasma extracts containing 50 ng/mL of drug enantiomers and internal standard by CE with optimized FESI injection. Capillary column, 57 cm x 50 μm) i.d.; applied voltage, +20 kV; temperature 25°C; buffer, formic acid-ammonia (pH 4, ionic strength 50 mM) and 3.5 mM HP-β-CD. FESI, hydrodynamic injection of water plug (5 s) then electrokinetic injection (+10 kV, 20 s) of racemic drug dissolved in a water-methanol (10/90 v/v) and 80 μM H_3PO_4 mixture. SPE pretreatment of plasma sample was carried out before FESI-CE. Reprinted from ref. [Grard et al., 2002].

In this Table we tried to emphasize briefly the most important features of the methods and purpose of sample preparation, i.e., sample clean-up, analyte preconcentration, analyte derivatization. Here, the selectivity and sensitivity gain of combining on-line sample preparation procedures with chiral CE for the determination of low concentrations of drugs in complex matrices was clearly exemplified.

From among those examples, selected applications in biological and model (presented here for a given sample preparation method only in the case when no bioapplication is available) samples are described in the text of this section in detail, emphasizing the practical aspects of the proposed methods via their performance parameters (precision, recovery, etc.). In this way, the usefulness of the various techniques in routine analysis can be clearly ascertained.

Field-enhanced sample stacking. The FESI-CE-UV method has been developed for the quantification of cationic enantiomers of the new adrenoreceptor antagonists in plasma samples, for the illustrative electropherogram see Figure 3.26 [Grard et al., S., 2002]. An excellent accuracy on retention times and peak efficiencies was found with relative standard deviations (RSDs) being generally less than 1% and 2%, respectively. The FESI method also provided good reproducibility of ratio between the corrected areas of enantiomer and of the internal standard, since the RSD never exceeded 3%. These experimental results attest to the reliability of the FESI method in analysing chiral drugs by CE.

The increase of sensitivity by applying a FESS procedure was necessary for the analysis of amphetamine and its metabolic products (3,4-ethylenedioxymethamphetamine, MDMA, 3-4-methylenedioxyamphetamine, MDA, 3,4-methalenedioxyethylamphetamine, MDE) in hair samples [Tagliaro et al., 1998]. The FESS-CE-DAD method allowed the chiral determination of the metabolites at concentrations occurring in real samples from ecstasy users, with the possibility of recording UV spectra of the peaks. The analytical precision was characterized by relative standard deviation values <0.8 % (≤0.15% with internal standardization) for migration times intra-day and <2.0% (≤0.54% with internal standardization) day-to-day; linearity, in the range 0.156-40 μg/mL, and accuracy were also satisfactory. Even when evaluated at the lowest concentration of the standard curve, 0.156 pg/mL (with a signal-to-noise ratio of 5 for MDMA and 3 for amphetamine), the between-days area reproducibility was acceptable, ranging from 7.93% RSD for MDMA, to 16.07% RSD for amphetamine. The intra-day RSDs of migration times were always <0.8%, while the between-days RSDs were <2.0%; for relative migration times, RSDs were ≤0.15% and ≤0.54%, respectively. Absolute peak area reproducibility was still acceptable at 20 pg/mL (RSD was about 5% intra-day and ≤8% inter-days), but at 0.2 pg/mL the variability was relevant (RSDs between 9 and 12%). The area normalization on the basis of an IS (D-methamphetamine) was helpful, but for MDMA, MDA and MDE, RSDs remained around 10%.

FESI as an on-line sample stacking method was employed in order to increase the detection sensitivity for six β-blockers, namely (±) carteolol, (±) atenolol, (±) sotalol, (±) metoprolol, (±) esmolol and (±) propranolol, in human serum samples [Huang L. et al., 2008]. The final serum sample solution had to be diluted before injection since the ion concentration in the sample solution strongly influenced the signal enhancement. When the human serum sample was not diluted, the signal enhancement was not great because of the matrix effect. For validation of the FESI-CE-UV method, sensitivity (Table 3.1), linearity and precision were evaluated. The study showed that the repeatabilities of migration times (intra-day RSD 5.5-6.1%, inter-day RSD 4.4-5.2%) and peak heights (intra-day RSD 1.5-12.9%, inter-day RSD 3.6-5.2%) for the enantiomers of β-blockers were satisfactory. Recoveries (calculated using the 5 mg/mL racemic standard solutions) ranged from 82.1 to 98.2%.

The FESI-CE-UV method was used to determine the concentration of sulindac (SU) and its two active metabolites, sulindac sulfide (SI) and sulindac sulfone (SO), in human plasma [Chen Y.L. et al., 2006]. During method validation, calibration plots were linear (r > 0.994) over a range of 0.3–30.0 μM for SU and SO, and 0.5–30.0 μM for SI. During intra- and inter-day analysis, relative standard deviations (RSD) and relative errors (RE) were all less than 16%. Compared to the peak area ratio of the standard analytes, the absolute recoveries for SU, SO and SI were about 82, 81 and 60%, respectively. This method was feasible for the investigation of a pharmacokinetic profile of SU in plasma after oral administration of one SU tablet (Clinoril, 200 mg/tab) to a female volunteer.

The LVSS-CE-DAD method was applied for the sensitivity enhancement of four basic racemic drugs (methoxamine, metaproterenol, terbutaline and carvedilol) in serum samples [Denola et al., 2007]. Accuracy ranges were 96.4–103.4% for the hydrodynamic injection and 102.3–115.5% for the electrokinetic injection. Better recovery values were achieved with

hydrodynamic injection wherein values ranged from 62.8–74.5% as compared to that of the electrokinetic injection in which recovery values were all lower (31.1–69.4%). The deviations in the resolution values were higher for the electrokinetic (RSD 3.6–6.0%) than for the hydrodynamic injection (RSD <1.9%). However, the repeatability of migration times was better when control samples were loaded electrokinetically (RSD 0.6–0.8%) rather than hydrodynamically (RSD 2.1–2.6%). Migration times do not vary significantly from those obtained with the control samples. Good repeatability in terms of migration times and enantioresolution was evident in the very low RSD values (migration time RSD ≤0.5; enantioresolution RSD ≤5.5) in both types of injections.

