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*Gerhardus de Jong*

**Capillary Electrophoresis–Mass Spectrometry (CE-MS)**

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# Capillary Electrophoresis–Mass Spectrometry (CE-MS)

Principles and Applications

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# 1
# Detection in Capillary Electrophoresis – An Introduction

*Gerhardus de Jong*

Capillary electrophoresis (CE) has been developed into a strong analytical technique. Separation is based on charge-to-mass ratio, and high efficiencies can be obtained with short analysis times. In principle, CE is suitable for charged compounds, but by using micelles in the background electrolyte (micellar electrokinetic chromatography, MEKC), neutral compounds can also be separated. Other additives can increase the selectivity of CE, for example, cyclodextrins for chiral separations. The consumption of solvents is small as flow rates are very low and mostly aqueous buffers are used. This latter aspect also means that the technique is biocompatible and is well suited for the analysis of intact proteins. Next to capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF) and capillary gel electrophoresis (CGE) are powerful for the analysis of biopolymers. The reproducibility and robustness of CE are often less than those of liquid chromatography (LC) and gas chromatography, but in the past years, this has been improved by reliable injection techniques and more stable electroosmotic flows. Only small sample volumes are needed, which is favorable for some applications. However, the injection of low volumes is a disadvantage for the concentration sensitivity. This can be compensated by on-line electrokinetic and chromatographic preconcentration of relatively large volumes [1, 2]. Moreover, sensitive detection can decrease the detection limits, which is important for trace-level analysis.

The detection volume should be small, and an efficient combination (or even integration) of the separation capillary and the detector is required. On-capillary ultraviolet (UV) and especially diode-array detection is mostly used. Fluorescence is another optical system, and high sensitivity can be obtained by fluorescence detection in CE. However, derivatization is often necessary for attachment of a fluorophore to the analytes. Electrochemical detection has also been developed for CE and can be divided based on three principles: potentiometric, amperometric, and conductometric. Today, potentiometric detection and amperometric detection are rarely used. In early CE work, conductivity detection was the standard approach, and this universal detector is still applied for compounds that are difficult to detect by UV absorption. Coupling of CE and mass spectrometry (MS) is important as MS is sensitive and selective. Moreover, it can provide structure

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information and identification of unknown compounds. Efficient interfacing has been realized and CE-MS is now well established. The design and potential of the main detection modes in CE will be shortly described in this introductory chapter.

## 1.1 UV Absorption

UV absorption is by far the most common detection mode in CE, and on-capillary detection is a part of commercial instruments. It is a universal principle as the use of fused-silica capillaries and aqueous buffers allows detection wavelengths below 200 nm up to the visible region of the spectrum. The use of low wavelengths offers a significant gain in sensitivity and wide applicability. The detector volume is very small, which means that band broadening is prevented. However, the design is critical and the optical path length is equal to the capillary diameter, which limits the sensitivity. Moreover, the linear detection range is limited due to the small size and the curvature of the capillary. The bubble cell, the *Z*-shape cell, and similar flow cells have been developed to increase the optical path length, but peak broadening may occur and the cells are not often employed.

For compounds that do not exhibit UV absorption, indirect detection can be applied. An absorbing co-ion is added to the background electrolyte (BGE), and this is displaced by the analyte. At the position of the analyte, a negative peak will appear. The displacement depends on the charges of the probe and the analytes and on their mobilities. Each fluctuation in the probe concentration is detected as noise. In principle, indirect UV detection is universal but optimization is rather complex [3]. The choice of the monitoring ion and other components of the BGE needs special attention. Examples of analytes are inorganic and simple organic ions and sugars. Typical detection limits are in the micromolar range. For detection of anions, monitoring ions such as chromate, benzoate, and phthalate can be used. For cations, for example, imidazole and pyridine are added to the BGE.

## 1.2 Fluorescence

Fluorescence is very sensitive, especially if a laser is used as excitation source. Excitation light should be focused on a very small detection volume with curved walls of the capillary. Furthermore, analyte emission should be effectively collected from the same volume. The inner and outer surfaces, which refract the excitation and emission light, cause scatter, which in turn can induce significant background noise. The fluorescence is emitted in all directions and only a small part is collected. Therefore, proper attention should be paid to the design of the optical configuration to allow sensitive detection in CE [4]. The analytes can be detected on-column (i.e., inside the capillary) or off-column. The determination of attomol and sub-ng $ml^{-1}$ levels has often been demonstrated. Because of its small

sample requirements, CE with laser-induced fluorescence is an excellent tool for single-cell analysis [5].

Many molecules do not exhibit fluorescence, and therefore, this detection mode is also selective. On the other side, a wide range of derivatization reagents have been developed to confer fluorescent properties to compounds that are intrinsically not fluorescent [6, 7, 8]. Various reagents are commercially available and can be easily applied. Recently much attention has also been paid to the possibilities of in-line derivatization (8). The appropriate excitation wavelength should be chosen for the analyte(s) of interest. Excitation sources that allow flexible wavelength selection are xenon, mercury–xenon, and deuterium lamps. By the use of a grating or filter, a suitable wavelength can be selected. Lasers emit monochromatic light with a high intensity and directionality. This facilitates focusing of the light onto the capillary, which is one of the main reasons for laser-induced fluorescence detection in CE. Unfortunately, there are only a few laser lines available in the deep-UV region. More recently, light-emitting diodes (LEDs) have become an attractive alternative for lasers as an excitation source due to their small dimensions, stable output, and low costs [9]. LEDs are semiconductive light sources and the wavelength is determined by the semiconductor material. Commercial LEDs are available from the deep-UV to near-IR region.

Detection of the emission light is most often performed using a photomultiplier tube in combination with a filter. In order to obtain spectral information, an imaging detector, for instance, a charge-coupled device, is required. When this is combined with a spectrograph, emission spectra of analytes can be monitored in the wavelength-resolved detection mode [10].

## 1.3 Conductivity

In the early stage of CE, conductivity was often used for detection. With the introduction of fused-silica capillaries, this was replaced by UV and fluorescence detection. However, there are still some strong points for conductivity detection in CE. The technique is universal and suitable for compounds such as inorganic ions. Contactless conductivity detection (CCD) is a very useful detection mode as electrodes do not contact the BGE in the on-capillary design [11, 12]. Two stainless-steel tubes mounted around the capillary act as the so-called actuator electrode and pick-up electrode. A capacitive transition is generated between the actuator electrode and the liquid inside the capillary. After passing through the gap between the electrodes, a second capacitive transition between the electrolyte and the pick-up takes place, and if the conductance changes by analytes, this will be measured by the pick-up electrode. The difference in conductance between the analyte zone and the BGE should be as high as possible. Moreover, the conductivity of the BGE is very important and should not be too high in order to prevent high noise and not be too low as this causes electrodispersion. A compromise is the use of amphoteric buffers at relatively high concentrations. Limits

of detection for cations and anions in the ng ml$^{-1}$ region have been obtained. CE-CCD has also been used for the separation and detection of saccharides and underivatized amino acids. The system has successfully been applied for the analysis of biological samples. CCD can easily be coupled to other detectors and is well suited for microfluidics CE [13].

## 1.4 Mass Spectrometry

MS is attractive for detection in CE as efficient separation is coupled with sensitive and selective detection of small and large molecules. Simultaneously, MS and MS/MS can be used for identification of compounds. Moreover, compounds that coelute in CE may easily be distinguished in MS. The development of reliable CE-MS took a rather long time. As in LC, the challenge is to combine a liquid-phase separation technique with a vacuum detection technique. Especially, this is difficult for MEKC, CIEF, and CGE as the run buffer contains high concentrations of nonvolatile components. However, an advantage of CE is the use of very low flow rates (nl min$^{-1}$). Furthermore, electrospray ionization (ESI) is well suited to ionize polar and charged substances separated by CE. The complexity for the interfacing is that both CE and ESI are based on an electrical field and the fields should be combined. Several approaches have been described in the literature, and after many years of development and optimization, CZE-MS can now be used in routine [14]. Recently, a collaborative study on the robustness of CE-MS for peptide mapping has been presented [15]. The results demonstrate that CE-MS is robust enough to allow method transfer across multiple laboratories. This is an important step for the technological maturity of CE, also with respect to LC-MS, which is a very strong analysis technique in various areas. The high complementarity of CE-MS has been demonstrated for metabolic and proteomic profiling [16, 17].

Principles and applications of CE–MS are described in this book. The next chapter will show the fundamental aspects of CE–MS and emphasize the interfaces. And the subsequent chapter will highlight the sheath-liquid interface, still the most important coupling between CE and MS. Separate chapters will describe the developments in microchip CE-MS and the potential of the on-line combination of sample preconcentration and CE-MS. The other chapters will focus on different application fields and show the wide applicability of CE-MS. Important technological information and many illustrative figures are presented. Both basic aspects and the state of the art of CE-MS are shown.

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# 2
# Electrospray Ionization Interface Development for Capillary Electrophoresis–Mass Spectrometry

*Jessica M. Risley, Caitlyn A.G. De Jong, and David D.Y. Chen*

## 2.1
## A Brief Introduction to the Development of CE-MS

Our intent in this chapter is not to offer a review of every configuration or application of various capillary electrophoresis–mass spectrometry (CE-MS) interfaces in the literature. Several reviews on this topic have been published [1–9]. Rather, we intend to describe and discuss several features of interface designs that are currently being used.

Capillary electrophoresis (CE) coupled to mass spectrometry (MS) was first established as an analytical tool in 1987 [10]. CE-MS combines the high separation efficiency of CE in liquid phase and mass spectrometry in gas phase. The orthogonal nature of these two separation techniques allows separation of unresolved analytes from CE and identification in MS [11]. This pairing allows for both quantitative analysis and structural elucidation.

The typical liquid-to-gas ion transformation method used for CE-MS is electrospray ionization (ESI), because analytes can be sprayed directly into the mass spectrometer from the CE at atmospheric pressure. There are other ionization techniques that have been used with CE-MS, including other types of spray, as well as gas phase and desorption ionization techniques [2, 6]. For the purposes of this chapter, we will only be addressing interface development related to ESI.

In a conventional CE setup, both ends of the separation capillary are inserted into vials containing background electrolyte (BGE). Electrodes are immersed in the inlet and outlet vials to produce a voltage gradient. Since mass spectrometry is an off-column detection technique, the CE outlet vial cannot exist in the typical manner [2]. For this reason, much creativity and scientific ingenuity have gone into interfacing CE-MS. Common challenges in coupling CE and MS include the following: the electrical current mismatch between CE and the ESI source; low flow rate of CE, which can restrict the geometry of the tip in order to maintain stable electrospray; and limitations on BGE choice in order to be compatible with both CE and MS [1, 6, 12].

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There are two commonly used strategies for coupling CE to MS via ESI. One type of the CE-ESI-MS interface uses a sheath liquid and can include a nebulizer gas. A subset of the sheath liquid interface uses a liquid junction to make up the flow required to maintain a stable ESI. Another type of interface is sheathless. Some interfaces can be used to generate nanospray ESI into the mass spectrometer. We will discuss several examples of both sheath-liquid and sheathless interfaces in subsequent sections of this chapter.

## 2.2
## Fundamentals of ESI and Electrochemical Reactions in CE-MS

### 2.2.1
### Principles of ESI: Converting Solvated Ions into Gaseous Ions

ESI gained widespread use in the late 1980s after Fenn *et al*. demonstrated its experimental use [13, 14], although Malcolm Dole had observed the technique in the 1960s [15]. ESI is an ionization technique that relies on desolvation of analyte ions, which are already formed in solution [2]. This means that large, nonvolatile biomolecules can be analyzed directly from the liquid phase [16]. The detailed operation principles of ESI have been discussed in several detailed reviews [16–22]. We will summarize the basic principles here to provide context for the interface designs used for CE-ESI-MS.

The study of ESI has divided the process into three key steps: production of charged droplets, droplet evolution, and formation of gas-phase ions from ions in solution [1, 20].

The first step involves the formation of stable electric-field-induced spray. A dilute analyte moves through the interface at a low flow rate ($0.1–10\,\mu l\,min^{-1}$) [16] that is subject to a high voltage (2–5 kV); the voltage can be positive or negative, depending on the analytes of interest. The voltage applied to the interface provides the electric field gradient needed at the liquid surface to allow for charge separation. This results in the outflow from the capillary being distorted into a Taylor cone, which emits a mist of fine droplets (Figure 2.1) [23].

The droplets emitted from the Taylor cone undergo rapid solvent evaporation. When organic modifiers are present in the bulk solution, the organic component tends to evaporate more readily [20]. The charge density then builds up on the surface of the shrinking droplets as they move toward the mass spectrometer until the surface tension of the solution is balanced by the Coulombic repulsion of the surface [16]. This is called the *Rayleigh limit* [24]. Droplets at the Rayleigh limit produce smaller highly charged offspring droplets through the process of jet fission, and the evaporation/fission process is repeated until the final generation of ESI droplets remain [23].

The last step is the formation of gas-phase ions. This has been described by several different models [20, 23]. According to the charge residue model (CRM) introduced by Dole *et al.* [15], Coulomb fissions continue to occur until

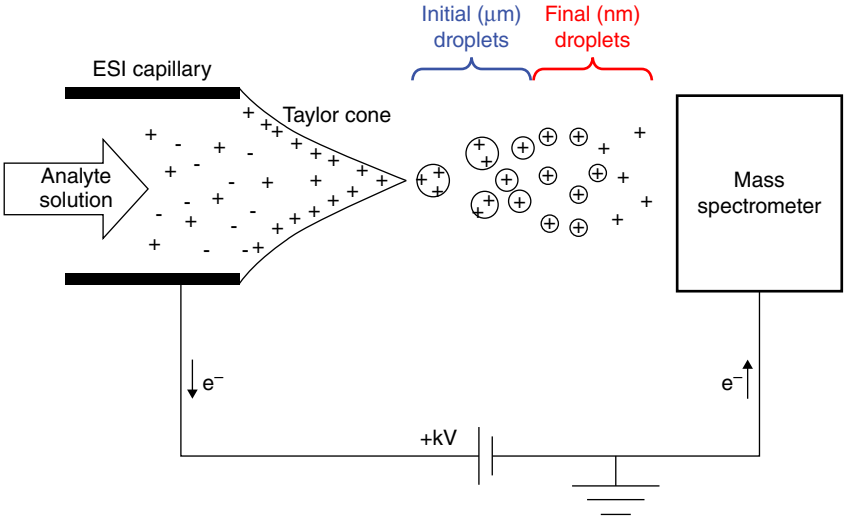


**Figure 2.1** Schematic of positive-mode electrospray ionization. Reproduced from Konermann *et al*. [23] with permission of American Chemical Society.

nanodroplets form, which contain only one analyte molecule per drop. After the desolvation process, the analyte retains the droplet's excess charge and becomes a free gas-phase ion [1]. Another mechanism, the ion evaporation model (IEM), was introduced by Iribarne and Thomson (1976). It is assumed in the IEM model that during the evaporation/fission process, there is a point where the charge density on the surface of the droplet is high enough to allow Coulombic repulsion to overcome the liquid surface tension. This allows the solvated ions to be ejected into the gas phase [2, 16]. By either mechanism, the ESI process results in gas-phase ions that can be analyzed for the mass-to-charge ratio by a mass spectrometer.

### 2.2.2 Considerations and Conditions for CE-ESI-MS Methods

Combining the tools of CE and MS via ESI is a natural strategy since both CE and ESI are well suited for the analysis of compounds that can be ionic in solution. However, CE requires a BGE. This can pose problems for ESI since high concentrations of buffer, including nonvolatile components, can result in analyte signal suppression. To mitigate this problem, low concentrations of volatile BGE solutions are used (e.g. acetic acid, formic acid, or ammonia) [2, 6]. Regarding the pH of the BGE, a typical starting point is to use a high pH BGE for negative-mode ESI or a low pH BGE for positive-mode ESI [26]. The addition of organic modifiers (e.g., methanol) to the BGE improves separation and, most significantly, MS detection. Typically, an organic modifier comprises 20–30% of the BGE.

An important consideration in CE is the electroosmotic flow (EOF). A consistent, reproducible EOF is essential for stable and reproducible electrospray for

several reasons. Reproducible migration times can be crucial for large-scale analysis using CE-MS for applications such as metabolomics and proteomics [26]. Furthermore, to achieve stable electrospray, a stable flow from the capillary is necessary, which can be facilitated by a stable EOF [11]. The stable electrospray allows for efficient analyte ionization. To control the EOF, the chemistry of the inner wall of the capillary is very important [4]. Many coatings have been developed to prevent adsorption of analytes and to enhance, stabilize, or suppress the bulk EOF [4, 11, 27].

### 2.2.3 Electrochemical Considerations in CE-MS

At the surface of each electrode immersed in aqueous buffer in the traditional CE system, a half-cell is formed. Therefore, expected half-cell electrochemical reactions occur; the major reactions are oxidation at the anode and reduction at the cathode. As a result of these processes occurring simultaneously at the cathode and anode, the pH of the solution decreases at the anode and increases at the cathode, with bubbles forming at each electrode [28].

$$O_{2(g)} + 4H^+ + 4e^- \leftrightarrow 2H_2O\, E^o_{red} = +0.816\,V(\text{vs SHE})$$

$$2H_2O + 2e^- \leftrightarrow H_{2(g)} + 2OH^-\, E^o_{red} = -0.413\,V(\text{vs SHE})$$

Different from the traditional CE system, the CE-MS coupling can be thought of as combining two controlled-current electrolytic cells, which form a three-electrode system: the CE inlet, CE outlet/ESI emitter, and MS inlet [1, 6]. What makes this three-electrode system unique is that the CE and ESI-MS circuits share an electrode [1]. Importantly, under CE-ESI-MS, the CE and the ESI-MS processes behave as two separate circuits, was demonstrated by Smith and Moini [28]. In addition, CE currents are at least an order of magnitude larger than the current for the ESI process [6]. This current mismatch between the separation and spray voltages means that the electrochemical reactions resulting from the CE circuit will dominate over those from the ESI circuit [6].

Within this shared electrode system, one side of the shared electrode is part of the CE circuit and the other side is part of the ESI-MS circuit. The electrochemical reactions that occur at the shared electrode can be both oxidative, one oxidative and one reductive, or both reductive. Which reactions occur will depend on the magnitude and the polarity of the voltage of the shared electrode compared to the CE inlet and the MS inlet [28]. Experimental results from Smith and Moini [28] state that electrolysis reactions pertinent to the CE circuit occur far upstream nearest to the relevant counter electrode, and ESI-related electrolysis occurs downstream at the emitter tip electrode [6, 28]. Therefore, measured current in the system will be representative of the difference between the currents of the oxidation and reduction processes taking place. In addition to the electrolysis reactions already stated, there are other reactions that simultaneously take place with the different metals or chemical species in the system.

Bonvin *et al*. [1] have summarized many of these reactions that can occur at the emitter tip.

To overcome some of the difficulties caused by some of the electrochemical reactions occurring at the interface, several approaches have been suggested. For instance, pH buffers are commonly used as the BGE to maintain the pH at the CE inlet, outlet, and ESI emitter. Smith and Moini [28] have also suggested the use of redox buffers to overcome problems with pH change, oxidation of analytes, and bubble formation.

## 2.3 Interface Designs

The coupling of CE to MS is not trivial due to several considerations, including the low and often varied flow rate of CE (nl $min^{-1}$range) [29] and the CE separation requirement of a closed electrical circuit [30, 31]. CE can be coupled to MS with sheath-flow or sheathless ESI interfaces. These two types of interface differ in whether or not a sheath liquid is applied and the establishment of an electrical contact [8, 32–34]. Currently, sheath-flow interfaces are most widely used [8], followed by sheathless interfaces. Nanospray ionization can be achieved using either sheath-flow or sheathless interfaces, generating electrospray at flow rates in the nanoliters (nl) per minute range [1, 35]. Some examples of these different types of interface will be discussed in the subsequent sections.

### 2.3.1 Sheath-Flow Interfaces

The typical configuration of sheath-flow interfaces is the coaxial sheath-flow interface (shown in Figure 2.2a), which was first developed by Smith *et al*. [36]. This configuration uses a triple-tube system in which the CE separation capillary is surrounded by a tube of a larger diameter through which the sheath liquid flows. The electrical contact for the CE terminal voltage is applied to the conductive sheath liquid in the outer tube. The sheath liquid then meets and mixes with the effluent that exits the capillary. A sheath gas is also commonly used as a part of this configuration and is applied through the outermost tube. Its role is to facilitate a more advantageous spray formation for ESI [26], although sensitivity can be reduced due to dilution by the sheath liquid [5, 8, 32, 34].

The application of a sheath liquid both helps to stabilize the electrospray [8, 33] and enables the use of BGEs that are more typical for CE [5, 8, 33]. Due to the modification of the CE effluent by the sheath liquid, the composition of the ESI spray can be made to be more MS compatible. However, the addition of sheath liquid results in dilution of the analyte [5, 8, 32, 34]. The presence of other chemical species in the electrospray can also reduce ionization efficiency due to competition [34].

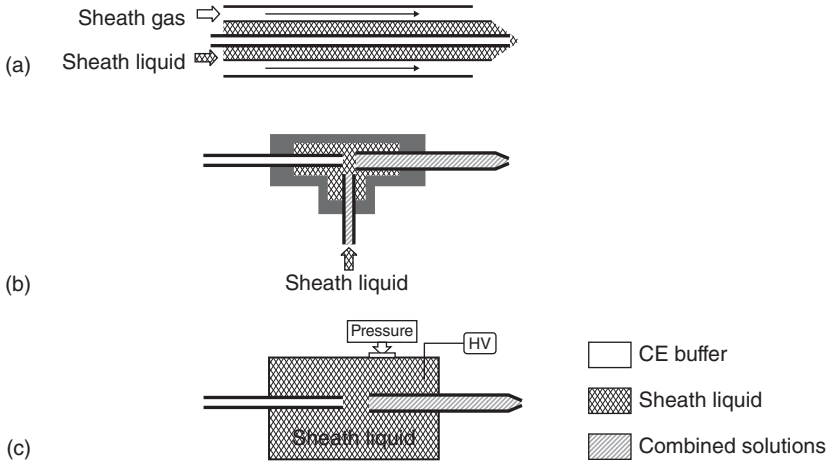


**Figure 2.2** Common sheath-flow interface arrangements. (a) coaxial sheath-flow interface with sheath gas, (b) liquid junction interface, and (c) pressurized liquid junction interface. Reproduced from Maxwell and Chen [6] with permission of Elsevier.

Liquid junction interfaces (see Figure 2.2b) may be defined as a separate type of interface, differing from sheath-flow interfaces by the location at which the sheath liquid and CE separation liquid meet and mix [8, 32]. However, in this chapter, liquid junction interfaces have been classified as a subset of sheath-flow interfaces due to inclusion of a sheath-liquid or -flow modifier and an electrical contact applied to the sheath-liquid or -flow modifier. Examples of sheath-flow interfaces that fall under the definition of a junction-at-the-tip interface design will be discussed, which is a type of liquid junction interface.

#### 2.3.1.1 Flow-Through Microvial Interface

The Chen group developed a flow-through microvial interface [5, 29, 30, 37], as shown in Figure 2.3. This interface uses a flow-through microvial to combine the flow of sample from the CE and a modifier liquid, which adjusts the BGE composition from CE to maintain a stable spray and increases ionization efficiency. The needle has an asymmetrical tip, which causes the electric field to focus at the sharpest point of the metal electrode. At the inner tip of the needle, there is a space formed by the inside wall of the needle and the capillary from the CE called the flow-through microvial, which acts as a cathodic electrode and the outlet vial for the CE. This is where the CE sample effluent and the chemical modifier liquid meet and mix. This interface typically operates at a flow rate of 100–400 nl $min^{-1}$ [30]. Several key features that serve to distinguish this interface from other types of interfaces include the ability to use standard unmodified CE capillaries [5, 30] and the increased spray stability at lower flow rates due to the beveled needle tip [29]. However, this interface does have several limitations; there is still a dilution factor due to the application of the modifier solution [5, 30, 37], and spray becomes unstable at a current higher than 15 μA [5, 30], resulting in a need for relatively

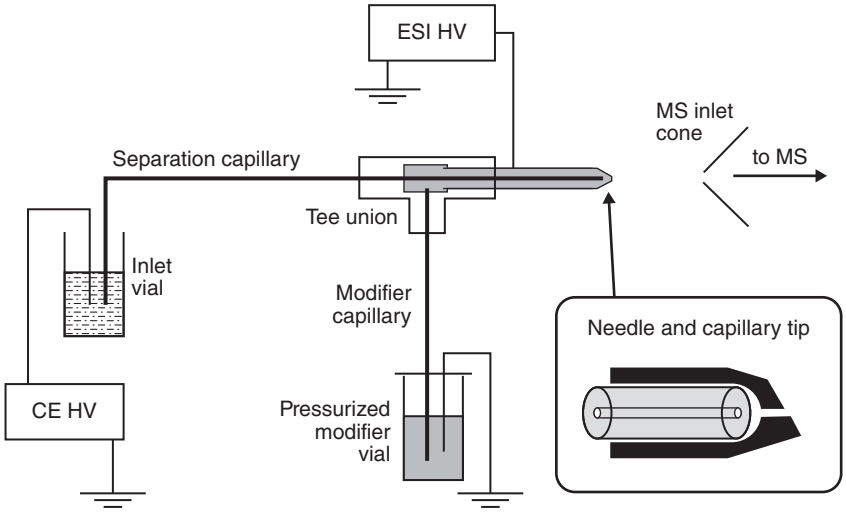


**Figure 2.3** Schematic illustration of the flow-through microvial interface apparatus, including a dissected view of needle tip with inserted capillary (inset). Reproduced from Maxwell *et al*. [30] with permission of Wiley.

low-conductivity BGE compositions. This interface has most notably been used in the development of cIEF-ESI-MS [38] and in the analysis of *N*-linked glycans [39–42].

#### 2.3.1.2 **Nanospray Sheath-Flow Interfaces**

Nanospray ionization operates at flow rates of 1–1000 nl $min^{-1}$ to generate a stable Taylor cone into the MS [1, 35], although currently, nanospray ionization typically refers to electrosprays generated at low nl $min^{-1}$ flow rates (tens to few hundreds) [43]. Currently used nanospray interfaces tend to have tip orifice diameters of 10–30 μm and use flow rates of 10–150 nl $min^{-1}$ to generate stable spray. Nanospray ionization interfaces can be sheath-flow or sheathless interfaces. The low flow rates of nanospray ESI mean that a reduced MS signal is produced, but this is balanced by increased ionization efficiency [4, 43].

#### 2.3.1.3 **Electrokinetically Pumped Sheath-Flow Nanospray Interface**

Dovichi's group has developed an electrokinetically pumped sheath-flow nanospray interface [5, 44, 45], as seen in Figure 2.4a. A plastic crosspiece is used to hold a separation capillary threaded into a borosilicate glass emitter and a tube that connects to a reservoir of sheath liquid. A stable sheath-liquid flow is produced by EOF generated at the inner wall of the glass emitter due to the application of a voltage on the sheath-liquid reservoir. Electrokinetically pumping in the sheath-flow solution results in a lower dilution of the analyte than is typical for a sheath-flow interface [44, 46]. In the most recent generation of the interface (bottom of Figure 2.4b), the diameter of the emitter orifice has been increased,

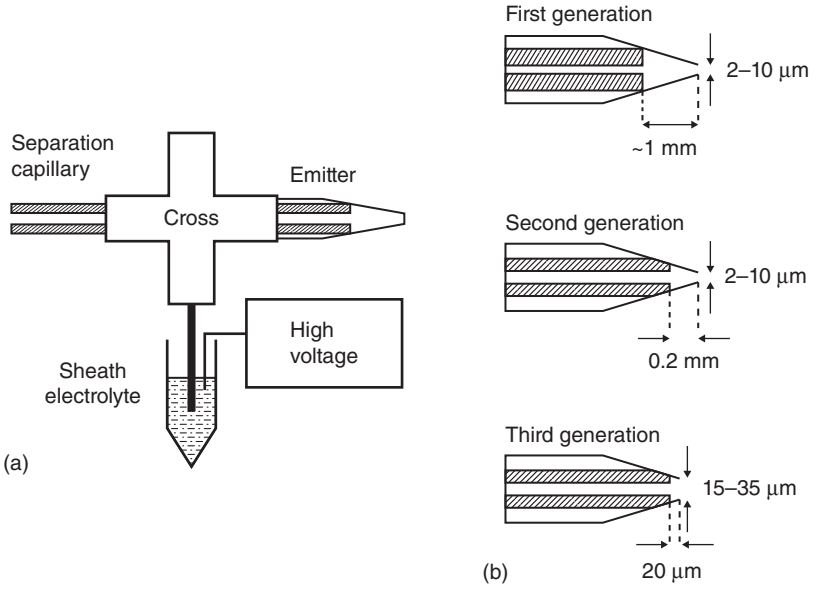


**Figure 2.4** Comparison of electrokinetically pumped nanospray CZE interfaces. (a) Overall design of the interface. The separation capillary is threaded through a PEEK cross (1.25 mm through hole) into a glass emitter. A tube (~1 mm i.d.) connects a side arm of the cross to a sheath electrolyte reservoir, which is connected through a platinum electrode to a high-voltage power supply. The mass spectrometer inlet is held at ground potential. (b) Designs of three generations of interface. The original interface uses a flat separation capillary, which is able to approach within ~1 mm of the emitter orifice; typical orifice diameters are 2–10 μm. The second generation uses a separation capillary with an etched tip, which approaches within ~200 μm of the emitter orifice; typical orifice diameters are 2–10 μm. The third-generation interface also uses an etched separation capillary but with a much larger exit orifice. The etched tip approaches within a few micrometers of the orifice; typical orifice diameters are 15–35 μm. Reproduced from Sun *et al*. [44] with permission of American Chemical Society.

and the space between the end of the separation capillary and the emitter orifice has been reduced, leading to greater sensitivities [44]. The flow rate due to EOF in the emitter is estimated to be approximately 50 nl $min^{-1}$, and the EOF flow rate from the separation capillary is approximately 20 nl $min^{-1}$, which causes the interface to be designated a nanospray interface. Dovichi's interface is reported to be sensitive [5, 44] and to produce reproducible results [44]. However, the emitter is borosilicate glass, which is less robust than fused silica and can lower reliability [5]. Also, similar to the Chen group's interface, current through the separation capillary should be limited to less than 10 μA in order to operate at the applied voltage value for electrospray [44]. Some notable applications of this interface include proteomics [44, 47–57] and multiple reaction monitoring (MRM) CE-MS [58, 59].

