4

Advanced Combinations of Detection and Electrophoresis

4.1 Detection in electrophoresis - introduction

In the past two decades, the on-line coupling of separation techniques and appropriate (e.g., spectral, electrochemic) detection, also referred to as hyphenation, has become an important topic in analytical chemistry [Ewing et al., 1989]. In most instances hyphenation is used to obtain more information on the identity of the sample constituents, to improve analyte detectability or to enhance the speed of analysis. Therefore, in the following sections on-line detection approaches will be discussed. A list of the detection techniques hyphenated with CE and several important characteristics of these techniques are given in Table 4.1.

Detection in CE is a significant challenge as a result of the small dimensions of the capillary. Poor concentration sensitivity of CE is due to the limited sample volume that can be introduced into the capillary (typically nanolitre volumes in 25-75 μm internal diameter capillaries) and the low optical path length when the most available and used UV-VIS absorbance detection is employed. This is even worse when combining photometric absorbance detection with the MCE, resulting in a preference for other detection techniques for the microfluidic devices (e.g., fluorescence, electrochemical) [Belder, 2006]. A review of the major on-line CE detection modalities with their advantages and limitations is presented by Swinney and Bornhop [Swinney & Bornhop, 2000]. Hempel [Hempel, 2000] aimed at ascertaining strategies for how to improve the sensitivity in CE for the analysis of drugs in biological fluids, discussing sensitive detection modes such as laser-induced fluorescence detection (LIF) and mass spectrometry (MS). Hernández et al. [Hernández et al., 2008, 2010] give a general view of the different strategies used in recent years (2005-2007) to enhance the detection sensitivity in chiral analysis by CE. Molecular fluorescence, phosphorescence and chemiluminescence spectrometry as very sensitive and selective detection techniques are presented by Powe et al. [Powe et al. 2010]. Alternatively to the sensitive detection techniques (advanced detection), an advanced CE (i.e., CE with on-line sample preconcentration) or, at best, a combination of both advanced approaches, has been proposed for a significant enhancement of the sensitivity in chiral CE analyses, as comprehensively illustrated in chapters 3 and 4 of this monograph.

Analyte identification aspects in CE and CEC separation techniques are discussed by Kok et al. [Kok et al., 1998]. Attention is paid to the spectrometric techniques covering UV-VIS absorption, fluorescence line-narrowing spectroscopy, Raman spectroscopy, nuclear magnetic resonance (NMR) and MS. The most important/applied detection techniques, regarding an on-line structural identification in chiral CE analyses, are comprehensively presented in chapter 4 of this monograph.

When performing chiral analysis based on the implementation of chiral selector into the separation system, potential interferences of the chiral selector with the detection technique have to be considered and avoided, in order not to deteriorate the detection signal. Therefore, great attention must be paid to the selection of the type of chiral selector and to studying the compatibility of the chiral separation system with the linked detection technique in each particular case. Thus, chiral selectors with suppressed absorption properties must be used in absorbance detection, chiral selectors with suppressed electrochemical activity in electrochemical detection, etc. Another possibility in avoiding detection interferences caused by a chiral selector is to utilize specific electromigration effects (countercurrent migration of charged chiral selector, manipulation of the selector velocity by EOF, see chapter 2) [Iio et al., 2005; Yang L. et al., 1997; Lu W.Z. et al., 1996; Fanali et al., 1998a; Schulte et al., 1998; Lu W., 1998] or techniques (the partial filling technique, a zone of the capillary where the enantiomeric separation takes place is filled with BGE containing the chiral selector while the zone close to the detector only contains buffer without chiral selector) [Rudaz et al., 2005; Schappler et al., 2006]. These approaches are used to prevent entering the selector into the detection space. In analogy, the compatibility of chiral selectors with on-line sample preparation techniques also has to be considered in advanced CE systems (those with on-line sample preparation), as discussed in chapter 3. Therefore, the most complex situation is given when considering chiral selector (or, generally, selector) vs. on-line sample preparation vs. on-line detection. This hyphenation requires sophisticated solutions based on a deep knowledge of the particular fields, which are presented in the chapters 2-4 of this monograph.

The most important and/or the most frequently used on- and end-column detection techniques and corresponding hyphenations suitable for (advanced) CE are critically described in the following sections, also giving examples of their utilization in chiral bioanalyses of drugs (section 4.6. and Tables 2.1 and 3.1). Other, more rarely used detection techniques for CE (radioisotope detection, nuclear magnetic resonance, photothermal refraction, refractive index detection, circular dichroism, Raman-based detection, electrochemiluminescence, etc.), with their description and utilization, can be found in review papers given above. Those rare detection techniques have been mostly applied in achiral analyses, while their chiral applications available in literature are given in Table 2.1.

4.2 Photometric absorbance detection

4.2.1 Single wavelength UV-VIS absorbance detection

UV–VIS absorbance detectors working at single wavelength are the most commonly used on-column detectors for microseparations, particularly for CE, due to their interesting features, such as commercial availability, simplicity, versatility, relatively low cost, and frequently used as universal detection techniques because many organics can be detected at 195–210 nm. UV-VIS absorbance detectors produce LODs in CE corresponding to a few femtomoles of analyte (at subpicomole levels), i.e., high mass sensitivity. However, due to the need for small volumes employed in CE to avoid peak broadening that decrease the efficiency of the separation (pl to nL volumes), such sensitivity is in the micromolar range, i.e., appears modest when expressed in terms of concentration (mass detection limits are in the range of 10^{-13}-10^{-15} mol while concentration LODs ranging from 10^{-5} to 10^{-7} M depending

upon the analyte being analysed). These LODs are clearly insufficient to solve many analytical problems, especially to analyse ultratrace analytes in complex matrices, being variable in qualitative and quantitative composition. Therefore, various strategies based on increasing absorbance signal (A=εdc) have been proposed to overcome the poor concentration sensitivity of UV-VIS-CE, such as (i) the selection of lower-UV wavelengths, 190–205 nm [Swinney & Bornhop, 2000], (ii) indirect absorbance detection for analytes with low values of molar absorptivity ε [Yeung, 1989], for the principle see Figure 4.1, (iii) increasing the pathlength d [Kaniansky et al., 1997; Grant & Steuer, 1990; Tsueda et al., 1990; Taylor & Yeung, 1991; Poppe, 1980; Mainka & Bachmann, 1997; Kaltenbach et al., 1997, Anaheim, CA 1997; Aiken et al., 1991] and (iv) derivatization of analytes [Mikuš & Kaniansky, 2000; Hu T. et al., 1995]. However, the strategies (i-iv) have several limitations and specific features that have to be carefully considered before being employed. These are (i) a relatively poor signal-to-noise ratio, chiral selector can also significantly absorb, (ii) use of strongly absorbing chromophores, inserted chromofores can influence complex equilibrium of analyte with chiral selector, (iii) use of capillaries with higher internal diameter, e.g., 300-800 μm (temperature dissipation and, by that, separation efficiency decrease with internal diameter), or properly designed detection window, e.g., bubble cells (see Figure 4.2a and a photo detail in Figure 4.3), Z-shaped cells (see Figure 4.2b), multireflection nanolitre scale cell (often accompanied by larger probe volumes and reduction in separation efficiency and resolution) and (iv) additional sample preparation step is needed, electrophoretic mobilities, as well as the complexing properties of derivatives, can differ from those of their native forms. Nevertheless, the detection sensitivity with UV-VIS absorbance detectors usually cannot be increased more than several tenths times, even if applying any of the presented approaches, so that sample preparation is required. On the other hand, an appropriate use of the on-line sample preparation procedures (as given in chapter 3) enables an application of UV-VIS absorbance detectors, even in ultrasensitive bioanalyses, as illustrated on several examples (in section 4.6. and Table 3.1).

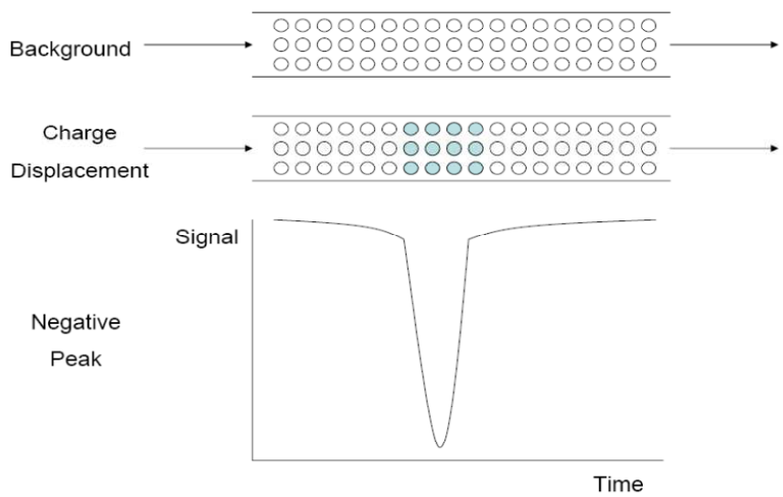

Figure 4.1. Schematic illustration of indirect absorption detection. The separation electrolyte additive provides a large background signal at the detector. Displacement occurs in the analyte zone and lower absorbance intensity is observed. White dots represent absorbing species, blue dots represent non-absorbing species. Reprinted from ref. [Yeung, 1989].

Method	Mass detection limit (moles)	Concentration detection limit (M)[b]	Advantages/ disadvantages
UV-VIS absorption	10^{-13} – 10^{-15}	10^{-5} – 10^{-7}	● Universal ● Diode array offers spectral information
Fluorescence	10^{-15} – 10^{-17}	10^{-7} – 10^{-9}	● Sensitive ● Usually requires sample derivatization
Laser-induced fluorescence	10^{-18} – 10^{-20}	10^{-10} – 10^{-16}	● Extremely sensitive ● Usually requires sample derivatization ● Expensive ● Post-column LIF is 3-6 orders more sensitive than direct on-column LIF
Amperometry	10^{-18} – 10^{-19}	10^{-10} – 10^{-11}	● Sensitive ● Selective but useful only for electroactive analyses ● Requires special electronics and capillary modification
Conductivity	10^{-15} – 10^{-16}	10^{-7} – 10^{-8}	● Universal ● Requires special electronics and capillary modification
Mass spectrometry	10^{-16} – 10^{-17}	10^{-8} – 10^{-9}	● Sensitive and offers structural information ● Interface between CE and MS complicated
Indirect UV, fluorescence, amperometry	1-2 orders less than direct method	–	● Universal ● Lower sensitivity than direct methods
LODs of other detection techniques [M]: Photothermal refraction (10^{-7}-10^{-8}), Potentiometric (10^{-7}-10^{-8}), Refractive index (10^{-5}-10^{-6}, capillary, 10^{-5}, chip-scale), Raman (10^{-3}-10^{-6}, preconcentration needed), Radiofrequency, NMR (10^{-3}), Radioisotope (10^{-10}), Laser-induced capillary vibration (10^{-8}, chemical derivatization, 10^{-5}, native)			

Table 4.1. Detection techniques in CE[a]
[a] adapted from refs. [Heiger, 2000; Swinney & Bornhop, 2000]
[b] assume 10 nL injection volume

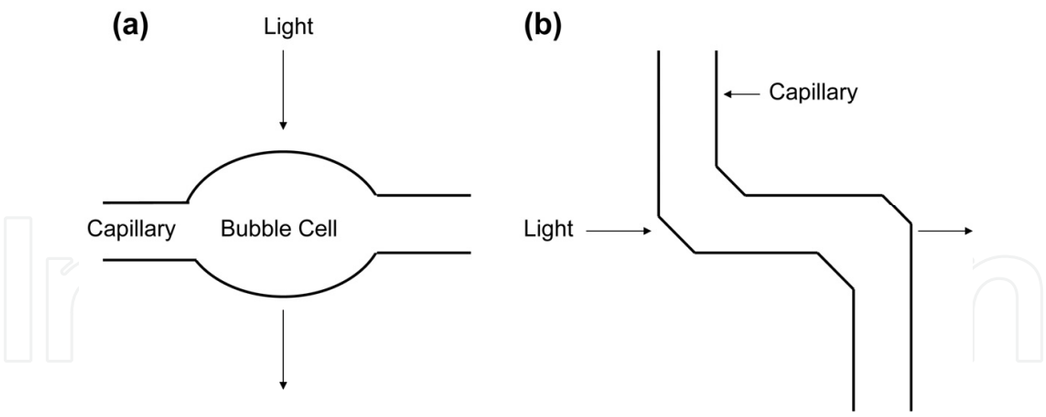

Figure 4.2. Techniques for increasing the pathlength of the capillary: (a) a bubble cell and (b) a z-cell (additional tubing). Reprinted from ref. [Skoog et al., 2007].

4.2.2 Multi-wavelength (spectral) UV-VIS absorbance detection

A diode array detector (DAD) serves as a typical multi-wavelength on-column CE detector providing UV-VIS spectra in the required interval of wavelengths [Heiger D.N. et al., 1994; Beck et al., 1993], for the instrumental scheme of the CE-DAD see Figure 4.3. Many organic compounds have characteristic spectra in the UV which can be used to help identify the substance passing through the sensor cell.

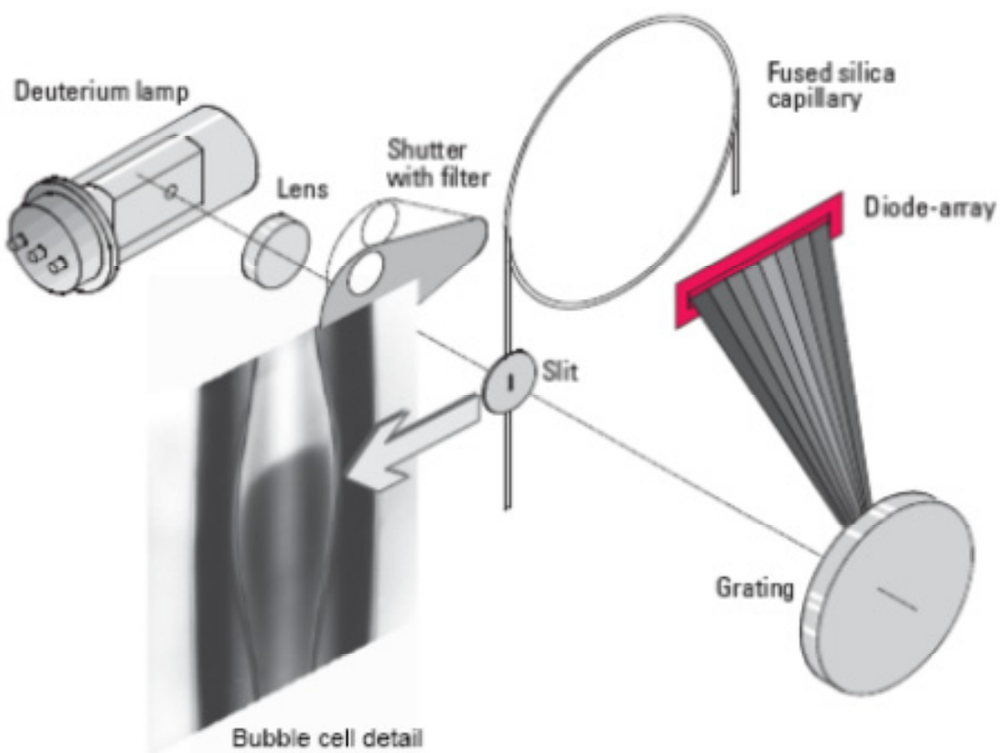

Figure 4.3. Schematic overview of the CE diode array detector (DAD) optics. Reprinted from ref. [Agilent Technologies, 2009].