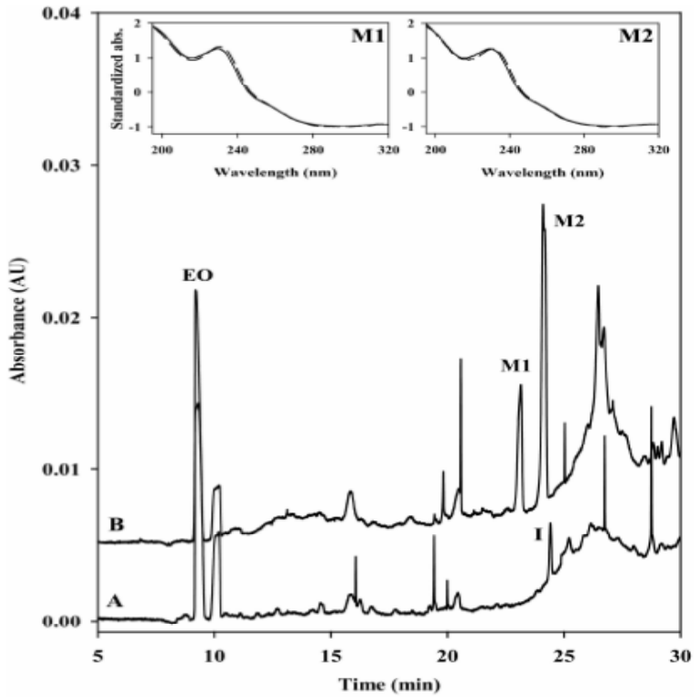

Figure 3.27. SWP-MEKC electropherograms obtained from a 30 psi x s injection of four pooled human liver microsomes incubations (A) without lorazepam (blank) and (B) with 50 μM lorazepam. Two insets show the comparison of blank subtracted, standardized spectra of lorazepam metabolites M1 and M2 (broken lines), and lorazepam (full line). Analytical conditions: detection was effected at 200 nm and, for solute identification purpose, the fast scanning mode (range: 195-320 nm, scanning interval: 5 nm) was employed, temperature 25°C, applied voltage 20kV (current about 28 μA). Separation electrolyte: 6 mM $Na_2B_4O_7$, 10 mM Na_2HPO_4, and 75 mM SDS, pH 9.1 (before addition of SDS). Reprinted from ref. [Baldacci & Thormann, 2006].

Transient isotachophoresis. tITP was used for the preconcentration of timolol and ephedrine in standard solutions and dosage forms [Hedeland et al., 2007]. tITP served for peak sharpening of S-timolol and therefore, the tITP-CE-DAD determination of the enantiomeric impurity (R-timolol) was possible with sufficient enantioresolution, as illustrated in Figure 3.28. For ephedrine, the tITP-CE-DAD method was validated. The intermediate precision for the quality control (QC) samples was in the interval 4.2–5.6% and the precision for each set of samples

was equal or less than 5.6% (for seven out of nine values), which is considered as acceptable for the application. The accuracy at 1.9% of the enantiomeric impurity for three different BGEs where the composition was varied (±1%) was in the range 91.3–99.7% and the precision was 1.7–8.7% (n = 2). Thus, the method is robust and small differences in the BGE composition should not have any major influence in the determination of the 1R,2S-ephedrine impurity.

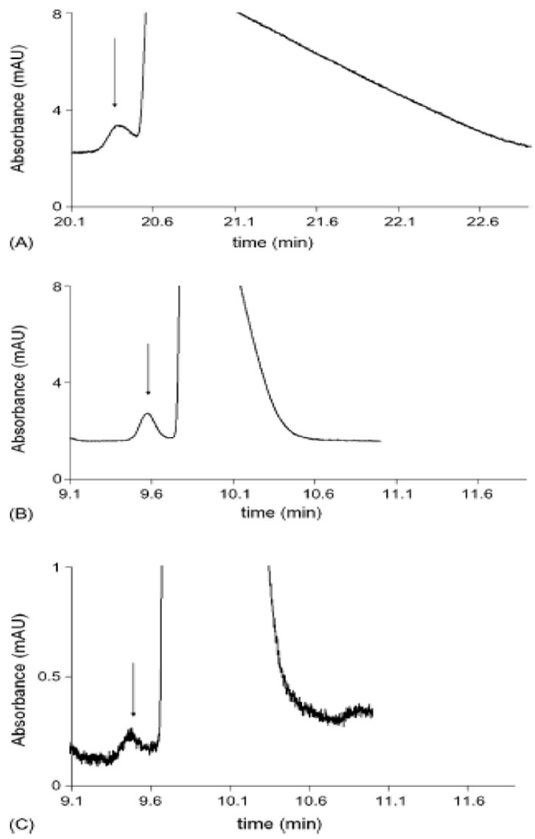

Figure 3.28. Peak sharpening of S-timolol by tITP. BGE: 100mM (+)-ketopinic acid and 40mM KOH in methanol:ethanol (3:2, v/v). 30 kV, L_{det} 23 cm. Sample: 2mM *S*-timolol and 0.05mM *R*-timolol (2.4%) dissolved in methanol. (A) Normal injection pressure injection at 35 mbar over 5 s, (B) tITP, leading electrolyte: 100mM sodium acetate in methanol (25 mbar over 1 s). Terminating electrolyte: 200mM triethanolamine in methanol (anodic vial). (C) tITP at LOD (0.2% *R*-timolol). Conditions as in (B). The arrow in the electropherograms (A–C) points out the position of the *R*-timolol peak. Reprinted from ref. [Hedeland et al., 2007].

Combined stacking techniques. A combination of LVSS and SWP, where LVSS involves a subcombination of field-amplified stacking and pH-mediated stacking, provided a very high sensitivity for the anionic cyanobenz[*f*]isoindole (CBI)-amino acids [Kirschner et al., 2007]. Average and standard deviation in peak area ratios (CBI-amino acid/ internal standard) for nine injections of 2.4 μM CBI-amino acid were as follows: CBI-L-Glu 0.461±0.070, CBI-D-Ser 0.400±0.085, CBI-L-Asp 0.358±0.036. A procedure for the LVSS-SWP-CE-LIF determination of CBI-D-Ser was applied for squirrel brain samples. This preconcentration technique was also applied to multicomponent mixtures of CBI amino acid derivatives without loss of resolution, see electropherogram in Figure 3.29.

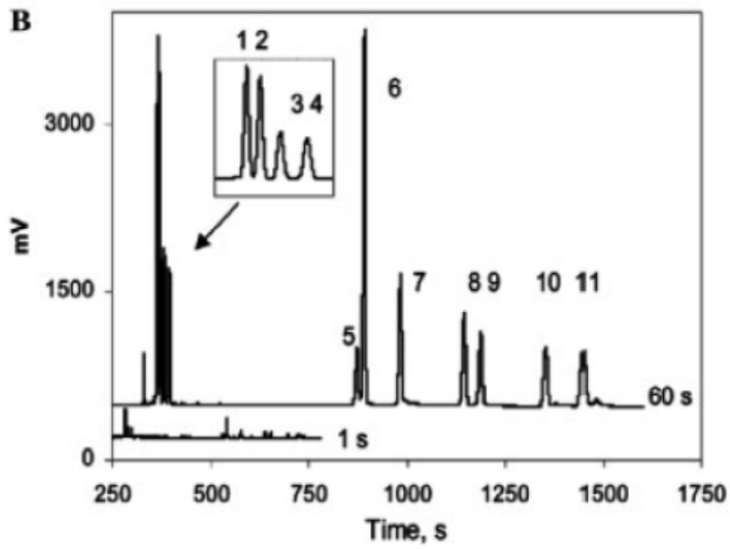

Figure 3.29. Electropherogram showing the potential of stacking/sweeping-EKC combination to the enantioseparation of a complex sample of CBI–amino acids (0.5 μM each). Electrophoretic conditions: fused-silica capillary, 70 cm total length (45 cm detector length) and 25 μm i.d.; separation buffer, 25 mM phosphate buffer (pH 2.0) containing 2% HS-β-CD; applied voltage, 230 kV; hydrodynamic injection, 380 mbar for 180 s. LIF detection with l_{exc} at 420 nm. Peak identification: 1, CBI-D-Arg; 2, CBI-L-Arg; 3, CBI-D-His; 4, CBI-L-His; 5, CBI-Gly; 6, CBI-L-Tyr; 7, CBI-L-Glu; 8, CBI-D-Ser; 9, CBI-L-Ser; 10, CBI-L-Glu; 11, CBI-D-Glu. Adapted from ref. [Kirschner et al., 2007].