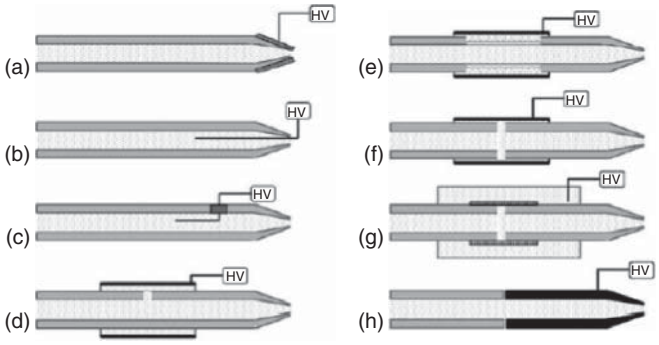


**Figure 2.5** Methods for creating electrical contact in sheathless interfaces. (a) Conductive coating applied to the emitter tip, (b) wire inserted at tip, (c) wire inserted through hole, (d) split-flow interface with a metal sheath, (e) porous, etched capillary walls in metal sleeve, (f) junction with metal sleeve, (g) microdialysis junction, and (h) junction with conductive emitter tip.Reproduced from Maxwell and Chen [6] with permission of Elsevier.

### 2.3.2 Sheathless Interfaces

The electrical contact for sheathless interfaces is established on or near the CE BGE directly at the outlet of the capillary [32, 34]. Figure 2.5 shows examples of different sheathless interface configurations. A potential may be applied to the capillary outlet in several different ways, depending on the design of the interface [32, 33]. Some interface designs coat the end of the capillary spraying tip with metal, while others attach a spray tip to the end of the capillary. Another method is to insert a wire electrode into or near the CE outlet.

Sheathless interfaces have the advantage of improved sensitivity due to a lack of dilution by a sheath liquid [32–34]. Disadvantages for sheathless interfaces tend to be specific to the individual design of the interface. The metal of the CE tip can degrade in interface designs where the capillary tip is coated. An attached spray tip leads to dead volumes, and gas bubbles formed through electrochemical reactions at the surface of an inserted wire electrode can interfere with spray stability.

#### 2.3.2.1 Porous-Tip Nanospray Sheathless Interface/CESI 8000

A nanospray sheathless interface with a porous tip was designed by Moini [5, 33, 60] and was further developed by Beckman Coulter and SCIEX to integrate CE and ESI [5] (see Figure 2.6). The commercially available interface from SCIEX is called the CESI8000, although the prototype developed by Beckman Coulter was called *the high-sensitivity porous sprayer* (*HSPS*). The end of the separation capillary is made porous by etching with hydrofluoric acid and allows for electrical contact due to the movement of ions and electrons [5, 60] without the movement of liquid [33]. A stainless-steel cylinder filled with BGE surrounds part of

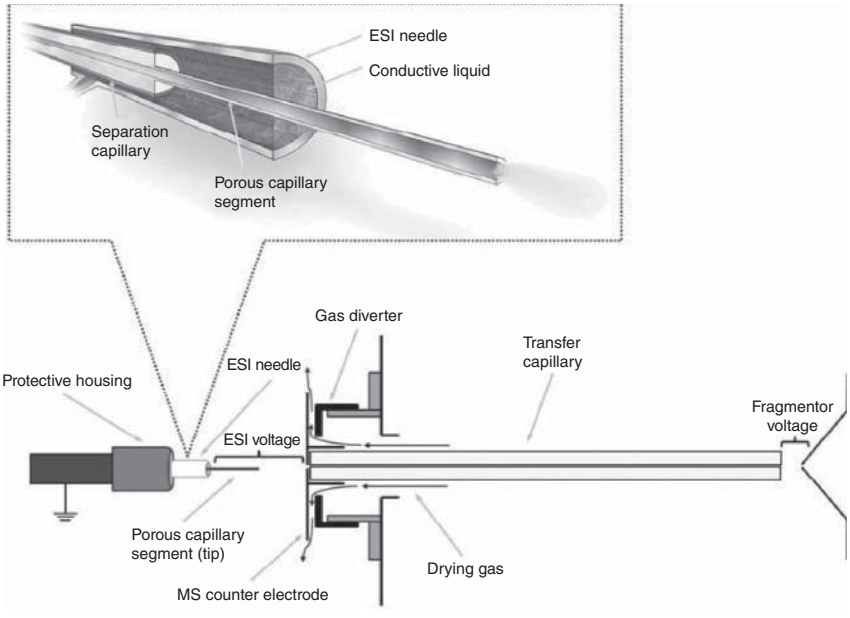


**Figure 2.6** Principle of the HSPS sheathless interface (Beckman Coulter)/CESI 8000 (SCIEX).Reproduced from Bonvin *et al*. [61] with permission of Wiley.

the porous capillary, while allowing a section of the tip to be extruded from the metal cylinder, and an ESI voltage is applied to the metal cylinder. The interface is sensitive [60], does not require a tapered emitter tip [33], and any electrolysis reactions occur in the conductive liquid inside of the ESI needle instead of inside the separation capillary. However, similar to the interfaces of the Chen group and Dovichi group, this interface operates best when currents of less than 10 μA are used [5]. It is also necessary to replace the conductive liquid inside the ESI needle every 3–4 h, as it may dry out or cause oxidation in the needle [60]. Some applications of this interface have been characterization of posttranslationally modified histones by the Linder group [62], as well as transient capillary isotachophoresis (cITP) [63], and phosphoproteomics [64] by the Mayboroda group.

#### 2.3.2.2 Sheathless Porous Emitter NanoESI Interface

A sheathless porous tip nanoESI emitter interface was developed by Wang *et al*. [65] as seen in Figure 2.7. Similar to the Moini/Beckman Coulter/SCIEX interface, this interface uses hydrofluoric acid to etch the end of a piece of capillary, making it porous [5, 65]. The difference is that in this interface, instead of the end of the separation capillary, a separate piece of capillary is made porous [5]. The porous piece of capillary is smaller in diameter (20 μm i.d., 90 μm o.d.) than the separation capillary (100 μm i.d., 360 μm o.d.), so the porous piece of capillary can be inserted into the separation capillary and glued in place [65]. The connected capillaries

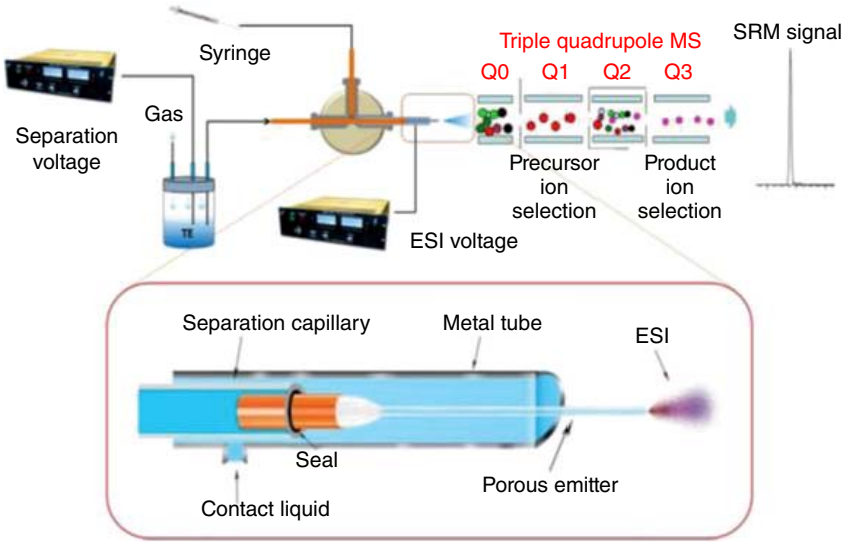


**Figure 2.7** Schematic of CITP/CZE-nanoESI-QQQ MS setup used in this study. The lower part shows a detailed view of the sheathless interface design (not to scale).Reproduced from Wang *et al*. [65] with permission of American Chemical Society.

are inserted into a metal tube, and the porous capillary tip emerges from the end of the metal tube. The metal tube is also filled with BGE, and the ESI voltage is applied to the metal tube, allowing an electrical contact with the porous emitter tip. The use of a separate porous emitter tip allows the interface to operate in the nanospray regime due to the narrow i.d. of the porous tip, while maintaining the ability to load a large sample volume in the separation capillary. As a sheathless interface, the sample is not diluted, and therefore, a better sensitivity is possible. Also, since the separation capillary and porous emitter tip are separate pieces of capillary, different diameters of either may be used, or the separation capillary may be coated, or the emitter tip may be easily replaced. However, the separation capillary needs to be neutrally coated to reduce EOF, and care must be taken to assemble the connection between the separation capillary and porous emitter tip to prevent leakages or blockages. This interface has so far been used in studies of cITP and selected reaction monitoring (SRM) [65, 66].

### 2.3.3
### Interface Applications/CE Mode of Separation

In this chapter, we are presenting four CE-ESI-MS interfaces that have been developed in the past years. It is important to note that these are not the only interfaces currently in use and that the coaxial sheath-liquid interface is still a standard interface used for many CE-MS analyses.

## 2.4
## Specific Interface Applications

### 2.4.1
### Capillary Isoelectric Focusing

Capillary isoelectric focusing (cIEF) was introduced by Hjertén and Zhu in 1985 and is a separation technique that offers high resolving power [67, 68]. In cIEF, amphoteric biomolecules (e.g., proteins) are separated according to their respective isoelectric points (pIs). This is done by forming a pH gradient through the application of a voltage across a capillary containing a mixture of carrier ampholytes (CAs). The mixture of CAs and amphoteric biomolecules fills the capillary, and one end of the capillary contains a basic catholyte solution, while the other end contains an acidic anolyte solution (Figure 2.8). When different CAs migrate to their p*I* between the anolyte solution and catholyte solution, they create the pH gradient; this allows the amphoteric biomolecules to be focused and separated into sharp zones according to their respective pIs [27, 69].

cIEF with MS is a powerful combination since it offers information about both the p*I* and the mass-to-charge ratio of the amphoteric analytes. The process of cIEF-MS always involves two steps: first, a focusing step with the mixture of CAs and second, a mobilization step. Some challenges associated with adapting cIEF for MS detection have been overcome by employing volatile organic catholytes and anolytes to establish the pH gradient and by creative focusing/mobilization strategies to deal with the lack of catholyte reservoir vial with a CE-MS setup [27].

Many strategies that overcome the absence of a catholyte reservoir have been described in several reviews [4, 27, 68, 70–72]. Recently, the Dovichi group used their interface for cIEF-ESI-MS for several biomolecule applications [73, 74]. In this system, the electrokinetically pumped sheath-flow nanospray interface (Figure 2.4) is used to couple a cIEF column to an orbitrap mass spectrometer. They overcome the lack of catholyte reservoir by employing a two-step process. First, the focusing step in the separation capillary is carried out. Following the focusing step, the cathode end of the capillary is inserted into the emitter of the ESI interface and chemical mobilization is performed with the sheath-flow buffer [73, 74].

Another cIEF-ESI-MS strategy was developed by the Chen research group using their flow-through microvial interface, which has been used for cIEF-ESI-MS analysis [38]. In this system, a catholyte plug or the flow-through microvial can be used to provide the catholyte (Figure 2.8). The capillary that is used to incorporate the sheath-flow modifier liquid allows the catholyte to be delivered during the focusing step or an acidic solution to be delivered to facilitate chemical mobilization in the second step. This interface system is advantageous since it allows for automation of the two steps in cIEF.

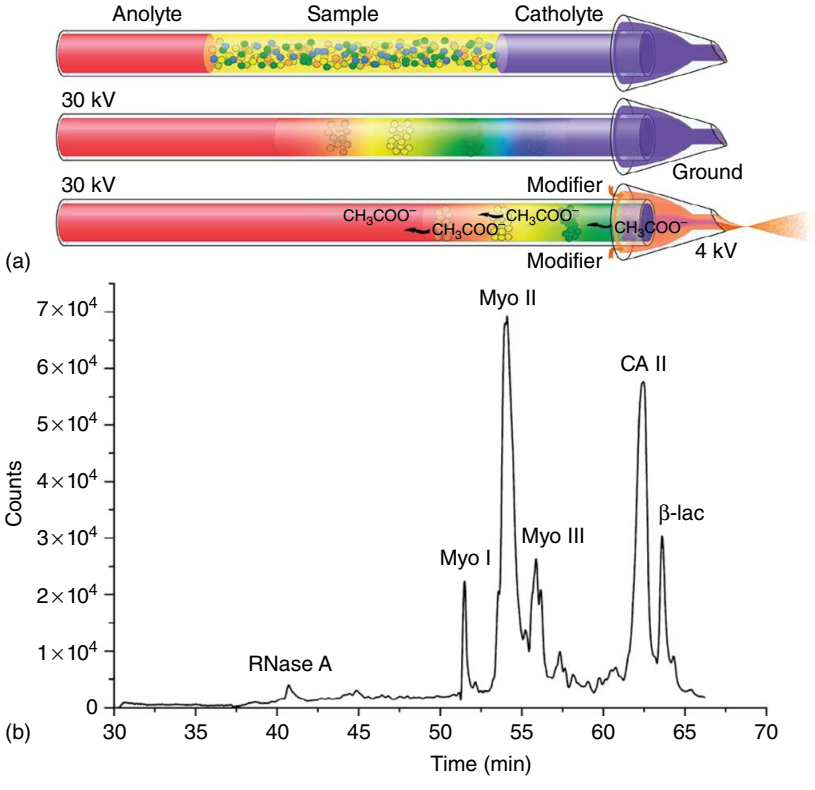


**Figure 2.8** (a) Schematic diagram of automated cIEF-MS. The flow-through microvial provides the electrical contact and chemical environment for cIEF. The CE-MS interface improves the ionization efficiency of the mobilized effluent without significant dilution. (b) Total ion chromatogram for a protein mixture separated in an N–CHO coated capillary. Reproduced from Zhong *et al*. [38] with permission of American Chemical Society.

### 2.4.2
### Glycan Analysis by CE-ESI-MS

Protein glycosylation is an important posttranslational modification, enabling functions such as cell–cell signaling and interactions [75, 76]. The structural analysis and identification of glycans from glycoproteins can be achieved using mass spectrometry, but due to the number and complexity of glycans that can be obtained, separation before MS analysis is often necessary [75]. Capillary electrophoresis has been shown to be a successful separation tool for glycan analysis [39–41].

The CESI 8000 interface has been used to analyze the glycosylation of the trypsin-digested fragment crystallizable (Fc) portion of human IgG [77], as seen in Figure 2.9. Transient cITP was used to focus the analytes and improve

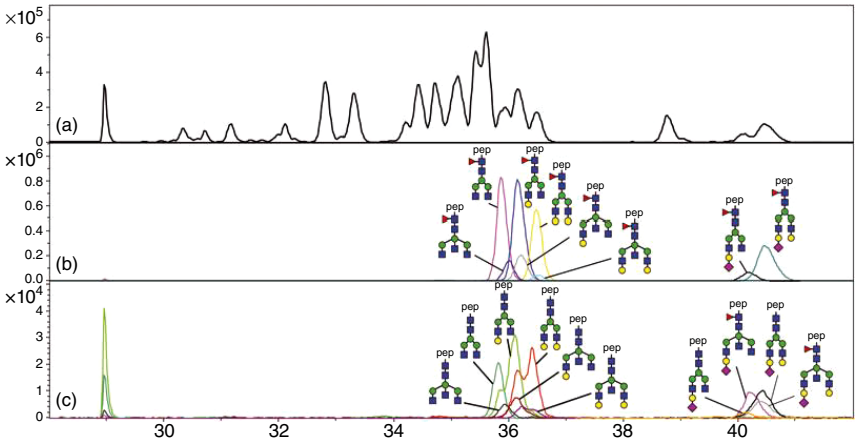


**Figure 2.9** (a) A base peak chromatogram of total IgG tryptic digest from human serum, (b) an extracted ion chromatogram of IgG1 glycopeptides of high concentration with assignments, (c) an extracted ion chromatogram of IgG1 glycopeptides of low concentration with assignments. The blue square is *N*-acetylglucosamine, the red triangle is fucose, the green circle is mannose, the yellow circle is galactose, and the purple diamond is *N*-acetylneuraminic acid.Reproduced from Heemskerk *et al*. [77] with permission of Wiley.

sensitivity. The results obtained, using this CE-MS interface, were comparable to those obtained using nano-RPLC-MS. Another study was performed using the CESI 8000 interface to characterize intact pharmaceutical glycoproteins [78]. During a 60 min analysis, hundreds of pharmaceutical protein isoforms were able to be detected and characterized.

The flow-through microvial interface developed by the Chen group was used in the development of a method to analyze carbohydrates that had been derivatized with a negatively charged fluorescent label [41]. Baseline resolution was obtained for glycans of 15 glucose units or fewer. The interface has also been used to characterize *O*-acetylated *N*-linked glycans found in fish serum [39]. Fish serum *O*-acetylated *N*-linked glycans are negatively charged due to high levels of sialylation and can be analyzed by CE-MS without derivatization. Isomeric glycans were able to be baseline separated. Another method developed using the same interface was able to separate and detect both neutral and negatively charged glycans that had not been labeled or derivatized [40]. This interface was also used to analyze mass-tagged glycans quantitatively [42].

## 2.5 Conclusion

The four CE-ESI-MS interfaces discussed in this chapter have been used to analyze a diverse set of target analytes, thus showing the flexibility of these interfaces

and the feasibility of their application to more complex samples. Despite the large number of applications, most of the interfaces presented here are in use by only one or two research groups. Only one of these interfaces (CESI 8000, AB SCIEX) is commercialized and is therefore more widespread in use, yet there has been significant growth in the field of CE-MS. Future commercialization of other CE-MS interfaces would allow for more widespread use by giving options for choosing an interface best suited for the kind of analysis one plans to do. In time, interfaces such as those presented in this chapter may begin to replace the coaxial sheath-liquid interface, which is still a standard interface used for many CE-MS applications. By continuing to develop and optimize CE-ESI-MS interfaces to make them easy to use and reliable, CE-MS will grow as a more common standard analytical tool.

Each of the interfaces presented in this chapter has its own unique features, depending on the target for optimization, such as sensitivity or spray stability. There are more factors than the interface design that determine or impact analytical characteristics and figures of merit, which makes it difficult to directly compare different CE-ESI-MS interfaces based on those metrics. Readers are advised to use Table 2.1 to make a more informed choice about which interface to use, based on multiple different parameters, such as the availability of the interface, and the analyte of interest. Readers should also carefully consider the need to balance several important parameters, such as sensitivity, spray stability, and the ability to manipulate the interface to suit laboratory, user, and/or analysis needs.

The interface is a key component in the ability to fully harness the potential of a CE-MS combination. The development of interfaces able to operate at low flow rates with increasing sensitivity has enabled CE-ESI-MS to be well suited to omics analysis, with many studies being conducted in proteomics in particular. The combination of these techniques lends itself well to proteomics since CE is ideally suited to charged molecules, ESI enables such large molecules to be ionized, and various MS techniques allow for greater coverage, separation, and insight into -omics biomolecule analysis.

The trend toward lower flow rate and higher sensitivity also lends itself well toward miniaturization (i.e., microfluidics and lab-on-a-chip). CE, as a nanotechnique, is well suited toward miniaturization, and a CE-MS interface that can operate within nanoliter flow rate ranges is invaluable. As CE-ESI-MS interface development continues to show promise in the areas of reduced flow rate and increased sensitivity, the incorporation of microfluidic separation platforms prior to MS analysis becomes more and more practical. A trend toward micro total analysis systems is valuable for volume-limited samples and progressing -omics studies.

Further interface development and optimization will promote future applications where CE-ESI-MS will be able to provide valuable insights into complex systems.

**Table 2.1** Areas of study and application have been listed for each interface described in Sections 2.3.1 and 2.3.2, along with CE separation mode, BGE, mass spectrometer used, and some analytical characteristics reported in each reference.

| Interface | Application | CE separation mode | CE BGE | Mass spectrometer | Analytical characteristics | Reference |
|---|---|---|---|---|---|---|
| Flow-through microvial interface | Cancer biomarkers in urine | CZE | 0.4% or 2.0% Formic acid in 50% MeOH | Finnigan LCQ*Duo ion trap MS and API 4000 triple-quadrupole MS | Intraday calibration correlation coefficient: 0.9951; Interday calibration correlation coefficient: 0.9990; LODs: 0.010–0.134 μM | [79] |
| | Proteins | cIEF | Anolyte: 50 or 125 mM formic acid in 30% glycerol/water; catholyte: 100 mM ammonium hydroxide in 30% glycerol/water | Finnigan LCQ*Duo ion trap MS and Micromass Q-TOF 1E MS | No remarks | [38] |
| | *O*-Acetylated *N*-glycans in fish serum | CZE | 0.2–2.0% Formic acid or acetic acid, 10–50 mM ammonium acetate, ammonium formate, or 25–50 mM ε-aminocaproic acid | Finnigan LCQ*Duo ion trap MS and API 3000 triple-quadrupole MS | No remarks | [39] |
| | Glycans from glycoproteins | CZE | 30–100 mM Ammonium acetate (pH 3.0–4.0) with 20% MeOH | API 4000 triple-quadrupole MS and maXis ultrahigh-resolution TOF-MS | Resolution ≈ 20 000 at $m/z$ 1334 | [40] |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Glycans | CZE | 2.0% Formic acid in 30% MeOH | Finnigan LCQ*Duo ion trap MS | No remarks | [41] |
| | Labeled glycans | CZE | 50 : 49 : 1 (v/v/v) MeOH/water/formic acid | Synapt G2 MS | No remarks | [42] |
| | Amine-containing metabolites | CZE | 0.2% Formic acid in 50% MeOH | Synapt G2 MS | LODs between 0.02 and 1.24 μmol $l^{-1}$ | [80] |
| | Cationic metabolomics | CZE | 10% Acetic acid | maXis qTOF-MS or 6550 iFunnel-Q-TOF-MS | LODs between 0.01 and 3.13 μM; sensitivity between 0.13 and $7.32 \times 10^3$ AU/μM | [81] |
| | Peptides and proteins | CZE | 100 mM $NH_4Ac$ (pH 3.1) with 10% MeOH; 1% formic acid in 25% MeOH; 0.2% formic acid in 50% MeOH | Xevo TQ MS and Finnigan LCQ*Duo ion trap MS | LODs: 4–50 ng $ml^{-1}$ for proteins, 0.1–3.4 μmol $l^{-1}$ for peptides | [30] |
| Electrokinetically pumped sheath-flow nanospray interface | Amino acids and peptides | CZE | Formic acid | LTQ XL MS and Q-Exactive MS | *S*/*N* of 30–440; LODs between 0.9 and 6.1 μM | [82] |
| | Peptides | CZE | 10 mM ammonium acetate | Finnigan LCQ ion trap MS | *S*/*N* of 350, 2000, and 3500; LODs of 1, 0.2, and 0.1 fmol; spray stability by RSD in TIC of 2% and 3% in EIC | [45] |
| | Peptides | CZE | 10 mM Ammonium acetate (pH 7) | Orbitrap Velos MS | Resolution of 60 000 at *m*/*z* 500; LODs of $10^{-10}$ to $10^{-6}$ M | [83] |

(*continued overleaf*)

**Table 2.1** (*continued*)

| Interface | Application | CE separation mode | CE BGE | Mass spectrometer | Analytical characteristics | Reference |
|---|---|---|---|---|---|---|
| | Proteins | CZE | 0.1% Formic acid | LTQ-Orbitrap Velos MS | Resolution of 30 000 at $m/z$ 400 | [84] |
| | Protein digest | cIEF | Anolyte: 0.1% formic acid; catholyte: 0.3% ammonium hydroxide | LTQ-Orbitrap Velos MS | Peak widths of 10 s or less; Resolution of 60 000 at $m/z$ 400; LODs of ≤7 nM | [73] |
| | Leu-enkephalin peptide | CZE | 5 mM $NH_4HCO_3$ | QTrap 5500 hybrid linear-ion-trap triple-quadrupole MS | Mass detection limits (at $S/N = 3$) were approximately 600 zmole and approximately 30 amole; migration time of less than 10 min | [59] |
| | RAW 264.7 cell lysate digest proteins | CZE | 10 mM ammonium acetate | QTrap 5500 hybrid linear-ion-trap triple-quadrupole MS | Reproducibility for: peak height RSD = 21% and migration time RSD = 1%; LOD = 60 pM | [58] |
| | *E. coli* proteomics | Dynamic pH junction preconcentration/CZE | 0.1% v/v Formic acid | LTQ XL MS | No remarks | [85] |
| | Proteomics | CZE | 10 mM Ammonium acetate (pH 5.7) | LTQ-Orbitrap Velos MS | No remarks | [47] |

| | | | | | |
|---|---|---|---|---|---|
| PC12 cell proteomics | cIEF | Anolyte: 0.1% formic acid; catholyte: 0.3% ammonium hydroxide | LTQ-Orbitrap Velos MS | Resolution of 30 000 at $m/z$ 400 | [74] |
| 30–80 kDa proteomics | CZE | 0.1% Formic acid | Q-Exactive MS | FWHM = 8–16 s; reproducibility of: migration time RSD < 3% and peak intensity RSD < 30% | [48] |
| Cellular homogenate proteomics | CZE | 10 mM $NH_4HCO_3$ (pH 8.0) | Orbitrap-Velos MS | Linear dynamic range of 12–750 nM; one LOD of 0.3 nM | [49] |
| Intact protein proteomics | CZE | 0.1% v/v Formic acid | LTQ-Orbitrap Velos MS | LODs ($S/N = 3$) of 20–800 amole; reproducibility of: migration time RSD < 3% and peak intensity RSD < 30% | [50] |
| HeLa proteomics | CZE | 5% v/v Acetic acid | Q-OT-qIT, Orbitrap Fusion MS | Peak capacity of approximately 300; median peak width of 8.5 s | [51] |
| Human cell proteomics | CZE | 0.5% v/v Formic acid | Q-Exactive MS | Linear dynamic range over 4.5 orders of magnitude; migration time reproducibility RSD < 4% | [52] |
| Proteomics | CZE | 5% v/v Acetic acid | LTQ-Orbitrap Velos MS | Reproducibility for peak area RSD = ~10% over 127 runs | [44] |

(*continued overleaf*)

**Table 2.1** (*continued*)

| Interface | Application | CE separation mode | CE BGE | Mass spectrometer | Analytical characteristics | Reference |
|---|---|---|---|---|---|---|
| | *E. coli* proteomics | CZE | 0.1% Formic acid in water | LTQ-Orbitrap Velos MS | No remarks | [53] |
| | *E. coli* proteomics | CZE | 0.1% v/v Formic acid | LTQ-Orbitrap Velos MS | Resolution of 30 000 at $m/z$ 400 | [56] |
| | Proteomics | Dynamic pH junction preconcentration/CZE | 50 mM Formic acid in water | LTQ-Orbitrap Velos MS | FWHH 6 s; $S/N$ ~$10^4$; mass concentration LODs approximated at low pg $ml^{-1}$ | [54] |
| | Proteomics | CZE | 0.1% v/v Formic acid | Orbitrap Elite MS | No remarks | [55] |
| | Proteomics | CZE | 5% Acetic acid | LTQ-XL MS | Resolution of 1.2–2.6 for protein separation using coated capillaries; reproducibility of: peak intensity RSD = 8.5% and migration time RSD = 1.2% | [57] |
| Porous tip nanospray sheathless interface/CESI 8000 | Therapeutic antibodies | cITP/CZE | 1.67 M Acetic acid | TripleTOF 5600+ MS | No remarks | [86] |
| | Enzymes from drug-resistant bacteria | CZE | 10% Acetic acid | maXis impact UHR-TOF-MS | No remarks | [87] |
| | Silk Dating | CZE | 30–60 mM solution of 18-C-6-TCA in water | Finnigan LCQ*Duo ion trap MS | | [110] |

| | | | | | |
|---|---|---|---|---|---|
| Herbal compounds | CZE | 1% Acetic acid in 1 : 1 v/v water : MeOH | Q-Exactive (QE) Orbitrap MS | LODs of 18–24 fg; reproducibility for migration time RSD = 0.7–1.0% | [88] |
| Herbicides | CZE | 10 mM Formic acid with 50 mM ammonia (pH 10.0) | MicrOTOF II MS | LODs of 0.57–7.6 pg; linear ranges of 0.1–50 and 5–100 $\mu g\,ml^{-1}$ | [89] |
| Histones | CZE | 0.1% Formic acid | LTQ Orbitrap XL MS | LODs ($S/N = 3$) of 5.96 and 11.46 amole; selectivity of 1.061 | [90] |
| Posttranslationally modified histones | CZE | 0.1–0.6% Formic acid | LTQ Orbitrap XL MS | No remarks | [62] |
| Cationic metabolomics | CZE | 10% v/v Acetic acid | Agilent 6220 accurate-mass TOF LC/MS | LODs ($S/N = 3$) of 0.004–0.8 $\mu mol\,l^{-1}$; reproducibility of: relative peak area RSD = 2.4–18% and migration time RSD < 1.4% | [91] |
| Mouse cerebrospinal fluid metabolomics | CZE | 10% Acetic acid | maXis impact UHR-TOF-MS | LODs ($S/N = 3$) of 0.7–12 nM; reproducibility of: peak area RSD < 14% and migration time RSD < 2% | [92] |

(*continued overleaf*)

**Table 2.1** (*continued*)

| Interface | Application | CE separation mode | CE BGE | Mass spectrometer | Analytical characteristics | Reference |
|---|---|---|---|---|---|---|
| | Urine metabolomics | CZE/transient cITP | 10% Acetic acid | micrOTOF MS | LODs of 11–120 nM; reproducibility of: peak area RSD <12% and migration time RSD < 2%; 2 orders of magnitude sensitivity improvement for t-ITP | [93] |
| | Mouse plasma metabolomics | CZE | 10% v/v Acetic acid | maXis 4G UHR-TOF MS | No remarks | [94] |
| | Therapeutic peptides | CZE | 50 mM Formic acid at pH 2.9 with 50 mM ammonium formate | UHR-Qq-TOF maXis 3G MS | LOD ~1 fmol | [95] |
| | Peptides | CZE | 50 mM Acetic acid and 50 mM formic acid with ammonia (pH 3.5) | micrOTOF MS | Intraday reproducibility of: peak area RSD < 9% and migration time RSD < 4%; LODs of 10 pg $ml^{-1}$; linear dynamic range between 0.05 and 5 ng $ml^{-1}$ | [96] |

| | | | | |
|---|---|---|---|---|
| Pharmaceuticals | CZE | 30 mM Ammonium formate (pH 3.0) | Single-quadrupole Agilent 1100 MSD | LODs ($S/N = 3$) of 250 pg ml$^{-1}$, 500 pg ml$^{-1}$, 5 ng ml$^{-1}$, and 10 ng ml$^{-1}$; reproducibility of migration time RSD < 1.5% | [61] |
| Phosphoproteomics | CZE/transient cITP | 10% Acetic acid | maXis 4G UHR-TOF MS | No remarks | [64] |
| Drug–lysozyme conjugates | CZE | 5% v/v 100 mM Acetic acid (pH 3.1, adjusted with 0.1% ammonium hydroxide) in 95% water/isopropanol | micrOTOF MS | No remarks | [97] |
| Protein–metal complexation | CZE | 10 mM Ammonium acetate containing 0.1% Polybrene (pH 6.9) | Q-TOF Premier MS | Resolution ~ 10 000 | [98] |
| Erythrocyte protein–protein and protein–metal complexation | CZE | 0.01% Acetic acid and ammonium hydroxide | Q-TOF Premier MS | Reproducibility of migration time RSD = 0.4% | [99] |
| Pharmaceutical protein glycoforms | CZE | Acetic acid and ammonium hydroxide | micrOTOF MS | Resolution of 20 000 above $m/z$ 1250; mass accuracy <5%; LODs of 2.8–9.5 ng ml$^{-1}$; reproducibility of: peak area RSD < 8.2% and migration time RSD < 1.4% | [78] |