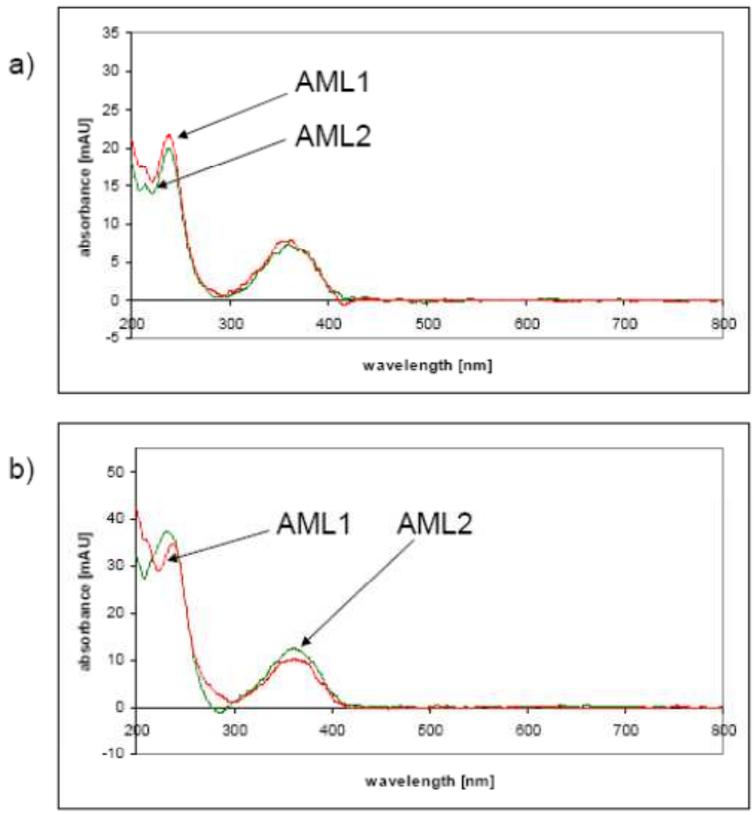

Figure 4.4. Processed UV-VIS spectra of amlodipine (AML) in different matrices. (a) AML present in demineralized water, serving as a reference spectrum, (b) AML present in 10 times diluted model urine, at a 500 ng/mL concentration level of the drug in the samples. Separating and other working conditions of the ITP-EKC-DAD method modified with (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD), serving as the chiral selector, were the same for (a) and (b). AML1, AML2 – migration positions of the first migrating enantiomer of amlodipine (red spectrum), the second migrating enantiomer of amlodipine (green spectrum), respectively. Reprinted from ref. [Mikuš et al., 2008a].

Although UV-VIS spectra are not as informative as those of NMR, MS or infra-red spectrometry, they still can serve for a preliminary confirmation of peak/analyte identity and validation of peak purity [Strašík et al., 2003]. The similarity of spectral profiles can be used for the indication of structurally related compounds separated from each other while differences in spectra can indicate impurity in analyte peak. As an example, the similar profiles of the UV-VIS absorption spectra of the amlodipine enantiomers recorded in the model water and urine samples, indicating an acceptable purity of the separated electrophoretic peaks of the amlodipine enantiomers in the complex matrices (urine), are shown in Figure 4.4. With respect to spectral analysis, DAD also has significant advantages over rapid scanning detectors such as the signal-to-noise ratio of spectral data being independent of the number of wavelengths acquired, the bandwidth of individual wavelengths not being predetermined and on-line spectra being available at all times [Heiger D., 2000]. CE-DAD provides comparable sensitivity and linear detection range, and

the same possibility to be combined with CE (e.g., on-line combination via optical fibres) when compared to the single wavelength UV-VIS absorbance detectors. Therefore, CE-DAD was proposed as an advantageous alternative to conventional CE-UV with the single wavelength detection and, at the same time, a pragmatic solution to the expensive CE-MS or CE-NMR, for many pharmaceutical and biomedical applications including those with the enantioselective aspects [Beck et al., 1993], see also examples in section 4.6 and Table 3.1.

4.3 Fluorescence detection

4.3.1 Conventional fluorescence detection

Fluorescence detection is a more sensitive and selective alternative to UV-VIS absorbance detection, and it is usually carried out in the on-column arrangement. For a schematic layout of on-column fluorescence detection see Figure 4.5. Mass detection limits are in the range of 10^{-15}-10^{-17} mol, while concentration LODs range from 10^{-7} to 10^{-9} M depending upon the analyte's fluorofore [Ewing et al., 1989].

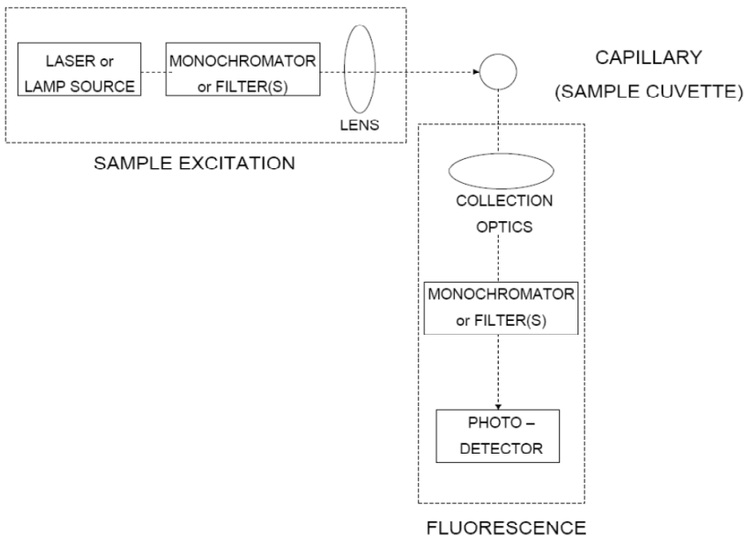

Figure 4.5. A schematic layout of on-column fluorescence detection. Adapted from ref. [Amankwa et al., 1992].

Generally, there is a considerably reduced number of compounds exhibiting natural fluorescence in comparison with UV-VIS absorbing compounds and therefore, this detection mode is very often linked with analyte derivatization. However, the complicated chemistry and time-consuming procedures associated with derivatization make fluorescence detection {conventional, as well as LIF (see below)} less attractive than universal detection methods. Another possibility to perform fluorescence detection is its indirect regime (universal). The concept of indirect fluorescence measurements is essentially the same as indirect absorbance measurements, except the background (buffer) fluoresces instead of absorbing, see Figure 4.1. Unfortunately, indirect fluorescence (likewise UV absorbance and other indirect methods) has 10-100 times less sensitivity than direct methods. Poor LODs for indirect fluorescence measurements are the result of the noisy fluorescence background. Therefore, it is not surprising that the indirect fluorescence mode has rare use in CE.

4.3.2 Laser-induced fluorescence detection

Laser-induced fluorescence (LIF), based on a fluorescence signal produced by the molecule after its illumination by a laser beam, is the most sensitive, extremely selective, small volume detection method, suitable for an on-line arrangement with the CE. It is also one of the most favoured detection techniques for microfluidic devices such as MCE. The first on-column LIF detection system was developed by Zare et al. [Gassmann et al., 1985]. For the scheme of the CE-LIF see Figure 4.5. The concentration LODs of the best CE/laser-based systems are in interval 10^{-14}-10^{-16} M with mass detection limits 10^{-18}-10^{-20} mol (individual molecules can be detected) [Ewing et al., 1989]. A variability (sensitivity, selectivity) of LIF detection is given by employing different lasers {the common argon laser (458 and 488 nm), He-Cd laser (440 nm), blue diode laser (420 nm), frequency-doubled Ar-ion laser (257 nm), frequency-doubled Kr-ion laser (284 nm)} [Hernández et al., 2008, 2010; Nie et al., 1993; Chang & Yeung, 1995; Craig D.B. et al., 1998]. To overcome problems associated with on-column detection, such as broad-band luminescence background and poor S/N ratios, Rayleigh and Raman light scattering from the capillary, several techniques based on the spatial or spectral separation of the fluorescence signal from the high background signal have been proposed (the best results have been reached with a sheath flow cuvette incorporated at the end of the capillary, performing an end-column LIF) [Cheng Y.F. & Dovichi, 1988; Chen D.Y. & Dovichi, 1996; Craig D.B. et al., 1998; Xue & Yeung, 1995]. For example, the schematic diagram of sheath-flow CE-LIF system with multichannel photomultiplier tube detection is shown in Figure 4.6.

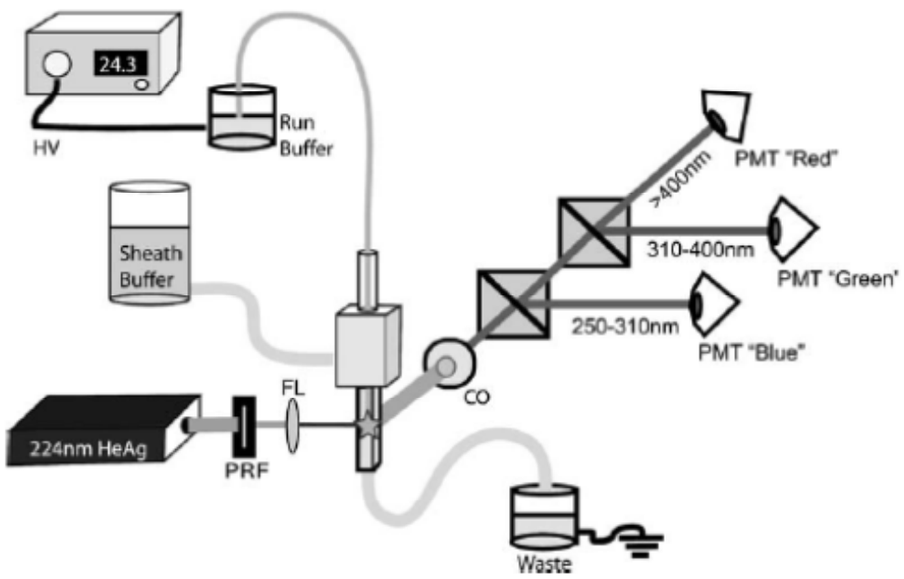

Figure 4.6. Schematic diagram of sheath-flow CE-LIF system with multichannel photomultiplier tube (PMT) detection. The beam from a HeAg laser is used for excitation of the analyte as it elutes from the capillary. Fluorescence is collected orthogonally to the excitation optics and transmitted or reflected by one or both of two custom dichroic beam-splitters. The spectrally distributed fluorescence is detected by one of three PMTs corresponding to wavelength ranges 250–310 nm (PMT "Blue"), 310–400 nm (PMT "Green"), and 400+ nm (PMT "Red"). Reprinted from ref. [Lapainis et al., 2007].

The application examples shown in section 4.6. and Tables 2.1 and 3.1 indicate that CE-LIF is a powerful tool, due to its excellent sensitivity, for the analysis of samples ranging from tissue extracts to single cells. However, one challenge for the chiral CE analysis of complex biological samples is the accurate peak identification in complex electropherograms, as often matching a migration time between an analyte and the corresponding standard may be insufficient to confirm the peak's identity. Therefore, sample preparation procedures, and, especially, their on-line modes (chapter 3), should be used to eliminate matrix interferents and, by that, simplifying the confirmation of the peak's identity, see e.g., [Cho et al., 2004] and Figure 3.33. Another interesting possibility to confirm the peak's identity reliably is based on the confirmation of analyte signal identity via a combination of single-step immunoprecipitation (specific elimination of analyte signal) and CE-LIF analysis [Miao et al., 2006], see Figure 4.7.

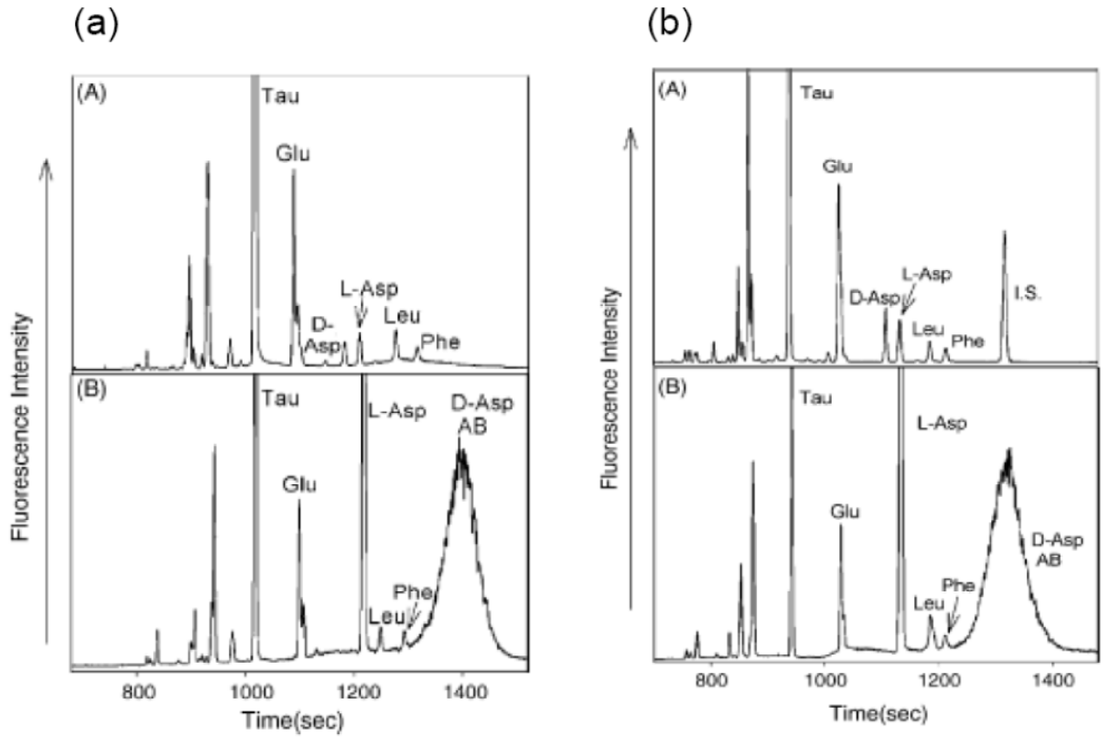

Figure 4.7. D-Asp antibody allows confirmation of analyte peak assignment in the CE-LIF analysis of complex biological samples such as nerve tissue (a) or single cell (b). Chemical information output of CE-LIF analysis is significantly enhanced by use of the antibody for signal identification. (a) Electropherograms of an anterior tentacular nerve sample before (A) and after incubation (B) with D-Asp antibody and L-Asp conjugate. (b) Electropherograms of an MCC neuron sample before (A) and after incubation (B) with D-Asp antibody and L-Asp conjugate. Increased intensity of the L-Asp signal and disappearance or decrease in the amplitude of the D-Asp peak are noticeable in (B). Internal standard (I.S.), L-cysteic acid. Adapted from ref. [Miao et al., 2006].

4.4 Electrochemical detection

Electrochemical detection for CE can be divided into three main categories: conductimetric (based on conductivity changes monitored by sensors during electrophoretic migration process) [Landers, 1997; Everaerts et al., 1979; Gebauer et al., 1997], potentiometric (based on classical ion-selective microelectrodes) [Haber et al., 1990, 1991] and amperometric (based on electrode vs. analyte redox reaction that produces a current, directly related to the analyte concentration) [Ewing et al., 1994]. Electrochemical detection provides an alternative solution for non-absorbing or non-fluorescing analytes. This detection approach offers both universal and selective modes, and provides sensitive measurements in an end-column, as well as an on-column, arrangement in the CE and MCE format [de Silva, 2003].