Sensitive CE methods are required for emerging areas of biochemical research such as the metabolome. In ref. [Britz-McKibbin et al., 2003], DPJ-SWP-CE-LIF was applied as a robust single method to analyse trace amounts of three flavin derivatives, riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), from several types of samples including bacterial cell extracts, recombinant protein and biological fluids. For the separation of flavins a chiral selector mediated CE separation system was needed. Submicromolar amounts of flavin coenzymes were measured directly from formic acid cell extracts of *Bacillus subtilis*. This method was also applied to the analysis of free flavins in pooled human plasma and urine without the need for laborious off-line sample preconcentration (e.g., SPE). The method was validated in terms of reproducibility, sensitivity (Table 3.1), linearity and specificity. The linearity of the method within a 100-fold concentration range was excellent as reflected by the correlation coefficient (R^2) of 0.999 for all three flavin calibration curves. However, significant nonlinearity was observed to occur at concentrations above 0.1 μM. Because of the lack of native fluorescent species in most biological matrices, the method is considered to be extremely specific with few chemical interferences. Reproducibility of the CE method was assessed by analysis of five replicate injections of formic acid cell extract (malate) sample performed on two consecutive days. Inter-day coefficients of variance (CV, n=10) for migration time and peak area of flavin coenzymes were determined to be 0.68 and 3.8%, respectively. The low reproducibility of this technique may be attributed in part to the long injection time (60 s) used for analysis,

whereas precision in CE is often limited by short hydrodynamic injections. Anyway, flavin analysis by DPJ-SWP-CE-LIF offers a simple, yet sensitive way to analyse trace levels of flavin metabolites from complex biological samples.

Transient isotachophoresis with field-enhanced sample injection, using β-CD as the chiral selector and tetrabutylammonium hydroxide (TBAOH) as the additive, was applied for on-line preconcentration and enantioseparation of three beta-agonists, namely, cimaterol, clenbuterol and terbutaline. Under the optimum conditions, the detection limits (defined as S/N = 3) of this method were found to be 1 ng/mL for all three pairs of beta-agonists enantiomers. Compared with conventional electrokinetic injection, the enhancement factors were greatly improved to be 250-fold. The proposed method has been applied for the analysis of human urine samples [Huang, L. et al., 2011].

Stacking techniques with derivatization. An on-line sample preconcentration approach coupled with in-capillary derivatization (SPCD-CE) has recently been applied, among others, to the (indirect) chiral separation of amino acids, whereby a special application represents the detection of D-amino acids as biomarkers in connection with bacterial growth, see Figure 3.30 [Ptolemy et al., 2006; Ptolemy & Britz-McKibbin, 2006]. In comparison with conventional CE, SPCD-CE provided a 100-fold improvement in concentration sensitivity, reduced sample handling and shorter analysis time. The reproducibility (n = 5) of the integrated SPCD-CE-UV technique was acceptable with average coefficients of variation of approximately 7.4 and 1.2% for quantitation (peak height) and apparent migration time, respectively [Ptolemy et al., 2006].

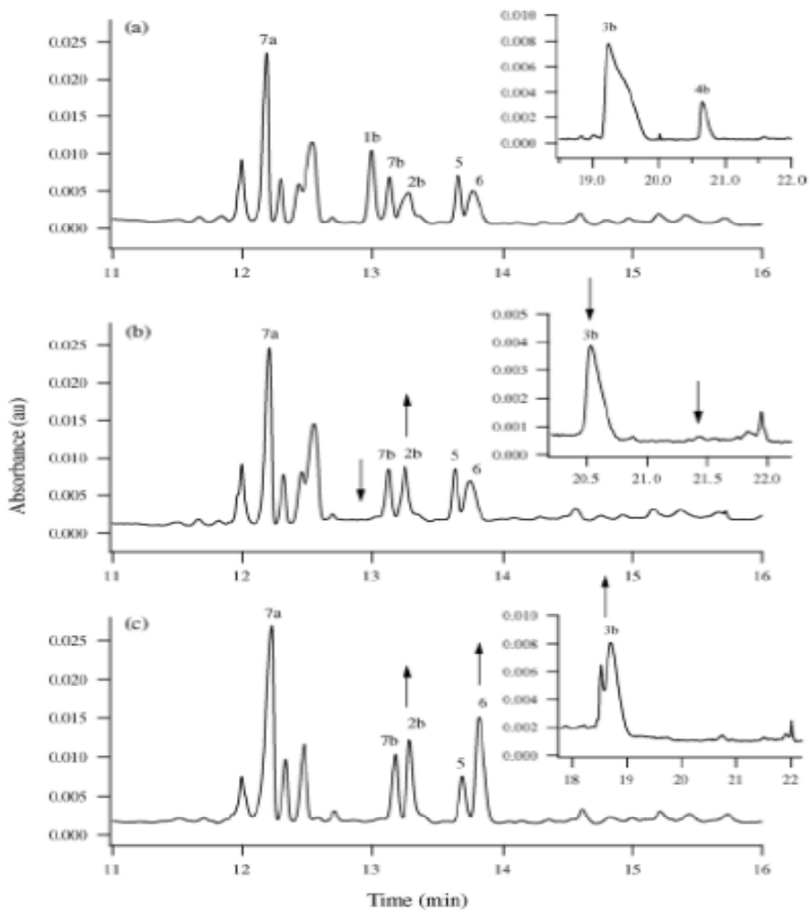

Figure 3.30. Direct enantioselective amino acid flux analyses in the extra-cellular medium of *E. coli* by SPCD-CE. Electropherograms represent the extra-cellular media with 5% seeding volume incubated for (a) 0 h (control), (b) 3 h and (c) 5 h. Other conditions: sample solutions contained 25 and 50μM of the D and L-amino acids, respectively, prepared in 40mM phosphate, pH 6.0 using a sample injection of 100 s. Conditions: 140mM borate buffer, pH 9.5; voltage 25 kV; capillary length 67 cm; internal diameter; 50μm; UV 340 nm. Analyte peak number corresponds amino acid-isoindole adducts, where 1a: D-Ser, 1b: L-Ser, 2a: D-Ala, 2b: L-Ala, 3a: D-Glu, 3b: L-Glu, 4a: D-Asp, 4b: L-Asp, 5: taurine (6 μM, internal standard), 6: Gly and 7a,b: L-Lys side-product. The direction of arrow indicates a net release (↑) or uptake (↓) of amino acid has been observed in the extra-cellular medium during bacterial growth. Note the rapid uptake of L-Ser and L-Asp and the steady-state enantioselective release of L-Ala. There was no detection of net efflux of D-Ala and D-Glu from *E. coli* into extra-cellular matrix during bacterial growth. Reprinted from ref. [Ptolemy & Britz-McKibbin, 2006].

Counter-flow gradient focusing. TGF has been shown to be effective for a 1200-fold concentration enrichment in 30 min for the baseline-separated enantiomers of glutamic acid, as illustrated in Figure 3.31 [Balss et al., 2004]. No validation data are available for this method.