(*continued overleaf*)

**Table 2.1** (*continued*)

| Interface | Application | CE separation mode | CE BGE | Mass spectrometer | Analytical characteristics | Reference |
|---|---|---|---|---|---|---|
| | Intact proteins | CZE | 100 mM Acetic acid (pH 3.1 adjusted with ammonium hydroxide) | micrOTOF MS | Reproducibility of: peak area RSD < 8.5% and migration time RSD < 0.74%; LODs of 0.50–1.28 nM | [100] |
| | Proteins | CZE | 0.1% Polybrene in 0.1% acetic acid | Finnigan LCQ MS | No remarks | [60] |
| | Quantitative proteomics | CZE | 10% Acetic acid | LTQ Orbitrap XL MS | Resolution = 60 000 at $m/z = 400$ | [101] |
| | *Pyrococcus furiosus* proteomics | CZE | 0.1% Acetic acid with 20% isopropanol | Orbitrap Elite MS | No remarks | [102] |
| | Proteomics | CZE | 5% Acetic acid with 5% isopropanol | LTQ Velos Orbitrap Elite MS | Resolving power of 120 000 at $m/z = 400$; $S/N$ of 11–629 | [103] |
| | Proteomics | CZE | 0.1% Acetic acid with 0.1% poly-E or poly-B | LCQ classic ion trap MS | No remarks | [104] |
| | Proteomics | Transient cITP/CZE | 95 mM HOAc–5% MeOH (pH 3.1) | LTQ XL MS | No remarks | [105] |
| | Antibody proteomics | CZE | 10% Acetic acid | micrOTOF-Q II MS | No remarks | [106] |
| | Monoclonal antibody biosimilarity | Transient cITP/CZE | 10% Acetic acid | 5600 TripleTOF MS | Resolution of 40 000 in MS ($m/z$ 485.251) and 25 000 in MS/MS ($m/z$ 345.235) | [107] |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Antibody primary structure and characterization | Transient cITP | 10% Acetic acid | 5600 TripleTOF MS | Resolution of 40 000 in MS ($m/z$ 485.251) and 25 000 in MS/MS ($m/z$ 345.235) | [108] |
| | Peptides | Transient cITP | 10% Acetic acid | maXis UHR-TOF MS | LODs of 1.4–8.4 nM | [63] |
| | IgG glycopeptides | Transient cITP-CZE | 10% Acetic acid | maXis 4G UHR-TOF MS | LOD of 20 attomol | [77] |
| Sheathless porous emitter nanoESI interface | Targeted peptides | cITP/CZE | 9 : 1 0.1 M Acetic acid: MeOH | Agilent 6490 QQQ MS | Reproducibility for concentration: CV < 24%; LOQ ≤ 50 pM | [66] |
| | Targeted peptides | cITP/CZE | 9 : 1 0.1 M Acetic acid: MeOH | TSQ Quantum Ultra triple-quadrupole MS | Linear dynamic range from 10 pM to 500 nM; LOQs 10 and 500 pM; reproducibility for: peak area CV = <22% and migration time CV = <10% | [65] |
| | Peptide mixture | Microchip CZE | 9 : 1 of 0.1 M Acetic acid in water/methanol | TSQ Quantum Ultra triple-quadrupole MS | No remarks | [109] |

### Abbreviations

| | |
|---|---|
| BGE | background electrolyte |
| CA | carrier ampholyte |
| CE-MS | capillary electrophoresis–mass spectrometry |
| CI | chemical ionization |
| cIEF | capillary isoelectric focusing |
| cITP | capillary isotachophoresis |
| CRM | charge residue model |
| CZE | capillary zone electrophoresis |
| EI | electron ionization |
| EOF | electroosmotic flow |
| ESI | electrospray ionization |
| IEM | ion evaporation model |
| LOD | limit of detection |
| MALDI | matrix-assisted laser desorption/ionization |
| MRM | multiple reaction monitoring |
| nL | nanoliter |
| p*I* | isoelectric point |
| SRM | selected reaction monitoring |
| *S*/*N* | signal-to-noise ratio |

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# 3
# Sheath Liquids in CE-MS: Role, Parameters, and Optimization

*Christian W. Klampfl and Markus Himmelsbach*

## 3.1
## Introduction

Only 6 years after the first paper on capillary electrophoresis (CE) by Jorgenson and Lukacs in 1981 [1], Smith's group described the coupling of CE with mass spectrometric detection (MS), using an interface without a sheath flow [2]. Just 1 year later, the same group presented an improved electrospray ionization (ESI) interface for CE-MS, this time involving the use of a sheath liquid (SL) [3]. The authors describe the benefits of the new interface design as follows: "The liquid sheath electrode allows the electrospray ionization interface to be operated for almost any buffer system of interest for CE. This includes aqueous and high ionic strength buffers that could not otherwise be electrosprayed. In addition, the interface provides simplicity of operation and day to day reproducibility not obtained previously" [3]. This early statement about a sheath-flow interface already includes important advantages of this design. One is the decoupling of conditions for separation from those for proper MS ionization. The sheath-flow interface provides free choice of background electrolyte (BGE) for separation and of the SL responsible for the ionization behavior of the analytes, so both may be optimized separately. In addition, sheath-flow interfaces excel with improved robustness and provide good reproducibility with respect to analyte signals – a fact that is of great concern in quantitative analysis. Another advantage is the fact that SL-based interfaces for CE-MS are easy to use, at least compared to most sheathless designs. Over the years, a variety of different interface designs have been reported, but the use of most of them remained restricted to a few publications, mostly by the developing group [4]. Anyhow, it still took several years before the first (almost) "plug-and-play" interfaces for CE-MS arrived as "early" sheath-flow interfaces still required substantial operational skills. For a long time, the sheath-flow interface was the only interface for CE-MS available commercially; only recently, a novel sheathless design from Moini [5] was introduced to the market by AB-SCIEX. This is also reflected in the literature with the vast majority of CE-MS publications reporting the application of sheath-flow interfaces. Detailed descriptions

*Capillary Electrophoresis – Mass Spectrometry (CE-MS): Principles and Applications,*
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and discussions of interface design and development are not the main aim of this chapter, but comprehensive information on this topic can be found in Chapter 2 of this book as well as from a series of review papers [6–11]. In this chapter, primarily SL-related chemical and physical parameters are discussed, which include SL composition as well as SL flow rate and closely related issues such as the nebulizer pressure.

## 3.2 Sheath-Liquid Functions and Sheath-Flow Interface Design

According to the review by Maxwell and Chen [6], the most important interfaces employing a sheath-flow or makeup liquid are the coaxial sheath-flow interface and the liquid junction interface. In both cases, the effluent from the separation capillary is mixed with an SL. Although, as stated earlier, this chapter is not specifically devoted to interface design, a brief overview of the basically different layouts of instrumentation belonging to the group of "sheath-flow interfaces" is necessary. In the following section, these two designs are briefly discussed particularly with respect to SL-related issues.

### 3.2.1 Coaxial Sheath-Flow Interface

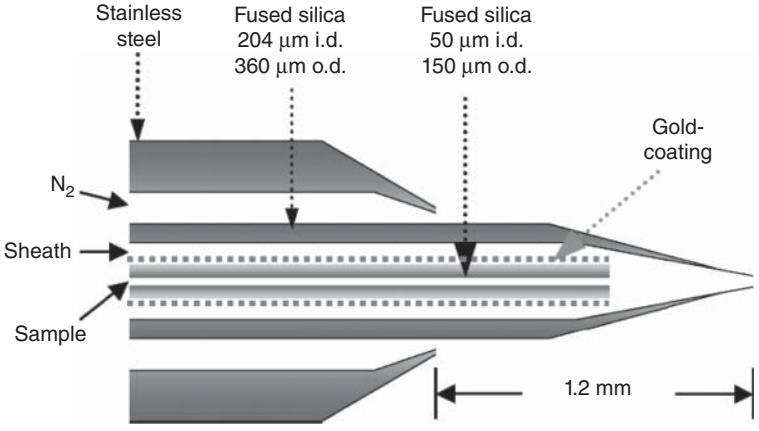
By far, the most widely used design is the coaxial sheath-flow interface. A schematic drawing of this type of interface is depicted in Figure 3.1.Generally, as shown in Figure 3.1a, the separation capillary is inserted into a double-walled metal tube allowing the flow of sheath gas (outer tube) and the SL (inner tube). Hereby, the SL takes responsibility of several important issues:

- Establishing the required electrical contact between the liquid inside the separation capillary and the metal tube acting as electrode
- Adjusting flow rates (particularly when HPLC-MS interfaces are used)
- Providing favorable conditions for ESI
- Enhancing ionization yields
- Overcoming eventual incompatibility between BGE and ESI processes.

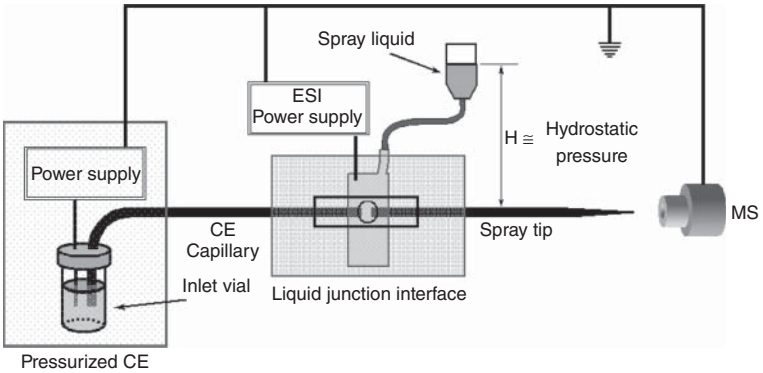
The middle part of Figure 3.1b depicts a miniaturized version of this design (developed by Liu *et al*. [12]), helping minimizing void volumes responsible for interface-caused peak broadening. This interface design required, in addition to the charged SL, a gold coating applied to the capillary tip for guaranteeing sufficient charge separation and thereby a smooth ESI process.

Conventional, that is, nonminiaturized versions of the coaxial sheath-flow interface are commonly operated at flow rates in the low $(1-10)\,\mu l\,min^{-1}$ range. These flow rates are needed, on the one hand, to guarantee a stable electrospray; on the other hand, flow rates are often adjusted to improve compatibility with CE-MS interfaces that have actually been developed and optimized for


(a)




(b)




(c)

**Figure 3.1** Different types of sheath-flow interfaces. Coaxial sheath-flow interface (a), miniaturized coaxial sheath-flow interface (b, reproduced from Liu *et al*. [12] with permission of Wiley), and miniaturized liquid junction interface (c, reproduced from Fanali *et al*. [13] with permission of Wiley).

HPLC-MS (with much higher flow rates). This situation is encountered for all sorts of atmospheric pressure ionization interfaces (ESI, atmospheric pressure chemical ionization (APCI), and APPI). So, as stated earlier, the SL can be seen as a "makeup flow" providing increased robustness of the spray but connected with dilution of the capillary effluent, a fact that in special cases (see discussion on non-ESI-compatible BGEs) might positively influence the performance of the system as will be discussed later. SL flow rates employed cover a wide range, with flow rates reported, in combination with a coaxial sheath-flow interface, as low as 0.1 µl $min^{-1}$ [14] but also up to 50 µl $min^{-1}$ [15]. In the majority of cases, spraying is assisted by the use of a sheath gas. As suggested by Banks, not only can sheath gas be used to enhance the spray formation but also electron scavenging gases, such as $SF_6$ or $O_2$, as sheath gas may help reducing the occurrence of corona discharge when operating in the negative ion mode [16]. Nevertheless, a few reports describing CE-MS with a commercially available (nonminiaturized) triaxial sheath-flow interface and spraying solely based on the application of an electric field can be found in the literature [14, 17–20]. One advantage of using no sheath gas is the reduction of suction effects that can negatively influence separation performance. It is obvious that this approach is only applicable when rather low SL flow rates (maximum of 4 µl $min^{-1}$) are used. An interesting option to combine the advantages of (almost) no sheath gas during separation and the positive influence of a sheath gas on the ion yield of the analytes in the ESI source has been presented by Domínguez-Alvarez *et al*. [21]. Applying only a minimum sheath-gas pressure (1 psi) during injection and separation allows avoiding sheath-gas-related problems such as introduction of air bubbles into the capillary during injection (a potential source for current drops during separation) and also helps minimize additional hydrodynamic flow due to suction effects during the separation process, thereby maintaining the electrophoretic resolution. Only when the first analytes reach the capillary outlet, the sheath-gas pressure is raised to 10 psi, a value that is in the range of conventional CE-MS experiments.

### 3.2.2
### Liquid Junction Interface

A second type of sheath-flow interface is the so-called liquid junction interface. A schematic drawing of such a device, in this case a miniaturized version developed by Fanali *et al*. [13], is depicted in Figure 3.1c. Also, with this interface, the SL takes responsibility of several important issues:

- Establishing the required electrical contact between the liquid inside the separation capillary and metal tube acting as electrode
- Providing favorable conditions for ESI
- Enhancing ionization yields
- Overcoming eventual incompatibility between BGE and ESI processes.

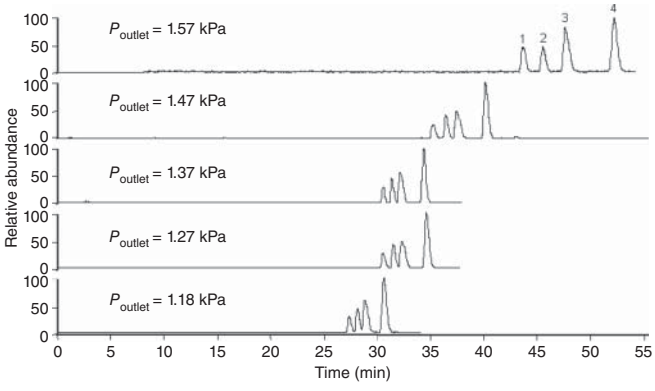



**Figure 3.2** Effect of hydrostatic pressure applied to the sheath-liquid reservoir in the miniaturized liquid junction interface (see Figure 3.1c) on migration times and resolution of four β-blockers. Reproduced from Fanali *et al*. [13] with permission of Wiley.

Comparing these points to the situation encountered with the coaxial sheath-flow interface, one main difference can be detected. While in both cases, establishment of an electrical contact is a key issue, all points related to the dilution effect of the SL have to be assessed differently when contrasting the liquid junction interface with the coaxial sheath-flow interface. This is due to the interface design, resulting in quite diverse SL flow rate regimes. While coaxial sheath-flow interfaces are (except from a few rare cases) operated at (sheath-) flow rates of at least several microliters per minute, these are in the ~100 nl $min^{-1}$ range for the liquid junction interface, thereby resulting in a much less pronounced dilution of the capillary effluent. Another point that has to be considered when using a liquid junction interface for CE-MS is the need to apply a minimum pressure to the separation capillary inlet for counterbalancing the pressure from the SL reservoir, thereby avoiding a backflow of the SL into the separation capillary. Both pressures, the one applied to the separation capillary inlet and the pressure arising from the SL reservoir (adjusted either by direct application of a certain pressure or by a height difference between reservoir and liquid junction), influence the resolution and the analysis time. An example for the effect of different SL flow rates resulting from a constant pressure at the inlet vial and changes in the pressure applied to the SL reservoir can be seen in Figure 3.2. Here, a pressure of about 2 kPa was applied to the capillary inlet and the pressure in the liquid junction was varied by raising or lowering the SL reservoir. Values lower than 1.17 kPa resulted in insufficient flow of the SL, leading to spray instabilities, so the SL reservoir was pressurized with a minimum of 1.18 kPa. As can be seen from this figure, excessive pressure from the SL reservoir not only increased resolution but also increased separation times unnecessarily. So a compromise value of 1.37 kPa SL pressure (corresponding to an SL flow rate of approximately 200 nl $min^{-1}$) was used for further experiments.

## 3.3 Sheath-Liquid-Related Parameters and their Selection

### 3.3.1 Sheath-Liquid Composition

The increasing popularity of CE-MS has led to a large number of publications describing the use of this technique for a variety of analytical problems. Focusing on interfacing- and coupling-related parameters, several review papers include extensive tables listing separation- and detection-relevant data such as BGE composition and also SL composition and flow rates [22–32]. Browsing through these tables, several findings regarding SL composition can be deducted. SL composition can be quite variable with simple solutions such as a pure organic solvent or mixtures as complex as those discussed later in Section 3.5.

Focusing on the use of a coaxial sheath-flow interface and ESI ionization, actually the most commonly employed combination in CE-MS, an SL has to comply with several requirements, of which the following two are the most prominent:

- Guaranteeing a stable electrical contact between the outlet electrode of the CE system (in this case, the sprayer needle of the interface)
- Providing an advantageous environment for the ionization and finally the transfer of the analytes into the MS instrument.

For the first task, the final liquid (i.e., the mixture of SL and eluate from the separation capillary) should show sufficient conductivity to allow unhindered charge transport. Focusing on this requirement, an optimum SL should contain some electrolyte, whereby this prerequisite might already be fulfilled by the contribution from the BGE. Anyhow, in most cases, additional electrolyte (mostly dissolved in water) enhances spray and current stability. Coming to the second point, the situation is often quite contrasting, as any increase in ion concentration also comprises an increased probability of ionization suppression, thereby leading to reduced signal intensities or increased baseline noise. So from this point of view, the use of pure organic solvents (ideally, small aliphatic alcohols) would be the best choice.

Several papers describe the use of pure methanol as SL [16, 33–36]. Methanol as a protic solvent is well compatible with the ESI process. On the other hand, pure acetonitrile is rarely used as solvent for generating of an ESI spray. This is reflected by the fact that only a single report using an SL solely consisting of acetonitrile exists, namely a work on the analysis of nitroaromatics and cyclic nitramine compounds by CE-MS [37]. Simo *et al*. separated synthetic polymers by CE-MS with a methanol/acetonitrile (87.5 : 12.5) mixture [38]. Starting with this simplest composition for SL, several issues might be discussed. Leaving out all requirements resulting from electrophoresis (mainly, the need to establish a stable electrical contact), pure methanol as SL may be a good choice as it often provides excellent signal intensities in combination with ESI. This has been tested in several experiments mimicking CE-MS, without any voltage applied and simple pumping through the sample zone and BGE by pressure. Nevertheless, in the case of real

CE-MS experiments, the lack of conductivity with the SL has to be compensated by fast and thorough mixing with conductive BGE, a situation that cannot always be realized particularly when delicate conditions (i.e., ESI without a sheath gas) are employed [39].

In the majority of reports, SLs made up of organic solvents (or less frequently, mixtures of organic solvents) mixed with a certain percentage of water are described. Regarding the organic solvents, small alcohols, either methanol or *iso*-propanol, are most frequently used. Rarely, acetonitrile or ethanol is the basis of an SL, whereas there are several examples for mixtures of methanol or *iso*-propanol with acetonitrile as SL. A comprehensive picture displaying the variety of mixtures used as SL can be obtained from reviews on CE-MS [22–32].

Evaluating all this information, one can notice that in the majority of CE-MS applications, SLs with quite similar compositions have been employed. In most cases, researchers have either applied SL compositions based on experience from previous work without further modification or used this knowledge as a starting point for SL optimization. Basic discussions on the choice of the best-suited organic solvent and the optimum percentage of water mixed with this solvent can be found in several papers [39–44]. For CE-MS with nonaqueous BGEs also practically nonaqueous SLs based on mixtures of organic solvents (methanol, *iso*-propanol, ethanol, acetonitrile) with traces of water introduced by addition of acids (mostly acetic acid or formic acid) and/or their ammonium salts have been employed [26]. On the other side, almost completely aqueous SLs with less than 2% of organic solvent have been described for pesticide analysis with CE-MS [45, 46]. In the majority of reports, to achieve optimum conditions with respect to CE- and ESI-related parameters such as viscosity and surface tension of the sprayed solution, the water content of SLs is maintained between 20% and 80%. Unfortunately, until now, no systematic study on SL composition involving chemometric tools exists.

Apart from organic solvents and water, additives also play an important role in the search for the best-suited SL composition. As already discussed, to guarantee a smooth ESI process as well as to ensure proper charge transport regarding the closure of the CE circuit, an SL with sufficient conductivity is needed. In order to improve spray stability and also to enhance ionization of solutes (for those showing improved ion yields at a more acidic or alkaline pH), small amounts of a volatile acid (in most cases, formic acid or acetic acid) or base (mostly alkyl amines or ammonia) are added to the SL. A second option for this purpose is the addition of low amounts (up to 5 mM) of a volatile salt, where mostly ammonium salts of formic acid, acetic acid or carbonates are employed. An overview of the different SL compositions reported so far (with respect to SL additives) can be obtained from the extensive tables on CE-MS applications provided in a series of review papers [22–32]; information on strategies to find the right type as well as concentration of additive is described in several research papers [39, 47–49]. When using additives in the SL, their interaction with ionic species from the BGE also has to be considered. As the MS detector is an end-column detector, the outlet buffer vial filled with BGE is *de facto* replaced by the interface "filled" with SL (when

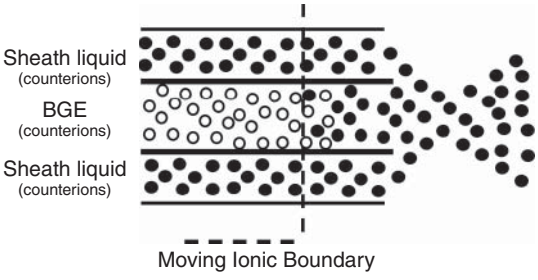



**Figure 3.3** Expanded view of the electrospray tip with an ionic boundary propagating into the CE capillary. The empty and filled circles correspond to the background electrolyte (BGE) and liquid sheath (LS) counterions, respectively. Reproduced from Foret *et al*. [51] with permission of American Chemical Society.

considering coaxial sheath-flow interfaces). To minimize negative effects originating from differences in ionic mobilities between ions present in the SL and in the BGE, the same counterion should be selected for both SL and BGE [50]. In their fundamental paper on liquid-sheath effects in CE-MS using an SL interface, Foret *et al*. investigated these issues with special emphasis on systems with a low or almost zero electroendoosmotic flow (EOF). Ionic migration phenomena leading to the formation of moving ionic boundaries in the end piece of the separation capillary were described [51]. When separation systems with a low or even zero electroosmotic flow are employed, sample ions and co-ions of the BGE exit the capillary at the ESI interface and, at the same time, counterions from the SL enter the column and migrate toward the injection end. If the BGE contains different counterions than the SL, a moving ionic boundary will develop inside the capillary (see Figure 3.3). This can substantially influence the electrophoretic behavior of analytes in the end zone of the separation capillary. Such a case has been investigated by Foret *et al*. using computer simulations for an SL containing 1% acetic acid combined with β-alanine/formic acid (pH 3.4) as BGE [51]. In their study, they calculated that in the end section of the capillary, the pH was increased by 0.6 units, leading to a dramatic reduction in conductivity.

A possibility to overcome or (at least) minimize this problem (when SL and BGE contain different counterions) is applying some pressure to the separation capillary inlet, thereby creating a forced flow out of the capillary end, preventing such backflow phenomena [52]. In the case of separations with zero EOF, even SL flow rates have to be considered as encountered by Soga and Heiger in their work on the analysis of amino acids by CE-ESI–MS [53]. For separating native amino acids, they used a 1 M formic acid electrolyte, thereby almost completely suppressing the EOF. Testing different SLs, all based on mixtures of water and methanol with addition of a small amount of (volatile) electrolyte such as ammonium formate or ammonium acetate, they encountered unexpected current drops when working at SL flow rates below 4 µl $min^{-1}$. This can be explained by the fact that in a zero-EOF system, anions (formate ions) migrate toward the injection end of the capillary, thereby leading to a zone at the separation capillary end lacking negative

charges. If, due to a too low SL flow rate, these negative charges are not provided continuously by the SL, current drops leading to system instabilities can occur.

A special case is encountered when a BGE with a non-ESI-compatible buffer component is used. Here, the basic rule that an SL should contain an electrolyte with identical counterions as used in the BGE cannot be followed. In this case, one task of the SL is dilution/replacement of these non-ESI-compatible counterions – so SLs with volatile ESI-compatible electrolyte ingredients are preferred. An example for this situation is described in the work published by van Wijk *et al*. [52]. Whereas in most cases, dilution of the capillary effluent due to an SL is seen as a weak point of sheath-flow interfaces for CE-MS coupling, this effect is advantageous when non-ESI-compatible BGEs are used. Many of these BGEs such as phosphate-, borate-, or tris(hydroxymethyl)-aminomethane (TRIS)-based electrolyte systems (just to name a few) are very popular due to their excellent performance with respect to separation and peak shapes. van Wijk *et al*. [52] investigated the impact of the SL on the separation obtained for four pharmaceutical compounds and an unknown impurity employing different BGE systems, some of them containing non-ESI-compatible ingredients. This situation implies that in order to enhance the quality of MS signals by improving the ESI compatibility of the system, SLs containing volatile salts or acids have to be employed. If the ions present in the BGE and the SL show different mobilities, migration of SL ions back into the separation capillary may occur. As can be seen from Figure 3.4, this effect can also trigger changes in migration selectivities. Using a 100 mM TRIS electrolyte (adjusted to pH 2.5 with phosphoric acid), the unknown impurity comigrated with SLV 308 (peak 3) as can be seen from electropherogram (a). A 100 mM TRIS electrolyte (adjusted to pH 3 with formic acid) allowed the separation of these two components (see electropherogram b). Simulating CE-MS experiments by replacing the BGE in the outlet vial by SL (methanol/water 1 : 1 with 0.2% formic acid) as shown in electropherogram (c), a moving boundary is formed due to migration of the formate ions into the separation capillary. This leads to change in separation selectivity for the 100 mM TRIS electrolyte (adjusted to pH 2.5 with phosphoric acid) BGE system. Due to the presence of the formate ions in the final part of the separation capillary, the peak for the impurity moves slightly forward in the electropherogram, appearing just in front of peak (2), which is a hybrid of the situation encountered with the 100 mM TRIS electrolyte (adjusted to pH 2.5 with phosphoric acid) BGE and the 100 mM TRIS electrolyte (adjusted to pH 3 with formic acid) BGE when BGE was present in both vials. When formate is present in both the BGE and the SL (as in electropherogram (D)) separation selectivities do not differ between UV and simulated MS experiments.

Not only the general type of interface employed (coaxial sheath flow or liquid junction) but also more minor differences in interface layout can lead to quite different results when optimizing the SL composition for a specific application. One example for such a case is investigations on the influence of SL composition on sensitivity enhancement in CE-(negative mode) ESI-MS for a series of common anionic metabolites. Employing a commercially available standard sheath-flow

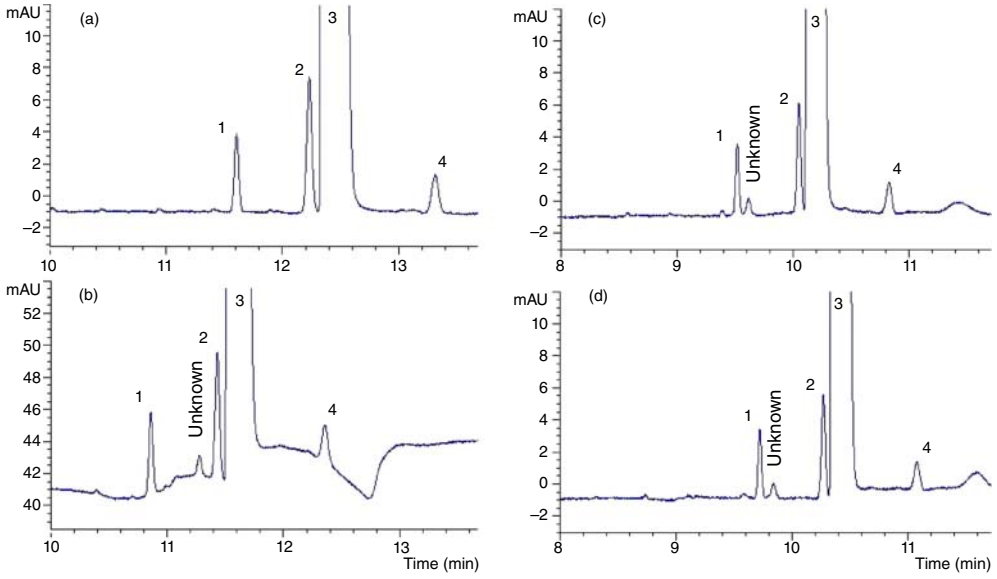



**Figure 3.4** Influence of liquid-sheath effect on selectivity. CE-UV electropherograms at 215 nm of a 1 mg ml$^{-1}$ SLV308 sample solution containing four impurities. Methanol/water 50 : 50% (v/v) containing 0.2% formic acid was used as sheath liquid (SL). Conditions: (a) 100 mM TRIS adjusted to pH 2.5 with phosphoric acid, inlet vial–outlet vial: BGE-BGE; (b) 100 mM TRIS adjusted to pH 2.5 with phosphoric acid, inlet vial–outlet vial: BGE-SL; (c) 100 mM TRIS adjusted to pH 3.0 with formic acid, inlet vial–outlet vial: BGE-GE; (d) 100 mM TRIS adjusted to pH 3.0 with formic acid, inlet vial–outlet vial: BGE-SL. Reproduced from Van Wijk *et al*. [52] with permission of Elsevier.

interface for CE-MS, Kok *et al*. tested acetonitrile-, methanol-, and *iso*-propanol-based SL with and without additives for ionization enhancement (aqueous ammonia, triethylamine, ammonium acetate) with respect to their performance for the analysis of anionic metabolites found in urine sample [44]. As a result of their studies, they found an optimum SL composition (adjusted to the BGE of 25 mM triethylamine) of methanol/water (1 : 1) containing 5 mM triethylamine.

Liu *et al*. performed a quite similar study but using a laboratory-made coaxial sheath-flow (nano)electrospray interface for CE-MS [39]. From simple infusion experiments, a clear trend toward simple mixtures of organic solvent with water leading to highest signals in MS could be detected. Nevertheless in CE-MS, SL optimization often means looking for a compromise between highest yields in ionization and sufficient stability of the CE current, a factor determining the quality of separation. So, working with a nebulizer-gas-free (nano)electrospray interface required addition of electrolyte to maintain spray stability, although this is accompanied with a reduction in relative signal intensities of up to 50%. Optimizing their SL (as well as BGE) composition with respect to these two key parameters (sufficiently stable electrical current and good ion yields), the following system (adjusted to 20 mM ammonium carbonate BGE) was found to be the best-suited: *iso*-propanol/water (1 : 1) containing just 0.2 mM ammonium carbonate.