The most difficult problem to solve in applying electrochemical detection in CE is the need to instrumentally eliminate the effect of the strong electric field used in electrophoretic separation from the conditions of electrochemical detections. The main drawback of electrochemical detectors in their implementation with CE is that it can be extremely difficult and tedious since sensor preparation can involve complicated fabrication and handling procedures, and accurate spatial alignment of the electrodes within the capillary [Swinney & Bornhop, 2000]. Moreover, limited sensor lifetime and required recalibration, strong adsorption of the intermediate reaction products of the analyte to the electrode surface reducing its activity/response (this can be overcome by the electronic cleaning of the electrode) [Haber et al., 1991; Ewing et al., 1994] are other limiting factors that should be carefully considered when electrochemical detectors are applied. These limitations can be pronounced, especially, when complex biological samples are analysed, where sample pretreatment (especially purification) is strongly recommended for reliable and reproducible detection.

Nevertheless, recent years have provided numerous new examples of applying flow-through electrochemical detectors in chemical analysis. The review by Trojanowicz [Trojanowicz, 2009], based on about 250 original research papers cited from the current analytical literature, presents their application in flow analysis and capillary electrophoretic methods. Potentiometric detection with ion-selective electrodes predominates in flow analysis carried out mostly in a flow injection system, while amperometric and conductivity detections are most commonly employed in capillary electrophoresis and they have been applied also in the chiral field. For application examples of electrochemical detection in chiral CE, see section 4.6. and Tables 2.1 and 3.1.

4.4.1. Amperometric detection

As with other electrochemical detection methods in CE, amperometric detection is also carried out with the use of microelectrodes because of the need to adjust their size to the diameter of capillaries. There are several most commonly reported constructions of various types used for amperometric detection cells in CE, as well as various types of working electrodes resulting in interesting application possibilities and very low detection limits obtainable, as reviewed by Trojanowicz [Trojanowicz, 2009]. Schemes of the amperometric detector coupled with the hydrodynamically open (conventional) and hydrodynamically closed (unconventional) CE systems are shown in Figure 4.8 and Figure 4.9, respectively.

Amperometric detection is based on electron transfer to or from the analyte of interest at an electrode surface that is under the influence on an applied DC voltage. The result of electron transfer is a redox reaction at the electrode that produces a current that is directly related to the analyte concentration. Therefore, chiral selectors giving a redox reaction at the electrode, comparable with the redox reaction of the analyte, cannot be employed for chiral separations with this type of detection and a modified chiral system has to be used.

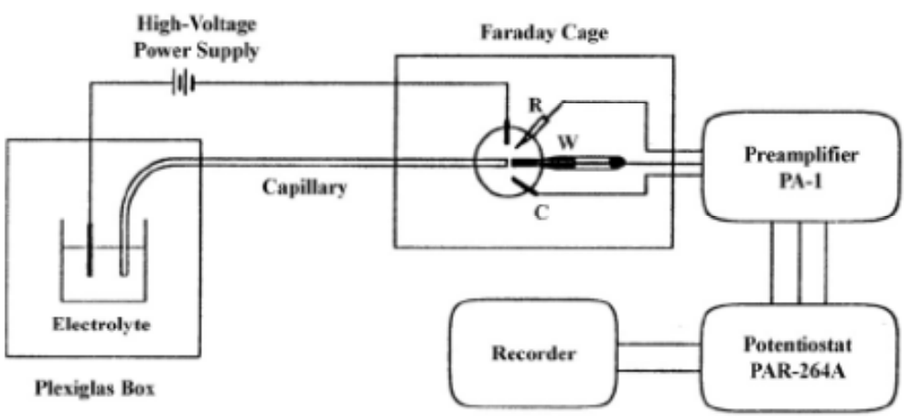

Figure 4.8. Schematic diagram of the capillary electrophoresis system with end-column amperometric detection: R, reference electrode; W, working electrode; C, counter electrode. Reprinted from ref. [His et al., 1997].

Pulsed amperometric detection (including the electrode cleaning step), an advanced detection mode, can reduce the major problem of this detection, linked with strong adsorption of the intermediate reaction products of the analyte to the electrode surface (reducing activity, i.e., electron transfer of the electrode and interfering with detection) [Ewing et al., 1994]. In this way, reproducibility of measurements, as well as sensitivity, can be considerably improved [Trojanowicz, 2009].

The conventional amperometric mode, usually used in the end-column configuration, is selective (restricted only for electroactive analytes) and can be tuned to the analyte of interest [O'Shea et al., 1993; Lu W.Z. & Cassidy, 1993]. On the other hand, the square-wave mode can be successfully applied for detection of typical nonelectroactive organic compounds, where currents associated with the non-Faradaic interfacial process due to analyte adsorption at a Pt microelectrode were used to provide the analytical signal [Gerhardt et al., 2000]. The detection limits obtained in such measurements are at least one order of magnitude lower than the ones that could be obtained by UV absorption. Moreover, such an amperometric detection mode is more universal and provides a wider application range.

Compared to the conductimetric mode, amperometric detection is much more sensitive, mass detection limits are in the range of 10^{-18}-10^{-19} mol corresponding to 10^{-10}-10^{-11} M concentration LODs [Ewing et al., 1989]. Thus, amperometric detection represents a good alternative to LIF for ultrasensitive CE determinations of another specific

group of analytes. For application examples of amperometric detection in chiral CE, see section 4.6. and Tables 2.1 and 3.1.

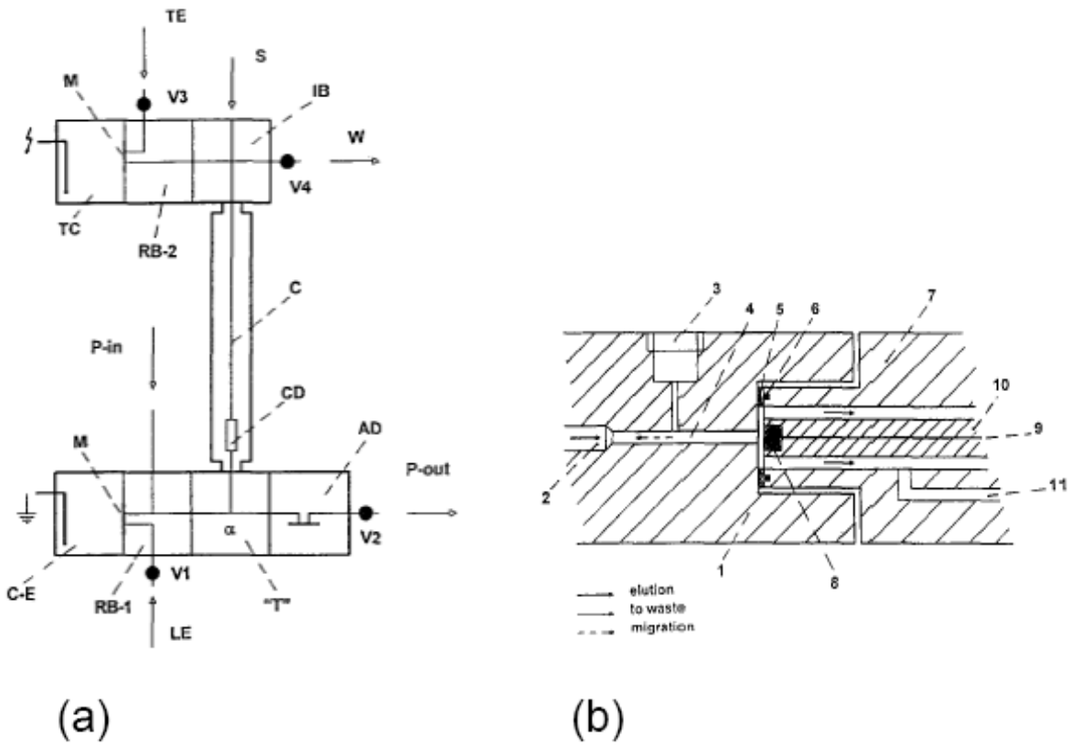

Figure 4.9. (a) Schematic diagram of the hydrodynamically closed CE separation unit with a post-column amperometric detector. IB = injection block for a microsyringe sample injection; C= capillary tube; CD =on-column conductivity detection cell; "T"=elution T-piece; α =bifurcation point; AD= amperometric detection cell; RB-1 = refilling block for the capillary tube (C); C-E=counter-electrode compartment; M = Cellophane membranes; LE = inlet for the leading electrolyte solution; P-in, P-out = inlet and outlet for the elution solution, respectively; RB-2 = refilling block for the terminating compartment (TC) and the injection block (IB); TE = inlet for the terminating electrolyte solution; W = waste; S = position for the injection of the sample; V1-V4 = valves. (b) Detail of the amperometric detection cell attached to the T-piece. l=T-piece; 2=channel to the counter electrode; 3= connection for the column; 4=elution channel (detection capillary) of 4 mm × 0.2 mm i.d.; 5=PTFE spacer (0.05 mm); 6=O-ring for a leak-proof connection; 7= amperometric cell; 8=working electrode; 9=connecting cable to the electronics of the detector; 10 = epoxy body of the electrode; 11 =connecting channel for the reference electrode. Reprinted from ref. [Kaniansky et al., 1995].

4.4.2 Conductivity detection

Conductivity detection, due to separation ions, charged particles or microorganisms in CE, has a special importance for capillary electrophoresis. The charged species are detected in the solution of electrolyte, which makes use of this detection process difficult. Nevertheless, the

practical importance of this detection is justified by the fact that commercially available detectors are on the market only for this electrochemical detection for CE [Trojanowicz, 2009].

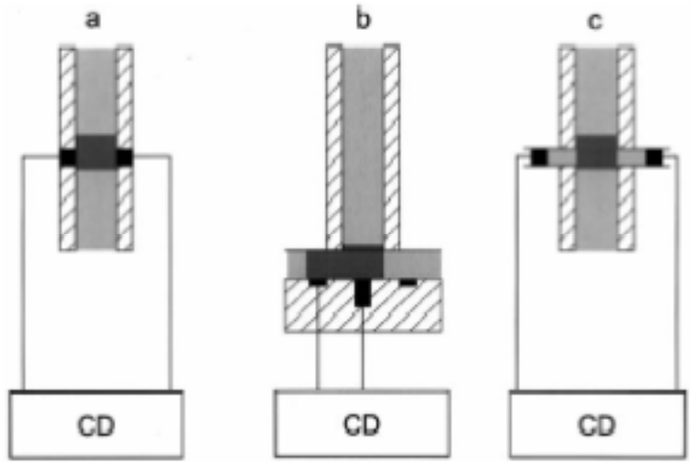

Figure 4.10. General schemes of the arrangements of the detection electrodes in contact conductivity detection cells for CZE. (a) An in-column placement of the detection electrodes exposed to direct contact with the separated constituents; (b) a post-column placement of the detection electrodes exposed to direct contact with the separated constituents; (c) an on-column placement of the detection electrodes with an electrolyte solution mediated contact with the detection compartment of the cell. CD, measuring circuitry of the detector. Reprinted from ref. [Bodor et al., 2001].

In general, conductivity detectors consist of two sensors (electrodes), in direct or indirect contact with the electrolyte solution, across which an electrical potential has been applied. General schemes of the arrangements of the detection electrodes in contact conductivity detection cells for CZE are shown in Figure 4.10. Conductimetric mode is attractive by providing universal detection in an on-line arrangement and it is the most frequently used electrochemical detection for CE. Therefore, it can be the first choice when analysing non-absorbing/fluorescing organic ions avoiding the derivatization step in the analytical protocol. However, sensitivity of the contact conductivity detection mode is moderate, mass detection limits are usually in the range of 10^{-14}-10^{-15} mol corresponding to 10^{-6}-10^{-7} M concentration LODs [Landers, 1997]. This is not sufficient for the majority of clinical and biomedical analytical problems when performed by conventional CE.

Improvement of detectability can be additionally achieved to the sub-ppb level by applying on-capillary preconcentration by electrostacking [Haber et al., 1998]. Another method of lowering the value of the detection limit in CE determinations with conductivity detection, following wide use in ion-chromatography, is to apply on-line suppressing of conductivity of BGE by the ion-exchange process [Avdalovic et al., 1993]. For example, the suppressor for determination of anions can be made of an ion-exchange sulfonated Teflon capillary tubing (1 cm long) placed between the separation capillary and the detector electrode in the wall-jet arrangement. This method can improve the detectability of about two orders of magnitude compared to indirect detection of non-UV-absorbing ions.

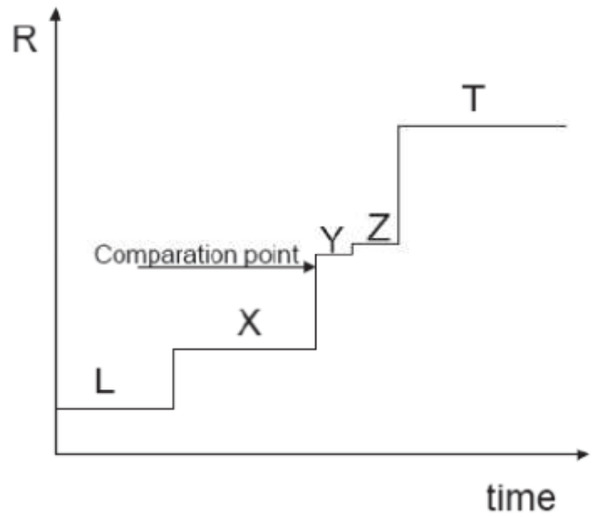

Figure 4.11. Graphical illustration of the principle of the electronic cutting of the zone of interest in the ITP stage of the ITP-CZE combination. L = leading ion, T = terminating ion, X = matrix compound(s), Y, Z = analytes, R = resistance. Reprinted from ref. [Tekeľ & Mikuš, 2005].

Jumps in voltage (conductivity) between neighbouring zones result in permanently sharp boundaries between zones and are very convenient for conductivity detection. Although extremely convenient to the detection of the ITP zones (the preferred detection in this field), the conductivity detection technique has a limited applicability in the CZE separations of enantiomers. It can be caused by the measurements of small conductivity changes due to the analyte zones on a relatively high-conductivity background of the carrier electrolyte. Therefore, when monitoring the enantioseparations by the conductivity detection, mobility difference of the analyte vs. chiral selector must be carefully considered.

On the other hand, one interesting possibility of the sensitive analyses of enantiomers in multicomponent ionic samples with the aid of conductivity detection is its implementation into column-coupled CE-CE systems, see examples in ref. [Gebauer et al., 2009, 2011; Mikuš & Maráková, 2009, 2010]. Conductivity detectors in ITP produce a staircase signal, resistance (or conductivity) vs. migration time, in which the relative high of step is constant for a given analyte under given separation conditions (qualitative parameter independent on analysis time). Such an ITP profile can be beneficial in the preliminary mapping of the composition of complex (e.g., biological) samples. This also gives a good possibility of an accurately "cut" (by electronic switching) desired zone (analyte) in the preseparation stage (ITP) of ITP-EKC combination and transferring it into the analytical stage (EKC) without potential interfering compounds from the matrices, avoiding any analytical capillary overloading by the major matrix constituents [Danková et al., 1999], see Figure 4.11. In this way, chiral analytes in multicomponent matrices (e.g., biological) can be monitored and on-line preseparated/pretreated in an achiral environment before a chiral CZE (i.e., EKC) step [Mikuš et al., 2006a, 2008a, 2008b; Marák et al., 2007, 2008; Mikuš, 2010]. Anyway, the conductivity detection can be advantageously used also for the detection of enantiomers in a chiral environment as it is well documented in the literature [Mikuš et al., 2006b; Kubačák et al., 2006a, 2006b, 2007].