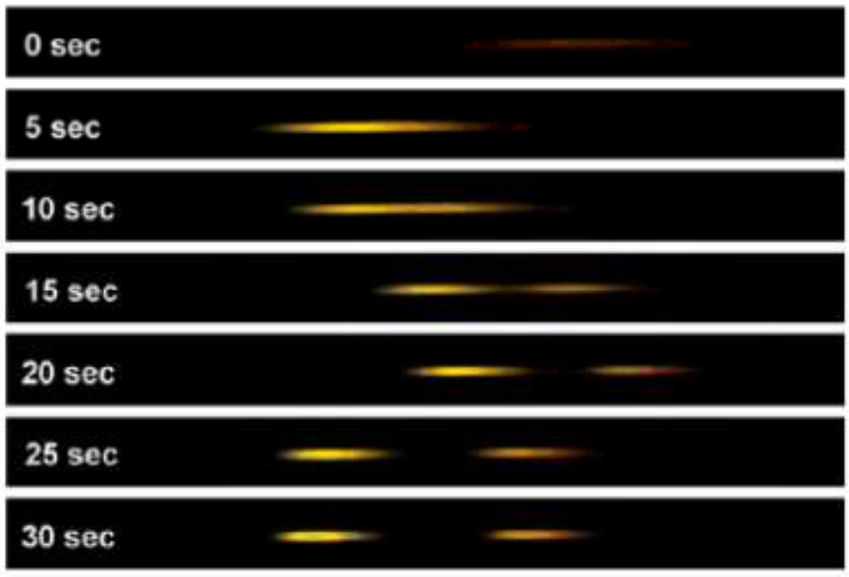

Figure 3.31. Example of TGF focusing and separation. Total image length is 1.9 mm. Real-time chiral TGF of DNS-D,L-Glutamic acid. Working conditions as in Figure 3.13. The D-enantiomer peak is to the left in the figure; the L-enantiomer is to the right. Reprinted from ref. [Balss et al., 2004].

Column-coupled electrophoretic techniques. An ITP-EKC-UV method was successfully applied for the determination of trace enantiomers of various drugs and their biodegradation products (pheniramine and its metabolites, dioxopromethazine, dimethindene, amlodipine) in model complex ionic matrices and clinical samples [Mikuš et al., 2006, 2008a, 2008b; Marák et al., 2007], see an example in Figure 3.32. RSD values of migration times were lower than 2.0%. The concentrations of the analytes in tested samples, corresponding to the quantitation limits, were determined with acceptable precisions (RSD values ranged in interval 3.95–4.54%, n = 5) and accuracies (relative errors ranged in interval 5.84–6.22%, n = 5) under the stated conditions. The mean relative errors (REs) indicated by the recovery tests, characterizing accuracy of the chiral method for pheniramine, dimethindene and dioxopromethazine, were 4.5, 4.8 and 3.6%, respectively [Mikuš et al., 2006a].

The ITP-EKC-DAD method was shown to be a powerful tool in enantioselective pharmacokinetic studies of the β-blocker drug, amlodipine, in clinical urine samples [Mikuš et al., 2008a] and the enantioselective metabolic study of cationic H_1-antihistamine, pheniramine, present in clinical urine samples [Marák et al., 2007]. Performance parameters of the ITP-EKC-UV/DAD methods were sufficient for the routine enantioselective biomedical analyses of unpretreated biological samples. Besides all the benefits of the ITP-EKC combination, as mentioned above, speed spectral evaluation of separated zones enabled preliminary characterization of their homogeneity (presence of mixed zones) and preliminary indication of structurally (spectrally) similar compounds (potential metabolites of the drugs) in unknown electrophoretic peaks. To complete the evaluation of the performance parameters, the selectivity of the ITP-EKC separation method can be examined by the spectral evaluation of purity of analyte zones.

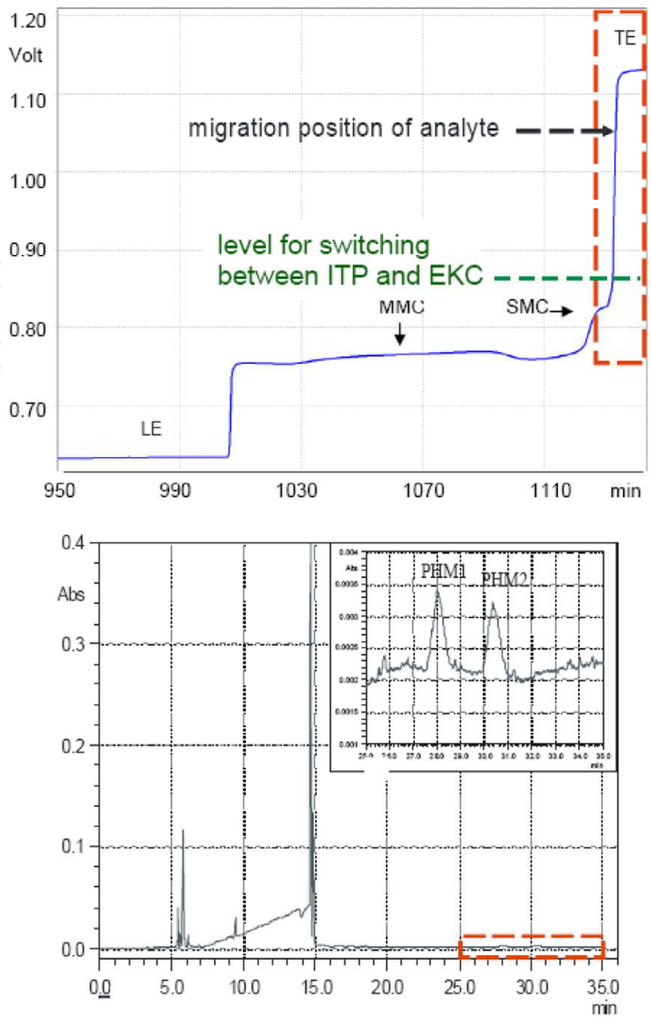

Figure 3.32. ITP-EKC method in column coupling configuration of the separation units for the direct analysis of unpretreated complex matrices sample; electrophoretic traces. Determination of pheniramine enantiomers in model urine sample demonstrates the effectivity of on-line sample preparation (removing matrices, preconcentration of enantiomers) in the first ITP stage (upper trace) and countercurrent separation mechanism (enantioseparation) in the second EKC stage (lower trace) of the ITP-EKC method. The separations were carried out using 10 mM sodium acetate - acetic acid, pH 4.75 as a leading electrolyte (ITP), 5 mM ε-aminocaproic acid - acetic acid, pH 4.5 as a terminating electrolyte (ITP), and 25 mM ε-aminocaproic acid - acetic acid, pH 4.5 as a carrier electrolyte (EKC). 0.1% (w/v) methyl-hydroxyethylcellulose served as an EOF suppressor in leading and carrier electrolytes. Carboxyethyl-β-CD (5 mg/mL) was used as a chiral selector in carrier electrolyte. Concentration of pheniramine was 7.10^{-8} M in the injected sample (a 10 times diluted spiked urine). The detection wavelength in EKC stage was 261 nm. The driving currents in the ITP and EKC stages were 200 μA and 80 μA, respectively. LE – leading cation; TE – terminating cation; PHM1, PHM2– migration positions of the first and second pheniramine enantiomer, respectively, MMC – major matrix constituents, SMC – semiminor matrix constituents. Reprinted from ref. [Mikuš et al., 2008b].