### 3.3.2
### Effect of Sheath-Liquid Composition on Molecular Structures

A fact that is of less importance for small molecules has to be considered when analyzing intact proteins or other large biomolecules with tertiary structures sensitive to their environment. As the SL composition determines the environment for the analytes after leaving the separation capillary, different SLs can also induce structural changes in such biomolecules. Stutz's group presented an interesting study on CE-MS of major birch pollen allergens focusing on SL optimization and variations in charge state distribution related to different SL compositions [54]. They tested SLs based on mixtures of water with either methanol or *iso*-propanol and minor amounts of formic acid for ionization enhancement (in the positive ion mode). Thereby, different effects were observed for methanol and *iso*-propanol. In contrast to methanol, where the solvent concentration in the SL had a strong effect on the charge state distribution of the investigated proteins (as can be seen from Figure 3.5), almost no influence was found for SLs with *iso*-propanol. Similar to the case of many other SL compositions reported so far, formic was used to enhance protonation of the solutes and with it increased ion yield. Nevertheless, it has to be taken into account that addition of any electrolyte to the SL will also result in ionization suppression, so only low amounts of formic acid (here 0.1%) are employed.

### 3.3.3
### Sheath-Liquid Flow Rates and their Optimization

One important parameter related to the use of SL interfaces in CE-MS is a careful choice of the best-suited SL flow rate. In this section, the discussion is restricted to ESI interfaces, atmospheric pressure photoionization (APPI) and APCI are reviewed in a separate section. Of course, discussion related to SL flow rates cannot be decoupled from the basic interface design, so the type (brand) of the interface employed for the corresponding studies always has to be included in these considerations.

Among the (commercially) available sheath-flow interfaces, the Agilent (type G1607A or G1607B) is by far the most widely employed one, as can easily be identified from the relevant CE-MS literature. As almost every CE-MS -related work includes (naturally to a varying extent) discussions on SL flow rates, there is also a substantial number of papers describing more detailed investigations on optimization of this parameter for this type of interface. Whenever effects based on different SL flow rates are examined, a second closely related parameter, namely the sheath-gas pressure or flow rate responsible for sufficient nebulization of the liquid, has to be taken into account. Although a very few reports on the use of this Agilent interface without sheath gas exist [e.g., 18–20, in which the first two use the old Agilent interface], in the majority of works, pressure-assisted spraying is preferred.

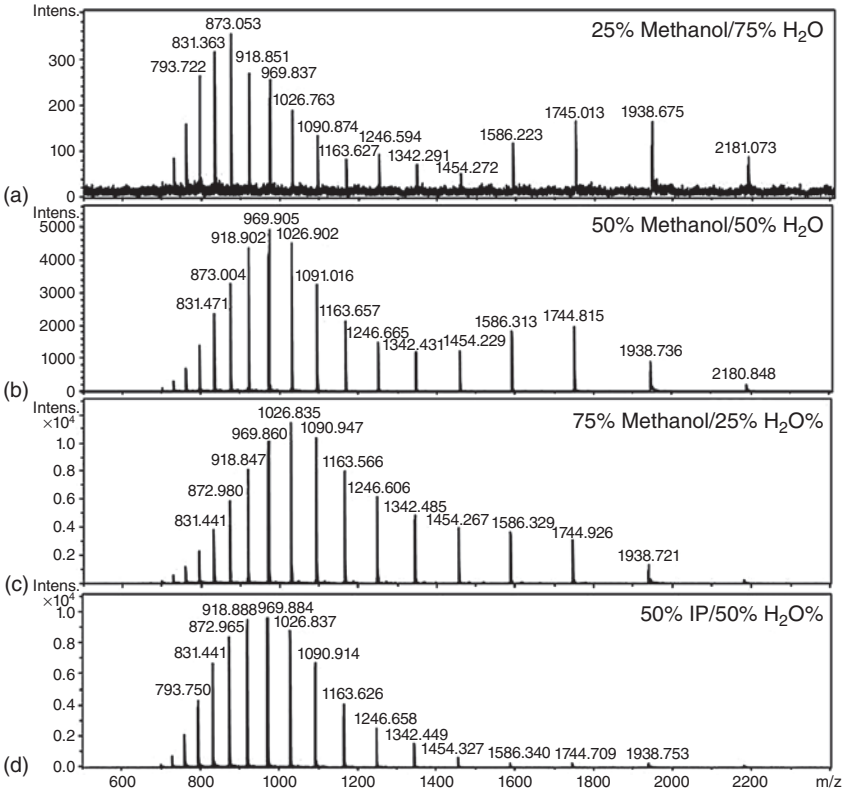



**Figure 3.5** Effect of methanol and *iso*-propanol content in SL on charge state distribution of Bet v 1a. BGE: 10 mmol $l^{-1}$ $NH_4HCO_3$, pH 7.50; SL composition: 0.1% v/v FA in ultrapure water with (a) 25%, (b) 50%, (c) 75% v/v methanol, and (d) 50% v/v *iso*-propanol. Reproduced from Gusenkov *et al*. [54] with permission of Wiley.

Already in 2004, Nilsson *et al*. published a study entitled "A chemometric study of active parameters and their interaction effects in a nebulized sheath-liquid electrospray interface for capillary electrophoresis mass spectrometry" [55]. Chemometric methods were applied to four parameters, namely ESI voltage, position of sprayer needle, nebulizer pressure, and SL flow rate using separation capillaries with internal diameters of 25 and 50 µm . Focusing on two more SL-related parameters, nebulizer pressure and SL flow rate, the following findings were observed: increased nebulizing pressure improved signal intensities but also increased the noise level, so $S/N$ ratios were not affected; regarding the preservation of separation efficiency, capillaries of smaller inner diameters together with low SL flow rates proved beneficial.

In a recent paper, Schmid *et al*. presented a (mostly) systematic approach for optimizing SL and SL-related parameters in combination with CE-MS using the Agilent G1607A interface [56]. MS parameters were optimized using the software Design-Expert® and a fractional factorial design ($2^{4-1}$ design). They searched for

optimum values (with respect to peak heights/areas) for the following parameters: drying gas temperature, drying gas flow, SL flow rate, and nebulizer pressure. Malondialdehyde (generated *in situ* by UV treatment of sugars under alkaline conditions) was the analyte for which these studies were conducted. SL composition was not included in these systematic studies, but was the subject of preliminary test, mainly experience from previous work. Several mixtures of *iso*-propanol, methanol, or acetonitrile with water were tested, of which *iso*-propanol/water 9 : 1 provided best results. Addition of small amounts of a base (ammonia) did not substantially improve signal heights/areas, but an improvement in system stability was observed when 0.01% of ammonia was added. Coming back to the more systematic investigations, a total of 12 experiments were performed, with the following ranges:

- SL flow rate: 1 – 10 µl $min^{-1}$
- Drying gas temperature: 150 – 250 °C
- Drying gas flow rate: 4 – 10 l $min^{-1}$
- Nebulizer pressure: 10 – 60 psi.

No significant correlation (*p*-values 0.9799 and 0.6619, respectively) was found between drying gas temperature and drying gas flow rate and the peak heights/areas obtained for malondialdehyde. With a *p*-value of 0.0733, the SL flow rate value was at the border between significant and insignificant influence. The most important parameter for optimizing sheath-flow-interface-related parameters (except for SL composition) found in this study was the nebulizer pressure showing a highly significant *p*-value of 0.0034. Two further sets of experiments with reduced ranges tested were performed in order to improve the precision when determining the optimum parameters. The corresponding plot for the two most influential parameters versus peak height achieved for malondialdehyde is depicted in Figure 3.6. As can be seen from this plot (recorded using an SL consisting of 90% (v/v) 2-propanol in $H_2O$ with 0.01% (v/v) $NH_3$, with a drying gas flow rate (nitrogen) of 7 l $min^{-1}$ and drying gas temperature of 200 °C), the nebulizer pressure was the crucial parameter in the optimization, whereas only minor differences (more pronounced when a low nebulizer pressure was selected) for the SL flow rate were observed. The optimization software finally resulted in highest peak intensities for a nebulizer pressure of 23 psi and an SL flow rate of 7 µl $min^{-1}$.

## 3.4 Sheath Liquids for Non-ESI CE-MS Interfaces

### 3.4.1 APCI and APPI

Although much less frequently used than the ESI interface, atmospheric pressure photoionization (APPI) is the second most widely employed ionization technique

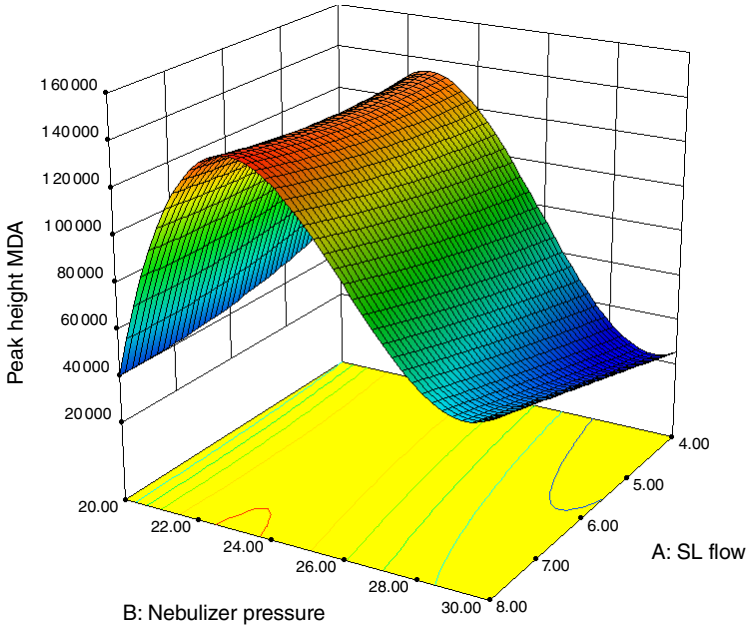



**Figure 3.6** Influence of nebulizer pressure and SL flow rate on the peak height of malondialdehyde (MDA) obtained from the third set of experiments (tested ranges reduced to 1–10 µl min$^{-1}$ for SL flow rate and 20–30 psi for nebulizer pressure for finding the optimum with higher precision) employed for optimizing the CE-MS interface parameters. Reproduced from Schmid *et al*. [56] with permission of Elsevier.

for on-line coupling of MS with electrophoretic [57–63] and electrochromatographic techniques [15, 64–68]. Generally speaking, the composition of SLs used in CE-APPI-MS is similar to those used in combination with ESI. There are only two distinct differences. APPI can basically be operated in two modes, namely ionization with or without the help of a so-called dopant, a substance added for increasing the yield of ions obtained within the ionization process [69]. Most commonly employed dopants are acetone, toluene, and anisole. In the case of electrodriven separation techniques, the simplest way to use such a dopant for ionization enhancement is its addition to the SL as a further component. There are several papers including in-depth investigations on the composition of SLs for CE-APPI-MS [58–60]. Parameters discussed include the mixture of solvents, addition of acids or bases, as well as the type and concentration of the dopant added to the SL. In their study, Mol *et al*. [58] investigated different SL compositions for the CE-APPI-MS analysis of a set of compounds comprising both relatively polar and nonpolar substances. For polar molecules such as mebeverine, purely nonaqueous SLs were preferred, whereas for less-polar analytes, SLs including water yielded better results. Formic acid (often used to increase the formation of $M-H^+$ ions) did not lead to any improvements, whereas the addition of dopants showed strong effects (mostly pronounced in the region between 0% and 1%),

where the nature of the analyte molecule determined whether acetone or toluene was preferable. A study published by the same group 2 years later extended these investigations to a group of pharmaceuticals [59]. Here, the results from the previous study were taken as a starting point. Nevertheless, a final SL composition consisting of acetonitrile/water/toluene/formic acid (75 : 25 : 5 : 0.1) was preferred for the following reasons: first, although purely nonaqueous SLs resulted in high signal intensities, the only mediocre robustness of such systems limited their use in more application-oriented work – an observation that is in accordance with many reports from CE-ESI-MS; secondly, the stability of signal intensities could further be improved by formic acid addition. A third study on SL compositions in CE-APPI-MS of basic pharmaceuticals was conducted by Veuthey's group [60]. Whereas SL composition was optimized employing the most widely used approach (based on experience from previous work, varying solvent/additive type and concentration within the usual margins), SL flow rate tuning was performed as part of a more systematic investigation employing a face-centered central composite design. Here, SL flow rate was optimized together with more source-related parameters such as drying gas flow rate, nebulizer pressure, drying gas temperature, vaporizer temperature, and MS capillary voltage. The fact that mainly polar substances were investigated led to an optimum SL consisting of *iso*-propanol/water/acetone (50 : 50 : 0.5). Figure 3.7 provides a summary of the data from the optimization experiments. As can be seen from these results, addition

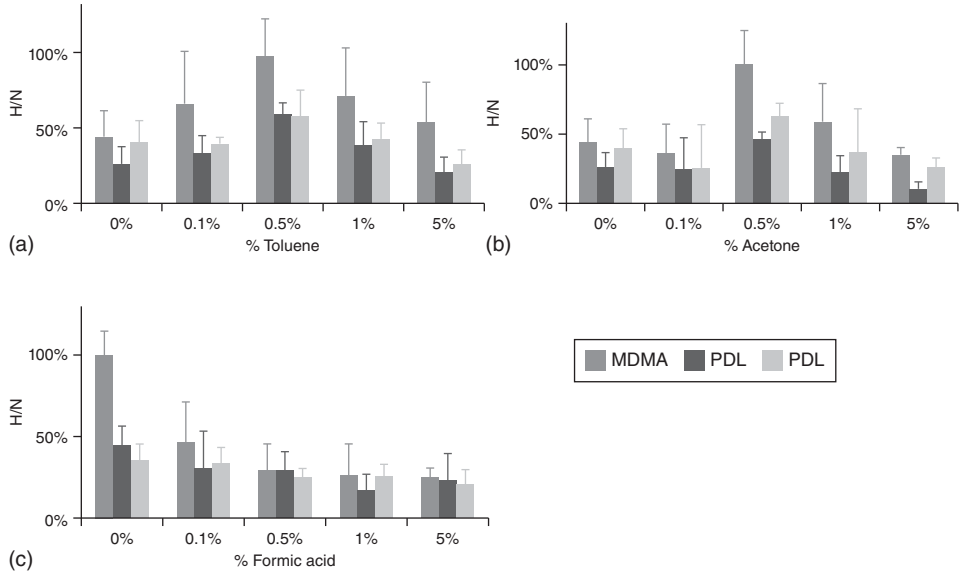



**Figure 3.7** Sheath-liquid composition optimization. Experimental conditions: FS capillary 65 cm × 50 μm i.d., injection 500 mbar s, CE voltage 30 kV, sheath-liquid composition *iso*-propanol/water (50 : 50 v/v) and flow rate 25 μl $min^{-1}$. Influence of sensitivity on peak height/noise (H/N) of (a) % toluene, (b) % acetone, and (c) % formic acid. Reproduced from Schappler *et al*. [60] with permission of Wiley.

of dopants should not exceed 0.5–1% and formic acid does not really improve ionization yield in APPI (at least not for the investigated set of compounds), a finding that is in agreement with the earlier paper by the de Jong's group [58].

The second difference compared to CE-MS using ESI interfaces is that the SL is delivered at slightly higher flow rates in APPI. This can be attributed to the fact that in APCI and APPI, optimum operational conditions are generally achieved at higher (overall) flow rates than those used in combination with an ESI source. Schappler *et al*. systematically investigated source-related parameters and SL flow rates for both CE-ESI-MS and CE-APPI-MS [60]. Thereby, an optimum SL flow rate of 3 µl $min^{-1}$ was found for ESI, whereas the value for APPI was 50 µl $min^{-1}$. As expected from theory [70], APPI ionization behaves as a mass-flow-sensitive technique, so the signals were not affected by a dilution effect. Mol *et al*. observed signal fluctuations for mebeverine using an APPI source when SL flow rates of 10 µl $min^{-1}$ [58] and less were used, so commonly, SL flow rates reported for CE-APPI-MS applications lie in the range of 15–50 µl $min^{-1}$.

One specific field of application of APPI is the combination of electrokinetic chromatography with pseudostationary phases such as MEKC and MEEKC with MS detection [64–68]. Here, one main task of the SL, which is added in large excess compared to the capillary effluent, is to overcome the intrinsic MS incompatibility of the most pseudostationary phase. This allowed the use of electrolyte compositions with up to 50 mM SDS in MEKC [64] or even 105 mM (3%) SDS in MEEKC [66]. Himmelsbach *et al*. have investigated the influence of the SL composition on the ionization efficiency observed for phenacetin under different electrical field strengths [66]. As can be seen from Figure 3.8, using an SL with dopant provided good signal intensities over a quite wide range of MS capillary voltages. Switching to a system without dopant maximum intensity was achieved at a capillary voltage of 1500 V with a substantial drop at lower and higher voltages.

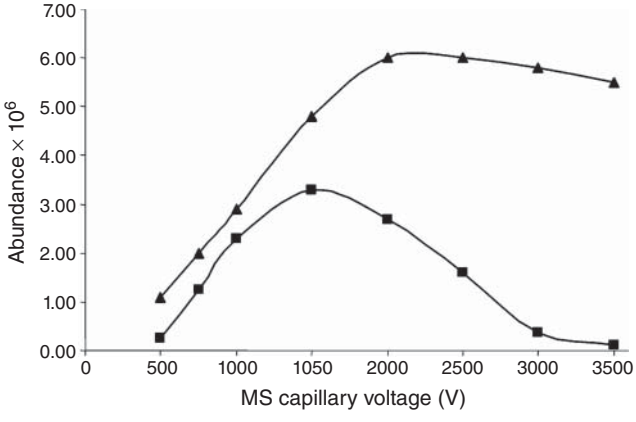



**Figure 3.8** Effect of the MS capillary voltage on the signal intensity obtained for phenacetin using a sheath liquid with 5% acetone as dopant (—▲—) and without dopant (—■—). Reproduced from Himmelsbach *et al*. [66] with permission of American Chemical Society.

As this has also been observed for a series of other substances, one can conclude that in CE-APPI-MS, SLs without dopant require a more careful optimization of the ion source parameters.

## 3.5 Sheath-Flow Chemistry

Sheath-flow chemistry, that is, the addition of reagents to the SL was first described by Bayer's group in 1999 [71]. Their approach, named coordination ion spray (CIS), involved the addition of reagents forming positively or negatively charged complexes with the analytes, thereby greatly enhancing the detectability of molecules that are commonly less suited for ionization by ESI. Reagents employed were mainly metal ions leading to positively charged complexes. Using capillary electrochromatography (CEC) coupled with CIS, $Ag^+$ ions were added to the SL for the separation and detection of unsaturated fatty acid esters, vitamins from the D-group, or estrogenic compounds, all substances providing only limited ionization yield when detected with ESI-MS [72]. In a later work, different metal ions ($Ag^+$, $Cu^{2+}$, $Li^+$, and $Co^{2+}$) were employed in chiral CEC–CIS-MS of several pharmaceuticals, thereby increasing the sensitivity as well as the selectivity (by different possibilities of complexation) of detection [73]. Besides these experiments (all carried out in the positive ion mode), CIS can be used in the negative mode with SL reagents such as $BF_3$, $H_3BO_3$, or $B(OCH_3)$ [72].

Deuterium–hydrogen exchange in the SL interface is the second example where the principle of sheath-flow chemistry is applied. Here, SLs based on deuterated solvents and additives are employed, leading to replacement of hydrogen by deuterium when analytes with exchangeable protons get in contact with the SL. The first report of hydrogen–deuterium (H/D) exchange in CE-MS employing a deuterated SL was already published by Palmer *et al*. in 2000 [74]. In 2008, Lau *et al*., presented an example of this approach for peptide analysis [75]. Just a year later, Klampfl *et al*. exploited H/D exchange as a tool for the unambiguous identification of melamine in milk powder by CE-QTOF-MS [76]. As can be seen from Figure 3.9, depicting the MS signal obtained for the melamine molecule, the peak corresponding to the fully deuterated species is by far the most intensive one. This is due to the fast exchange kinetics as deuterated reagents were only added to the SL, so the time for exchange was rather short.

Such fast reaction kinetics is generally an unconditional requirement in sheath-flow chemistry and must be considered when selecting reagents for sheath-flow reactions, as the time available for the reaction is extremely short. SL composition has in this case been mainly influenced by the availability of suitable (protic) deuterated solvents. For this reason, in H/D exchange, mainly mixtures of $D_2O$ and MeOD have been employed. The addition of low concentrations of nondeuterated (mainly due to cost reasons) acid such as acetic acid or formic acid did not significantly influence the results obtained [76]. Further examples for the use of H/D exchange can be found in the recent literature [77, 78]. The last two papers

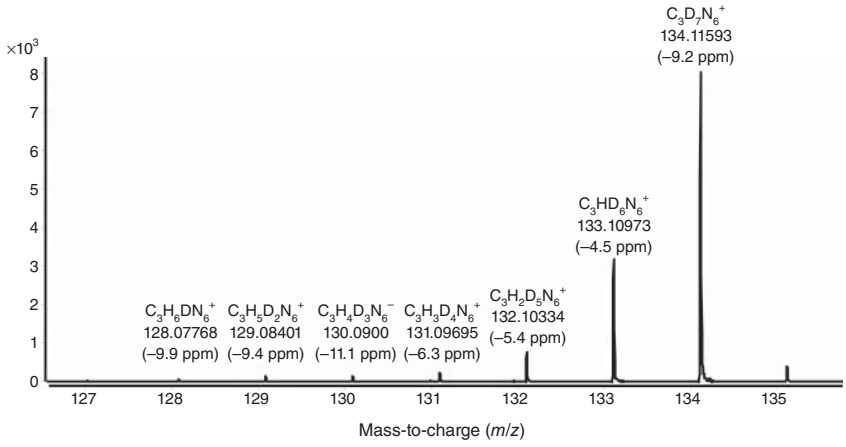



**Figure 3.9** Mass spectrum for melamine from a CZE-ESI-Q-TOFMS run using a deuterium oxide/mono-deuterated MeOH (80 : 20), 0.1% formic acid sheath liquid. Numbers in parentheses refer to mass error in ppm. Reproduced from Klampfl *et al*. [76] with permission of Wiley.

also include a further step toward the realization of more complex reactions in the SL. In this case, a stable radical (in this case, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$)) was added. Monitoring the products from reactions of analytes (first separated by CE) with the stable radical allowed screening of compounds with antioxidant potential. Hereby, a further dimension was added to the analytical system. Information available was now besides electrophoretic mobility and the corresponding mass spectrum, also the presence of reaction products from in SL reactions with DPPH$^{\bullet}$. Two examples for such reaction products can be seen in Figure 3.10, depicting the results obtained from the analysis of *Rosmarinus officinalis* and *Melissa officinalis* by CE-MS with SL reaction. In both cases, substances (rosmarinic acid and caffeic acid) with *ortho-* or *para*-diphenolic substitution patterns are oxidized by DPPH$^{\bullet}$, resulting in the corresponding quinone species [78].

The concept of sheath-flow reactions was also exploited by Bonvin *et al*. in their study on protein supercharging. The charge state distribution of proteins is modulated by the addition of specific supercharging reagents. Examples of such substances are *ortho-*, *meta-*, and *para*-nitrobenzyl alcohol and sulfolane. Employing the strategy of sheath-flow chemistry enables to perform protein supercharging experiments without hampering separation by the addition of supercharging reagents to the SL. In their paper, Bonvin *et al*. used the aforementioned reagents in concentrations between 0.25% and 1% added to an SL consisting of *iso*-propanol/water/formic acid [79]. The effect of *meta*-nitrobenzyl alcohol and sulfolane in the SL on the charge state distribution of the human growth hormone can be seen from Figure 3.11, proving that sheath-flow chemistry is also a suitable tool for supercharging experiments.

Summing up, sheath-flow chemistry can not only be used for enhancing the detectability of (commonly not so effectively ionizable) substances but also for

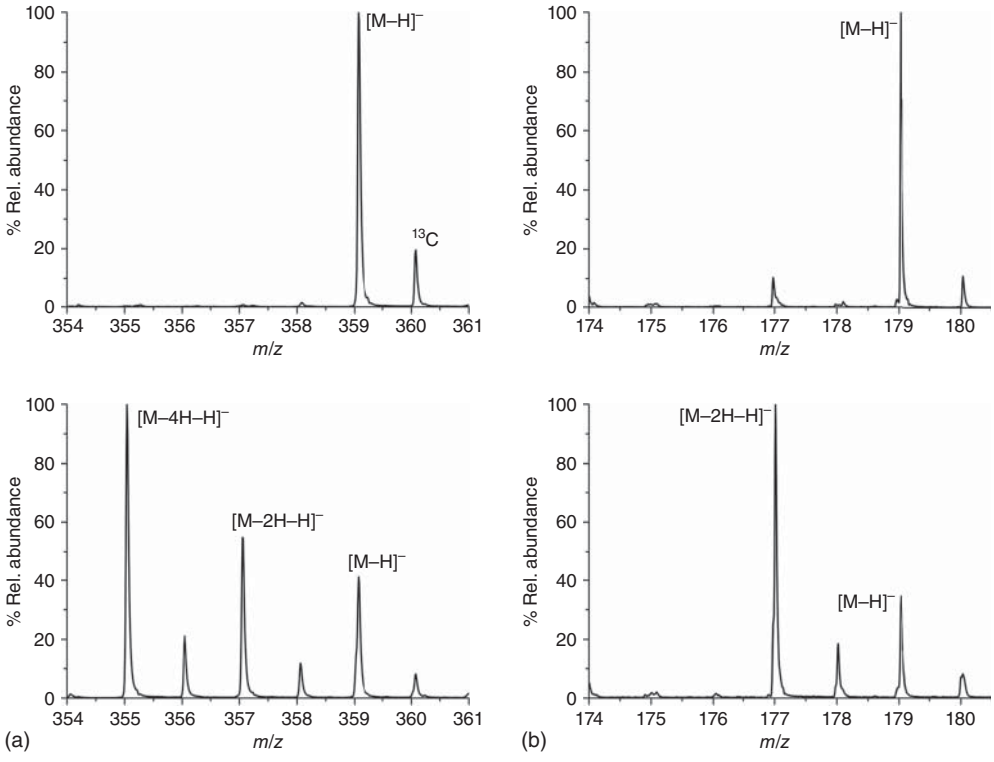



**Figure 3.10** Effect of online DPPH• reaction experiments for selected components: (a) rosmarinic acid found in *Rosmarinus officinalis* extract and (b) caffeic acid found in *Melissa officinalis*. Reproduced from Maringer *et al*. [78] with permission of Wiley.

increasing the information content of an analytical experiment. The cases presented, H/D exchange, reaction with stable radicals, and addition of supercharging agents are good examples for such a strategy providing information on the number of exchangeable protons, the antioxidative potential of the separated analytes, or shifting the average charge state of proteins to higher charge levels, respectively.

## 3.6 Conclusions

The sheath-flow interface is the most widely distributed device for coupling CE to MS detection. The main features responsible for the success of this interface are its ease of use and particularly its robustness in operation. Nevertheless, the performance of the sheath-flow interface is significantly governed by the selection of the appropriate SL. Browsing through the review papers on CE-MS applications, extensive tables listing a series of SL-related parameters such as SL composition, SL flow rate, and sheath-gas pressure or flow rate can be found [22–32]. Starting

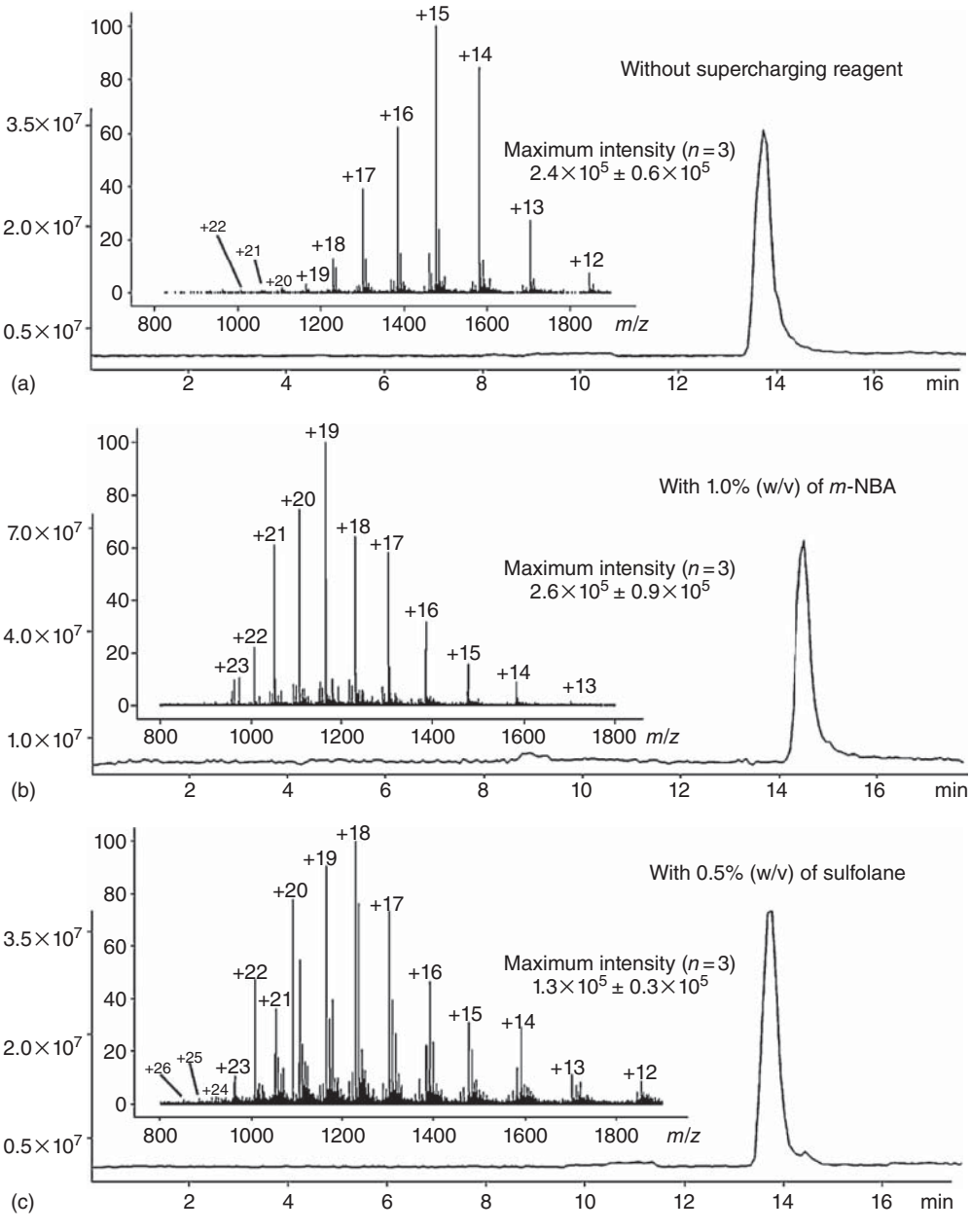



**Figure 3.11** Charge state distribution of human growth hormone with the respective electropherogram obtained in scan mode ($m/z = 850–2000$) with an acidic BGE (75 mM ammonium formate pH = 2.5 + ACN 20%, v/v) and a sheath liquid composed of (a) isopropanol–water–formic acid (49.5 : 49.5 : 1, v/v), (b) m-NBA 1.0% (w/v) in isopropanol–water–formic acid (49.5 : 49.5 : 1, v/v), and (c) sulfolane 0.5% (w/v) in isopropanol–water–formic acid (49.5 : 49.5 : 1, v/v). Reproduced from Bonvin *et al*. [79] with permission of Elsevier.

with the first parameter, the typical SL is based on a mixture of an aliphatic alcohol with water; often small amounts of acid/base or volatile salts are added to enhance spray stability. Although several studies are available on the selection of the appropriate SL ingredients, no fundamental study on this topic (employing chemometric tools for optimization) exists so far. Almost in all cases, experience from previous work is taken as the starting point for further investigations. SL flow rate and sheath-gas flow rate/pressure have been studied more systematically with at least two papers describing a more fundamental approach for optimizing these two parameters [55, 56]. Finally, specifically designed SL compositions can also be used for tasks beyond providing an appropriate environment for CE-MS coupling, as demonstrated in Section 3.5. Addition of reagents for "on-the-fly" reactions upon mixing of the analytes leaving the separation capillary with the SL is triggered.