Chiral selectors, as well as sample matrix constituents, can influence contact conductivity detection. To reduce a decrease in detection sensitivity and reproducibility caused by a modification of the electrode surface in an ionic environment, various approaches have been proposed. A contact conductivity detection cell for CZE with an electrolyte solution mediated contact of the separated constituents with the detection electrodes (ESMC cell) was proposed to eliminate detection disturbances due to electrode reactions and adsorption of the separated constituents when these are coming into direct contact with the detection electrodes, see Figure 4.12 [Bodor et al., 2001]. Despite precautions in the construction of sensing and measuring parts of the conductivity detector, the detection in the ITP runs can be accompanied by enhanced noise of the detection signal and some detection disturbances when injecting common real samples, i.e., complex ionic samples (Figure 4.13a). Here, an electrochemical cleaning of the detection electrodes as be recommended for some conventional conductivity detection cells [Kaniansky, 1981; Kaniansky et al., 1983] provided a simple and effective solution also in microchip format [Masár et al., 2001]. Here, a pair of 100-V pulses of reversed polarities is applied between the electrodes of a particular sensor along with a stream of the leading electrolyte solution. An overall effect of this cleaning procedure is clearly illustrated by isotachopherograms in Figure 4.13a and b.

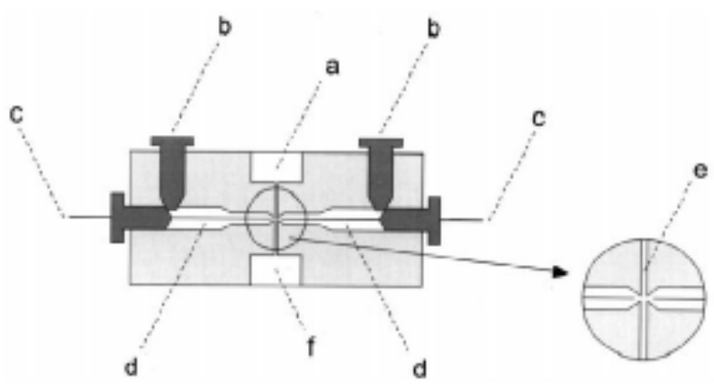

Figure 4.12. A schematic drawing of the conductivity detection cell with an electrolyte solution mediated contact of the separated constituents with the detection electrodes. (a) A connection of the cell to the separation capillary; (b) holes with screw plugs for filling the electrode channels (d) with the detection electrodes (c); (e) the detection compartment; (f) a connection of the cell to the counter-electrode compartment. A body of the cell was made of poly(methyl methacrylate). Reprinted from ref. [Bodor et al., 2001]

A contactless conductivity detector (instrumental scheme see in Figure 4.14), although a bit less sensitive than contact conductivity detector, is an advantageous alternative for the analyses of complex samples as it does not suffer from the contamination of the conductivity sensors by compounds from the electrolyte or sample (adsorption, electrochemical reactions) and, hence, the high reproducibility of detection signal can be maintained [Mikkers et al., 1979; Gebauer et al., 1997; de Silva et al., 2002; Kaniansky et al., 1999]. This detection approach was successfully applied also in ITP-EKC analyses of enantiomers in multicomponent ionic matrices, see refs. [Mikuš & Maráková, 2009, 2010] and references cited therein.

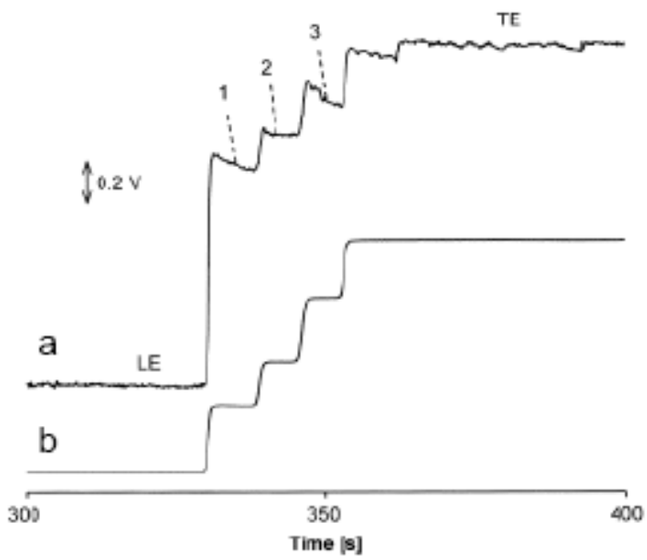

Figure 4.13. Impacts of cleaning of contaminated surfaces of the detection electrodes on the responses of the conductivity sensors in the ITP separations on the CC chips. Isotachopherograms from the separations of a three-component test mixture of anions as obtained by a particular sensor (a) before and (b) after electrochemical cleaning of its electrodes. LE=Leading anion; 1=succinate; 2=acetate; 3=benzoate; TE=terminating anion. The concentrations of the analytes in the test sample were 300 μM. The driving current was 10 μA. Reprinted from ref. [Masár et al., 2001].

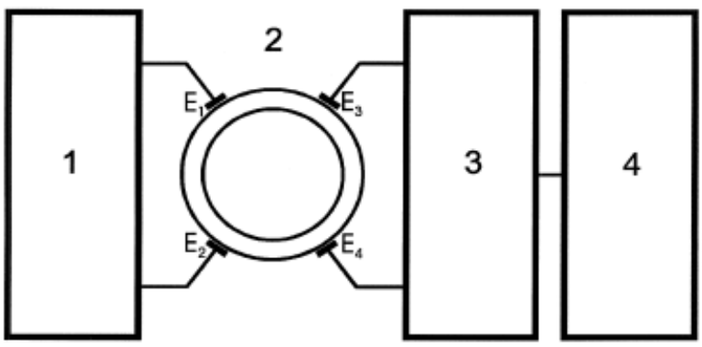

Figure 4.14. Block scheme of a high-frequency contactless conductivity detector used in this work. 1, high-frequency generator (consisting of a 4-MHz oscillator, an amplifier and inverter, phasing elements and output voltage amplifiers); 2, four-electrode (capacitive) detection cell; 3. receiver (consisting of a pre-amplifier, a narrow-band amplifier, a selective filter, a high-frequency rectifier, a d.c. amplifier and a low band-pass filter); 4, a data acquisition unit; E –E , detection electrodes. Generated from refs. [Gaš et al., 1980; Vacík et al., 1985].

4.5 Mass spectrometry

An on-line combination of CE and MS (representative scheme of CE-MS is in Figure 4.15) is a highly effective technique that separates analytes according to differences in their electrophoretic mobilities and then provides information on molecular masses and/or

fragmentation of analysed substances. In this way, the on-line CE–MS technique has been established as a powerful separation and identification analytical tool. Moreover, this end-column hyphenation may enhance the sensitivity of certain compounds depending on their characteristics such as proton affinity, absorptivity, etc. In the last 10 years there have been many significant developments in CE-MS instrumentation and applications that have made CE-MS a competitive tool.

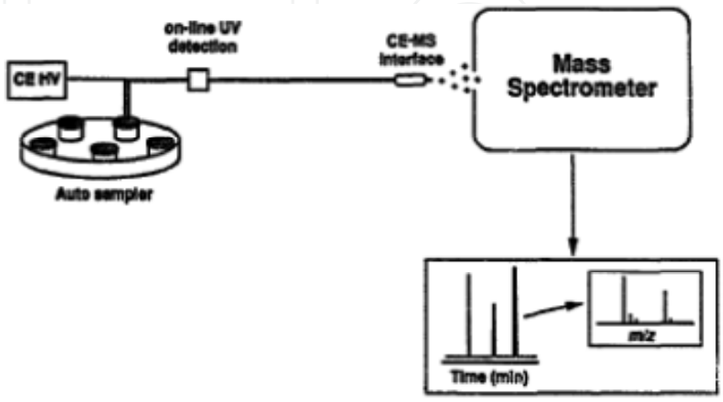

Figure 4.15. Generic illustration of a CE-MS instrumental arrangement. HV= High voltage. Reprinted from ref. [Cai & Henion, 1995].

Several recent review papers have been devoted to a combination of CE-MS, applied to the structural identification of analytes and trace analytes. Surveys on developments and applications of CE-MS in the years of 2003-2004 and 2005-2006 are given by Schmitt and Englmann [Schmitt-Kopplin & Englmann, 2005] and Gaspar et al. [Gaspar et al., 2008], respectively. The reader will rapidly find the mostly used CE-MS combinations, as well as applications classified as forensic, environmental, bioanalytical, pharmaceutical and metabolomics. Ramautar et al. [Ramautar et al., 2009] pay attention to the utilization of CE-MS in metabolomics, considering papers published between 2000 and 2008. The authors presented CE-MS to be a powerful technique for the profiling of polar metabolites in biological samples. The combination of CE with MS was chosen as a review topic also in conjunction with chiral separations in aqueous and non-aqueous media [Cai & Henion, 1995; Shamsi, 2002; Scriba, 2007]. Methodological aspects of CE–MS for quantitation were summarized and evaluated by Ohnesorge et al. [Ohnesorge et al., 2005]. Pantučková et al. [Pantučková et al., 2009] reviewed electrolyte systems for on-line CE-MS with respect to detection requirements and separation possibilities. Smyth and Rodriguez [Smyth & Rodriguez, 2007] summarized recent studies of the electro spray ionization (ESI) behaviour of selected drugs and their application in CE-MS and HPLC-MS. For application examples of chiral CE-MS, see section 4.6 and Table 3.1.

4.5.1 CE stage in CE-MS

The electrolyte systems in CE must be carefully selected regarding all the requirements of the on-line coupled MS analyser where terms such as the analyte ionization and evaporation play the key role (see section 4.5.2 CE-MS interfaces). It is obvious that both electrophoresis BGE and the solvent must support the ionization process and evaporation of droplets (i.e.,

volatile and low ionic strength buffers, such as acetic acid, formic acid and their ammonium salts, buffers with carbonate as a volatile anion, and alkylamines as volatile cations), otherwise, the MS signal suppression due to salt deposits on the source and decreased response due to ion-pairing with the analyte could occur (e.g., citrate, phosphate, phthalate, morpholine, Tris buffers). Operational regions in CZE recommended for use in practice for on-line CE–MS are shown in Figure 4.16. The limited choice of electrolytes that are suitable for online CE–MS brings considerable limitations not only to the CE separation itself, but also to the potential sample stacking [Pantučková et al., 2009]. For example, separation properties of typical suitable ITP systems are given in Table 4.2. Due to the high volatility and low surface tension of organic solvents like methanol or acetonitrile, the use of non-aqueous media in NACE-ESI-MS may enhance ionization, resulting in improved detection limits compared to separation in aqueous buffer systems [Servais et al., 2006].

Although numerous publications have appeared on CE-MS, this technique is still developing to be widely accepted for routine use. The major limitation of CE is the limited sample volumes that can be analysed without compromising separation efficiency. Consequently, the concentration detection limit for CE can be several orders of magnitude higher than that of chromatographic methods. Using the currently available instrumentation, CE-MS detection limits for some applications can be too high, making it unlikely to be used in routine analysis. For example, a study was conducted comparing the performance of CE-MS with that of microbore LC-MS in the determination of endogenous amounts of leucine-enkephalin and methionine-enkephalin in equine cerebrospinal fluid using identical sample clean-up and enrichment procedures [Muck & Henion, 1989]. CE-MS was found to be limited in its concentration detection capacity owing to its much smaller injection volume. Another drawback with CE-MS is that migration times tend to fluctuate with a change of temperature in the environment. Although some manufacturers have incorporated a temperature controlling system into their CE instruments, these devices cannot be effectively utilized in CE-MS applications because a large portion of the CE capillary is extended between the CE instrument and the mass spectrometer. For applications such as regulatory work or those involving unknown components in a mixture, the use of a suitable internal standard would be necessary [Pleasance et al., 1992; Henion et al., 1994]. The chemical condition of the CE capillary inner walls also plays an important role in CE separation. The reproducibility and ruggedness of CE-MS are not currently as good as those of LC-MS. Like LC-MS, the use of non-volatile buffers in CE-MS is generally avoided. Compromises are often made in choosing appropriate operational conditions for CE-MS. Sensitivity limitations, as well as ion source plugging problems, created by the use of non-volatile buffers have impeded the direct transfer of CE separation conditions to on-line CE-MS. Non-volatile additives, such as cyclodextrins, are widely used in the separation of closely related analytes including optical isomers [Ward, 1994; Novotny et al., 1994], however, the concentrations of these additives are often limited by practical restrictions [Varghese & Cole, 1993]. The MS sensitivity may deteriorate as the bulk flow of surfactant enters the mass spectrometer source region.

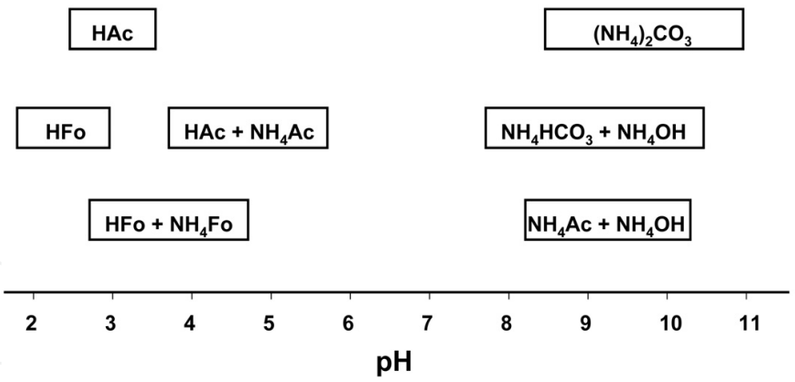

Figure 4.16. Operational regions in CZE recommended for use in practice for on-line CE–MS. The concentration of NH_4Ac usually ranges from 0.01 to 0.3M and the concentration of NH_4Fo from 0.02 to 0.9M. The pH of the buffer in the acidic range is adjusted with either HAc (0.01–1.2 M) or HFo (0.02–2 M) according to the co-ion used. Alkaline pH is usually adjusted by adding ammonia solution or alkylamine. Separation in alkaline pH is sometimes done in NH_4HCO_3 or $(NH_4)_2CO_3$ buffers in concentrations between 0.01 and 0.1 M. Reprinted from ref. [Pantučková et al., 2009].

	LE	*L*	$u_{eff,L}$ a)	pH_L	TE	*T*	$u_{eff,T}$ a)	$pH_{T,adj}$ b)
	Cationic ITP							
1	10mM NH_4Ac + 5mM HAc	NH_4^+	76.2	5.06	10mM HAc	H^+	14.7	3.25
2	10mM NH_4OH + 20mM Asp	NH_4^+	56.3	8.80	10mM imidazol + 20mM Asp	$Imid^+$	7.8	7.91
	Anionic ITP							
3	10mM $(NH_4)_2CO_3$	CO_3^{2-}	-47.8	9.19	10mM EACA + 20mM NH_4OH	$EACA^-$	-7.2	10.32
4	10mM NH_4Fo + 5mM HFo	Fo^-	-38.5	4.06	10mM MES + 10mM NH_4OH	MES^-	-11.2	5.92

Table 4.2. Separation properties of examples of ITP systems
a) Effective mobility (u_{eff}) of leading (*L*) or terminating (*T*) ion, in $10^{-9}m^2V^{-1}s^{-1}$.
b) pH adjusted.
Reprinted from ref. [Pantučková et al., 2009].