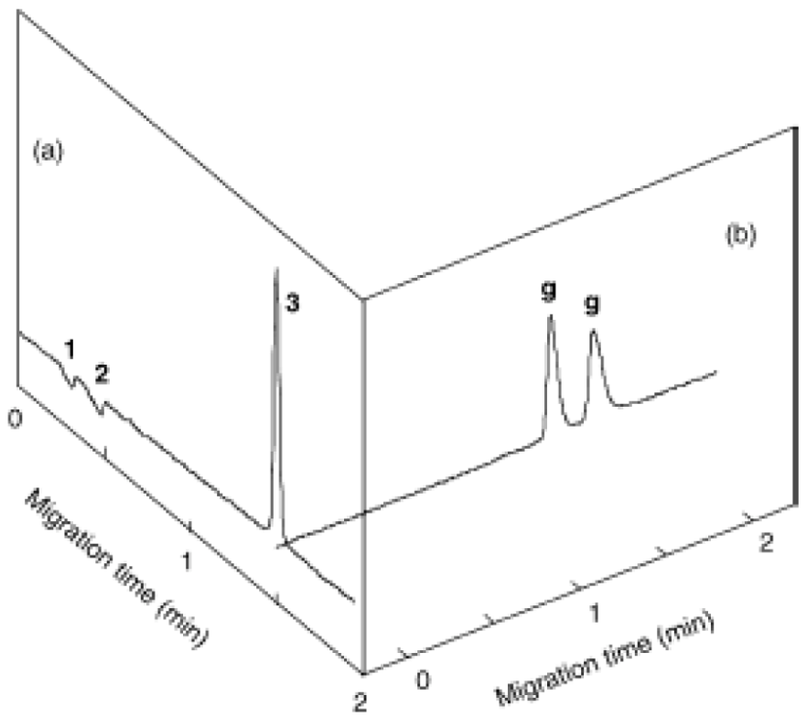

Figure 3.33. Chiral separation of gemifloxacin dissolved in a urinary solution with microchip electrophoresis. Peaks: (1) K^+; (2) Na^+; and (3) gemifloxacin racemate. Peaks for gemifloxacin enantiomers are denoted by 'g'. (a) The removal of metal ions was performed in the first separation channel. Run buffer: (50mM Bis–Tris + 10 μM quinine)/Citric acid of pH 4.0. (b) The chiral separation was performed in the second separation channel using a run buffer of 50mM Bis–Tris/Citric acid containing 50 μM 18C6H4 (pH 4.0). A urinary solution was five-fold diluted using 50mM Bis–Tris/Citric acid (pH 4.0). Gemifloxacin was 100 μM in five-fold diluted urinary solution. Gemifloxacin was injected into the second separation channel by floating reservoir D, with reservoir F grounded, for 15 s right after the analyte passed the first detection point to ensure that all gemifloxacin was introduced into the second channel. For the analysis in the second channel, the applied voltage was 3.0 kV at reservoir E with reservoir F grounded and all other reservoirs floating. For the instrumental scheme, see Figure 3.15. Light source: He–Cd laser (325 nm); indirect laser-induced fluorescence detection at 405 nm using a photomultiplier tube. Analytes were detected at 32 and 38mm from the first and second injection crosses, respectively. Reprinted from ref. [Cho S.I. et al., 2004].

A channel-coupled microchip electrophoresis device was designed to clean-up alkaline metal ions (interfering with chiral selector) from a sample matrix for the chiral analysis of gemifloxacin in urine, see Figure 3.33 [Cho S.I. et al., 2004]. The total analysis time of directly injected urine samples was less than 4 min with micromolar amounts of chiral selector (50 μM CWE). No validation data are available for this method.

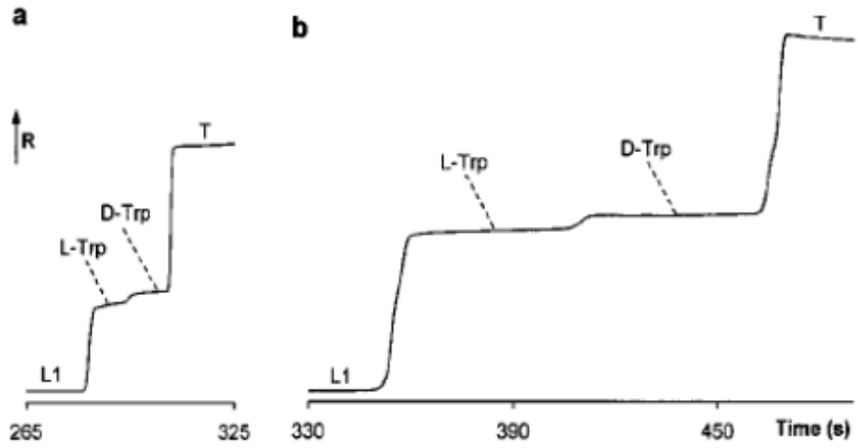

Figure 3.34. Isotachopherogram from the separations of racemic mixture of tryptophan enantiomers on the CC chip. (a) Single-column separation in the first channel using C1 to monitor the situation. The separation was carried out in the electrolyte system L1 with 8.5 A driving current. The injected sample contained the racemate at a $6x10^{-4}$ M concentration. (b) Separation in the tandem-coupled separation channels using C2 to monitor the separation. The separation was carried out in the electrolyte system L1 placed in both separation channels. The driving current was 8.5 A during the run in the first channel and it was reduced to 5 μA during the separation in the second channel. The injected sample contained the racemate at a $1.5x10^{-3}$ M concentration. R, increasing resistance; L1 leading anion (propionate), T, termination anion (ε-aminocaproate). Reprinted from ref. [Ölvecká et al., 2001].

The use of a poly(methylmethacrylate) chip, provided with a pair of on-line hyphenated separation channels and on-column conductivity detectors, to isotachophoresis (ITP) separations of optical isomers was investigated by Ölvecká et al. [Ölvecká et al., 2001]. Single-column ITP, ITP in the tandem-coupled columns, and concentration-cascade ITP in the tandem-coupled columns were employed in this investigation using tryptophan enantiomers as model analytes, see Figure 3.34. Although providing a high production rate (about 2 pmol of a pure tryptophan enantiomer separated per second), single-column ITP was found suitable only to the analysis of samples containing the enantiomers at close concentrations. The best results in the respect of the resolution were achieved by using a concentration-cascade of the leading anions in the tandem-coupled separation channels.

Extraction. A miniaturized SPE (1-3 mm capillary of 200 μm internal diameter packed with C18 alkyl-diol silica and capped by glass-fibre filters for the sorbent retaining) coupled on-line with CE significantly enhanced the concentration sensitivity for terbutaline [Petersson et al., 1999]. Experimental arrangement of the extractor and speed (10 min) sample enrichment procedure for the terbutaline enantioselective analysis are illustrated in Figure 3.18. The chiral application of the SPE-CE-UV method for model sample, as well as achiral application of the same method for plasma sample, is shown in Figure 3.35 [Petersson et al., 1999].