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# 4
# Recent Developments of Microchip Capillary Electrophoresis Coupled with Mass Spectrometry

*Gerard Rozing*

## 4.1
## Introduction

Soon after capillary electrophoresis (CE) became established as a high-resolution, liquid-phase microseparation, instrumental analysis technique by the end of the 1980s, first efforts were reported to couple CE with mass spectrometry. Despite specific difficulties involved in the coupling of CE with MS (see Chapter 2 of this book), this has proven to be a very successful endeavor. In particular since around the same time, electrospray ionization (ESI) became established as the preferred technique for coupling HPLC with MS. Since the volumetric flow rates involved in CE separations are in the ideal regime required for ESI, coupling CE with MS promised to be an excellent match. With the commercial introduction of the sheath-solvent flow interface by Hewlett-Packard in 1995, the technique has been available as a robust standard for interfacing CE with MS for more than 20 years to the broad community of users of CE.

At the same time in the late 1980s and early 1990s, initial efforts were reported to leave the tubular format of CE and establish the electrophoretic separation in equally sized rectangular channels in particular on a glass substrate. With the successful introduction on the market of systems for electrophoresis on a chip in the late 1990s (Agilent Bioanalyzer [1], Caliper LabChip Series, and Bio-Rad Experion), microchip capillary electrophoresis (MCE) has become a commercially successful platform technique.

Soon after CE-MS interfaces based on CE separations carried out with fused-silica capillaries and microchip electrophoresis have become feasible, contemporary attempts to couple the microchip separation (pressure and electrical field driven as well) by ESI interface to MS were reported.

These efforts though did not and still have not resulted in a comprehensive commercial product for microchip capillary electrophoresis coupled with mass spectrometry (MCE-MS) in contrast to the field of liquid chromatography where microfluidic chips for packed-column HPLC with an integrated sprayer

*Capillary Electrophoresis – Mass Spectrometry (CE-MS): Principles and Applications,*
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for ESI are coupled with MS have been commercially available on the market since 2005.[1)2)3)]

Therefore, MCE-MS is still in a "proof-of-principle" stage and has remained an active area of research for several groups worldwide. Since a comprehensive description of the MCE-MS from the beginning in the early 1990s is not very useful for the readership of this monograph, the focus this chapter is on developments of MCE-MS published since the middle of the past decade.

## 4.2 Microchip Capillary Electrophoresis

### 4.2.1 Brief Retrospective

Capillary electrophoresis is fundamentally limited by Joule heating in the conduit, causing radial temperature gradients, and thereby velocity differences across the cross section of the conduit occur, which disperse the analyte zone during separation. The magnitude of Joule heating depends on the cross sectional surface area of the conduit.

Therefore, performing CE on a chip with flat hydraulic channels in the range of 10–100 μm (height and width) dimension is a very favorable environment since the small dimensions of flat channels reduce the Joule heat generated and the high aspect ratio of a flat channel, that is, the large width-to-height ratio, favors fast heat dissipation out of the narrow channel. Therefore, higher field strength can be applied, which accelerates sample zone movement and reduces analysis time.

In addition, the axial flow velocity profile and therefore the concentration profile of the analyte zones in an electric-field-driven separation conduit are flat and independent of the shape of the conduit in contrast to pressure-driven liquid-phase chromatographic separations in open square or rectangular tubes. Furthermore, different analytical functions in the workflow of a chemical analysis such as sample preprocessing, preconcentration of analytes, separation, postcolumn operations, and detection are in principle easily integrated on planar devices.

Early on, it was the work of Manz *et al*. that demonstrated theoretically and experimentally the feasibility of electrical-field-driven separations on flat triangular or rectangular channels [2–7]. Other workers who were involved early were Ramsey *et al*. [8].

This early work has resulted in significant miniaturization of the analytical system for which Manz has coined the term μ-total analysis systems (μTAS). Since this is a unique and widely used terminology intended to comprise all steps of a chemical analysis, capillary electrophoresis on a microfluidic chip is usually

1) Agilent HPLC Chip.
2) Waters HPLC Chip.
3) Eksigent HPLC Chip.

named Microchip Capillary Electrophoresis (MCE), a terminology used by most scientists in the field.

### 4.2.2 Principle of Operation of MCE

In its simplest format, an MCE device consists of two flat channels ($80 \times 20\,\mu m$) that cross perpendicularly. Each channel ends in two small reservoirs, which are open at their ends. These reservoirs serve as sample inlet, sample waste, and buffer reservoirs. Electrodes can be placed in the reservoirs to control movement of liquids and direct the path of the sample to the point of injection and into the separation channel. Towards the end of the separation channel, there will be a point of detection by single-wavelength light absorption or fluorescence. Such a device is depicted in Figure 4.1.

The dimensions of the separation channel typically range from 10 to 50 µm in height and 50 to 100 µm in width. Depending on the material and preparation method used to make the chip (see the following sections), the channel may be rectangular, square, triangular, or semiellipsoid. The length of the separation will range from 10 to 100 mm. Meandering of the separation channel will allow longer lengths.

Typically, voltages up to ±1 kV are applied for sample injection and up to ±3 kV for separation. Voltages may become applied as suggested in Figure 4.1, although these may change position to have better control over sample movement. Sample injection is by driving a part of the sample solution crossing the separation channel into the channel (pinched injection [9–11]) (Figure 4.1a) or as in Figure 4.1b by

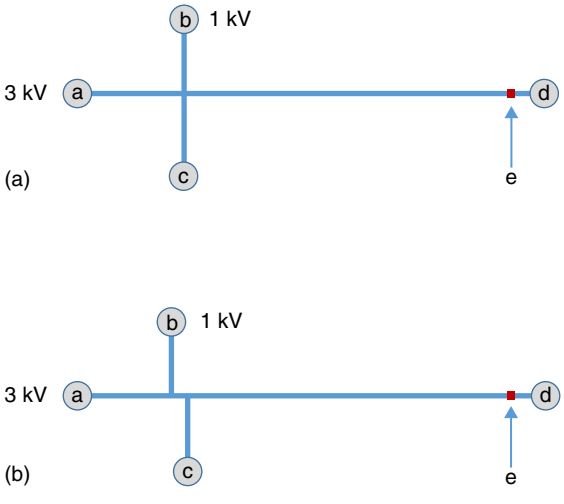



**Figure 4.1** Principle layout of a chip for capillary electrophoresis; a, buffer reservoir; b, sample reservoir; c, sample waste reservoir; d, outlet reservoir; e, point of detection. (a) Design for pinched sample injection. (b) Design for gated sample injection.

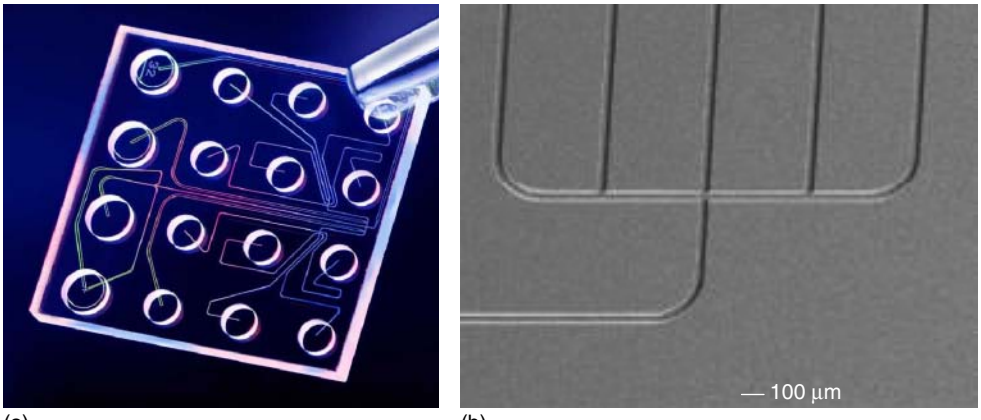
filling a short zone of the separation channel with the sample solution and driving this zone into the separation channel (gated injection [5]). Optical detection by fluorescence is the only viable option given the short optical path length (only the height of the separation channel) involved.

There is no theoretical difference between operation of electrophoresis separation in a capillary, in a rectangular conduit or in a conduit of any other shape. The mechanisms of separation do not change, but the way the samples are handled. All modes of capillary electrophoresis such as capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), and even free-flow electrophoresis (FFE) are feasible and applied in a microfluidic device whereby the first two modes are most ubiquitously used.

The essential difference with operation of electrophoresis in a capillary is the way how different parts of the workflow of a chemical analysis are executed. For example, sample manipulations, preprocessing and injection, pre- and postseparation operations, and detection. The construction of chips, the volumetric scale, and the application of the electrical driving force and the speed of the separation will hamper manual operation and hinder automation unlike with capillary electrophoresis and place stringent constraints on the analytical protocol.

As a realistic illustration of a microfluidic chip for capillary electrophoresis, a photograph of the Agilent LabChip™ is shown in Figure 4.2a. The fluidic chip is below the cover plate. The separation channel is seen in the middle of the chip and is connected to 12 sample wells and 4 buffer and waste wells on the cover plate. All channels are interconnected. An electrode is placed in each well to contain the samples. The point of detection by confocal laser-induced fluorescence is at the end of the separation channel on the right hand side of Figure 4.2a. Figure 4.2b shows details of the size and shape of the channels.

A simpler, commercially available microfluidic chip for capillary electrophoresis is shown by Micronit [12]. From these pictures, it will be obvious that the interface




(a) (b)

**Figure 4.2** (a) Photograph of the Agilent LabChip™. (b) Channel detail of LabChip™. Reproduced with permission of Agilent Technologies.

of a microfluidic chip for capillary electrophoresis with the outside world will be more essential than the working principle alone.

### 4.2.3 Preparation and Availability of Microfluidic Chips for Capillary Electrophoresis

Microfluidic chips for capillary electrophoresis can be manufactured from different substrates. Glass, in particular soda-lime glass, is most commonly used since fabrication methods such as etching are well understood, have widespread use, and are readily applied in practice. Moreover, glass is transparent, allowing for monitoring of the separation process on-line by optical means (mainly fluorescence microscopy), thereby optimizing the layout, geometry, and dimensions of the channels. The surface chemistry of glass channels is also well known, allowing modification of its properties that can be mandated by the separation process as in capillary electrophoresis (see, e.g., [13]).

In a detailed paper, Lee *et al*. [14] describe a simplified process for the preparation of microfluidic on soda-lime glass, which can be regarded as illustrative of the manufacturing of microfluidic chips. In Figure 4.3, their principle process is shown.

Instead of using an expensive metal or polysilicon/nitride layer as an etch mask, a thin layer of a positive photoresist is used for etching the channels in soda-lime glass. A microfluidic channel with a depth of $35.95 \pm 0.39\,\mu m$ is formed after 40 min etching in an ultrasonic bath. The resulting channel has a smooth profile

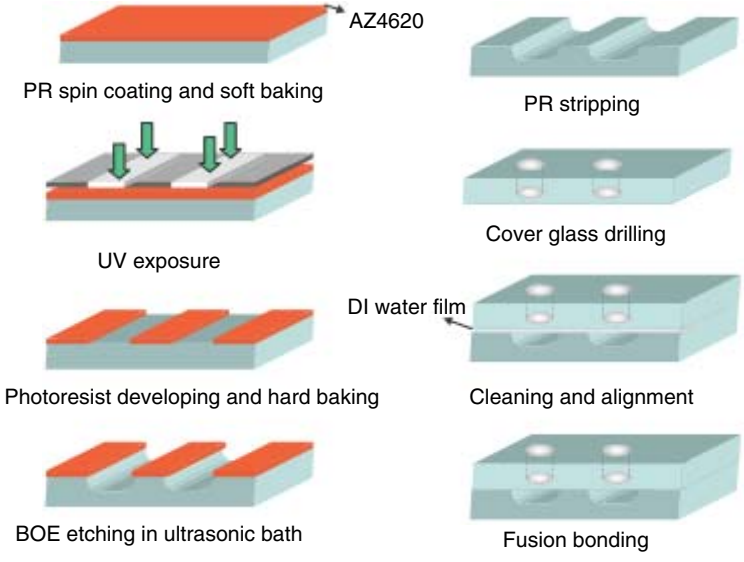



**Figure 4.3** Simplified fabrication process for fast prototyping of microfluidic chips on soda-lime glass substrates. Reproduced from Lin *et al*. [14] with permission of Institute of Physics.

with a surface roughness of less than 45.95 ± 7.96 Å. Glass chips with microfluidic channels are then bonded at 580 °C for 20 min to seal the channel while a slight pressure is applied. Successful sampling and separation of Cy5-labeled bovine serum albumin (BSA) and anti-BSA were achieved.

There have been less reports on quartz-based microfluidic chips since the first report by Ramsey *et al*. [15]. This may be partly because soda-lime glass with coated photoresist is readily available, omitting the tedious, sophisticated operations to deposit the photoresist. Moreover, etching quartz requires HF and proceeds in an anisotropic manner, which makes the channel shape more difficult to control.

Other materials have found to be more useful in practice, such as polydimethylsiloxane, polymethylmethacrylate (PMMA), polyimide (PI), and polycarbonate (PC). These organic polymers have the advantage of simpler preparation by injection molding [16], laser ablation [17], or hot embossing [18]. Recently, cyclo-olefin polymer (COP) has been a commercially available polymeric material, which is increasingly used [19]. COP exhibits several superior characteristics such as high UV transparency, good molding ability, high thermal resistance, biocompatibility, ease of metallization, and high resistance to common solvents such as methanol, acetonitrile, acids, and bases.

Recent reviews provide extensive details about the usage of the aforementioned materials for the preparation of microfluidic devices for capillary electrophoresis [20, 21].

The majority of the readership of this monograph will not have the infrastructure, tools and resources, or experience to prepare microfluidic chips for electrophoresis or MCE devices that can interface to an MS system.

In the appendix on page 96, an overview of sources of microfluidic chips and chips for MCE-MS is given. All of them will provide custom-designed chips, interfacing and packaging services, and eventual volume manufacturing. Almost all of the companies mentioned here described their technology, services, and products as microelectromechanical systems (MEMS) and view microchips for capillary electrophoresis as a part of their business. Exhaustive overviews of all manufacturers involved in MEMS are provided by FluidicMEMS on their website [22] and on Wikipedia [23].

It needs to be mentioned at this stage that none of the manufacturers mentioned in the table and these overview are explicitly claiming to provide custom-made microfluidic chips for capillary electrophoresis coupled to mass spectrometry.

## 4.3 Reviews on MCE and MCE-MS

Initially, MCE evolved quite slowly. Although the basic work was done in the early 1990s, and despite the rapid development of Lab-on-a-Chip technology, MCE took almost a decade to get established. On introduction of the first commercial MCE instruments by Agilent Technologies (see [1]) and Bio-Rad, these

manufacturers gave these instruments trade names, thus avoiding to mention the base technique that was used, namely capillary electrophoresis.

Dolnik was the first to comprehensively review MCE in 2000 [24] and 2004 [25]. Landers and coworkers considered microchip electrophoresis as an emerging technology for molecular diagnostics in their assessment of the technology in 2000 [26]. Their assessment was spurred by the contemporary introduction of the Agilent, Caliper, and Bio-Rad MCE devices, but at the same time, they called for even integration of more functions on the chip such as DNA purification and PCR to make the devices even more attractive. Although there have been attempts to integrate more functionalities to MCE devices or develop high-throughput versions, one can easily recognize that commercial providers have been unsuccessful in doing this and the current MCE systems on the market still use the technology as introduced in the early 2000s.

In a very detailed but comprehensive review, Lin and coworkers assessed the status of the MCE field until 2008 [27]. They dealt with considerations for the design of microfluidic channels for capillary electrophoresis and materials that can be used for their fabrication. Given the larger surface/volume ratio of rectangular channels compared with cylindrical conduits, especially surface coating and deactivation of different materials used to prepare chips were discussed. Different modes of CE on microfluidic chips, the usage of electrokinetic sample concentration, on-chip preseparation labeling steps, and modes of detection were reviewed and their results were described. The application of MCE for protein separation by different modes of CE including two-dimensional separations is discussed.

Lin finalized this review in 2008 with the expectation that, with the integration of sample preprocessing and improved detection, MCE will grow into a widely used tool for many applications. This conclusion is similar to that by Landers' group 7 years earlier!

Detection in MCE was reviewed in detail in 2007 by Götz and Karst [28] and by Garcia *et al*. [29] in 2010. The review by Götz focuses on development in the early 2000s and covers features and performance of optical detection in microfluidic capillary electrophoresis in great detail. As mentioned before, fluorescence detection, which delivers very high sensitivity and selectivity, is the most widely used method of detection. Instruments utilize laser (LIF), and light-emitting diodes (LEDs) are used as excitation sources. Since microfluidic chips allow for easy light path extension (e.g., by illumination from the side), applications of UV-Vis spectrophotometric detection are also referred.

Garcia *et al*. cover a more recent work on detection in MCE and in capillary electrophoresis, from the first decade in the 2000s. Besides optical detection, they review extensively the usage of electrochemical detection. They describe prototype systems for CE and MCE that are portable, which can be used, for example, in outer space.

They conclude that outstanding progress has been achieved with regard to the development of microfluidic systems that can handle a wide variety of samples. They hope that future developments in instrumentation will fill the gap between

the current prototype technologies and system evolving to true lab-on-a-chip devices for broad analytical needs.

The most recent review on MCE was published by Castro and Manz [30]. The review covered the most recent work on instrumental aspects, different modes of capillary electrophoresis on a chip, and applications. Manz can be regarded as the father of technology in the field of MCE. Therefore, this is a comprehensive and detailed review and a place to start accessing the most recent MCE work.

A monograph dealing exclusively with MCE has been published [31]. Henry's monograph is an issue in the "Methods in Molecular Biology" series and excels in describing a lot of experimental details. Both deal with fabrication methods of microfluidic chips from diverse starting materials, surface deactivation and modification, on-chip detection or coupling with external detectors, and application of MCE for the separation of DNA, proteins, and peptides. This book, although published in 2006, is still a good starting point to learn the basics of MCE.

The books by Landers [32] and Garcia *et al.* [33] deal both with capillary electrophoresis and microchip electrophoresis in one edition and they are more recent ones. Especially, the Handbook of Capillary and Microchip Electrophoresis provides many application examples beyond nucleic acids, proteins, and peptides. Key contributors in the field describe or review the integration of sample preparation and preconcentration steps, other CE separation modes and cell separations.

Apart from the review articles referred before that specifically deal with MCE and MCE-MS, many review articles dealing with coupling of microfluidic devices, microchip HPLC, and capillary electrophoresis with MS contain information pertaining to MCE-MS. Table 4.1 lists these reviews and a synopsis.

In addition, the journal Electrophoresis has frequently published special issues on CE-MS, which also include reports on MCE-MS. These can be easily retrieved [52].

## 4.4 Principal Requirements for MCE-MS

### 4.4.1 Electrospray Ionization

Yamashita and Fenn [53] and Aleksandrov *et al.* [54] were the first to describe atmospheric pressure ESI as an inlet method for MS. This method allows the formation of a spray of (sub)micrometer-sized charged droplets under the influence of an electrical field from which eventually charged molecules (ions) enter the MS via the inlet orifice. This first work showed that AP-ESI works best at flow rates of $1-10\,\mu l\,min^{-1}$ with aqueous solvents.

Volumetric flow rates in CE in a 50 µm i.d. capillary typically can be in the range of $0-500\,nl\,min^{-1}$ depending on the presence and the magnitude of the electroosmotic flow. In MCE, volumetric flow rates in the separation channel typically will be 5× lower.

**Table 4.1** Review articles on microfluidic device coupling with MS.

| Authors | Title | Pub. year | Reference |
|---|---|---|---|
| R.D. Oleschuk, D.J. Harrison | Analytical microdevices for mass spectrometry | 2000 | [34] |
| Andrew J. de Mello | Chip-MS: Coupling the large with the small | 2001 | [35] |
| W.-C. Sung, H. Makamba, S.-H. Chen | Chip-based microfluidic devices coupled with electrospray ionization–mass spectrometry | 2005 | [36] |
| I.M. Lazar, J. Grym, and F. Foret | Microfabricated devices: A new sample introduction approach to mass spectrometry | 2006 | [37] |
| F. Foret and P. Kusy | Microfluidics for multiplexed MS analysis | 2006 | [38] |
| A.D. Zamfir | Recent advances in sheathless interfacing of capillary electrophoresis and electrospray ionization mass spectrometry | 2007 | [39] |
| J. Lee, S.A. Soper, and K.K. Murray | Microfluidic chips for mass-spectrometry-based proteomics | 2009 | [40] |
| F. Kitagawa, K. Otsuka | Recent progress in microchip electrophoresis–mass spectrometry | 2011 | [41] |
| S. Ohla and D. Belder | Chip-based separation devices coupled to mass spectrometry | 2012 | [42] |
| S.-L. Lin, T.-Y. Lin, M.-R. Fuh | Recent developments in microfluidic chip-based separation devices coupled to MS for bioanalysis | 2013 | [43] |
| D. Gao, H. Liu, Y. Jiang and J.-M. Lin | Recent advances in microfluidics combined with mass spectrometry: Technologies and applications | 2013 | [44] |
| S.-L. Lin, T.-Y. Lin, M.-R. Fuh | Microfluidic chip-based liquid chromatography coupled to mass spectrometry for determination of small molecules in bioanalytical applications: An update | 2014 | [45] |
| V. Kašička | Recent developments in capillary and microchip electro-separations of peptides (2011–2013) | 2014 | [46] |
| X. Feng, B.-F. Liu, J. Li, and X. Liu | Advances in coupling microfluidic chips to mass spectrometry | 2015 | [47] |
| Hankemeier *et al*. | Lab-on-a-chip hyphenation with mass spectrometry: strategies for bioanalytical applications | 2015 | [48] |
| M.G. Roper | Microfluidics-to-mass spectrometry: A review of coupling methods and applications | 2015 | [49] |
| V. Kašička | Recent developments in capillary and microchip electro-separations of peptides (2013–middle 2015) | 2015 | [50] |
| S. Štěpánová, V. Kašička | Recent developments and applications of capillary and microchip electrophoresis in proteomic and peptidomic analyses | 2015 | [51] |

In two seminal papers [55, 56], Wilm and Mann demonstrated theoretically and experimentally the electrospray process to generate smaller droplets at volumetric flow rates below 1 µl $min^{-1}$. This process was called *nano-ESI*. Optimal flow rates for nanoelectrospray is in the range of 20 – 100 nl $min^{-1}$, which is within the flow rate range where MCE devices operate. Their work has been corroborated by many others demonstrating significant gain of sensitivity in the nanoflow-rate regime [57, 58], as well as reduction of the extent of ion suppressions during ionization.

Given the excellent match of the volumetric flow in MCE device with nanoelectrospray, the main challenge is to provide the required electrical connections for applying the CE field and the electrospray voltage. For CE-MS coupling, the requirements are described in detail in Chapter II of this monograph. A very fortunate circumstance in MCE-MS will be that the CE current and the electrospray current will not be as largely different as in sheath-flow CE-MS interfaces.

Essential though for coupling of MCE to MS is to establish a stable ion nanospray. At the low-volumetric scale, the MCE device operates, the spray needle (or equivalent) needs to match these nanoscale dimensions and will be consequently extremely tiny and difficult to fabricate.

### 4.4.2 Principle Layout of MCE-MS Devices

In principle, different approaches for generating a nanoelectrospray from a microfluidic device have been described in the literature. Belder *et al*. [42] summarized these approaches in five types as shown in Figure 4.4.

As the basic chip layout given in the previous section, microfluidic devices for MCE-MS will have a connection with access holes acting as micro vials containing background electrolyte (BGE), sample, and sample waste in the cross or double-T configuration described before. There will be a separation channel of typical lengths of 20 – 150 mm (the longer ones will be meandering on the chip) and cross section of 10 – 20 by 50 – 80 µm. The substrate material of which the chips are fabricated will be glass such as borosilicate or a polymer such as Polydimethylsiloxane (PDMS) or other.

The separation channel will be open since it is desired to form an electrospray plume of the liquid leaving the separation channel. As will be illustrated later, it is mandatory that the liquid exits the chip from a narrow orifice such as a very sharp hollow needle.

As is indicated in the lower part of Figure 4.4, these authors describe a generic microfluidic separation device where the analytes and buffer are driven by hydraulic force or an electrical field and separation based on chromatographic retention or electrophoretic mobility.

In addition, these authors illustrate the connection of a makeup flow that connects to the separation channel, which is shown in Figure 4.4. It will be shown that one or two channels delivering a makeup flow are of huge importance for generating a stable electrospray but also for connection with the electrospray voltage or to common ground (depending on the brand of MS instrumentation used). Further details follow later in this chapter.

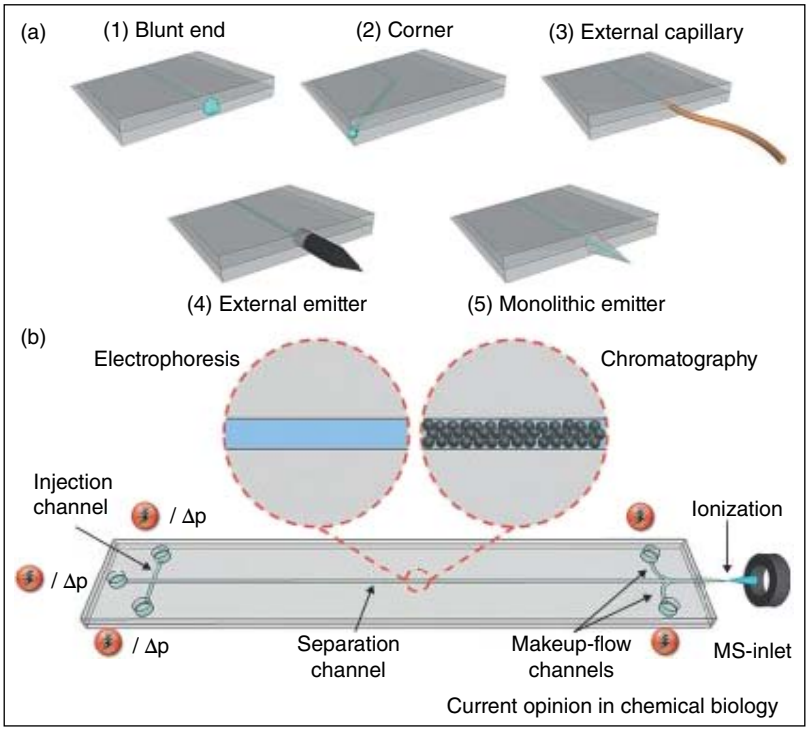



**Figure 4.4** MS coupling of microfluidic chips; (a) different approaches to generate the nanospray; (b) microfluidic device for HPLC or CE separation. Reproduced from Ohla and Belder [42] with permission of Elsevier.

Essential though is the way of formation of the spray. When looking into the work published in the literature, this process has found different implementations. Although five methods for interfacing MCE with MS are described in Figure 4.4, interface approaches for MCE-MS can be reduced to three methods essentially:

a) Spraying directly at the exit of the separation channel (blunt-end approach)
b) Spraying through a connected capillary or microfabricated emitter
c) Spraying with a monolithic emitter, that is, fabricated as an integral part of the chip or using a sharp corner of the chip as emitter.

These three approaches are addressed in the following sections.

## 4.5 MCEMS by Direct Off-Chip Spraying

Driven by the development of nano-ESI by Wilm and Mann and the excellent match of the volumetric flow rate of MCE devices, Ramsey *et al*. were the first

to demonstrate the successful formation of an electrospray by electro-osmotic pumping of the solvent directly to the exit of the separation channel [59].

The layout of their microfluidic chip resembled a standard design for MCE devices shown in Figure 4.4, with a cross-channel to deliver the sample. The exit hole was created by cutting the dead end of the separation channel and polishing the side. The channel dimensions were 60 µm wide and 10 µm high. The length of the separation channel was 33 mm. The side-arm channel had been coated with linear polyacrylamide to suppress or reduce electro-osmotic flow. The separation channel remained uncoated. Electrical potentials were applied to the solutions in the top and side-arm reservoirs. The chip was placed 3–5 mm in front of the MS aperture, which was at ground potential.

The neutral coating on the side channel was essential to propagate the buffer from the inlet to the exit hole and avoid crossflow. But the buffer in the side-arm channel must be conductive to allow the electrospray voltage to be applied to the exit of the separation channel. A voltage of 6 kV was applied to the uppermost reservoir and 4 kV was applied to the side-arm reservoir, providing approximately 1.2 kV potential drop for electro-osmotic pumping within the uncoated channel segment and approximately 4.8 kV potential for electrospray formation. Alternatively, the side arm can be at ground potential, in which case the MS inlet aperture must provide the electrospray voltage.

In a second paper, these authors reported problems including that all the liquid leaving the separation channel had a tendency to spread over the surface of the chip, thereby reducing stability of the spray and reducing sensitivity [60]. Therefore, they abandoned this approach. They modified their chip by not breaking the chip but drilling an access hole to the separation channel from the backside of the chip and connecting a nanospray tip (50 µm i.d., 360 µm o.d., 5 µm i.d. orifice) with a length of 2 cm to the exit hole. Effectively, the nanospray tip is oriented perpendicular to the backplane of the microfluidic chip. Initial results of the MCE-MS separation of a set of test peptides were reported.

## 4.6 MCE-MS with Connected Sprayer

Given the results of Ramsey *et al*., other researchers started using disposable or reusable nanospray tips as a means to generate the electrospray. Harrison *et al*. took the same approach as Ramsey described in the previous section and also used a side arm on the chip layout [61–63]. In this case though and in order to obtain a stable spray, the side arm was used to deliver a makeup solvent to the tip at an overall volumetric flow rate of 150 nl $min^{-1}$. A very narrow and short fused-silica capillary was used as nanospray tip (1 cm length, 0.06 mm o.d., and 0.015 mm i.d.). Although the sprayer tip was glued in the exit hole of the chip, the authors claimed it to be exchangeable. The separation channel in the chip was 40 mm long (Figure 4.5).