Fortunately, a CE methodology improvement for advanced CE-MS hyphenation can be performed in several ways. A number of on-line sample preparation techniques have been introduced to improve the concentration sensitivity of CE, as given in chapter 3. One of the approaches for on-line analyte concentration for CE hyphenated with MS is the chromatographic method. This concept has been incorporated into CE-ESI-MS applications [Tomlinson et al., 1994]. The pre-column was made of a small bed of HPLC packing attached

to the inlet of the CE capillary. It serves as an on-line device for sample clean-up such as desalting and preconcentration for subsequent analysis by CE-ESI-MS. The capability of the ITP-CZE-ESI-MS system was demonstrated for the trace analysis of a complex matrix such as calf urine extracts. Several research groups have evaluated the ITP-CZE-ESI-MS approach [Kelly et al., 1994; Severs & Games, 1994; Moseley, 1994]. On-line ITP preconcentration has lowered the concentration detection limits of CE-MS by two orders of magnitude [Kelly et al., 1994; Moseley, 1994.]. Such an improved CE system provided much better separation efficiency than conventional LC systems. The two dimensional ITP approach based on a large volume sample injection (30 μl injection volume) and large volume ITP preseparation (800 μm i.d.) was applied for a very efficient sample preconcentration and analyte injection into a hyphenated MS stage [Tomáš et al., 2010]. The experimental arrangement of this ITP-ITP approach is illustrated in Figure 4.17. Although it has not been used for any enantiomeric separation so far, the ITP-CE approach is very promising for advanced hyphenation with the MS detection, overcoming many of the practical limitations of the CE-MS combination.

The use of small-ID capillaries was found to have improved the absolute sensitivity of CE-ESI-MS [Wahl et al., 1992, 1993]. This improvement in sensitivity is due to the high ionization efficiency associated with the very low bulk flow-rate from the small-ID capillary into the mass spectrometer. Since the ESI ion current is nearly independent of flow-rate, ESI-MS will operate as a mass-sensitive detection system when the ESI current is limited by the flow-rate of the charged species in the solution to the ESI source [Wahl et al., 1994]. For example, a 25- to 50-fold increase in absolute sensitivity can be obtained by reducing the i.d. of CE capillary from 100 to 10 μm [Wahl et al., 1993]. Another instrumental development is the use of coated capillaries. Coated capillaries are often used in the analysis of biological samples to minimize band broadening and peak tailing due to adsorption of analytes onto the capillary walls [Kohr & Engelhardt, 1993]. Moreover, the elimination of sample adsorption (mainly proteins) and EOF considerably increases reproducibility of the measurements. Several types of coated capillaries have been used in CE-MS applications such as aminopropyl silylated fused-silica capillaries [Goodlett et al., 1994; Wahl et al., 1993], capillaries coated with polyacrylamide [Thompson, T.J. et al., 1993], different hydrophilic derivatized capillaries [Cole et al., 1994] and non-covalent coated CE capillary with an overall positive charge [Thibault et al., 1991]. The use of coated capillaries to eliminate the electroosmotic flow may also be useful in minimizing the flow of non-volatile additives into the mass spectrometer [Kirby et al., 1994]. This may be of particular importance in applications such as chiral separations by CE-MS.

One solution to improving the sensitivity of CE-MS is the development of alternative types of mass spectrometers which offer the potential for greater sensitivity, as discussed in section 4.5.3.

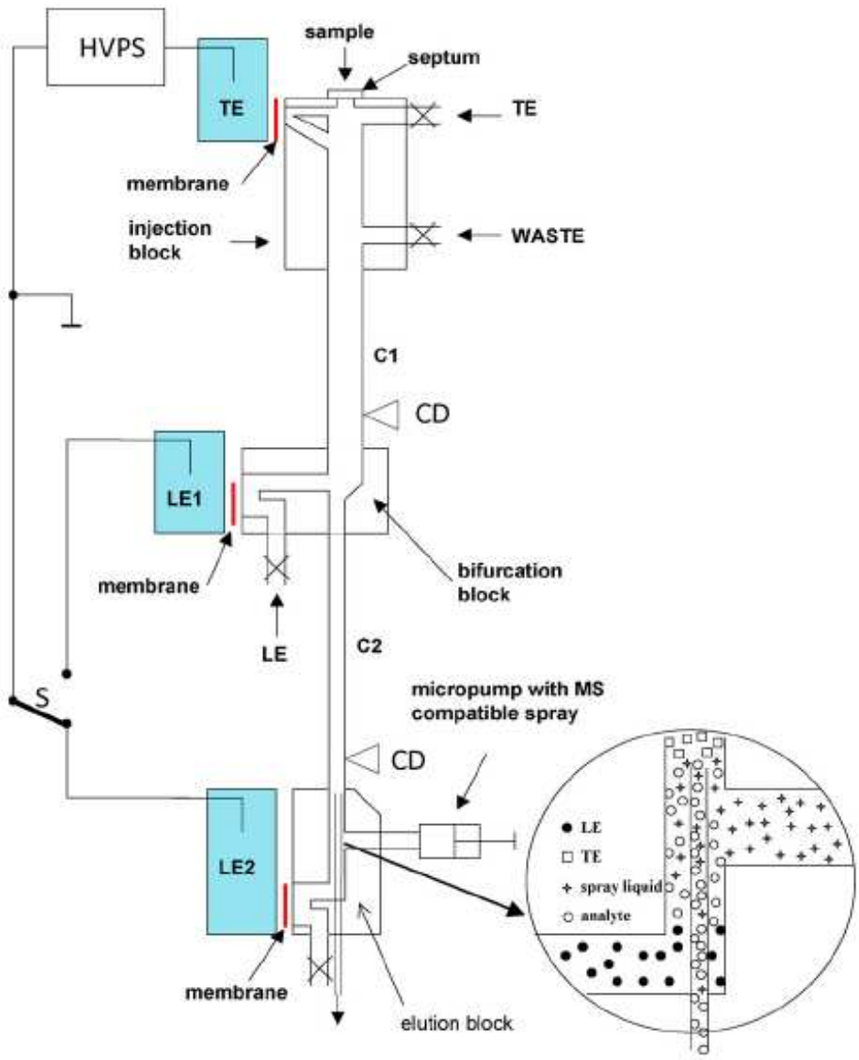

Figure 4.17. Scheme of the hydrodynamically closed isotachophoretic system. X, valves; HVPS, high voltage power supply; LE, TE, leading and terminating electrolytes and electrode reservoirs, respectively; C1, C2, preseparation and separation capillaries; CD, conductivity detectors; S, high voltage switch. Reprinted from ref. [Tomáš et al., 2010].

4.5.2 CE-MS interfaces

The transfer of analytes from the liquid phase of CE to the gas-phase for MS is facilitated via interfaces, where practically all solvent and BGE are selectively removed, and the MS inlet is reached by analytes in the gas-phase with only some rests of the BGE. Several ionization methods have been used for the hyphenation of CE with MS. These include electro spray ionization (ESI), ion spray or pneumatically assisted electro spray ionization, and continuous-flow fast atom bombardment [Cai & Henion, 1995]. These are the on-line interfaces applied for the small, as well as large, molecules. Schematic diagrams of these interfaces are illustrated and described in the next subsections. On the other hand, desorption MS interfaces are preferred for the large non-volatile molecules like proteins,

DNA etc. The coupling of CE to desorption MS can usually be off-line, although advanced (on-line) hyphenations have also recently appeared. The off-line coupling of CE with matrix-assisted laser resorption ionization and ^{252}Cf plasma desorption MS using fraction collection are reviewed by Cai and Henion [Cai & Henion, 1995]. In the recent review by Huck et al. [Huck et al., 2006] the most important techniques developed to hyphenate CE to matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) are summarized. The principles of the different interfaces and ways to solve the hyphenation problem are explained and discussed in detail. Very recently, modified matrix-assisted ionization techniques have been adapted also for the analysis of low molecular weight compounds like drugs and their metabolites [Wang et al., 2011]. Therefore, this interface is principally applicable also in chiral CE-MS and a special analyte treatment is briefly mentioned here. A schematic diagram of the mechanism of MALDI is shown in Figure 4.18.

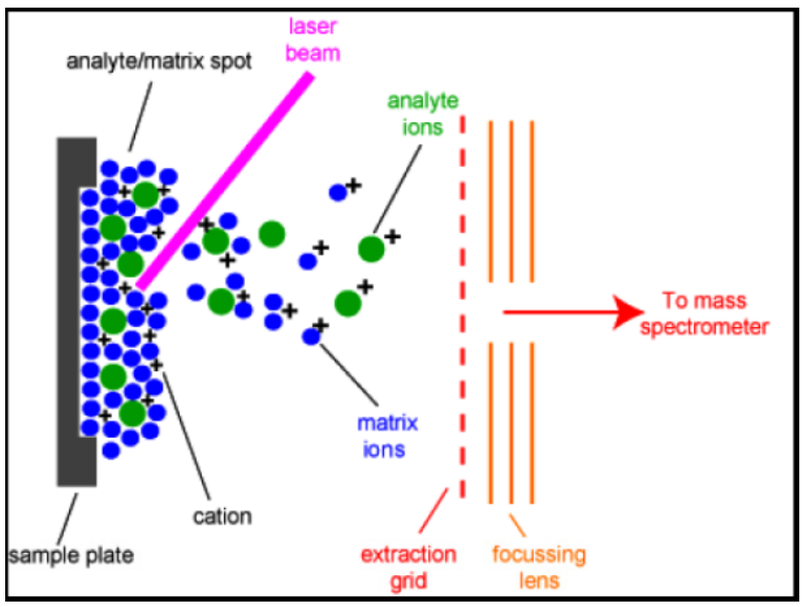

Figure 4.18. A schematic diagram of the mechanism of MALDI. The mechanism of MALDI is believed to consist of three basic steps: (i) *Formation of a 'Solid Solution'*: this is essential for the matrix to be in access thus leading to the analyte molecules being completely isolated from each other. This eases the formation of the homogenous 'solid solution' required to produce a stable desorption of the analyte. (ii) *Matrix Excitation*: the laser beam is focused onto the surface consist of the matrix-analyte solid solution. The chromaphore of the matrix couples with the laser frequency causing rapid vibrational excitation, bringing about localised disintegration of the solid solution. The clusters ejected from the surface consist of analyte molecules surrounded by matrix and salt ions. The matrix molecules evaporate away from the clusters to leave the free analyte in the gas-phase. (iii) *Analyte Ionisation*: the photo-excited matrix molecules are stabilized through proton transfer to the analyte. Cation attachment to the analyte is also encouraged during this process. In this way the characteristic $[M+X]^+$ (X= H, Na, K etc.) analyte ions are formed. These ionisation reactions take place in the desorbed matrix-analyte cloud just above the surface. The ions are then extracted into the mass spectrometer for analysis. Reprinted from ref. [http://www.chm.bris.ac.uk/ms/theory/maldi-ionisation.html].

4.5.2.1 Ion spray interface

Ion spray (ISP) is closely related to the commonly used electro spray ionization interface (ESI), the difference being the application of a nebulizing gas which permits stable electro spray operation at flow-rates up to 1 mL/min [Hopfgartner et al., 1993], whereas pure electro spray has been restricted to flow-rate below 10 μl/min [Whitehouse et al., 1985]. ISP provides a mild ionization via an ion evaporation process which results in primarily molecular mass information in the form of singly and multiply charged ions. For the schematic diagram of CE-ISP-MS interfaces see Figure 4.19.

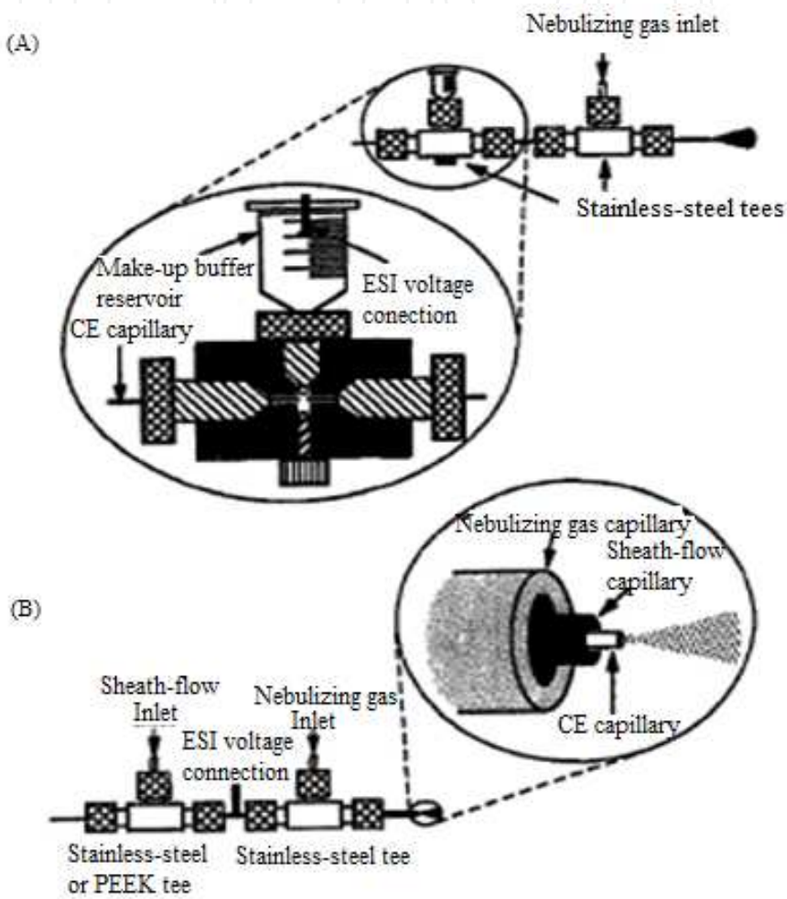

Figure 4.19. Schematic diagram of CE-ISP-MS interfaces. (A) Liquid junction and (B) coaxial sheath-flow configurations. Reprinted from ref. [Cai & Henion, 1995].

4.5.2.2 Continuous-flow fast atom bombardment interface

The continuous-flow fast atom bombardment (CF-FAB) technique can dramatically extend the capability of MS for the determination of fragile and polar compounds [Minard et al., 1988; Moseley et al., 1989a, 1989b]. For the schematic diagram of CE-CF-FAB-MS interfaces see Figure 4.20. There are several attractive features of CE-MS using the ESI interface in comparison with CF-FAB interface. Since larger-ID capillaries can be used for the ESI interface, the loading capacity should be greater than those using the coaxial sheath-flow CF-FAB interface where only small-ID capillaries (ca. 10 μm) can be used due to the

pressure drop at the source region which leads to hydrodynamic flow within the CE capillary distorting the plug profile of electroosmotic flow and causing band broadening. The ESI interface also gives reduced background noise and higher sensitivity [Deterding et al., 1991; Edmonds et al., 1991].