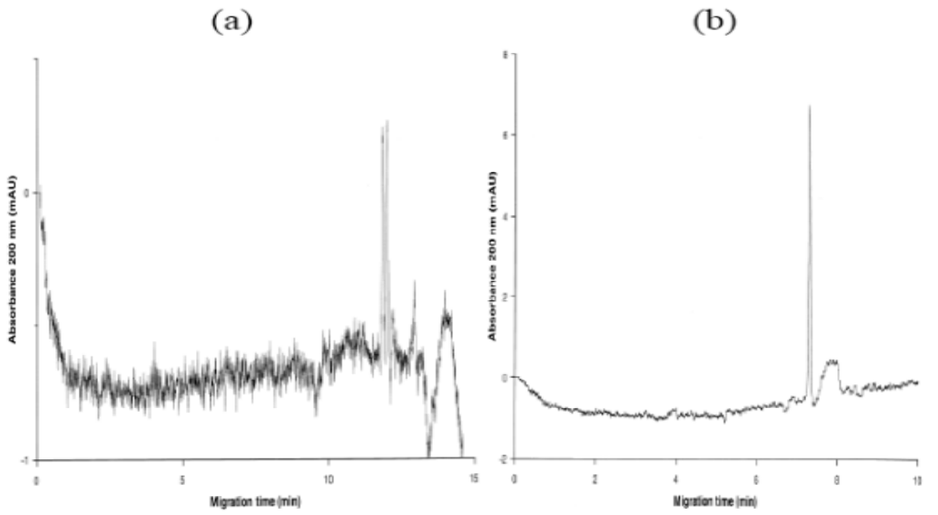

Figure 3.35. Miniaturized on-line SPE for enhancement of concentration sensitivity in CE. (a) Terbutaline enantiomer separation with on-line enrichment. A resolution of 1.6 and a separation efficiency of 300 000 plates were obtained. Enrichment capillary: L_t 58.0 cm, L_d 51.2 cm, L_i 5.5 cm, l_e 2.5 mm, wash: water x 1.6 min x 140 kPa, wetting: methanol x 2.4 min x 140 kPa, conditioning: water x 2.4 min x 140 kPa, injection: 100 nM *rac*-terbutaline in water x 1.0 min x 140 kPa, wash/ filling: 40 mM potassium phosphate (pH 6.4) x 0.1 min x 140 kPa, 15 mM dimethyl-β-CD in 40 mM potassium phosphate (pH 6.4) x 0.7 min x 140 kPa, desorption: acetonitrile x 40 s x 3.4 kPa followed by 15 mM dimethyl-β-CD in 40 mM potassium phosphate (pH 6.4) x 4.0 min x 3.4 kPa, voltage: 14 kV, detection wavelength: 200 nm, temperature: 25°C. (b) Direct injection of terbutaline in plasma with on-line enrichment. The enrichment capillary had not been subjected to plasma samples before this run. Enrichment capillary: L_t 58.0 cm, L_d 51.2 cm, L_i 5.4 cm, l_e 1.25 mm, wash: water x 1.4 min x 140 kPa, 200 mM SDS in 100 mM sodium borate (pH 9.0) x 3.5 min x 140 kPa, wetting: methanol x 7.0 min x 140 kPa, conditioning: water x 2.1 min x 140 kPa, injection: 2 mM terbutaline in water–bovine plasma (3:1) x 0.1 min x 140 kPa, wash: water x 1.4 min x 140 kPa, filling: 40 mM potassium phosphate (pH 6.4) x 0.8 min x 140 kPa, desorption: acetonitrile x 30 s x 3.4 kPa followed by 40 mM potassium phosphate (pH 6.4) x 3.0 min x 3.4 kPa, voltage: 20 kV, detection wavelength: 200 nm, temperature: 25°C. Adapted from ref. [Petersson et al., 1999].

The separation efficiency was 300 000 plates. The relative average deviation from the mean was 17% for the enrichment capillaries. Within each enrichment capillary the relative average deviation from the mean was below 2% (n=6). Fouling of the capillary wall with plasma protein during the analysis was prevented by an SDS washing step. However, partial clogging of the enrichment capillary was observed after repeated plasma injections.

Electro membrane extraction as a new microextraction method was applied for the extraction of amlodipine (AML) enantiomers from biological samples [Nojavan & Fakhari, 2010]. During the extraction time of 15 min AML enantiomers migrated from a 3 mL sample solution through a supported liquid membrane into a 20 μl acceptor solution presented inside the lumen of the hollow fibre. The driving force of the extraction was 200 V potential

with the negative electrode in the acceptor solution and the positive electrode in the sample solution. 2-Nitro-phenyl octylether was used as the supported liquid membrane. Using 10 mM HCl as background electrolyte in the sample and acceptor solution, enrichment up to 124 times was achieved. Then the extract was analysed using the CD modified CE method for separation of AML enantiomers. The best results were achieved using a phosphate running buffer (100 mM, pH 2.0) containing 5 mM hydroxypropyl alpha CD. The range of quantitation for both enantiomers was 10-500 ng/mL. Intra- and inter-day RSDs (n = 6) were less than 14%. The limits of quantitation and detection for both enantiomers were 10 and 3 ng/mL respectively. Finally this procedure was applied to determine the concentration of AML enantiomers in plasma and urine samples.

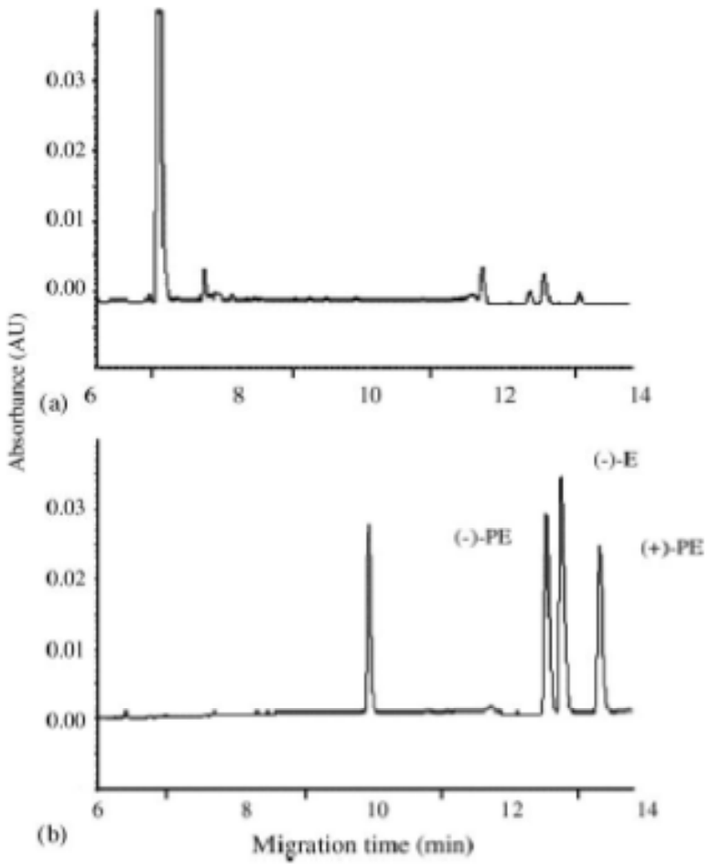

Figure 3.36. Separation of spiked urine sample (a) with directly injected (concentration of each analyte: 5.00 μg/mL); (b) after SPME-FESI (concentration of each analyte: 0.25μg/mL) followed by CE separation. The optimized SPME conditions: temperature of 90°C, time of 30 min, 2.00 g sodium hydroxide and 0.50 g sodium chloride for headspace extraction; 20% acetonitrile aqueous phase solvent with desorption time of 20 min for desorption. CE conditions: the run buffer contained 17.5mM β-CD and 150mM phosphate (pH 2.5) in the running voltage 25 kV at 20°C. Sample injection: 7 kV×10 s. (1R,2S)-ephedrine ((−)-E), (1R,2R)-pseudoephedrine ((−)-PE) and (1S,2S)-pseuodephedrine ((+)-PE). Reprinted from ref. [Fang H.F. et al., 2006b].