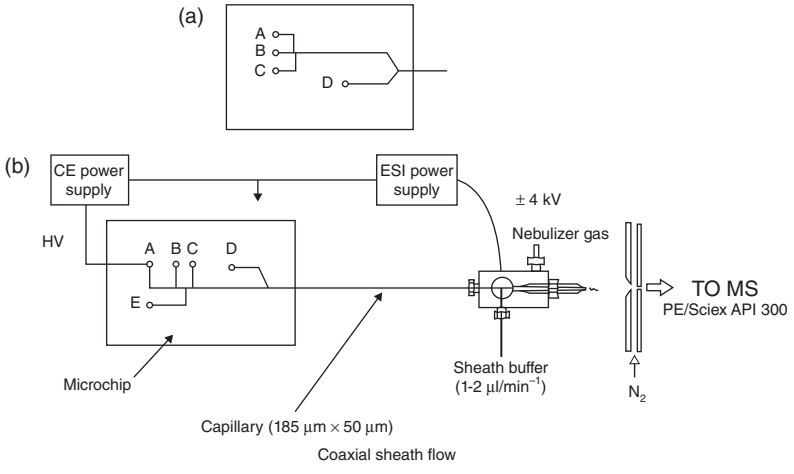



**Figure 4.5** Schematic representation of the chip-CE configuration using (a) a disposable nanoelectrospray emitter or (b) a sheath-flow ESMS interface. Reproduced from Li *et al*. [62] with permission of American Chemical Society.

Alternatively, in this work, the separation chip was connected via a fused-silica capillary (10 cm long, 0.185 mm o.d., 0.050 mm i.d.) pulled out to a spray tip, then to a sheath flow, pneumatically assisted spraying device. This capillary also acts as an additional electrophoresis channel and enhances the resolution of the analyte bands. The capillary was fed through a stainless-steel needle where the ES voltage and a sheath solvent at $1-2\,\mu l\,min^{-1}$ were applied.

These authors concluded that the direct coupling of nanoelectrospray emitter to their MCE device delivered modest performance in terms of separation efficiencies ($N \approx 500-3500$) but provided an efficient means of removing mass spectral interferences that can be present in complex protein digests with a substantial reduction in analysis time. Alternatively, by using longer CE transfer, separation efficiency ($N \approx 26\,000-58\,000$) is enhanced, allowing more complex mixtures to be separated.

In further work and in collaboration with Henion *et al*. [64], these authors reported the adaptation and connection of a miniaturized version of Henion's ion spray interface for ESI LC-MS interfacing [65] to their microfluidic chip as described earlier (Figure 4.6). In the electrospray interface, a makeup solvent is delivered. The electrospray voltage was applied via a sheath flow, and spray formation was aided and stabilized with a nebulizing gas. By the addition of a solid-phase extraction zone on the chip or the usage of electrokinetic stacking, low- to sub-nanomolar detection limits were achieved.

Given the need for high-precision drilling of the exit hole, the difficulties in (re)placing the emitter or transfer CE capillary in and out of the exit hole of the microfluidic chip with possible clogging of the tip and sample contamination, Karger *et al*. opted to use a liquid junction [66–68]. This junction was created

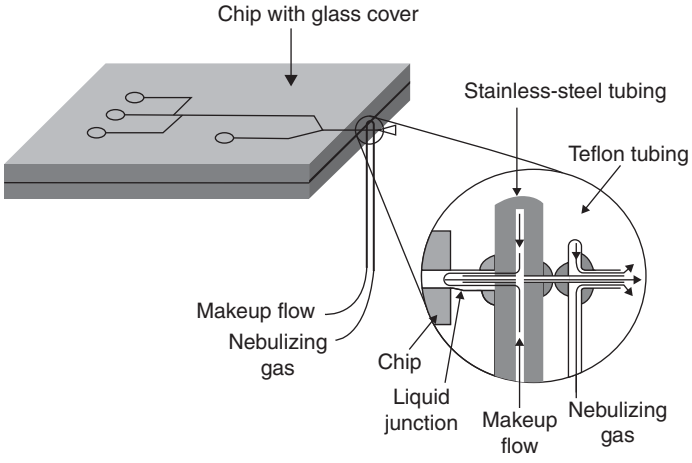



**Figure 4.6** Glass chip–based CE/MS apparatus and the expanded view of the coupled microsprayer. Reproduced from Wachs and Henion [65] with permission of American Chemical Society.

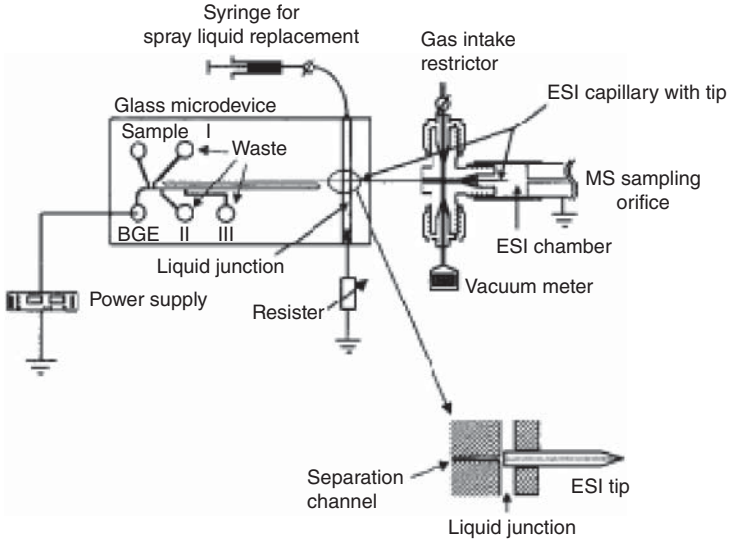



**Figure 4.7** Diagram of the MCE device with a subatmospheric ES interface. The expanded view shows the coupling of the ESI tip with the separation channel in the liquid junction. Reproduced from Zhang *et al*. [67] with permission of American Chemical Society.

by an additional cross-channel on the chip, which left a small gap between the end of the separation channel and the inlet of the emitter tip (Figure 4.7). The cross-channel was flushed with a conductive liquid and grounded. The electrospray voltage was applied to the emitter, which used the junction channel as electrical ground as well.

The emitter capillary was standard fused silica of 25 mm long, 380 µm o.d., and 25 µm i.d., which was etched down at the end with hydrofluoric acid to a tapered shape. The emitter capillary was held by a cross and centered with a septum on the outlet side. The other end of the emitter was positioned with an $X-Y-Z$ translation stage into the exit hole (400 µm i.d.) of the chip. No glue or similar substance was used.

The authors showed feasibility of their MCE-MS system in Figure 4.7 with test mixtures of proteins and a tryptic digest of BSA in less than 1 min of analysis time. Eventually, they expanded their MCE device to comprise pressure injection of the sample and elongated the separation channel to a length of 11 cm. Plate numbers up to 40 000 and LOD of 0.1 µM were reported.

In a later publication, these authors demonstrated the coupling of this interface with sampling from 96-well plate showing feasibility of their approach for high-throughput sampling.

Nevertheless, the connection of the emitter with the chip remained a very critical step in fabrication and practical operation of the MCE-MS interface. Otsuka and Terabe came up with a robust solution for this problem [69, 70]. They used the same simple basic design of the MCE device with a liquid junction crossing the separation channel at the end and a tapered emitter used to generate the electrospray. The separation channel was $50 \times 50$ µm with a length of 22.9 mm. The emitter used was a commercially available fused-silica capillary (New Objective) cut to a length of 25 mm (i.d. 20 µm) with an orifice of 10 µm attached to the MCE device using a polyether ether ketone (PEEK) screw without glue. ESI voltage was applied via the liquid junction (because conductive coatings on the spray nozzle outside have shown extremely short lifetime).

In comparison of the peak widths obtained with a neutral single analyte by LIF detection before the liquid junction and MS detection, they still found a significant increase in the peak width by the interface. The separation and MS detection of simple basic drugs and peptide was feasible at the 100 ppm concentration level.

In their second paper, these authors used a longer separation channel (68.5 mm) to increase the separation efficiency but also to reduce the suction effect, which was found to occur in their initial work, which in turn added a hydraulic flow component to the overall flow in the device. In addition, an SMIL (successive multiple ionic layer) coating as described by Katayama [71] was applied to the chip. Separation of peptides was demonstrated at low-micromolar concentration (Figure 4.8). Stable electroosmotic flow (EOF) was obtained and electrokinetic gated injection became possible under low pH conditions using SMIL-coated quartz microchip. These authors identified the need for on-line concentration techniques for MCE-MS with these devices.

Conversely, Markides *et al*. have described a microfabricated emitter for ESI coupling with MS, which was coupled with a CE capillary [72]. Their device consisted of a PDMS block of $60 \times 20 \times 5$ mm with a 50 µm channel, the inner side of which was deactivated with a cationic polymer Poly E-323. The device was connected to the CE separation capillary on the inlet side and to a carbon-coated fused-silica capillary as the emitter on the other side. The emitter was grounded

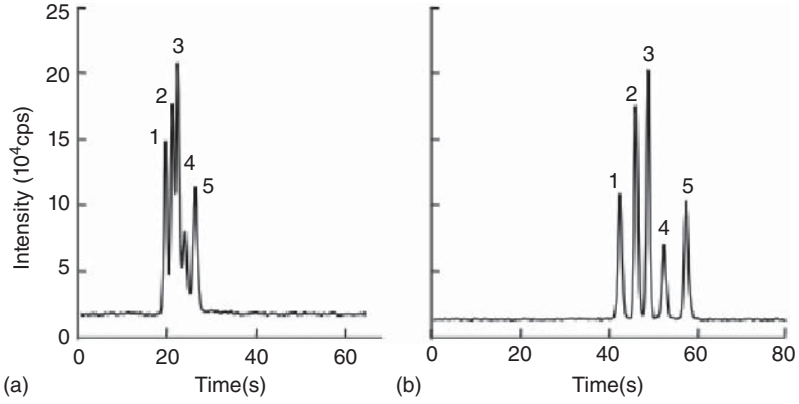



**Figure 4.8** Separation of bradykinin, angiotensin I, angiotensin II, [Sar1, Ala8]angiotensin II, and [Val4]angiotensin III five ions). (a) Short chip and (b) long chip. Separation buffer, 50 mM acetic acid–ammonium acetate buffer (pH 5.7) containing 50% (v/v) acetonitrile. Reproduced from Tachibana *et al*. [70] with permission of Elsevier.

and the ESI voltage delivered from the MS. No nebulizing gas or other spray-forming aids were used. With this elegant approach, the authors demonstrated high-efficiency separations of peptides at the low-micromolar level.

Her *et al*. initially used a similar approach to that of Markides to create a connection between the chip and the fused-silica emitter capillary [73]. Their chip was fabricated from PMMA with a separation channel of $50 \times 50$ μm and length of 45 mm. A metal wire 30 μm o.d. was shoved through a fused-silica emitter capillary (0.05 mm i.d.) and positioned in the separation channel on the chip. A short gap (300 μm) remained between the fused-silica capillary and the chip, which was filled with a fast-curing epoxy resin. After removal of the wire, a short epoxy resin channel remained. The fused-silica capillary was drawn out to generate a tip of 10 μm i.d. and the outside was coated with a conductive rubber. Feasibility of the device was shown at a concentration level of 20 μM of a set of test peptides.

In their further work, the authors reported to add a sheath-solvent flow connection to the device [74]. Instead of spraying of the chip directly, fused-silica connection capillary tapered at the end to 40 μm o.d. was connected to the exit of the separation channel. The chip was positioned on a PMMA holder where the connection capillary was placed in the spray needle (700 μm pulled out to a tip of 10 μm) as much forward as possible. The annular space between the connection capillary and sprayer capillary connected to a reservoir from which a sheath solvent was delivered and the electrospray voltage applied.

With this MCE-MS device, the CE voltage was decoupled from the electrospray voltage. Moreover, ion suppression by cyclodextrin and phosphate in the BGE was suppressed, leading to a significant reduction of the LOD. The authors reported feasibility of the device by the analysis of histamine antagonists and peptides. Since the 10-mm ESI sprayer is not fixed on the CE microchip, sprayer replacement

can readily occur if the sprayer becomes clogged or damaged. Consequently, this interface is more robust than a sheathless design.

In further work [74], these authors connected their device and coupled it directly with a CE sprayer capillary while leaving a short cleft as a liquid junction. The liquid junction was connected to a reservoir with phosphate buffer.

Zheng *et al*. [75] reported on connecting the exit of their MCE device to a fused-silica capillary (150 mm × 75 µm). The microchannel was measuring 100 × 25 µm and a length of 57 mm. On another microfluidic device, a pneumatically assisted, micrometal sheath-flow sprayer was fabricated. Electrospray voltage was applied to the metal sprayer needle. Background electrolyte was ammonium acetate at pH 8.5, and the sheath solvent used was acidified aqueous methanol. The device was applied for the analysis of *O*-glycans obtained by beta-elimination from a glycopeptide.

Nevertheless, as connecting a sprayer to a microfluidic separation chip has appeared not to be simple, it has not become used as a practical routine method. Approaches with an integrated sprayer are favored and are described in the next section.

## 4.7 MCE-MS Devices with Integrated Sprayer

Karger *et al*. realized (see [66]) that only spraying of a microfluidic chip would result in significant broadening of the eluting, separated analytes since droplets (20–200 nl depending on the surface properties and BGE composition) are formed prior to spray formation. They connected a side-arm conduit toward the end of the separation channel (semicircular-shaped, 29 × 80 µm, length 100 mm) to deliver a makeup solvent. In addition, two channels delivered a nebulizing gas (nitrogen 0.3 l $min^{-1}$) to support and stabilize spray formation. Effectively, this device resembles a microfluidic copy of the conventional pneumatically assisted sheath-solvent flow interface as described by Chen in Figure 2.2.

A very narrow orifice and a needle-like geometry have proven to be essential for spray formation. Solvent velocity increases with narrowing of the orifice. The strong surface curvature at the end of the needle causes a higher density of field lines and stronger ionization of the droplets in the aerosol. Given the delicate handling required for working with an attached spray needle, MCE devices with integrated sprayer have become more of interest despite the putative disadvantage of plugging of the spray tip, which would render the MCE device unusable.

At the same time, successful efforts in coupling of HPLC on a chip with mass spectrometry have been reported [36, 38, 76, 77]. Dedicated HPLC-MS chips and interfaces became available on the market by Agilent Technologies [78] and Waters Inc. The Agilent HPLC chip was based on a polymeric material (Kapton™), which had a sprayer needle integrated on the chip. The Waters HPLC chip was a ceramic microfluidic chip equipped with an exchangeable sprayer needle. The coupling of such an HPLC chip to MS is putatively simpler since

there is only one electrical circuit required that drives spray formation. The solvent is propagated by hydraulic force, which also acts as a fluid connector to the grounded HPLC instrument.

Belder *et al*. were the first to report a monolithic microfluidic chip for MCE-MS [79–81]. Their MCE device was a simple, commercially available Borofloat glass chip. At the exit side of this chip, the glass was milled away to leave a triangular-shaped flat cone, which was concentrically aligned with the separation channel. The end of this cone was drawn out to a very narrow diameter of few micrometers. After the drawing, the needle tip was cut and treated with HF to remove any debris.

By a detailed comparison of their MCE device with commercially available nanospray needles (Figure 4.9), these authors found that comparable spray performance and sensitivity are achieved with similar emitter diameters. Eventually, they demonstrated the separation of some low-molecular-weight (LMW) solutes in less than 100 s at 50–200 ppm level concentration (Figure 4.10).

In a later work, Belder *et al*. reported applications of their MCE-MS device that they described earlier. In this work, the MCE-MS was equipped with additional


**Figure 4.9** Schematic drawing of the MCE device with integrated nanospray emitter. SO, sample outlet; SI, sample inlet; BI, buffer inlet; MS, mass spectrometer. Reproduced from Hoffmann *et al*. [79] with permission of Wiley.

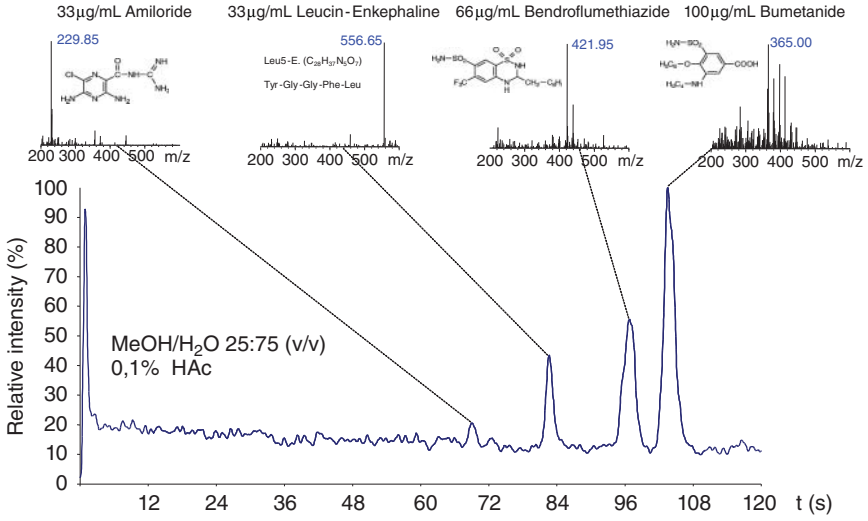



**Figure 4.10** Separation and mass spectrometric characterization of 4 LMW test solutes in <100 s at low-micromolar level. Reproduced from Hoffmann *et al*. [79] with permission of Wiley.

side channels to deliver a makeup solvent [82]. The solvent was regarded necessary in order to assist in spray formation without affecting the CE separation. Moreover, the voltage for electrospray can be applied via the makeup channel. The dimension of the separation channel was 50 × 20 mm, with a length of 78 mm. The BGE used was 5 mM ammonium acetate at pH 8, and the makeup solvent was aqueous methanol. A mixture of cardiac drugs was used to optimize the separation parameters and composition of the sheath solvent. The authors concluded that the combination of an MCE device with makeup flow offers the advantage of being a more reliable approach for MCE-MS coupling.

In their initial work in coupling microfluidic capillary electrophoresis with ESI/MS, Ramsey and coworkers generated the electrospray at the exit of the separation channel on the planar edge of the microfluidic chip (see [49]). But the fluid exiting the opening had a tendency to spread over the glass surface and form large droplets, resulting in excessive band broadening. Moreover, higher electrospray voltages are required to form the spray. A sharp contour as the point where the spray is formed is a mandatory requirement.

Therefore, many other groups have opted to connect fused-silica nanospray tips (self-made or commercially available from New Objective Inc., Woburn, MA, USA) to the microfluidic chips. However, great craftsmanship is required to make the connection, leaving concern about extra-column dispersion in the connection.

Given the problems and concerns mentioned, Ramsey *et al*. modified their microfluidic CE chip such that the separation channel ends at the corner of the rectangular microchip, thus providing a sharp two-dimensional feature for an electrospray tip [83]. In order to avoid issues with a liquid junction that is required for electrical, a side channel was fabricated, which connected to the separation channel just less than 200 μm before the exit of the separation channel.

The separation channel was coated with a polyamine layer (PolyE-323), which renders the separation channel positively charged at neutral to acidic pH of the background electrolyte producing a stable anodic EOF while repelling positively charged analytes. The surface of the side channel was not coated, resulting in a small cathodic EOF in the side channel avoiding the cationic analytes to enter the side channel and cause zone broadening. The electrode for providing the electrospray voltage was connected to the side channel. A schematic drawing of the device is shown in Figure 4.11.

Figure 4.12 illustrates the formation of the spray from the edge of the microfluidic chip. With the long channel chip, these authors demonstrated the separation of a tryptic digest of BSA at 5 μM concentration level. The plate count for the device was 200 000.

In following work, the authors could demonstrate the continuous on-chip lysis of erythrocytes with real-time electrophoretic separation and ESI of the liberated hemoglobin [84]. They expanded the functionality by doubling the emitter so that, on the one hand, a CE separation was achieved and simultaneously from a second channel, reference masses were fed into a second spray with sequential entry of analytes and reference masses into the mass spectrometer [85]. The basic design,

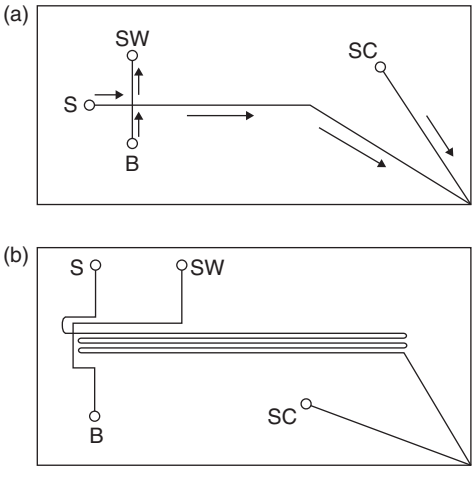



**Figure 4.11** Schematic diagrams of the short-channel (a) and the long-channel (b) CE-ESI-MS chips. The length of the separation channel (measured from the injection cross to the outlet) was 4.7 cm for the short-channel chip and 20.5 cm for the long-channel chip. For both chips, the channels were all 75 μm wide at full width and 10 μm deep. The reservoirs are labeled S (sample), B (buffer), SW (sample waste), and SC (side channel). The direction of electro-osmotic fluid flow is indicated by the arrows in (a). Reproduced from Mellors *et al*. [83] with permission of American Chemical Society.

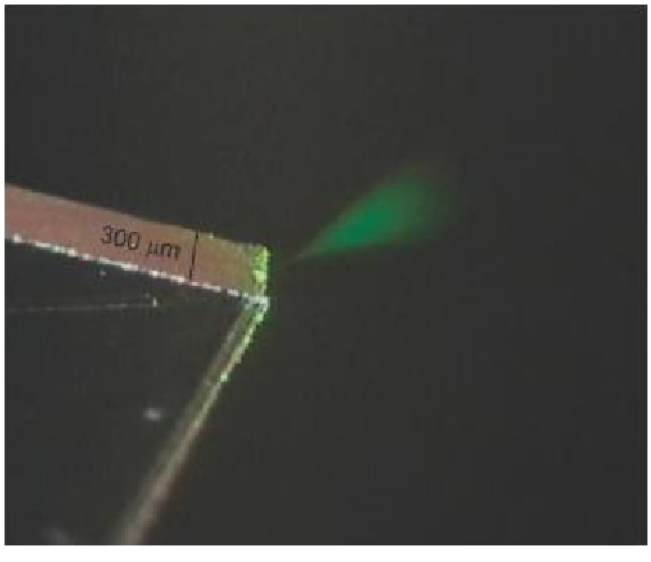



**Figure 4.12** Image of the electrospray plume generated from the corner of a CE-ESI-MS chip acquired with a CCD camera. The liquid being sprayed was 50/50 (v/v) methanol/water with 0.2% acetic acid. The voltages applied to the microchip reservoirs raised the potential at the spray tip to 3.5 kV above that of the mass spectrometer inlet and caused the liquid to be pumped out of the chip at a flow rate of ~40 nl $min^{-1}$. Reproduced from Mellors *et al*. [83] with permission of American Chemical Society.

dimensions, and surface treatment of the chip were essentially similar to those published in [67].

Most recently, this group discovered that the coating procedure with a polyamine resulted in a relatively nonhomogeneous surface layer that caused eddies and backflow in the separation channel, which resulted in additional band spreading [86]. With the same microfluidic chip as described earlier, they proceeded with elaborate chemical vapor deposition of amino propyl silane, followed by deposition of aminopropyl trialkoxysilanes. This resulted in a homogeneous coating with a positive charge under neutral and acidic conditions. The side channel (see Figure 4.11) was flushed with a strong base to remove the coating from the surface so that an electro-osmotic flow is generated toward the common exit. Separation of a tryptic digest of enolase at 5 µM concentration level on the long channel chip showed an efficiency of +500 000 plates with the resolution of 60 substances in 90 s.

Ramsey *et al*. used the MCE-MS device described in [85], for the separation and characterization of charge variants of intact monoclonal antibodies (Infliximab) [87].

As an alternative to an electro-osmotic delivery of solvent, Li *et al*. have described the usage of a pressure-driven makeup flow [88]. The chip design was similar to that of Ramsey's group, with a side channel merging with the CE separation channel at an angle of 45° (Figure 4.11). The chip itself was made up of glass but covered with a PDMS lid. The separation channel had a length of 25 mm to the merging point, with dimensions of 670 × 60 µm. The spray needle was constructed from a fused-silica capillary (100 µm i.d.) drawn out to a tip of 20 µm i.d. This capillary was connected to the exit channel at the corner of the glass chip (no details given). The MUF channel was connected with a syringe pump delivering the sheath solvent (methanol/1% acetic acid) at 0.1 ml $min^{-1}$. The BGE was ammonium acetate at pH 5.5.

Voltage (3.5 kV) was applied to the inlet buffer vial while the mass spectrometer entrance was left at ground. So no additional ES voltage was required. The voltage served the electrophoretic movement and spray formation.

With this system, these authors were able to demonstrate separation and mass spectrometric detection of amino acids. Liu *et al*. used this MCE-MS device for separating an amino acid mixture containing glutamine, serine, threonine, phenylalanine, and glutamic acid [89]. All of them were baseline separated from each other within 3 min. Plate numbers of >10 000 (on a 2.5 cm MCE separation channel) were obtained. The concentration was at the low-micromolar level.

Tuomikoski and Franssila demonstrated the usage of SU-8 polymer as a scaffold for microfluidic devices [90], which was subsequently applied by Kostiainen's group at Helsinki University to fabricate an MCE device based on SU-8 [91] . They were the first to use SU-8 for MCE and characterized its electrokinetic properties such dependence of the EOF on the pH of the BGE.

Nordman *et al*. [92] and Sikanen *et al*. [93] used a microfluidic device with an analogous layout as described by Ramsey (see [67]). Using SU-8 allows multilayered fabrication creating a separation channel ending in a triangular sprayer and a side channel connecting to the separation channel. The side channel delivered

a sheath solvent toward the end of the separation channel and served to connect the electrospray voltage. The effective dimensions of this channel were 50 × 50 μm, with a length of 20 or 40 mm. 30 mM ammonium acetate or formate was used as BGE, while methanol–water (80 : 20) solution with 1% acetic or formic acid was used as sheath liquid.

Separation and identification of the peptides from tryptic digests of some standard proteins were possible with very good precision at the 20–100 μM concentration level in <10 min. The authors considered the relative ease of fabrication and potential for large-volume production as promising features in 2010.

In order to improve the LODs, this group has combined on-line sample enrichment methods on the MCE-MS device: for example, by on-line liquid-phase microextraction [94, 95] on the microchip similarly as described earlier, which was compared with off-line SPE. The estimated detection limits of a tramadol metabolite, for example, were 2 μM and 4 nM in full-scan and SRM MS modes, respectively. These authors viewed the advantages of the combination of LPME with MCE-MS in terms of speed of analysis and the ability to handle much smaller sample volumes.

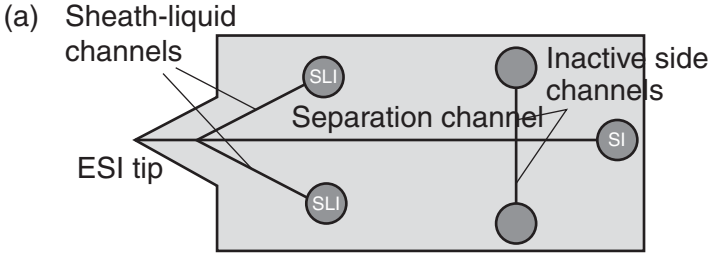
In a more recent work, this group combined CIEF on their chip with MS coupling. Toward that end, two chip designs were investigated (Figure 4.13) [96].

In the first design, the channel was filled with a sample of peptides in 2% ampholyte solution (pH 3–10) while the side channels were filled with methanol/water/1% acetic acid. After applying the sample, the inlet vial was filled with 1% ammonium hydroxide solution, which acts as the catholyte, whereas the sheath solvent acts as the anolyte. After focusing, when the current decreased and stabilized, the solvent in the inlet vial was replaced with BGE and the zones moved toward the outlet. The dependence of the EOF on the pH was a special advantage of using SU-8 as the base material for the chip. At low pH, the EOF (close to the anode) the flow direction is anodic and opposing to the cathodic flow observed at high pH (close to the cathode). Thus, the net velocity during cIEF approaches zero, yet electrokinetic flow for mobilization occurs after changing the inlet solvent and the voltage.

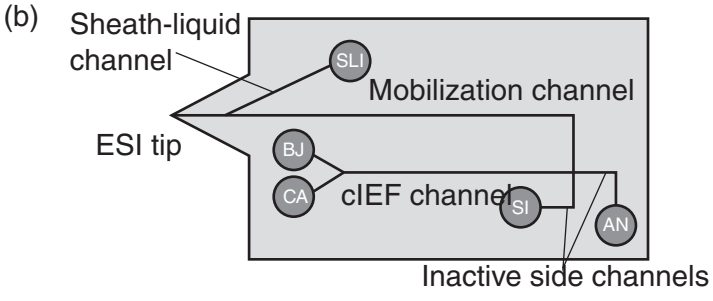
In the second MCE device, the focusing step was decoupled from mobilization by adding an isotachophoresis step.

Nordman *et al*. used the microfluidic CE-MS chip described earlier (see [73] and [74]) for the separation and characterization of the tryptic digests of simple protein test mixture and of the lysate of the human muscle cells [92]. The SU-8 microchip allowed rapid (<10 min) CZE separation of tryptic peptides with high plate number (approaching $10^6$ $m^{-1}$) from the test mixture and their high-resolution MS identification at the femtomole level. From the lysate of the human muscle cells, seven proteins were identified. Besides the excellent analytical performance, these authors claim that microfluidic chips based on SU-8 are an excellent alternative to glass, given the relative simple fabrication, accurately defined microstructures, and the low costs of highly reproducible mass production of the devices.

As an alternative approach to obtain preconcentration, these authors placed a highly porous, cross-linked methacrylate ester monolith at the intersection




| Steps | SI | SLI | SLI |
|---|---|---|---|
| Focusing (60 s) | Ground | 6.0 kV | 6.0 kV |
| Mobilization | 4.0 kV | 2.0 kV | 2.0 kV |




| Steps | BI | CA | SI | AN | SLI |
|---|---|---|---|---|---|
| Loading (100–120 s) | Ground | Float | 1.5–3 kV | Float | Float |
| eIEF (200–270 S) | Float | Ground | Float | 1.5–6 kV | Float |
| Mobilization | 6 kV | Float | Float | Float | 3.5 kV |

**Figure 4.13** Schematic views of the SU-8 microchips used for direct coupling of cIEF to on-chip ESI/MS (a) and for multiplex cIEF-tITP separation coupled to on-chip ESI/MS (b). Reproduced from Nordman *et al*. [96] with permission of Elsevier.

of the injection and separation channels in the SU-8 chip device as described earlier [97]. As a result, approximately 20-fold enrichment factors were obtained at loading (preconcentration) times as short as 25 s at low-micromolar concentration levels.