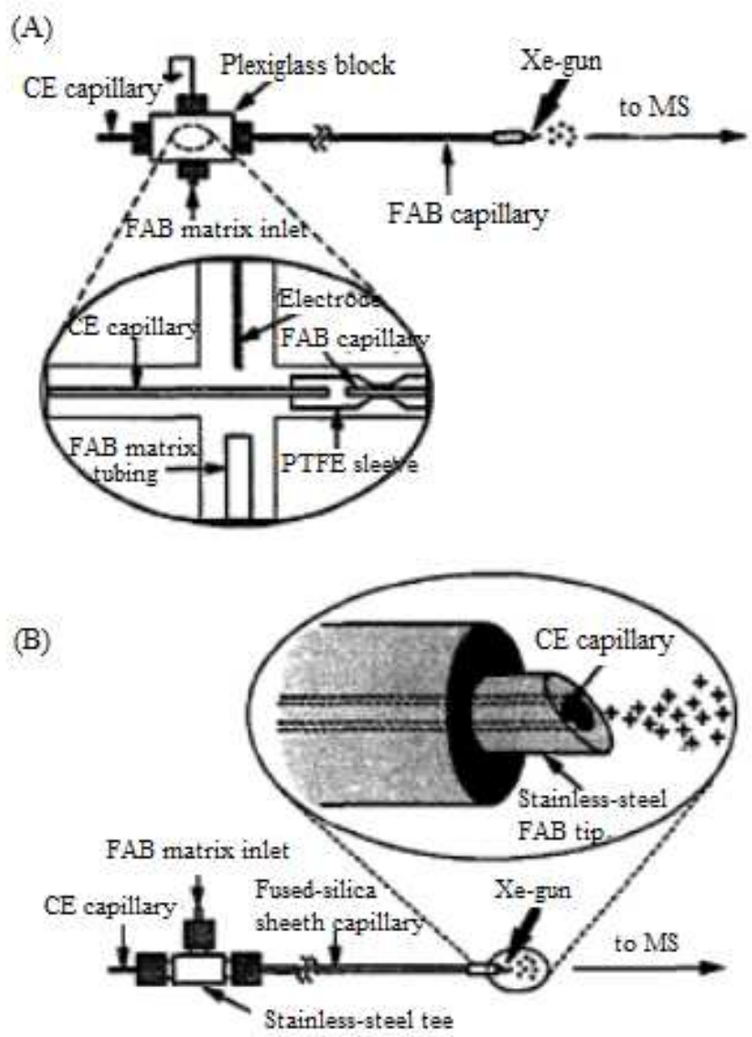

Figure 4.20. Schematic diagram of CE-CF-FAB-MS interfaces. (A) Liquid junction and (B) coaxial sheath-flow configurations. Reprinted from ref. [Cai & Henion, 1995].

4.5.2.3 Electro spray ionization interface

The ESI source is currently the preferred interface for CE-MS, as it can produce ions directly from liquids at atmospheric pressure and with high sensitivity and selectivity for a wide range of analytes. A schematic of an ESI source is in Figure 4.21. ESI is based on electrostatic effects on solution starting with the nebulization of a sample into electrically charged droplets and finishing with the formation of the gas-phase ions. A schematic of the mechanism of ion formation in ESI is in Figure 4.22.

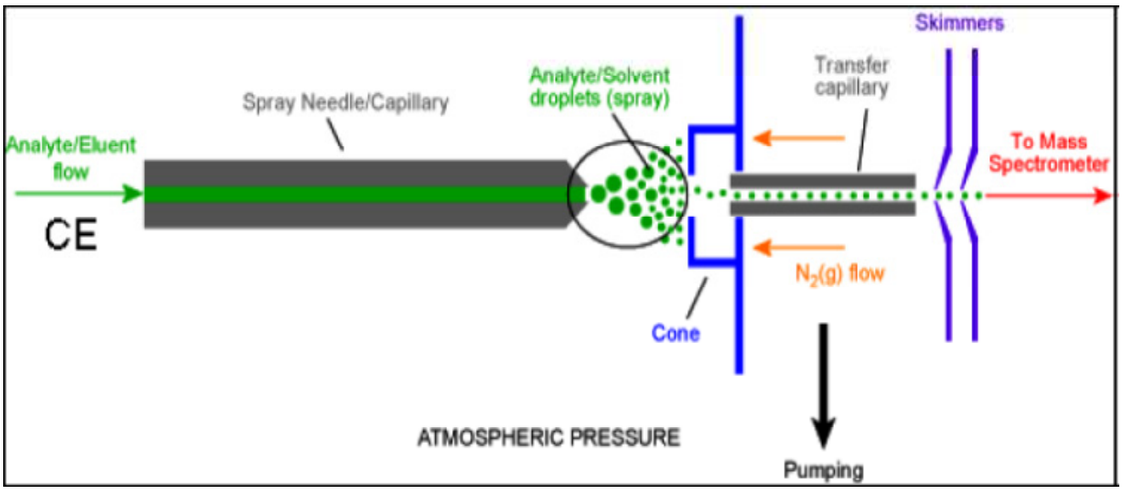

Figure 4.21. A schematic of an ESI source. The analyte is introduced to the source in solution as the eluent flow from the CE analyser. Flow-rates are typically of the order of 1µl min^{-1}. The analyte solution flow passes through the electro spray needle that has a high potential difference (with respect to the counter electrode) applied to it (typically in the range from 2.5 to 4 kV). This forces the spraying of charged droplets from the needle with a surface charge of the same polarity to the charge on the needle. The droplets are repelled from the needle towards the source sampling cone on the counter electrode (shown in blue). As the droplets traverse the space between the needle tip and the cone and solvent evaporation occurs. This is circled on the Figure 4.21 and enlarged in Figure 4.22. Reprinted from ref. [http://www.chm.bris.ac.uk/ms/theory].

Nearly all commercially available CE–MS interfaces are nowadays based upon electro spray ionization (ESI) [von Brocke et al., 2001; Schmitt-Kopplin & Frommberger, 2003]. For the schemes of ESI interfaces see Figure 4.23. Here, various configurations of ESI have been proposed in order to eliminate dead volume at the capillary terminus (Figure 4.23A) and to produce and maintain a stable electro spray signal during a CE-ESI-MS experiment (Figure 4.23B,C). Gale and Smith [Gale & Smith, 1993] compared the performance of the two ESI interfaces (i.e., sheath and sheathless) using pressure infusion. Their study showed several advantages of the sheathless version, including great sensitivity, low required flow-rates and long-term stability. In another study [Wahl et al., 1994], the performance of a gold-coated sheathless interface was compared with that of a sheath-flow interface regarding their dependence on a buffer system and concentration, as well as capillary i.d. The sheathless interface turned out to offer better analyte detectability. It was found that the sheathless configuration also eliminated the possible interferences from the sheath solvents such as charge state distribution shift [Kriger et al., 1994].

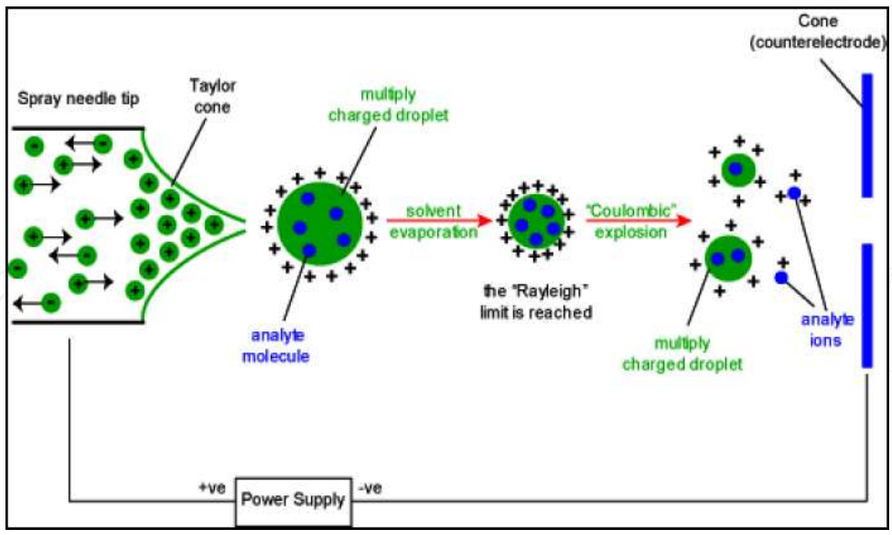

Figure 4.22. A schematic of the mechanism of ion formation in ESI. As the solvent evaporation occurs, the droplet shrinks until it reaches the point that the surface tension can no longer sustain the charge (the Rayleigh limit) at which point a "Coulombic explosion" occurs and the droplet is ripped apart. This produces smaller droplets that can repeat the process as well as naked charged analyte molecules. These charged analyte molecules (they are not strictly ions) can be singly or multiply charged. This is a very soft method of ionisation as very little residual energy is retained by the analyte upon ionisation. This is why ESI-MS is such an important technique in biological studies where the analyst often requires that non-covalent molecule-ligand (e.g., protein) interactions are representatively transferred into the gas-phase. By this technique very little (usually no) fragmentation is produced. For structural elucidation studies, this leads to the requirement for tandem mass spectrometry where the analyte molecules can be fragmented. Reprinted from ref. [http://www.chm.bris.ac.uk/ms/theory].

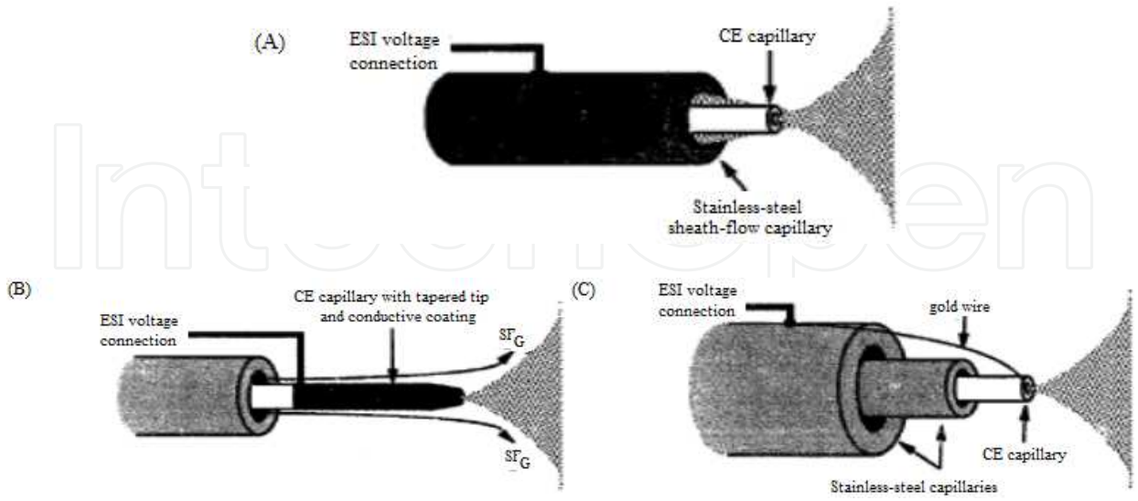

Figure 4.23. Schematic diagram of CE-ESI-MS interfaces. (A) Coaxial sheath-flow configuration, (B) sheathless interface with tapered and conductive coated CE capillary and (C) sheathless interface using a gold wire electrode. Reprinted from ref. [Cai & Henion, 1995].

4.5.3 Mass spectrometry detection approaches

So far, the triple quadrupole (TQ) and ion trap (IT) have been the most commonly used mass analysers in CE-MS for the analysis of low-molecular-weight compounds (including drugs and their metabolites) in biological samples [Soga, 2007]. A schematic of the basic set-up of a TQ mass analyser is shown in Figure 4.24 while that of IT is in Figure 4.25. These MS instruments provide (i) high sensitivity (mainly TQ) with (ii) the capability to obtain structural information on unknown compounds (mainly IT). However, a disadvantage of these mass analysers, especially with respect to fast and highly efficient CE separations, is the relatively slow scanning process, therefore, they are not able to obtain sufficient data points across a very narrow CE peak to accurately define it [Soga et al., 2003]. In addition, the mass resolution of these instruments is limited providing lower selectivity for distinguishing analysed compounds. In general, TQ and IT mass analysers are more suited for targeted metabolomic studies by CE-MS [Ramautar et al., 2006; Monton & Soga, 2007].

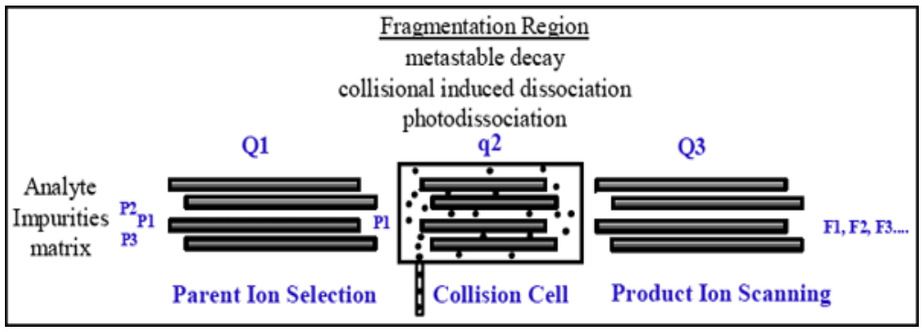

Figure 4.24. Conceptual view of tandem mass spectrometry with a tandem-in-space triple quadrupole mass analyser. The first mass analyser (Q1) selects the precursor ion of interest by allowing it only to pass while discriminating against all others. The precursor ion is then fragmented, usually by energetic collisions, in the second quadrupole (q2) that is operated in transmissive mode allowing all fragment ions to be collimated and passed into the third quadrupole (Q3). Q3 performs mass analysis on the product ions that compose the tandem mass spectra and are rationalized to a structure. Reprinted from ref. [www.noble.org/PlantBio/Sumner/metabolomics.html].

The limitations of TQ- and IT-MS can be overcome by the time-of-flight (TOF) mass analysers where ions are accelerated by an electric field [Bajad & Shulaev, 2007; Lacorte & Fernandez-Albaz, 2006]. The working principle of a linear time-of-flight mass spectrometer is illustrated in Figure 4.26. The high scan speed (e.g., 10 spectra/s), high mass accuracy and high mass resolution of TOF-MS makes this method very suitable for non-targeted metabolomics studies [Soga et al., 2003]. Despite the increased resolution of TOF analysers, potential interferences from solvent ions, adducts and compounds with the same nominal mass as the metabolites in the biological sample can still disturb the analysis. Therefore, efficient separations prior to MS analysis are of key importance. These problems could also be circumvented by using Fourier transform ion cyclotron resonance (FT-ICR) MS, which has a mass resolution of >500 000 and provides very accurate mass measurements with sub-ppm errors. However, accurate mass measurements with FT-ICR MS are relatively slow which can compromise the analysis of narrow CE peaks, and faster analysis may also lead to reduced sensitivity in FT-ICR MS [Baidoo et al., 2008].