Extraction + stacking. A sufficient selectivity and sensitivity enhancement of two orders of magnitude was achieved for ephedrine derivatives {(1R,2S)-ephedrine, (1R,2R)-pseudoephedrine, (1S,2S)-pseudoephedrine} in human urine samples using an on-line combination of headspace SPME with FESI, see ref. [Fang H.F. et al., 2006b]. Electropherogram from the SPME-FESI-EKC method applied on spiked urine samples is shown in Figure 3.36. The intra-assay relative standard deviations were between 4.38 and 7.76% (concentration of analytes: 0.3 μg/mL; n = 5). Recovery was in range 88.7-97.5%. The performance parameters indicate a good potential of the SPME-FESI-CE-DAD method for its routine use.

The back LLE integrated with FESI and supported by centrifuge microextraction (CME) was applied for a significant increase in sensitivity for (1*R*, 2*R*)-pseudoephedrine, (1*R*, 2*S*)-ephedrine, (1*S*, 2*S*)-pseudoephedrine and (*S*)-(+)-methamphetamine in spiked serum [Fang H.F. et al., 2006a]. In this method, the CME effectively combined removal of macromolecular contaminants and other interfering components, desalting and preconcentration into one single step. Performance parameters of the CME-LLE-FESI-CE-DAD method indicate good potential of the proposed method for its routine use. The relative standard deviations (RSD, n=6) of all of the analytes were between 8.7-17.7% on the basis of peak areas. Utilizing (-)-pseudoephedrine as an internal standard, RSD results showed significant improvement (5.3-8.9%). For a 0.1 μg/mL concentration of the ephedrine derivates, recoveries were in the range of 97-114%. Relative recoveries, defined as a ratio of CE peak areas of two different sets of spiked urine extracts, were calculated to evaluate the effect of the matrix. The relative recoveries for all target drugs were from 90-112%. This means that the matrix had little effect on the method, particularly on the CME step.

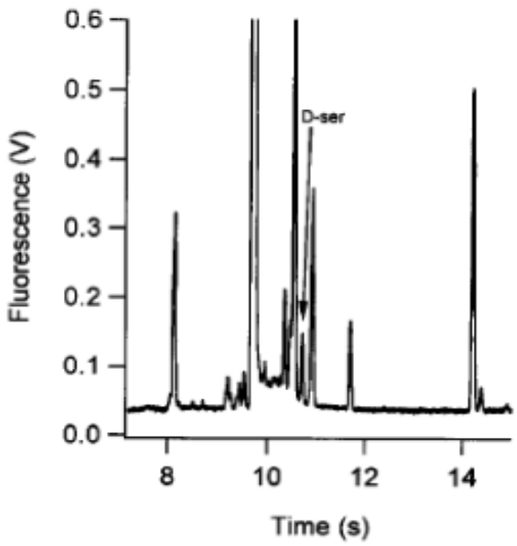

Figure 3.37. On-line microdialysis-CE-LIF analysis of the supernatant over an intact larval tiger salamander retina. A single salamander retina was incubated for 3 h in 50 μL Amphibian Ringers solution. D-Serine (5.2±2.1 μM, standard error, *n* = 4) was detected in the supernatant. Separation buffer, 50 mM borate, 20 mM HP-β-CD, pH 10.5. Separation distance, 8 cm. Reprinted from ref. [O'Brien et al., 2003].

Dialysis. A microdialysis coupled on-line with CE has been used for the rapid determination of Asp enantiomers in tissue samples from rats [Thompson, J.E. et al., 1999]. Filtration and deproteination was carried out inserting a microdialysis probe into a homogenized tissue sample. The variability that was observed by the microdialysis-CE method was due to variations in the sample as the same sample analysed repeatedly gave a RSD of 2.6%. A similar set-up for the on-line microdialysis-CE was used for the enantioselective analysis of Ser (separated from other primary amines commonly found in biological samples) in tissue homogenates [O'Brien et al., 2003], see electropherogram in Figure 3.37. Recovery was in the range of 71.5-86.8%.

Dialysis + stacking. An EKC analysis method designed for use with microdialysis sampling and electrophoretic sample stacking has been developed for the determination of isoproterenol enantiomers [Hadwiger et al., 1996]. The half-life of isoproterenol is less than l0 min, therefore, a 1 min sampling frequency, with a dialysis perfusion flow-rate of 500 nL/min, was needed to sufficiently define the pharmacokinetic curve. The optimized chiral CE method was applied to the analysis of intravenous microdialysis samples collected following administration of racemic isoproterenol. A typical electropherogram of a microdialysis sample collected over the first 5 min after dosing is shown in Figure 3.38. The enantiomers of isoproterenol are resolved from each other and from all endogenous compounds. Unfortunately, the detection limits were not sufficient to follow the concentration of isoproterenol for long enough to establish pharmacokinetic parameters. However, no off-line sample preconcentration was possible because of very low sample volumes for the analysis. Using an on-column concentration technique (*pH-mediated peak stacking*, i.e., injecting a plug of acidic solution directly after the sample) essential for analysing highly ionic sample, i.e., the microdialysis perfusate of plasma, the pharmacokinetic data/curve for the enantioselective elimination of isoproterenol could be obtained. A five-fold increase in sensitivity was achieved inserting an in-capillary stacking preconcentration of microdialyzed plasma samples, as shown in Figure 3.39. Using a catecholamine-based internal standard with similar electrochemistry (important for the electrochemical detection, EC) to isoproterenol, the precision of analysis increased from 3.2% RSD to 1.4%. This example illustrated a practical situation where the on-line sample preparation essentially replaced an off-line procedure.

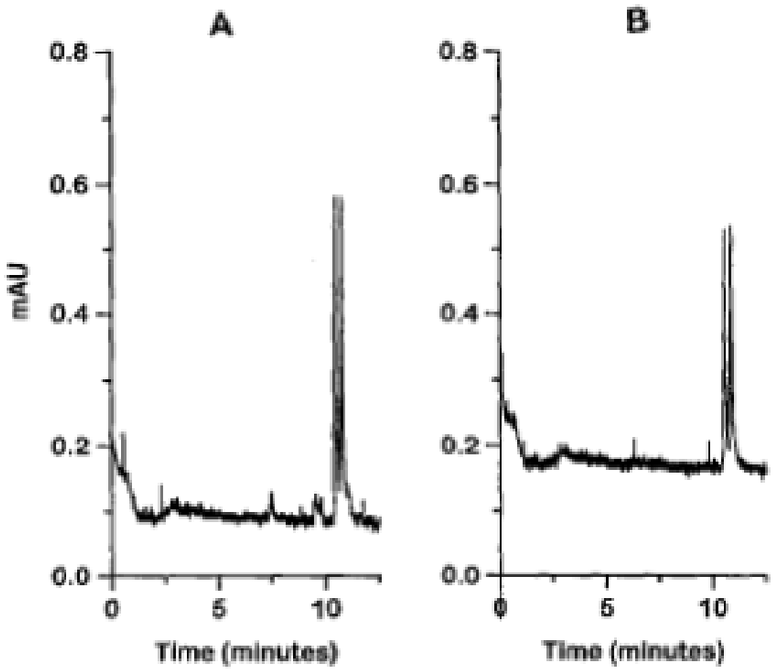

Figure 3.38. CE-UV electropherograms of isoproterenol obtained with CE and CE-microdialysis method. (A) Standard containing 100μM of (-)- and (+)-isoproterenol (ISP) bitartrate dissolved in Ringers-8.0 mM Na_2EDTA-97 μM $NaHSO_3$; (B) Microdialysate acquired from a rat 5 min after dosing. Reprinted from ref. [Hadwiger et al., 1996]