Alternatively, these authors also investigated the feasibility of ORCOMER®s as base material for their microfluidic devices [98]. ORCOMER®s are alkoxysilanes, which are copolymerized with acryl, vinyl, or epoxy silanes. The polymer is easily formed by embossing and hardened by UV light or heat. For example, such ORCOMER®s are used by dentists for tooth filling. Microfluidic chips with similar design and dimensions as described earlier by these authors were fabricated. The authors believed that ORCOMER®s have advantages over glass as substrate from a manufacturing point. Standard proteins were readily separated without modification of the conduit surface. But the device was lagging in efficiency, compared with fused-silica-coated capillaries.

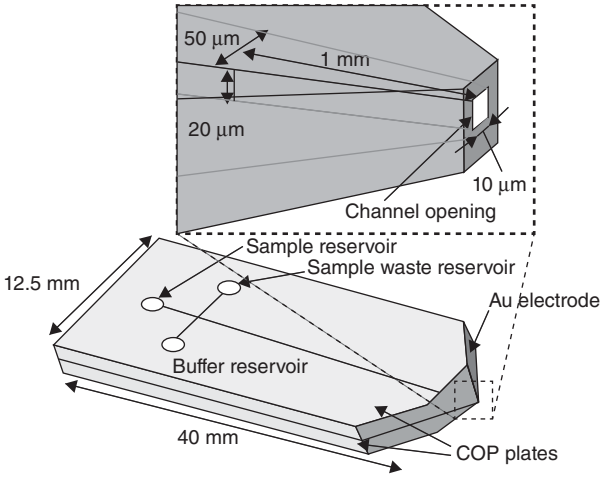



**Figure 4.14** Schematic diagram of the polymer microchip. Dimensions of the channel are 50 μm of width, 20 m of depth, and 30 mm of length. The width of the outlet channel is tapered from 50 to 10 μm. (Designations of the sample reservoir and buffer reservoir are incorrect in the figure. The buffer reservoir is at the beginning.) Reproduced from Shinohara *et al*. [99] with permission of Elsevier.

Apart from this manuscript, no further reports about using ORCOMER®s from this group was published, so one may assume that this pathway is less promising.

Otsuka and coworkers use a cyclo-olefin copolymer (COC, trade name ZEONEX®) as the substrate for microfluidic devices [99]. ZEONEX® is a copolymer of norbornene and ethane with an appearance similar to that of glass. The microchannel detail can be prepared by hot embossing after construction of the appropriate stamp. The microstructure is closed with ZEONEX cover and sealed. Access holes were drilled onto and the side of the chip with the exit hole machined to a triangular shape. The tip was coated with a gold layer by electron beam evaporation. The dimensions are shown in Figure 4.14.

The chip was positioned in front of the MS inlet. Separation voltage was applied to the buffer reservoir (1 – 1.3 kV) while the electrospray voltage (1.8 kV) was applied to the tip. Background electrolyte was 10 mm ammonium acetate. With this device, a stable flow rate was established (100 nl $min^{-1}$) and LMW solutes (1 mM) could be separated and detected in MS.

## 4.8 Multidimensional MCE-MS Devices

Based on their earlier work in the development of an MCE-MS device (see [79]) and their work to design a multidimensional MCE device [100], Ramsey and coworkers designed a device based on borosilicate glass (0.150 mm thick) incorporating a sample-trapping region, an LC channel packed with 3.5 μm porous particles, and a CE channel [101]. The LC channel had a length of 100 mm

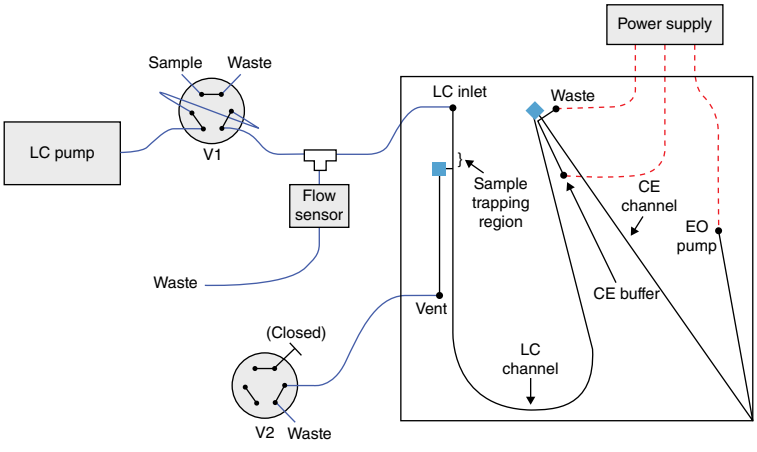



**Figure 4.15** Schematic for the microchip-based LC--CE-MS system. The blue squares on the microchip denote the location of weirs that were used to retain the packed particles. Valve 1 (V1) was used to perform LC injections, and valve 2 (V2) was used to open and close the vent line. Valves are shown in the "sample loading" configuration. Electrospray was performed from the lower-right corner of the microchip. Reproduced from Chambers *et al.* [101] with permission of American Chemical Society.

while the CE channel was 50 mm long. The LC channel was 180 × 25 µm and the CE channel 50 × 8 µm. Effluent from the LC channel is transferred to the CE dimension by the use of electrokinetically gated injections. The complete layout of the device is shown in Figure 4.15.

In the study of the separation of a tryptic digest of a mixture of BSA and enolase, the authors found a potential increase in peak capacity by a factor of 30 (provided the usage of a faster mass spectrometer detector). In subsequent work, these authors found complications involved with incorporation of the LC column on the chip. Packing the columns was time-consuming and the glass chip had limited pressure resistance (200 bar). Consequently, the separation column (length 150 mm, diameter 75 µm, packed with 1.7 µm BEH130 C18 particles) was moved off the chip as a separate column but connected with a short narrow transfer line and a self-designed ZDV coupling to the MCE-MS device [102]. On the chip, a flow splitter was incorporated so that one-third of the solvent from the LC column would be delivered to the CE-channel injection point.

With this setup, the authors were able to demonstrate with a tryptic digest of a five-protein mixture a peak capacity of 1400 and unravel the peptide map of a large protein-like IG2.

## 4.9 Conclusions and Perspectives

Especially regarding the vast amount of fundamental knowledge about fabrication of microfluidic chips for capillary electrophoresis, channel surface preparation,

integration of flow control, sample handling functions, and sophisticated detection methods, one would have expected that soon after the first MCE devices and systems became available commercially, MCE would have become a tsunami taking over all the conventional capillary electrophoresis applications and markets.

On personal experience, – the author has been deeply involved in both development of capillary electrophoresis in the 1990s at Hewlett-Packard and the MCE work for the Agilent Bioanalyzer in the early 2000s – from a manufacturing company involved in device and system development (HP/Agilent Technologies) and in other companies such as Caliper, Bio-Rad, Aclara, Orchid, Nanogen, efforts were begun to transfer CE applications to MCE platforms. Sales numbers were projected that promised exponential growth of the business.

These expectations appeared to be unrealistic. The anticipated growth of the MCE-based business did not materialize. Actually, the course that MCE business took looked very similar to the well-known *Gartner hype cycle* about technological innovation [103] as initial expectations and enthusiasm exploded in the late 1990s and created an "inflated hype" [104]. Soon after the introduction of commercial MCE systems by Agilent, Bio-Rad, and others, sales numbers started to trail expectations and showed only gradual increase in the business of the commercial products. On the other hand and masked by the MCE hype, conventional capillary electrophoresis systems and applications continued to remain a healthy business for few manufacturing companies.

So why did MCE not generate a tsunami in the bioanalytical instrumentation business, and why did MCE not become a global bioanalysis technique in the hands of many users? The same questions apply to MCE-MS.

Assessment and comparison of essential properties of both techniques will illustrate differences between them and may help to understand why there has not been an MCE tsunami and why MCE has not become a broad bioanalytical measurement technology. These general properties have been summarized in Table 4.2.

For example, one will easily realize that CE is an ultrahigh-efficiency "micro" separation technique while MCE will have lower separation power due to the shorter separation channel length rendering the separation much faster though. Instrumental CE is an open-platform technique without barriers for its application, which has been the essential reason for its widespread usage. Open platform means that access to the instrumentation and methods is unhindered by proprietary constraints from the manufacturer/supplier such as the usage of dedicated microfluidic chips that fit only in the manufacturer's instrument, reagents, standards, and instrument control and data handling software. As an open-platform technique with standard fused-silica capillaries (365 μm o.d.), adaptation of available CE methods to a new separation problems can be readily dealt with by changing conditions, background electrolyte composition, additives, dynamic capillary wall coating, and, for example, capillary length.

Conversely, only few successful efforts have been published, for example, to develop CE applications on commercial lab-on-a-chip instruments. Work by Smejkal *et al*. can serve as an illustrative example allowing comparison between

**Table 4.2** Properties of capillary electrophoresis and microchip capillary electrophoresis.

| Capillary electrophoresis | Microchip capillary electrophoresis |
|---|---|
| Ultrahigh efficiency in long separation capillaries | Moderate efficiency in short separation channels |
| Moderate speed and analysis time | High speed, short analysis time |
| Volume of the separation capillary typically 1–10 µl | Volume of separation channel typically 10–100 nl |
| Volumetric flow rate 0.2–2 µl $min^{-1}$ (dependent on capillary i.d.) | Volumetric flow rate 0.05–0.5 µl $min^{-1}$ (dependent on channel cross section) |
| Single separation channel (multicapillary systems) | Potential for parallel separation channels |
| Commercial open-platform instruments allowing multiple modes of CE separation | Closed instrument platforms, which only allow methods offered by manufacturers commercially |
| Separation capillaries and consumables are readily available commodities | Mostly application-specific chips and consumables from the manufacturer. Barely general-purpose chips, interfaces, and instrumentation available |
| Surface properties of the capillaries are under control or simply modified (e.g., SMIL) | Different base materials used, differ in surface properties. Surface/volume ratio is higher |
| Suited for charged, ionizable, polar, and neutral LMW → HMW analytes | Targeted by manufacturers on HMW biomolecules, for example, DNA sizing, SDS-PAGE of proteins |
| Broad range of applications and well-supported by literature and manufacturers | New applications are developed by suppliers or are research topics by groups with proper infrastructure |
| Nowadays, robust, reliable, precise, and broadly approved technology for new drug approval applications (e.g., US Pharmacopeia) | Leading methods in specific areas (e.g., RNA quality assessment) |
| High degree of automation possible and available | Low degree of automations especially regarding sample application |
| Integration of analysis workflow steps such as sample preconcentration is difficult | Integration of unit operations is feasible but requires large upfront efforts |
| Practical human–instrument interface. Easy handling of samples and capillaries | Cumbersome human–instrument interface requiring customization |
| CE-MS interfaces established and commercially available | Potential MCE-MS described but not commercially available |

CE and MCE. These authors showed the separation of APTS-labeled glycans on the Agilent Bioanalyzer in comparison with conventional CE [105] (Figure 4.16). The length of the separation channel on the Agilent Bioanalyzer is 14 mm, while the CE capillary used had a length of 350 mm. The two traces speak for themselves.

Capillary electrophoresis is widely established as a routine method with a broad field of applications from LMW to HMW analytes, for ionized, polar, and neutral molecules. It has found its way as an approved method for new drug submissions by many agencies, for example, US Pharmacopeia.

The level of automation of MCE devices has been hampered by difficulties in reaching to the very small access positions, by the need to use disposable devices or the need to include washing and cleaning protocols after analysis (Agilent

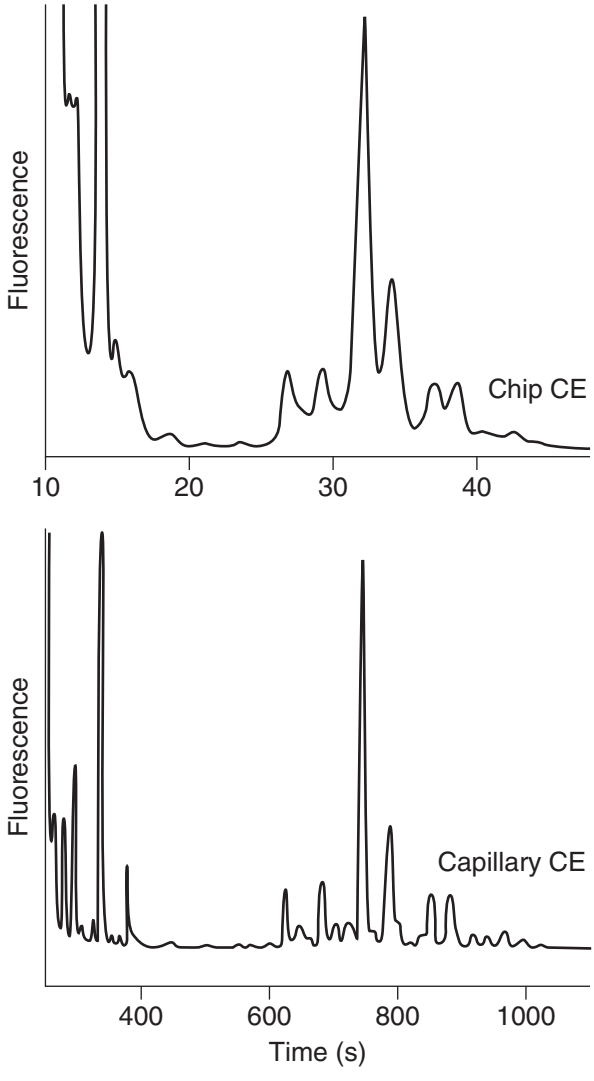



**Figure 4.16** Electropherogram of glycans released from plasma glycoproteins using standard CE equipment with 35 cm separation length and MCE with a separation distance of 14 mm. Upper and lower axes correspond to the time (seconds) of the CE and microchip electrophoresis separation, respectively. Reproduced from Smejkal *et al*. [105] with permission of Elsevier.

Technologies approach to a fully automated MCE system, the Automated Liquid Processing Unit 5100 series, was introduced in early 2005 and withdrawn from the market only 20 months later). Multiple separation channels can be realized but have remained a technological challenge. Multicapillary instruments, on the other hand, dominate in certain application areas for CE (clinical, DNA sequencing).

Although it seems very attractive to implement unit operations in sample preparation on a chip, one should keep in mind that structuring of the device is a large specialized effort, complicates the flow paths on the chips, affects manufacturing cost and reliability, and renders a microfluidic chip useful for that particular purpose only.

CE allows several detection methods besides spectrophotometry. Laser-induced fluorescence or contactless conductivity detection is provided by ancillary devices that are easily coupled. Coupling of CE with MS is now offered as commercial solution in different implementations. However, in the author's view, this is the area that may help MCE to have a breakthrough.

With these considerations in mind, it is not surprising that commercial MCE instrumentation has made a modest impact on the analytical instrumentation market. As other scientists have expressed, MCE will be successful only by seamless integration in an analytical workflow thereby integrating operations from conventional workflows on the microfluidic format.

Even more so, this applies to the integration of MS detection on this platform. To the author, rather than having a large-sized CE system coupled via a long transfer capillary to a MS it is more attractive and appealing to have a comprehensive, CE-driven microfluidic separation device as an inlet to a mass spectrometer. Such an exclusive, intimate, and seamless application of MCE as an MS sample introduction device would pair high speed of analysis and moderate separation efficiency with convenience and simplicity of the system. The previous sections and other work have more than delivered a proof of principle of the MCE-MS technology.

However, in this market, progress in chemical analysis instrumentation is not driven by new technological and methodological abilities but by the instrumentation industry leaders who look at addressable market size, putative customer needs, competitive threats, and, in particular, return of investment considerations. Compared to the WW business volume of (U)HPLC (instruments only, 1528 M$ in 2014), the CE instrumentation market is a niche (CE 116 M$ and MCE, 51 M$ in 2014). To be successful in a niche market, one has to offer irrefutable advantages over general-purpose (U)HPLC offerings that are adapted to a particular, important analytical question.

Commercial, analyzer-type MCE solutions (such as the Agilent Bioanalyzer) can be regarded an innovation by serving highly specific applications in life science research excellently (for a technology to be called an innovation, its application must provide a better solution for solving user problems and customer requirements or open a pathway to solving unmet problems and become widely accepted and commercially successful).

Conversely, for a new analytical instrumentation technology to be introduced on the market and to become available to the community of (bio)analytical scientists, it must meet the most essential customer needs such as analytical figures of merits, (LODs, accuracy, and precision), ease of method development, excellent diagnosis tools and/or supplier support, reliability, ease of use, match with lab infrastructure and staff, and a good fit with sample preparation protocols and

sample size. But most importantly, it must be able to answer an unmet or poorly solved analytical question.

From this assessment, and without commercial introduction of MCE inlet device for MS, MCE-MS will remain an attractive field for research by skilled schools and institutions and a promise for the clientele in the market.

## Appendix

**Table A.1** Overview of the most important relevant sources of microfluidic chips for capillary electrophoresis.

| Company | Technologies, materials and products | Location and website |
|---|---|---|
| Micronit Microfluidics | Design, prototyping, and interfacing microfluidic chips for capillary electrophoresis; fabrication of glass, silicon, polymer chips; packaging and interfacing | The Netherlands, http://www.micronit.com/technologies/lab-on-a-chip-platforms/chips-electrophoresis |
| Micralyne | Development and manufacturing of custom-designed microfluidic chips; technologies for glass and silicon chips; packaging and interfacing | Canada, http://www.micralyne.com/microfluidic-chips/ |
| Microfluidic ChipShop | Development and fabrication of customized microfluidic chips; glass, silicon, ceramics, metal; interfacing and packaging | Germany, http://www.microfluidic-chipshop.com |
| microLIQUID | Design, development of microfluidic chips; polymer chips (SU-8, PMMA, PC, PDMS, COC); integration of electrodes, interfacing, and packaging | Spain, http://www.microliquid.com |
| MicruX Technologies | Design, development, and interfacing of lab-on-a-chip devices; polymer (SU8)/glass hybrid devices with electrochemical detection; chips for capillary electrophoresis | Spain, http://www.micruxfluidic.com |
| iX-factory GmbH | Design, development, and interfacing of microfluidic chips | Germany, http://www.ix-factory.com |

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# 5
# On-Line Electrophoretic, Electrochromatographic, and Chromatographic Sample Concentration in CE-MS

*Joselito P. Quirino*

## 5.1
## Introduction

Mass spectrometry (MS) is a selective and sensitive analytical technique. Capillary electrophoresis (CE) is a high-efficiency microscale analytical separation technique with several modes available for the separation of small-to-large molecules. Thus, CE with an MS detector or CE-MS has become a powerful hyphenated analytical technique that has been implemented for many types of analysis including those in the environmental and biological fields. The most commonly used interface is the electrospray ionization (ESI), which is standard in most commercial MS instruments for CE and liquid chromatography (LC).

The amount of sample loaded into the column in CE is typically very small (i.e., nanoliter range) and this sample requirement causes problems with detection even with the MS. Off-line sample concentration methods such as liquid–liquid extraction or other extraction techniques are commonly used to solve this issue as well as to clean up the sample prior to CE-MS. The target analytes are transferred or transformed from a large to a small volume of sample solution. The enrichment can be controlled by the volume ratio of the initial and final samples. Another way to improve detection in CE-MS is by on-line sample concentration. The amount of sample loaded or target analytes injected into the column is at least 10× larger than the typical, in order to achieve at least an order of magnitude improvement in concentration sensitivity. Larger sample volumes are, however, often required to achieve concentration factors that will be relevant for the analysis of trace analytes in real samples.

On-line sample concentration for CE-MS in this chapter is divided into two sections, electrokinetic and chromatographic. Electrokinetic sample concentration is achieved by manipulation of the analyte electrophoretic or effective electrophoretic velocities. This could be achieved by changing the pH, conductivity or by adding additives of/to the sample or separation solutions in CE-MS. The velocity of the analytes is different in the sample and separation solutions. The difference in the analyte velocity at the two different solutions is responsible for the preconcentration effect. This area is reviewed every 2 years by Breadmore

*Capillary Electrophoresis–Mass Spectrometry (CE-MS): Principles and Applications,*
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and colleagues in the journal Electrophoresis, not only for CE-MS applications but also for CE with other detectors such as UV [1–3]. The advancements in electrokinetic sample concentration could be regarded as one of the drivers in the successful application of CE for trace analysis, which was probably not imagined due to the inherent poor concentration sensitivity of CE. Chromatographic sample concentration is solid-phase extraction (SPE) integrated into the CE-MS platform. As in typical off-line SPE, the sample is enriched in the SPE adsorbent, the adsorbent is washed, and then the enriched analytes are desorbed using an appropriate solvent/diluent. For in-line or on-line integration, the enriched analytes in the solvent stream is directed to the CE-MS for analysis. The area of coupling SPE to CE is also reviewed every 2 years by de Jong and colleagues in the journal Electrophoresis [4–6].

## 5.2 Electrophoretic and Electrochromatographic Sample Concentration or Stacking

Electrophoretic and electrochromatographic sample concentration is performed by the effective manipulation of the sample and separation solution chemistry, additives, and organic solvents [7]. In the scientific literature for CE, this is known as *stacking*, where the target analytes in the sample are concentrated by accumulation into the so-called stacking boundary. The boundary is formed between discontinuous systems, and this is simply achieved by changing the solution chemistry of the sample and/or separation solutions. After stacking, the analytes in the boundary are eventually separated and brought to the detector by electro-osmotic flow (EOF) and/or electrophoretic velocity. The translation of stacking techniques in CE is restricted by the choice of chemistries amenable to ESI and MS. The components entering the ESI and MS should normally be volatile [8, 9]. The electrophoretic stacking techniques, which have been demonstrated for CE-MS, are transient isotachophoresis (t-ITP), field amplification/enhancement, and dynamic pH junction. The electrochromatographic stacking techniques are sweeping and micelle-to-solvent stacking (MSS)/analyte focusing by micelle collapse (AFMC). The fundamental basis of these techniques and some applications are described here.

### 5.2.1 Electrophoretic Stacking Techniques

Electrophoretic stacking techniques are used for ionized and ionizable analytes. These are based on isotachophoretic (ITP) effects, changes in the electrophoretic mobility of the analytes by pH effects (dynamic pH junction), and increases in the electrophoretic velocity of the analytes in the sample zone inside the capillary by preparing the sample in a low-conductivity solution (field amplification). ITP and field amplification are amenable for both ionized and ionizable analytes while dynamic pH junction is only for ionizable analytes.

#### 5.2.1.1 Transient Isotachophoresis or t-ITP

t-ITP is a powerful stacking technique in CE-MS and was first demonstrated by Foret and coworkers with improvement in concentration detection limits by two orders of magnitude, which translates to a sensitivity enhancement factor (SEF) of 100 [10]. Stacking by t-ITP is for ionized and ionizable analytes. Isotachophoresis or ITP is a mode of CE with two basic components, the leading electrolyte (LE) and terminating electrolyte (TE). The leading and terminating ions (either negative or positive) have the highest and the lowest electrophoretic mobility, respectively. A counterion is normally chosen to control the pH. The capillary is filled with the LE and then the sample ions with mobilities between the leading and terminating ions is injected. A suitable voltage is applied with the LE and TE in the inlet and outlet ends of the capillary, respectively. The sample ions arrange in the order of decreasing mobility. The analyte with the highest mobility is at the back of the leading ion, while the analyte with the lowest mobility is at the front of the terminating ion. The sample ion zones are directly adjacent to each other and thus are detected as steps with an on-line photometric detector. An interesting characteristic of the ITP zones is that the concentration of all ions adjusts to the concentration of the leading ion as dictated by the Kohlrausch regulating function [11]. Thus, there is normally an increase in the sample ion concentration in ITP, and this characteristic has been exploited for sample concentration. For sample concentration, the ITP is conducted at the start of the run or right after sample injection (see Figure 5.1a). The ITP stacking boundary is initially found directly adjacent to the LE. The electrophoretic conditions are controlled such that after the sample ions are concentrated by ITP (see Figure 5.1b), the ITP state disappears and the sample ions separate by electrophoretic mobility (see Figure 5.1c). This is the reason why the stacking technique is called *t-ITP* since ITP only lasts for a short time.

The electrolytes directly amenable for use as separation solutions in CE-MS with an ESI are limited [12]. The use of nonvolatile electrolytes or additives requires additional care. With this in mind, the choice of leading and terminating electrolytes for t-ITP is also limited. Table 5.1 taken from Ref. [9] lists the ionic species suitable for CE-MS. As a simple strategy, the neutralization of $H^+$ or $OH^-$ by the

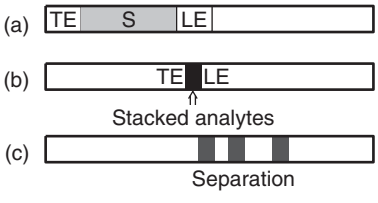


**Figure 5.1** Simplified schematic of t-ITP stacking in capillary electrophoresis. (a) The leading electrolyte (LE) is injected before the sample solution (S). The terminating electrolyte (TE) is injected. The electrophoretic mobility of the leading ion is faster than the sample and terminating ions, all having the same sign of charge (+ or −). (b) Voltage is applied and the sample ions are stacked behind the leading ion. This ITP state is transient and dissipates with the continued application of voltage. (c), The t-ITP stacked sample ions separate by electrophoresis.

**Table 5.1** Ionic species suitable for CE-MS and their $pK_a$ values and ionic mobilities.

| | $u^{a)}$ | p$K$ |
|---|---|---|
| $H^+$ | 362.5 | – |
| $NH_4^+$ | 76.2 | 9.25 |
| $Imid^+$ | 52.0 | 7.15 |
| $OH^-$ | −202.5 | – |
| $Fo^-$ | −56.6 | 3.75 |
| $Ac^-$ | −42.4 | 4.76 |
| $HCO_3^-$ | −46.1 | 6.35 |
| $CO_3^{2-}$ | −71.8 | 10.33 |
| $EACA^-$ | −28.8 | 10.8 |
| $MES^-$ | −28 | 6.1 |
| $Asn^-$ | −31.6 | 9.03 |

a) $\ln 10^{-9}\ m^2\ V^{-1}\ s^{-1}$.
Reproduced from Pantůčková *et al.* [9] with permission of Wiley.

appropriate counterion in the leading electrolyte can be utilized for t-ITP. For example, in t-ITP of cationic analytes, the leading ion could be ammonium with $H^+$ as the terminating ion formed by neutralization with acetate included in the leading electrolyte [13]. This system was used by Mayboroda and coworkers on enhancing the coverage of the urinary metabolome [14] and Lee and coworkers on the analysis of trace peptides in proteome analysis [15]. In the case for anionic analytes, the leading ion could be acetate with $OH^-$ as the terminating ion formed by the neutralization with the basic counterion [16].

Figure 5.2 shows the t-ITP work of Xia and coworkers on the analysis of brain and intestine peptides [17]. As shown in Figure 5.2a, or without t-ITP, most of the peptides were detected as broad peaks. With t-ITP, the peptides were detected as sharp peaks with $H^+$ (see Figures 5.2b) or β-alanine (see Figures 5.2c) as terminating ion. The leading ion (ammonium in 50 mM ammonium acetate, pH 4.8) was injected into the capillary previously filled with 50 mM acetic acid at pH 3.1. The sample cations were electrokinetically injected, and separation was conducted with 50 mM acetic acid at pH 3.1. In Figure 5.1b, the t-ITP occurred with the $H^+$ from the separation electrolyte in the inlet vial as terminating ion, which was neutralized by the acetate ions inside the capillary. This is more convenient than the use of β-alanine as terminating ion, which was also an additional injection step as shown in Figure 5.2c.

The neutralization of $H^+$ or $OH^-$ for t-ITP stacking in CE-MS seems to be the most popular and convenient approach to increase detection sensitivity of cationic analytes [18–21]. There were a few reports on the use of other terminating ions for t-ITP stacking of anionic analytes. Gareil and coworkers used acetate and glycine as the leading and terminating ions for the analysis of nerve agent degradation products [22]. Sensitive determination (LODs between 4 and 75 ng $ml^{-1}$) was

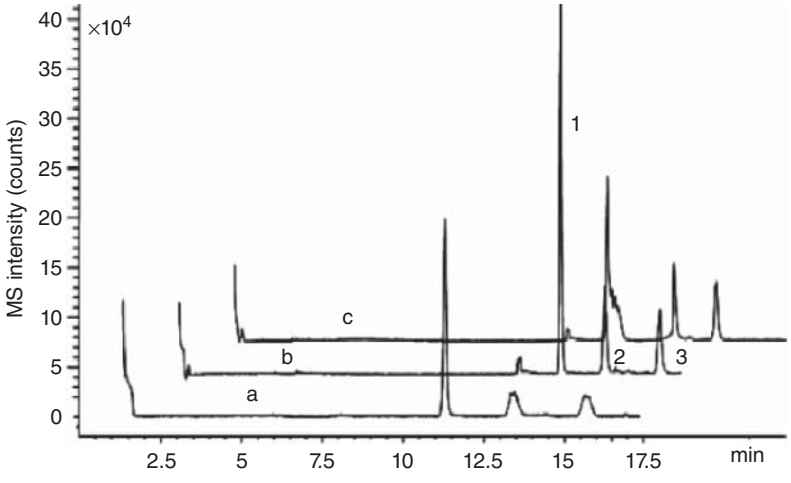


**Figure 5.2** t-ITP CE-MS of cationic peptides. Conditions: Bare fused-silica capillary 104 cm × 50 mm; Separation voltage, 25.0 kV; LE, 50 mM ammonium acetate (pH 4.8); Separation electrolyte, 50 mM acetic acid (pH 3.1); Sample, the three peptides mixture in 25 mM acetic acid; Sample injection, 28 kV for 40 s; (a) Without t-ITP; (b) t-ITP with $H^+$ as terminating ion where 50 mM ammonium acetate (pH 4.8) was injected (50 mbar for 60 s) prior to sample injection; (c) t-ITP with β-alanine as terminating ion where 50 mM ammonium acetate (pH 4.8) was injected (60 s at 50 mbar) prior to sample injection, 40 mM β-alanine (pH 3.6 with acetic acid) was then injected (50 mbar for 20 s). Reproduced from Xia *et al*. [17] with permission of Wiley.

performed for the analytes in soil extract and rat urine. Breadmore and coworkers used bicarbonate and 3-(cyclohexylamino)-1-propane-sulphonic acid as leading and terminating anions for the t-ITP stacking of hypolipidemic drugs with LODs as low as 180 ng $ml^{-1}$ for drinking and wastewater samples [23]. This level of sensitivity was achieved by the combination of t-ITP after field-enhanced sample injection, which is described in the next section. This combined technique is called *electrokinetic supercharging* as suggested by Hirokawa and coworkers [24].