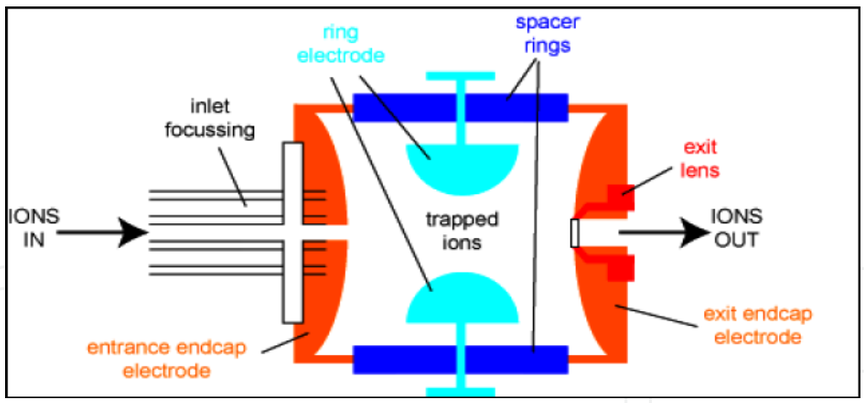

Figure 4.25. A schematic (cutaway view) of a quadrupole ion trap mass analyser. The ions, produced in the source of the instrument, enter into the trap through the inlet and are trapped through the action of the three hyperbolic electrodes: the ring electrode and the entrance and exit endcap electrodes. Various voltages are applied to these electrodes which results in the formation of a cavity in which ions are trapped. The ring electrode RF potential, an a.c. potential of constant frequency but variable amplitude, produces a 3D quadrupolar potential field within the trap. This traps the ions in a stable oscillating trajectory. The exact motion of the ions is dependent on the voltages applied and their individual mass-to-charge (*m/z*) ratios. For detection of the ions, the potentials are altered to destabilize the ion motions resulting in ejection of the ions through the exit endcap. The ions are usually ejected in order of increasing *m/z* by a gradual change in the potentials. This 'stream' of ions is focused onto the detector of the instrument to produce the mass spectrum. The very nature of trapping and ejection makes a quadrupolar ion trap especially suited to performing MS^n experiments in structural elucidation studies. It is possible to selectively isolate a particular *m/z* in the trap by ejecting all the other ions from the trap. Fragmentation of this isolated precursor ion can then be induced by collision-induced dissociation (CID) experiments. The isolation and fragmentation steps can be repeated a number of times and this is only limited by the trapping efficiency of the instrument. Reprinted from ref. [www.chm.bris.ac.uk/ms/theory/qit-massspec.html].

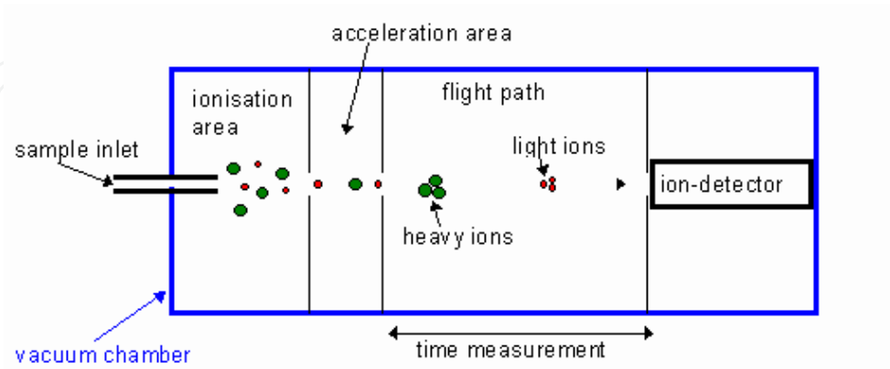

Figure 4.26. The working principle of a linear time-of-flight mass spectrometer. Time-of-flight mass spectrometers identify molecules by measuring the time that sample molecules, all starting with the same kinetic energy, require to fly a known distance. Reprinted from ref. [www.kore.co.uk/tutorial.html].

4.5.4 Chiral CE-ESI-MS

The key factor in manipulating enantioselectivity is the usage of suitable chiral selectors (see section 2.3). They are generally non-volatile molecules, whose introduction into the MS system should be avoided because they damage electro spray efficiency, and increase background noise, decreasing the sensitivity of detection [Shamsi, 2002]. It can be generalized that buffer components that interact strongly with the sample degrade the sensitivity of MS detection. For example, amphiphilic molecules give rise to intense signals in both positive and negative ion ESI, presenting a major barrier for MEKC-MS applications. The main target in such cases is to circumvent these difficulties. Therefore, an important aspect to be considered when a chiral analysis is going to be performed by CE-MS is the type of (i) chiral selector and (ii) separation mode, minimizing introduction of chiral selector into ESI-MS.

As for the selection of the proper type of chiral selector, micelles vs. micelle polymers can serve as a model example. Assuming that the enantioselective capability is comparable, micelle polymers provide several advantages over conventional micelles for MEKC-ESI-MS hyphenation (see section 2.3.8.) resulting in less background noise and higher obtainable S/N [Akbay et al., 2005; Hou et al., 2006; Rizvi et al., 2007].

As for the selection of proper separation mode minimizing the introduction of chiral selector into ESI-MS, the first possibility is immobilization of chiral selectors (CEC mode) which does not affect MS sensitivity (for CEC see section 2.2.2). The second possibility is based on the movement of the selector in the opposite direction toward the detector that can be provided by the selector's charge or by EOF in CE [Iio et al., 2005; Yang et al., 1997; Lu W. Z. et al., 1996; Fanali et al., 1998a; Schulte et al., 1998; Lu W. & Cole, 1998]. For advanced separation mechanisms see section 2.2.1.2. For example, EOF manipulation was used for micelles [Yang L. et al., 1997] and the countercurrent approach was used for antibiotics [Fanali et al., 1998a] and CD derivatives [Iio et al., 2005; Schulte et al., 1998] as chiral selectors. The partial filling of the capillary with the separation media containing the chiral selector in combination with its countercurrent migration can enhance the overall effect [Rudaz et al., 2005; Schappler et al., 2006]. Then, the concentration LODs obtainable by EKC-ESI-MS can be 10^{-8}-10^{-9} M corresponding with mass detection limits of 10^{-16}-10^{-17} mol [Ewing et al., 1989].

4.6 Applications

Absorbance detection. Mikuš et al. [Mikuš et al., 2006a] demonstrated that advanced CE systems based on (i) on-line coupled columns (ITP-EKC), enabling efficient on-line sample preparation (preconcentration of analytes, elimination of matrix constituents) and providing enhanced sample loadability (30 μl sample injection volume), and (ii) countercurrent migration of chiral selector (carboxyethyl-β-CD), allowed the use of a conventional UV detector (240 or 265 nm wavelengths were applied) for a highly efficient (~$8x10^4$ theoretical plates), selective (baseline enantioresolution and complete resolution from matrix compounds) and ultra-trace (LODs were in the concentration range of 1.1-4.8 ng/mL) direct determination of enantiomers of various drugs (H_1-antihistamines like pheniramine, dimethindene, dioxopromethazine) in the multicomponent ionic matrices (urine samples). The method was successfully applied for enantioselective metabolic study of pheniramine in the externally unpretreated urine samples showing its analytical potential in clinical research.

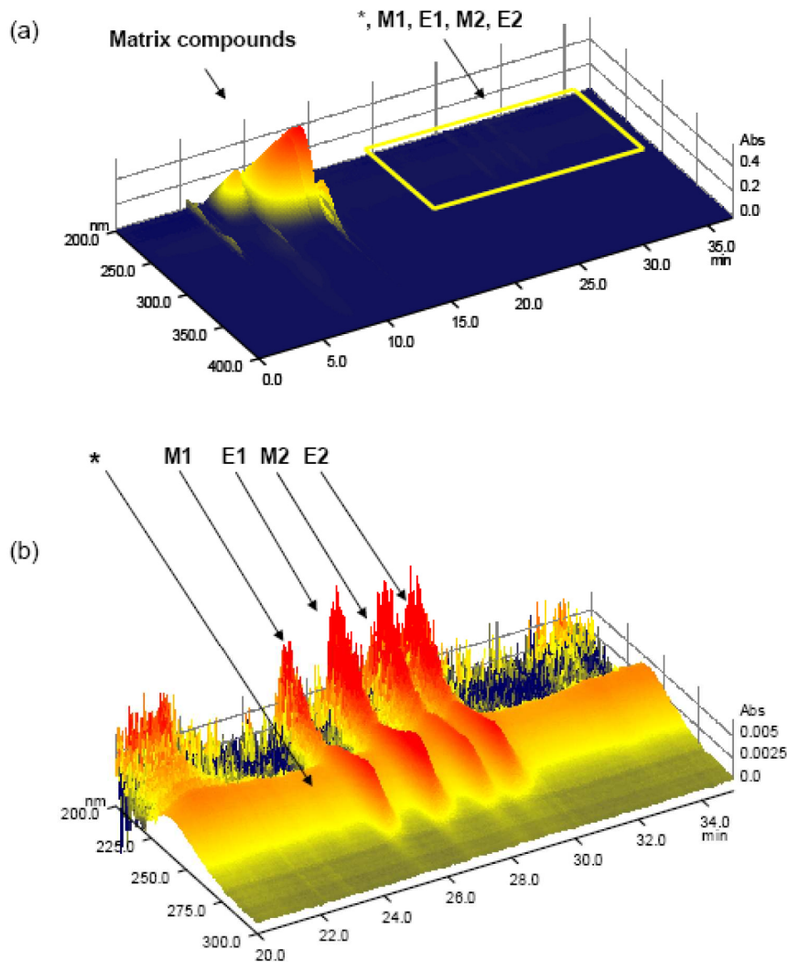

Figure 4.27. ITP-EKC-DAD method for the direct sensitive determination of enantiomers in a sample of unpretreated complex matrices with spectral characterization of electrophoretic zones. 3D traces were obtained combining electrophoretic (EKC) and spectral (DAD) data where the spectra were scanned in the interval of wavelengths 200-400 nm. (a) 3D trace illustrating the whole EKC enantioseparation of pheniramine and its metabolites in the on-line pretreated clinical urine sample (spectra of matrix constituents, well separated from the analytes, are pronounced), (b) detail on the 3D spectra showing the migration positions of pheniramine enantiomers (E1 and E2) and their structurally related metabolites (M1 and M2). The spectrum of the little unknown peak marked with the asterisk differed from the pheniramine spectrum significantly and, therefore, it was not considered as a pheniramine biodegradation product. The urine sample was taken 8.5 hours after the administration of one dose of Fervex (containing 25 mg of racemic pheniramine) to a female volunteer and it was diluted 10-fold before the injection. 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. Reprinted from ref. [Marák et al., 2007].

A very complex solution in applied biomedical analytical research was demonstrated by the hyphenation of the ITP-EKC (on-line sample preparation plus countercurrent chiral selector migration) with the spectral detector such as diode array detector (DAD). A DAD spectral characterization/identification in combination with the ITP-EKC separation was successfully applied for the enantioselective determination of drugs and their potential metabolites in unpretreated biological samples that were utilized in enantioselective pharmacokinetic and metabolic studies. Spectral monitoring of the real urine samples, taken after oral administration of 20 mg of amlodipine, carried out over a 54 hour time span, revealed the time vs. concentration profiles typical for the elimination of amlodipine enantiomers in the human body [Mikuš et al., 2008a]. These pharmacokinetic dependences clearly indicated the maxima of excluded amlodipine enantiomers and revealed the differences in elimination between the two enantiomers (enantioselective elimination). High reliability of the results was based on the fact that each point in the pharmacokinetic curves was characterized through spectra and only zones/peaks with acceptable purity (spectral homogeneity) and that matched to the reference spectra were further considered for the pharmacokinetic curve. In another work [Marák et al., 2007], ITP-EKC-DAD enabled potential pheniramine metabolites present at lower ng/mL concentration levels in real urine samples (taken after oral administration of pheniramine tablets to a female volunteer) to be distinguished, see Figure 4.27. Calculated match factors (reference vs. real spectra) gave a high statistical probability of peak identity of pheniramine enantiomers, as well as their potential metabolites. Two unknown peaks migrating close to pheniramine enantiomers peaks (Figure 4.27b) were supposed to be N-desmethyl pheniramine enantiomers, taking into account the former findings concerning the pheniramine metabolism and excretion, as well as migration times of the native and metabolized compounds. The DAD spectral analysis confirmed the high separation selectivity (i.e., producing of spectrally homogeneous analytes zones) of the ITP-EKC method with possible direct injection of unpretreated urine samples. Thus, the ITP-EKC-DAD method illustrates the benefits in (i) chiral field {countercurrent (enantio)resolution enhancement}, (ii) sample preparation (on-line preconcentration and purification) and (iii) detection (sensitivity enhancement and preliminary identification aspect) in analysis of a chiral drug and its metabolites in complex matrices.

Fluorescence detection. LIF was used for the chiral determination of many biologically active compounds [Hernández et al., 2008, 2010]. For example, CBI derivatized baclofen was determined in human plasma with LOD $5x10^{-8}$ M using the CD-EKC-LIF method (highly sulfated-β-CD as chiral selector, excitation wavelength was 442 nm, emission wavelength was 500 nm) [Kavran-Belin et al., 2005].

The main application of LIF in CE has been the analysis of amino acid enantiomers, including also biological samples [Quan et al., 2005; Zhao S.L. et al., 2005a, 2005b; Kirschner et al., 2007; Miao et al., 2005, 2006; Sheeley et al., 2005]. In all cases, the methods involved precapillary chiral derivatization of the amino acids. EKC-LIF (2-hydroxypropyl-β-CD and 2-hydroxypropyl-γ-CD as chiral selectors, excitation wavelength was 457.9 nm) was used for the chiral separation and detection of Ser, primary endogenous amino acid that binds to the Gly site of *N*-methyl-D-Asp receptor involved in a variety of physiological functions and disorders. The sensitive detection of this amino acid is essential, because it is at low

concentration in biological samples. D-Ser (NBD-F or CBI derivatives) was detected in rat brain achieving LODs of about 10^{-7} M [Quan et al., 2005; Zhao S.L. et al., 2005a]. The EKC-LIF method (a highly sulfated-β-CD as chiral selector, excitation wavelength was 420 nm) for CBI-Ser was improved (and applied also for other amino acids like aspartate and Glu) by on-line preconcentration procedures. Subsequently a very high sensitivity (LODs up to 10^{-10} M) was achieved in squirrel brain samples [Kirschner et al., 2007]. Derivatized amino acids, such as CBI-Ser and NDA-Asp, were also enantioseparated and detected in invertebrate tissue (mollusc neurons) [Quan et al., 2005; Miao et al., 2005; Zhao S.L. et al., 2005b] where LODs ranged from ~10^{-7}-10^{-10} M using EKC-LIF and excitation wavelength 457.9 nm. An effectivity of the dual chiral selector system and LIF detection for the enantioseparation and identification of CBI-D/L-serine enantiomers in real biological matrices is shown in Figure 4.28 [Zhao S.L. et al., 2005b]. In this way, for the first time, peaks corresponding to L-serine and D-serine were well identified and sensitively detected (LOD was 3x10^{-8} M) in *Aplysia* ganglian (a sea mollusc widely used as a neuronal model) homogenates. Miao et al. [Miao et al., 2005] demonstrated an approach for the quantitative investigation of the biochemical composition of subcellular regions of single neurons. Such investigations were possible thanks to the excellent LOD (5x10^{-10} M) of NDA-D-Asp and powerful separation system (CD-MEKC).