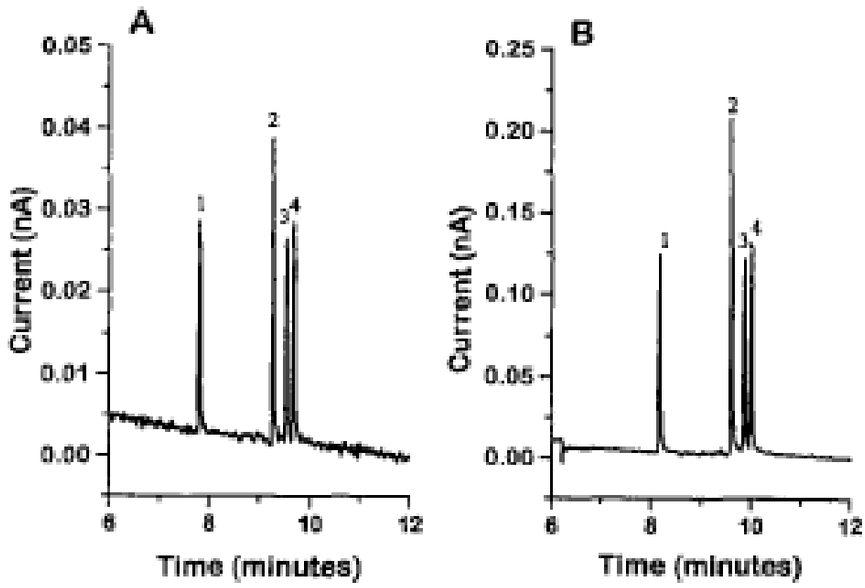

Figure 3.39. CE-EC electropherograms of standard solutions using normal electrokinetic injection (A) and acid stacked electrokinetic injection (B). Peaks: I=DHBA; 2=5NMHT; 3=(-)-iso -proterenol (ISP); 4=(+)-ISP. Reprinted from ref. [Hadwiger et al., 1996].

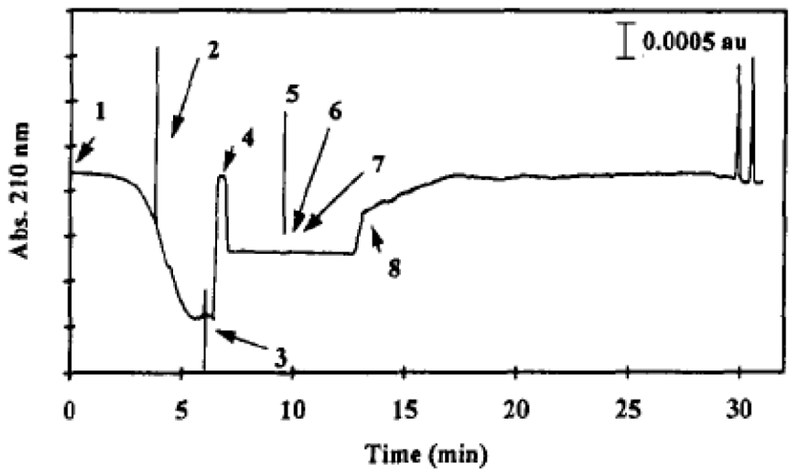

Figure 3.40. Electropherogram obtained after using the double stacking procedure followed by CE enantiomer separation. 3 μL of 400 nM *rac-terbutaline* (200 nM of each enantiomer) was injected. Arrows indicate the different events. (1) Stacking step one begins; (2) stacking peak of positive species; (3) voltage off, backpressure on; (4) zone of 5 mM phosphate buffer pH 7.5; (5) back-pressure off; (6) voltage on, stacking step two at the inlet end of the capillary; (7) back-pressure on; (8) back-pressure off, final separation step begins. The plasma samples were pretreated by on-line MLC before double stacking and CE enantioseparation. Reprinted from ref. [Pálmarsdóttir & Edholm, 1995].

Chromatography + stacking. The supported liquid membrane technique coupled on-line with CE through a MLC interface and additionally combined with a double-stacking preconcentration has been applied to a sensitive enantioselective determination of bambuterol in human plasma [Pálmarsdóttir et al., 1996, 1997]. No validation data are available for this method.

Plasma samples were pretreated and the concentration sensitivity increased by on-line MLC before double stacking and CE enantioseparation of terbutaline, see the electropherogram in Figure 3.40 [Pálmarsdóttir & Edholm, 1995]. Microlitre volumes of the cleaned sample from the MLC were concentrated directly in the electrophoresis capillary without significant loss of separation performance. The whole procedure was performed with a high degree of precision. Reproducibility of the double stacking procedure at different concentrations was as follows: RSD of migration times was 0.5-1.5%, peak areas 0.7-2.4% and peak heights 1.4-3.9% for enantiomer 1, and RSD of migration times 0.6-1.0%, peak areas 0.9-3.5%, peak heights 2.8-3.3 % for enantiomer 2. RSD of resolution was in the range of 1.1-1.9%. Other enantiomers of chiral drugs, namely bambuterol, brompheniramine, propranolol, ephedrine, were also separated using the same procedure.

Flow injection. An automatized system with on-line FI derivatization coupled to chiral CE has been developed for the enantiomeric separation of carnitine [Mardones et al., 1999]. This method allowed the determination of D-carnitine in a large excess of L-carnitine (1:100, D:L) in synthetic samples, see Figure 3.41. The reproducibility of the migration time was about 2.3%.

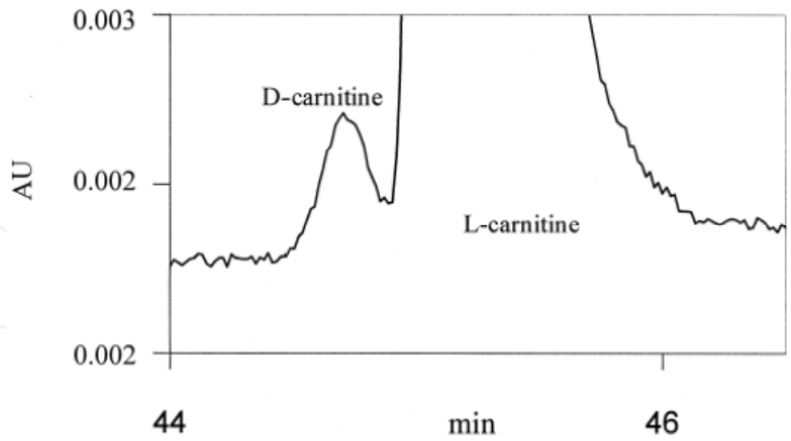

Figure 3.41. FI-CE derivatization and separation of carnitine. Electrophoretic separation of a synthetic sample containing a D-:L-carnitine ratio of 1:100, obtained with automatized FI-CE system based on FMOC derivatization and 2,6 dimethyl-β-CD as chiral selector. Reprinted from ref. [Mardones et al., 1999].

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