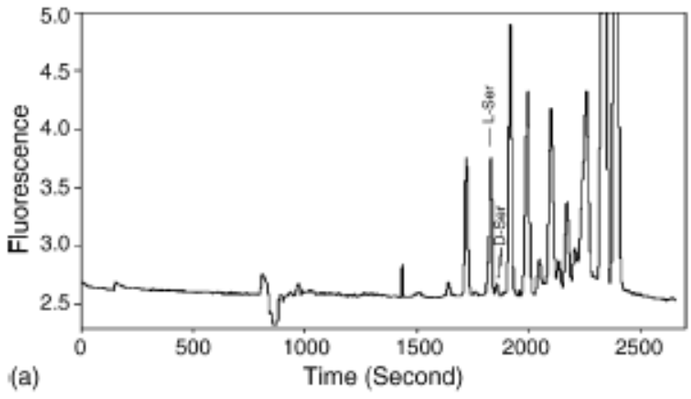

Figure 4.28. Separation and detection of CBI-Ser enantiomers in biological matrices. Separation conditions: 100mM borate (pH 9.5), 30mM β-CD and 60mM sodium deoxycholate (SDC). Electropherogram obtained from analysing an *Aplysia* pedal ganglion homogenate. Capillary was 50 μm i.d.×50 cm effective length. Voltage applied was 15 kV. Reprinted from ref. [Zhao S.L. et al., 2005b].

Electrochemical detection. Amperometric detection was introduced by Schwarz and Hauser in chiral MCE for the separation of basic drugs and biomarkers [Schwarz & Hauser, 2001, 2003]. Plate numbers of up to 20 000, chiral resolutions of 2.5 and detection limits of the order of 10^{-7} *M* were achieved. All separations were completed in less than 3 min. Detection was carried out with a two electrode amperomatric detector, eliminating the need for individual counter and reference electrodes. Because the electrochemical oxidation of catecholamines is pH-dependent, higher detection sensitivities could be obtained at higher pH values. Direct electrochemical oxidation of ephedrine and pseudoephedrine was achieved at a gold electrode at a high pH of 12.6. As the basic compounds are uncharged at

this pH, a charged cyclodextrin derivative had to be used for chiral resolution. It was observed that the detection sensitivity is also affected by the type and concentration of chiral and achiral additives applied for achieving resolution. As mentioned by the authors, electrochemical detection has the advantage that derivatization can be omitted. On the other hand, the freedom of buffer choice is limited as conditions have to be arrived which satisfy the requirements for both separation and detection. Another important aspect is that electrochemical detection lacks universality.

In another paper, the detection limits using UV absorbance detector were found to be too high to determine the concentration of isoproterenol (with a speed elimination pharmacokinetics) in intravenous microdialysis samples for a sufficient time following administration to establish the pharmacokinetics. The LODs of this catecholamine were decreased three orders of magnitude (to 3 ng/mL) by using an amperometric detector. Moreover, further sensitivity enhancement (to 0.6 ng/mL) was achieved by inserting in-capillary stacking preconcentration of microdialyzed plasma samples. This provided conditions suitable for obtaining pharmacokinetic curves of isoproterenol enantiomers in plasma, see Figure 4.29 [Hadviger et al., 1996].

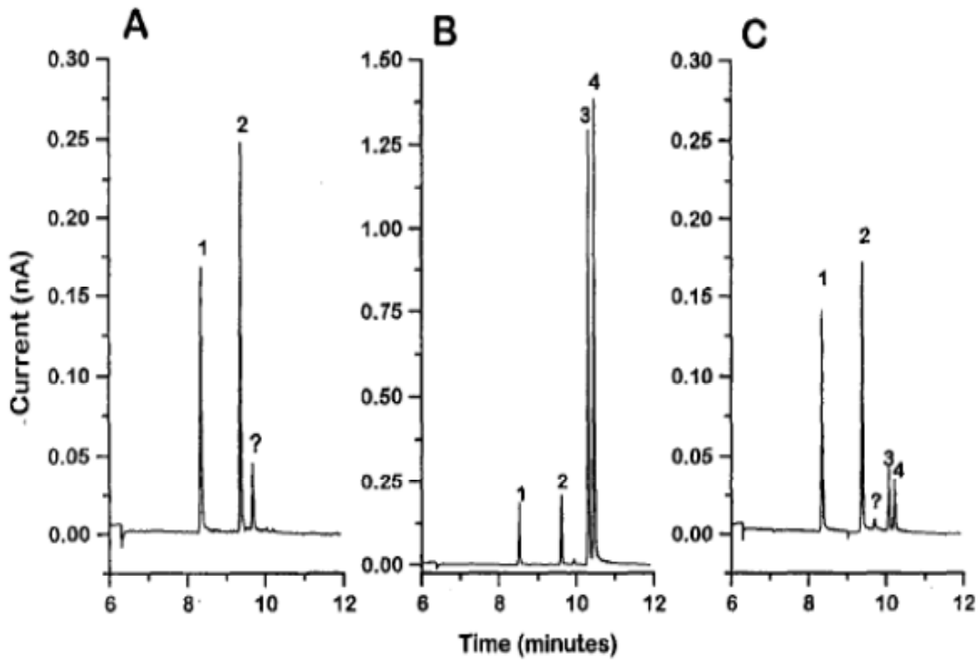

Figure 4.29. Typical CE-electrochemical detection (CE-EC) electropherograms from a pharmacokinetic experiment using on-line sample pretreatment combining dialysis and stacking. (A) Blank sample acquired prior to administering an ISP dose; (B) microdialysate acquired 6 min after dosing and (C) microdialysate acquired 54 min after dosing. Peaks: 1=3,4-Dihydroxybenzylamine(DHBA); 2=5NMHT; 3=(-)-isoproterenol (ISP); 4=(+)-ISP. Reprinted from ref. [Hadwiger et al., 1996].

While conductivity detection is regarded as rather intensive in zone electrophoresis, it can be very attractive in isotachophoresis due to the on-line focusing effect during analyses.

Mikus et al. used conductivity detection with a chiral ITP for the enantioseparation of various H1-antihistaminic drugs in dosage forms [Mikuš et al., 2006b; Kubačák et al., 2006a, 2006b, 2007]. Chiral isotachophoresis in microfluidic devices with on-column conductivity detection was successfully demonstrated by Olvecka et al. [Ölvecká et al., 2001] for the chiral separation of tryptophan enantiomers, however, only in model samples. For this purpose they used a coupled separation channel configuration. In other works, contact, as well as contactless, conductivity detection was successfully used for preliminary scanning of sample profile in the ITP stage of the ITP-EKC method when analysing various drugs present in biological samples. Thus, amlodipine, pheniramine (plus its metabolites), dioxopromethazine, and dimethindene could be easily transferred into the EKC stage for the final enantioseparation without undesired matrix constituents present in urine samples [Mikuš et al., 2006a, 2008a, 2008b; Marák et al., 2007]. The methods were applied in enantioselective metabolic [Mikuš et al., 2006a, 2008b; Marák et al., 2007] and pharmacokinetic [Mikuš et al., 2008a] studies of the drugs, see e.g., Figure 4.27.

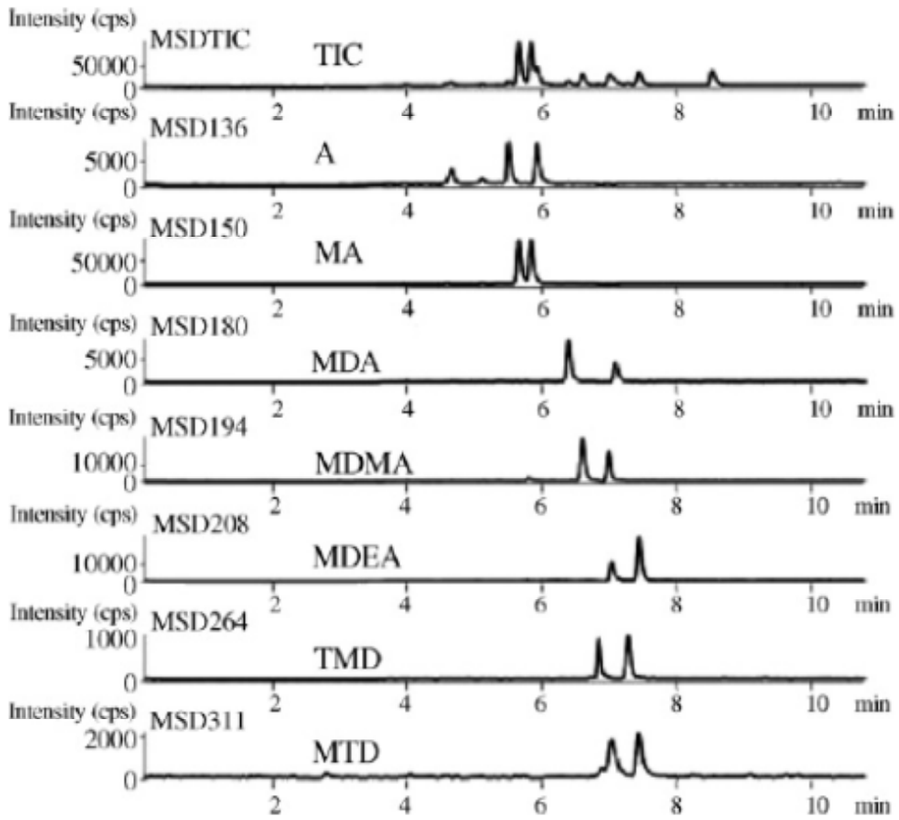

Figure 4.30. Chiral analysis of amphetamine (A), methamphetamine (MA), methylenedioxyamphetamine (MDA), methylenedioxyamphetamine (MDMA), methylenedioxyethylamphetamine (MDEA), tramadol (TMD) and methadone (MTD) amphetamine enantiomers at 0.5 ppb in plasma after LLE with EK injection. Experimental conditions: FS capillary of 75 cm and 650mm (ID); 25 kV; EK injection at 10 kV 610 s; 50% capillary filled with HS-γ-CD 0.15% in BGE 2% capillary filled with BGE post-plug; on-line CE–ESI/MS in SIM mode and extracted ion current (XIC). Reprinted from ref. [Schappler et al., 2006].

Mass spectrometry. Rudaz et al. [Rudaz et al., 2005; Schappler et al., 2006] used the EKC-MS with countercurrent migration of the negatively charged highly sulfated-γ-CD (ammonium formate electrolyte) and the partial filling technique to achieve the enantioseparation of amphetamine derivatives. The use of this strategy with a low concentration of the chiral selector allowed the rapid stereoselective separation of seven amphetamine derivatives in spiked plasma samples in analysis times of less than 6 min and concentration LODs ranging from ~$7x10^{-7}$-$3x10^{-6}$ M [Rudaz et al., 2005]. An enhancing of the detection sensitivity was accomplished with an electrokinetic injection accompanied by the introduction of an appropriate buffer plug length between the zone containing the chiral selector and the analyte injection (to overcome drawbacks linked with electrokinetic injection in the presence of a charged chiral selector) [Schappler et al., 2006], see Figure 4.30. When such complex matrices are analysed, MS signal suppression or enhancement effects are generally not reproducible and can compromise results using CE–ESI-MS. Therefore, the matrix effect was investigated with a commercially available coaxial sheath liquid ESI interface used as the post-capillary infusion system. It was found that with PP, signal suppression was observed while for LLE, no relevant matrix effect occurred in all experiments. Concentration LODs were improved by up to $4x10^{-9}$ M of each enantiomer present in plasma samples after LLE and detected by a single quadrupole MS as a detection device.

Anionic CD, heptakis(2,6-diacethyl-6-sulfato)-β-CD, migrating from the detector to the inlet of the capillary, was used for the simultaneous chiral separation of drugs [Iio et al., 2005]. This selective EKC-ESI-MS method (formic acid electrolyte) was applied to the analysis of methamphetamine, 3,4-methylenedioxyamphetamine and amphetamine in clinical human urine samples pretreated by LLE. Sufficient sensitivity was obtained when concentration LODs ranged from ~$6x10^{-8}$-10^{-7} M.

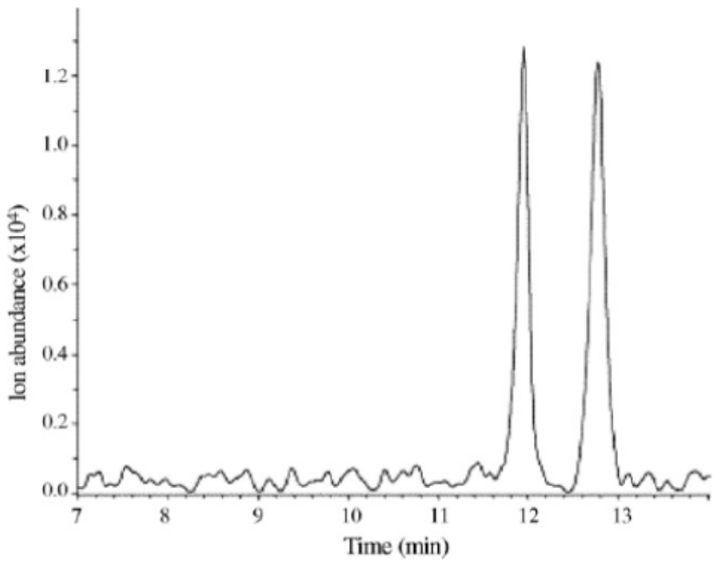

Figure 4.31. Typical electropherogram of salbutamol enantiomers at a concentration close to the LOQ obtained using SPE–NACE–ESI-MS (EIC at 240.4 *m/z*) using a BGE of 10mM ammonium formate and 15mM HDAS-β-CD in methanol acidified with 0.75M formic acid. Reprinted from ref. [Servais et al., 2006].

Micelle polymers are a promising class of chiral selectors for MS, which combine powerful chiral recognition in MEKC and high sensitivity of MS. A sensitive MEKC-MS method using poly(sodium *N*-undecenoxy carbonyl-L-leucine) sulfate (triethylamine + ammonium acetate + acetonitrile buffer) was developed for enantioselective analysis of pseudoephedrine in human urine [Rizvi et al., 2007]. The concentration LOD obtained was ~$2x10^{-6}$ M. LOD at pH 2.0 was 16 times lower than that obtained at pH 8.0, because the ionization efficiency of the MS, in the positive ionization mode, is higher when acidic solutions are used and the sulfated polymeric surfactant was effective also at low pH because of its permanent charge.

The on-line coupling of NACE and ESI-MS was used for the determination of low levels of the enantiomers of a basic chiral drug (salbutamol) in biological samples (human urine) pretreated by SPE [Servais et al., 2006]. The selected BGE (methanol acidified with formic acid containing heptakis(2,3-di-*O*-acetyl-6-*O*-sulfo)-β-CD offered good possibilities to be directly applied for MS coupling since the BGE contained volatile solvents and the non-volatile CD migrated toward the capillary inlet (countercurrent technique) away from the MS detector. After optimization of several parameters, such as sheath liquid composition and flow-rate, nebulizing gas pressure, CE counterpressure and position of the CE capillary outlet, LOQs of 18 and 20 ng/mL were obtained for the salbutamol enantiomers. The RSD at a concentration of 30 ng/mL was below 7% for both enantiomers and *R*-values were 0.9988 and 0.9966. This paper therefore proposed an easy to use and relatively sensitive NACE–ESI-MS method to determine enantiomers of a basic chiral drug in biological fluids preceded by SPE as sample clean-up. Figure 4.31 presents a typical electropherogram of the salbutamol enantiomers at a concentration close to the LOQ using SPE–NACE–ESI-MS. It is noted that, compared with the LOQ of the NACE-UV method (i.e., 375 ng/mL for an injection time of 15 s), the use of an MS detector (with an injection time of 30 s) gives rise to a significant increase in sensitivity.

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