#### 5.2.1.2 Field-Amplified/Enhanced Stacking

The concentration capability of a high conductivity separation electrolyte in CE was first described by Mikkers in 1979 [25]. The change in the concentrations during the electrophoretic process is ruled by the Kohlrausch relations and Ohm's law. If the conductivity of the sample solution (S) that was introduced inside the capillary is lower than the background separation solution (BGS), there will be inhomogeneity in the electric field profile across the capillary (see Figure 5.3a). The field in the S ($E$(S)) will be higher than in the BGS ($E$(BGS)), and thus, the electrophoretic velocity ($v_{ep}$) of the analytes is lower in the BGS since $v_{ep} = E \times$ electrophoretic mobility or $\mu_{ep}$ of the analyte. The cationic and anionic analytes will be stacked in the boundary that separates the *S* and the BGS at the cathodic and anodic sides of the *S* zone (see Figure 5.3b). In the literature, this is known as *field-amplified* or *-enhanced stacking*. The term *field-enhanced*

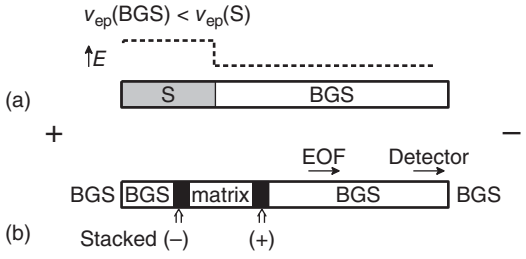


**Figure 5.3** Simplified schematic of field-enhanced or -amplified stacking in capillary electrophoresis with a fused-silica capillary and cathodic electro-osmotic flow (EOF). (a), The sample solution (S) is injected after conditioning the capillary with background solution (BGS) or electrolyte. If a voltage is applied, the field strength in the S > BGS and thus the electrophoretic velocity of the analyte ($v_{ep}$) will be faster in the S than in the BGS. (b) The cations (+) migrated quickly into the right or cathodic boundary between the matrix and BGS. The velocity decreased in the boundary and caused the stacking of the analytes. The same process occurred with the anions (–) but at the anodic boundary.

stacking seems more appropriate since the total electric field is constant and equal to the voltage applied divided by the length of the capillary. The research groups that applied this form of stacking to CE-MS were that of Vouros [26] and van den Eeckhout [27], for the sensitive analysis of DNA adducts. Stacking by field enhancement is for ionized and ionizable analytes.

The concentration of the sample ions can be increased by a factor approximately equivalent to the conductivity ratio (separation electrolyte/sample) [28]. To reach the maximum increase in detection sensitivity, the sample injection must also be increased by a factor equal to this ratio. For example, if the conductivity ratio is 10, the sample must be injected 10 times longer. The problem encountered with field-enhanced stacking is the broadening effect caused by the mismatch in the local EOF velocities. The bulk EOF is constant throughout the capillary, but the higher EOF velocity of the *S* zone pushes slower moving BGS zone. Thus, a high conductivity ratio of $10^4$ will not provide an SEF = $10^4$.

Field-enhanced stacking is performed by hydrodynamic (pressure) or electrokinetic (voltage) injection. This is called *field-enhanced/amplified sample stacking* (FESS/FASS) and *field-enhanced/amplified sample injection* (FESI/FASI), respectively. In FESS, the maximum SEF is around 10 due to broadening discussed earlier but can be increased to 100 or more if the low-conductivity sample matrix is removed prior to separation [28]. The removal of the sample matrix is difficult since there is no electrolyte at the outlet end to replenish the matrix that is pumped out of the column. Nevertheless, the removal of sample matrix in CE-MS was demonstrated by Chung and coworkers [29], by placing a BGS vial at the outlet of the capillary during the stacking. However, each analysis would require the manual manipulation from stacking to actual CE-MS analysis.

In FESI, the sample ions loaded is much larger compared to FESS and the low-conductivity sample matrix introduced into the capillary is also minimized. The injected amount is not limited by the sample volume and thus the SEF values

obtained with FESI are typically $\geq 10^2$. The implementation of FESI in CE-MS would normally require no-EOF or co-EOF conditions. Co-EOF where the both EOF and analyte velocity are directed to the MS detector provides stability in the ESI due to the steady flow of liquid to the spray. Analytes of the same sign of charge (positive or negative) are injected and there could be a bias due to differences in electrophoretic mobility.

Field-enhanced stacking in CE-MS is quite simple and thus has been popularly used. FESS was used by Duan and coworkers to detect and evaluate the antioxidant activity of four hydrophilic active components, danshensu, salvianolic acid B, caffeic acid, and lithospermic acid, in plant-derived samples [30]. FESI has been used by more groups since 2010. Desiderio and coworkers analyzed arginine, monomethylarginine, and (symmetric and asymmetric) dimethylarginines in the human plasma [31]. Ye and coworkers analyzed brain–gut peptides after SPE with detection capabilities at the very low μM level [32]. Hung and coworkers analyzed five haloacetic acids (HAAs) in tap water in combination with flow injection analysis [33]. Figure 5.4 shows the effective FESI (at 3.4 kV for 20 s of sample in water) CE-MS (total ion count) analysis of the studied HAA standard sample. The SEFs for the HAAs dichloroacetic acid (DCAA), trichloroacetic acid (TCAA), monochloroacetic acid (MCAA), dibromoacetic acid (DBAA), and monobromoacetic acid (MBAA) were up to $10^3$. A more recent and interesting application of FESI is by de Jong and coworkers as shown in Figure 5.5 [34]. Using FESI, an average SEF for all potentially genotoxic alkyl halides tested (except for

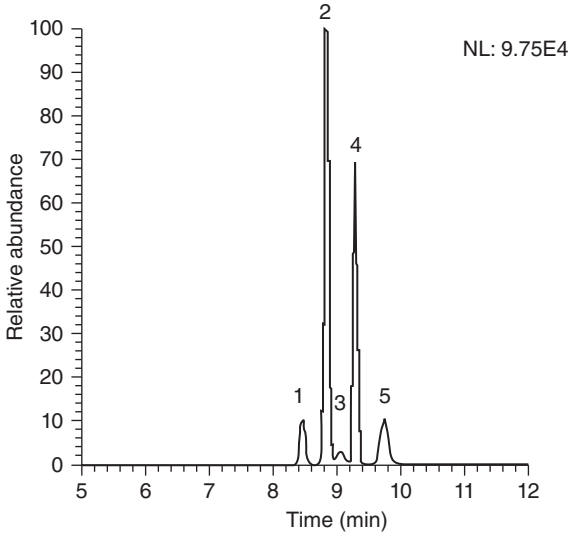


**Figure 5.4** FASS-CE-MS/MS analysis of 10 ppb HAAs in DI water. Separation electrolyte: 50% MeOH containing 2.5% ammonium acetate buffer at pH 3.5. Peak designation: 1, DCAA; 2, TCAA; 3, DBAA; 4, MCAA; 5, MBAA. Reproduced from Hung and Her [33] with permission of Wiley.

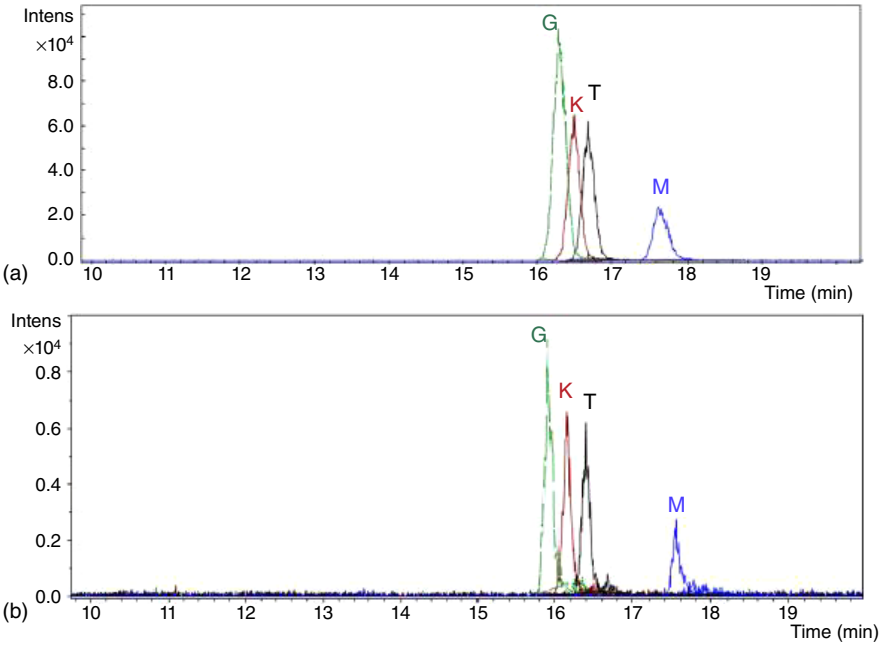


**Figure 5.5** Overlaid extracted ion electropherograms of 50 mg kg$^{-1}$ butyl 1-(pyridinyl-4yl) piperidine 4-carboxylate derivative test mixture of potentially genotoxic alkyl halides (G, K, T, and M) using FESI at 10 kV for 150 s (a) of 500 mg kg$^{-1}$ BPPC derivative test mix using typical hydrodynamic injection (b). Reproduced from van Wijk *et al*. [34] with permission of Elsevier.

alkyl halide L) of around 100-fold is obtained relative to typical injection. Note that the concentration shown in Figure 5.5a is 10 times lower compared to b.

FESI was also used by He and coworkers for cationic drugs clenbuterol, salbutamol, terbutaline, and formoterol in spiked urine [35]. Galceran and coworkers analyzed amprolium in eggs at low concentrations, which are far below the MRL-legislated values after SPE [36]. Ito and coworkers developed a very sensitive CE-MS method with FESI and in combination with a sheathless interface, and MS and tandem MS ($MS^2$) measurements with narrow mass range, repeated high-speed switching; SEF in the order of $2 \times 10^4$ was obtained [37]. The method was used to structurally characterize four kinds of pyridylaminated (PA) oligosaccharides, that is, lactose (Lac)-PA, globotriose ($Gb_3$)-PA, globotetraose ($Gb_4$)-PA, and $IV^3$ αGalNAc-$Gb_4$ (Forssman antigen)-PA, derived from neutral glycosphingolipids.

#### 5.2.1.3 Dynamic pH Junction

The use of pH to concentrate proteins and peptides is classically known in isoelectric focusing [38]. These biomolecules with positive and negative functionalities have an isoelectric point where the net charge is zero. For example, if a weak acid and a weak base are used as anodic and cathodic electrolytes, respectively, a pH gradient is generated. The analytes inside the capillary will migrate by

electrophoresis until it encounters a pH zone that is equal to its isoelectric point. The analyte molecules stop to migrate at this zone since it loses its charge. This leads to sample concentration of the proteins or peptides at different parts of the capillary. A similar analogy can be formulated for weakly ionizable analytes. Consider a weak acid with an acid dissociation constant ($pK_a$) of 7, and this acid is basically neutral at pH 5 and thus will focus in a zone inside the capillary with a pH $\leq$ 5.

The use of a sample diluent with a different pH than that of the BGS for sample concentration was described in CE by Aebersold and Morrison [39] for peptides. This approach was then termed as dynamic pH junction by Britz-McKibbin and Chen [40] for ionizable analytes with electrophoretic velocities (mobilities) that significantly change at the pH of the *S* and BGS. Stacking with dynamic pH junction is strictly for ionizable analytes. The pH of the S or BGS must be $\geq pK_a$ of each analyte $\pm$2 such that the analyte will be neutral in one of the solution zones. Amphoteric analytes can also be concentrated due to their isoelectric points and ability to reverse charge at two completely different pH values.

For example, if the pH of the *S* that was introduced inside the capillary is much lower than the BGS, there will a pH boundary that will be formed between these zones. The analyte is weakly acidic and prepared in a sample diluent with a pH = $pK_a$ (of the analyte) – 2. Upon application of voltage, the *S* zone is titrated by the hydroxide ions from the BGS zone. This causes the ionization of the neutral acidic analytes in the *S* zone. These ionized analytes migrate into the acidic *S* zone where they are protonated and become neutral again. The analytes are accumulated at the pH boundary that will eventually sweep through the injected S zone. Once all the acid in the *S* is neutralized, the analytes will separate by electrophoresis because of the basic BGS.

It is noted that the conditions for the normal dynamic pH junction is similar to t-ITP with $H^+$ and $OH^-$ neutralization. The $OH^-$ acts as the terminating electrolyte as it sweeps through the *S* zone. Therefore, it is highly possible that the stacking of ionizable analytes via the use of pH (i.e., dynamic pH junction) is also contributed by ITP effects. In the case where the weakly acidic analyte is prepared in a high pH diluent, the analytes will accumulate as neutrals in the low-pH BGS, and this is somewhat reminiscent of isoelectric focusing. This was also referred to a reversed dynamic pH junction and no separation of the stacked analytes can occur.

Ye and coworkers reported a stacking CE-MS method with LODs in the 0.2–2.0 nM range for four peptides with similar p*I* values (p*I* approx. = 5.5) [41]. The BGS and sample matrix were 0.5 M formic acid at pH 2.15 and 50 mM ammonium acetate at pH 7.5, respectively. The sample *S* was hydrodynamically injected as a long plug, and separation was at positive polarity where the peptides were analyzed as cations. During stacking, the protons from the acidic BGS in the inlet vial penetrated the injected S zone. This caused protonation of the peptides that were initially negatively charged in the *S* zone. The reversal in the electrophoretic mobility due to the change in the pH caused the peptides to stack at the pH boundary as per the definition of dynamic pH junction. There should also be an additional stacking effect due to t-ITP, whereby the ammonium ion in

the *S* and the protons from the inlet vial acted as leading and terminating ions, respectively. The t-ITP effect could be verified from their study on the effect of ammonium acetate concentration on the stacking effect. The increase in the concentration of ammonium acetate has led to sharper and taller peaks as shown in Figure 5.6. Note that in t-ITP, a higher concentration of leading ion (in this case, ammonium) provides a stronger focusing effect. Bai and coworkers used a very similar approach for the CE-MS analysis of systemin, an important group of plant peptide hormones that participate in the regulation of plant defense responses [42]. The sample was prepared in 60 mM ammonium formate at pH 6.7 while the BGS was acidic 3.5% formic acid. SEFs for the six target systemins ranged from 90 to 127 and LODs were <0.5 nM. For analysis of real samples (e.g., tomato and tobacco), sample preparation by SPE was required prior to stacking CE-MS.

Dovichi's research group recently published a few papers on the use of dynamic pH junction for improved detection in CE-MS for proteomic analysis. In one of their reports [43], dynamic pH junction injection of a 0.1 mg $ml^{-1}$ *Escherichia coli* (*E. coli*) digest has been shown to identify 527 peptides and 179 proteins. For comparison, a typical injection of a 10× more concentrated sample identified 508 peptides and 199 proteins. In the same paper, an SEF of 10 was also obtained for three intact proteins by a stacking injection. They combined the use of an on-line strong cation-exchange SPE strategy with dynamic pH junction [44]. Tryptic digest of *E. coli* was concentrated in the SPE sorbent and was eluted by a pH gradient step (all with a higher pH and lower conductivity than the BGS). The use

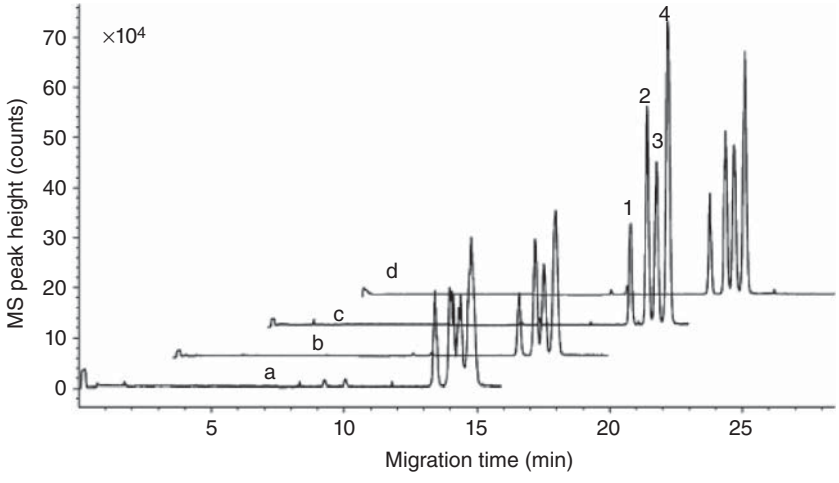


**Figure 5.6** Effect of sample matrix concentration on focusing using dynamic pH junction and t-ITP. Conditions: bare fused-silica capillary, 90 cm × 50 mm; BGS, 0.5 M formic acid at pH 2.15; sample matrix, ammonium acetate at pH 7.5 with concentration varied (a) 10 mM, (b) 25 mM, (c) 50 mM, and (d) 75 mM; injection, 30 s of a mixture containing 1 mmol $l^{-1}$ of each peptide; sample i.d., (1) L-Ala-L-Ala, (2) L-Leu-D-Leu, (3) Gly-D-Phe, (4) Gly-Gly-L-Leu. Reproduced from Ye *et al*. [41] with permission of Wiley.

of an acidic BGS for capillary zone electrophoresis (CZE) separation allowed for further focusing by dynamic pH junction of the eluted sample. An improved number of peptide and protein identifications were performed, and the performance of the total SPE-stacking-CE-MS method was comparable to a nanoLC-MS method. A similar system [45] was applied to the analysis of bovine serum albumin tryptic digest where 109 proteins and 271 peptides were identified in a 20 min separation.

### 5.2.2 Electrochromatographic Sample Concentration

Electrochromatographic stacking techniques are used for ionized, ionizable, and neutral analytes. These techniques are based on the interaction of the analytes with an additive (sweeping and AFMC), micellar collapse (AFMC) and the effect of organic solvents on the interaction between analyte and additive (MSS). Sweeping and AFMC are amenable to both charged and neutral analytes while MSS is only for charged analytes. These stacking techniques are not popularly used in CE-MS probably due to the use of an additive that could affect the ESI-MS performance. In addition, electrokinetic chromatography (EKC) methods require a pseudostationary phase (PS) or additive (e.g., micelles) to affect the separation. The use of advanced EKC mode of partial filling (PF-EKC) is required for ESI-MS detection in order to prevent the entry of the additive into the detector source.

#### 5.2.2.1 Sweeping

The main condition for zone narrowing in sweeping is that no PS is in the injected analyte mixture [46]. When a voltage is applied after sample injection, the PS penetrates the sample zone and picks up the analyte molecules due to their interaction with the PS. The analytes are concentrated at the front of the PS. Complete stacking is achieved once the PS completely migrated through the sample zone. The length of the swept zone is predicted to be narrowed by $1/(1 + k)$, where $k$ is the retention factor; thus, high SEFs can be obtained for analytes with high affinities to the PS used.

Sweeping as a preconcentration technique in EKC with MS was first shown by Isoo and coworkers, with an atmospheric-pressure chemical ionization interface [47]. They obtained SEFs in the order of $10^2$ for the studied organic pollutants. Amundsen and coworkers performed sweeping of neutral endogenous steroid hormones in PF-EKC with sodium dodecyl sulfate (SDS) micelles as PS. The EKC separation was important because the ESI-MS behaviors of testosterone and epitestosterone were identical [48]. The performance of sweeping with online UV was found to be superior compared to ESI-MS detection. In contrast, SEFs from $10^1$ to $10^2$ were obtained for dialkyl phthalates by sweeping PF-EKC with UV or ESI-MS [49]. The poor ionization efficiency of some compounds with ESI-MS may account to the poorer SEFs compared to UV detection.

Some additives or PS may be introduced together with the analytes into the ESI-MS source. For example, ethylenediaminetetraacetic acid was used as sweeping and separation additive for the CE-ESI-MS analysis of some metal

ions [50]. Palmer and coworkers used latex nanoparticles synthesized using *ab initio* reversible addition–fragmentation chain transfer in emulsion polymerization in EKC without partial filling [51]. This nanoparticle PS has been shown to provide efficient and selective separations with no measurable effect on the separation current and minor effects on analyte ionization efficiency during ESI. The PS was effectively used as sweeping agent that yielded low detection limits (10–16 ppb), particularly for more hydrophobic compounds studied.

#### 5.2.2.2 Analyte Focusing by Micelle Collapse or AFMC

AFMC and MSS are related techniques developed in our laboratory where surfactants are used to transport micelle-bound analytes to the stacking boundary or sb where the analytes are concentrated. The procedure and mechanism for neutral AFMC in PF-EKC with ESI-MS are shown in Figure 5.7 [52]. The sample prepared in a micellar sample matrix or *S* is injected after injection of a micellar solution (boxed zone) that is for PF-EKC separation (see Figure 5.7a). The capillary was initially filled with BGS that is compatible for ESI-MS. The electrolyte used to prepare the BGS is also used in the preparation of the sample matrix and micellar solution for separation. The concentration of the surfactant micelles in the sample matrix is lower than in the separation solution. To induce micelle collapse at the sb, an organic-solvent-rich or low-conductivity electrolyte plug is injected after the *S*. For example, if 10 mM SDS is used in the sample matrix, 30% acetonitrile can be used as organic-solvent-rich plug since the critical micelle concentration (cmc) of SDS in 30% acetonitrile is ~12 mM [53]. SDS micelles will not survive in

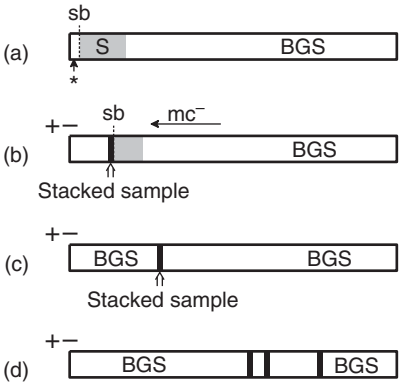


**Figure 5.7** Simplified schematic of analyte focusing by micelle collapse or AFMC in PF-EKC with a cathodic EOF. (a) The S of neutral analytes prepared in a micellar solution is injected after conditioning the capillary with BGS (nonmicellar) and injection of micellar solution for separation. The symbol * represents a low-conductivity electrolyte or organic-solvent-rich solution for micelle collapse. (b) Application of voltage caused the analytes to accumulate at the sb. (c) A final AFMC zone is formed when all the analytes are transported and released into the sb. (d) The micelles from the micellar solution penetrated the AFMC formed zone and caused them to separate by EKC principles. Note that the EOF is to the cathode where the detector is also located.

30% ACN plug because the concentration of SDS entering the solvent-rich plug is below the cmc.

When a voltage is applied (see Figure 5.7b), the micelles will transport the analytes to the sb where the micelles collapse. The collapse of the micelles releases the transported analytes and a more concentrated zone of the analyte is formed when all the micelle-bound analytes reached the sb (see Figure 5.7c). Injection of the sample matrix (without the analytes) after injection of the micellar solution for separation may also be required for low $k$ analytes; this is to improve their transport to the sb. The micelles from the boxed zone also reach the sb, but these micelles will not collapse because the concentration of the surfactant is high. The analytes then separate by EKC principles and migrate out of the micellar zone and then into the ESI-MS detector (see Figure 5.7d).

#### 5.2.2.3 Micelle to Solvent Stacking or MSS

MSS relies on the reversal in the effective electrophoretic mobility of the charged analyte in the sb [54, 55]. The sb or micelle-to-solvent stacking boundary (MSSB) is between a micellar and an organic-solvent-rich solution. The charged sample and surfactant micelle must not have the same sign of charge, that is, cationic and anionic analytes are focused by anionic and cationic micelles, respectively. The direction of the mobility is dictated by the micelles in the micellar zone. In an organic-solvent-rich zone, the influence of the micelles to the mobility is significantly reduced such that the sign of the mobility is dictated by the charge of the analyte.

Selective stacking can be easily achieved by MSS in co-EOF CZE ESI-MS. The concentrated analytes migrate to the ESI-MS by electrophoresis and EOF. The micelles electrophoretically migrate in the other direction; thus, the surfactants do not interfere with analyte detection. There is also some flexibility in the preparation of sample since MSS can be by preparation of the sample in the micelles or in organic-solvent-rich solution [56]. The improved sensitivity of some β-blockers drugs by MSS in CZE-MS is shown in Figure 5.8. The SEFs reported for MSS translates to one order of magnitude improvement in concentration sensitivity; however, when combined to other stacking modes, SEF approaching $10^3$ is highly plausible. For example, Wuethrich and coworkers obtained SEF of 500 when MSS was combined with FESI for the CZE chiral analysis by PF with 2-hydroxypropyl-β-cyclodextrin of a racemic drug (i.e., chlorpheniramine) [57].

## 5.3 On-line/In-line SPE with CE-MS

This section deals with the (on-line and in-line) integration of SPE to CE-MS where there is a direct stream of liquid connecting the two analytical schemes (SPE and CE-MS). In the on-line integration, the direct transport of liquid from the SPE device to the CE-MS is by the use of connecting columns or capillaries. In the in-line integration, which is the highest level of integration of sample preparation

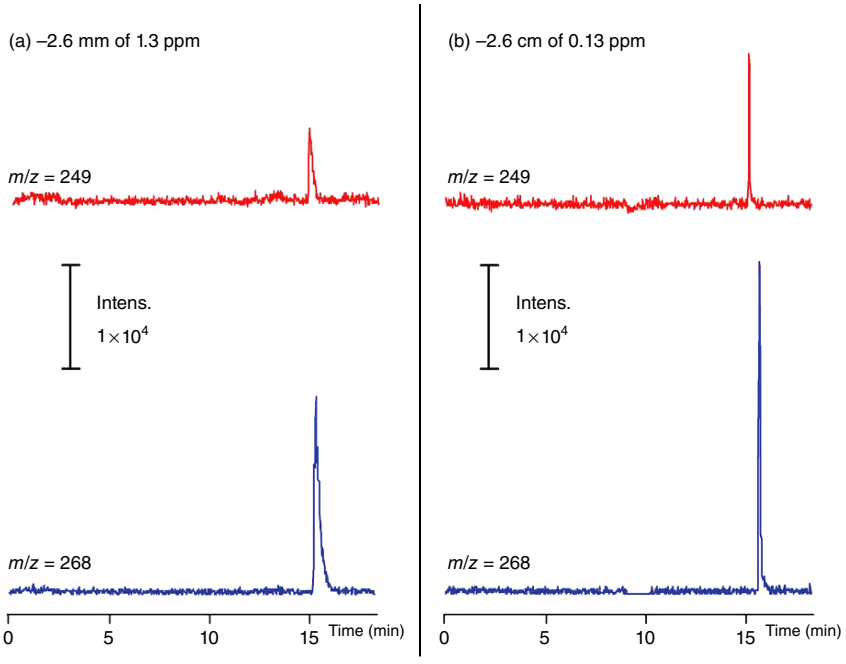


**Figure 5.8** Extracted base peak electropherograms for pindolol ($m/z = 249$) and metoprolol ($m/z = 268$) analyzed by CZE-ESI-MS (a) and MSS-CZE-ESI-MS (b). BGS = 30 mM ammonium acetate, pH 5 with 20% acetonitrile; sample matrix = BGS (a) and 10 mM SDS with 13 mM ammonium acetate, pH 5 (b); $S = 1.3\ \mu g\ ml^{-1}$ (a) and $0.13\ \mu g\ ml^{-1}$ (b) of pindolol and metoprolol; injection scheme: 2.6 mm (6 s at 50 mbar) of sample solution (a) and 0.6 cm (15 s at 50 mbar) of sample matrix followed by 2.6 cm (60 s at 50 mbar) of sample solution (b). Reproduced from Quirino [54] with permission of Elsevier.

and separation, the SPE device and separation capillary in CE-MS formed one analytical unit.

### 5.3.1 On-line SPE

The general design for on-line integration of SPE to CE-MS is to have a connecting column between the two independent systems. The advantage of such a design is that analyte concentration and washing in SPE can be performed independently. The connecting column could be switched to/from a waste reservoir/CE column, and thus, only the target concentrated fraction could be selectively analyzed by CE-MS. Her and coworkers reported the on-line integration of SPE to CE-MS where the system was evaluated for the analysis of peptides as mixtures or from protein digests [58, 59]. Figure 5.9 shows the schematic of their system, which consisted of a two-leveled two-cross PDMS interface, which connected the SPE to the analytical capillary [59]. The two-cross design allowed the independent operation

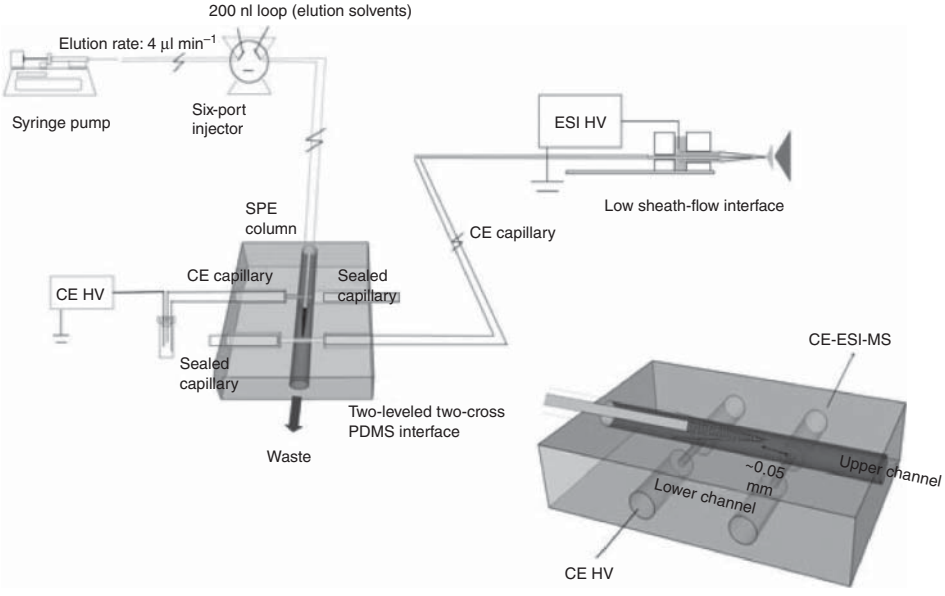


**Figure 5.9** Schematic of on-line SPE CE-MS. Reproduced from Lee *et al*. [59] with permission of Wiley.

of the SPE without unexpected flow through leakage to the analytical capillary. As shown on the top left of the figure, a syringe pump was used to deliver the BGS. One end of the SPE column was connected to a six-port injector, and the tapered end was inserted into the interface. The outlet of the SPE column was positioned right before the separation channel. A 200-nl loop was fabricated using a fused-silica capillary (360 µm o.d., 50 µm i.d.) for sample injection. Two sealed capillaries were used as blocking plugs within the interface. Also in the interface were the buffer delivery column and the section of the separation capillary, which was then attached to the low sheath flow and MS. The test peptide signals were enhanced up to 100-fold as shown in Figure 5.10 [58]. Note that, as shown in Figure 5.10a, the concentration of the peptides was 50× higher than that shown in Figure 5.10b. With the developed on-line SPE CE-MS and by a multistep (via sequential or staggered CE injections) elution process, the identification of peptides from protein digests was improved. The staggered CE injection gave the same performance as the sequential injection but with ~50% reduction in analysis time [59].

### 5.3.2
### In-line SPE

In in-line SPE, all the components of the sample solution loaded in the SPE pass through the analytical CE-MS column. The elution and separation conditions in SPE and CE-MS, respectively, must therefore be carefully selected in order to get the desired analytical performance. Since 2009, majority of the efforts have been

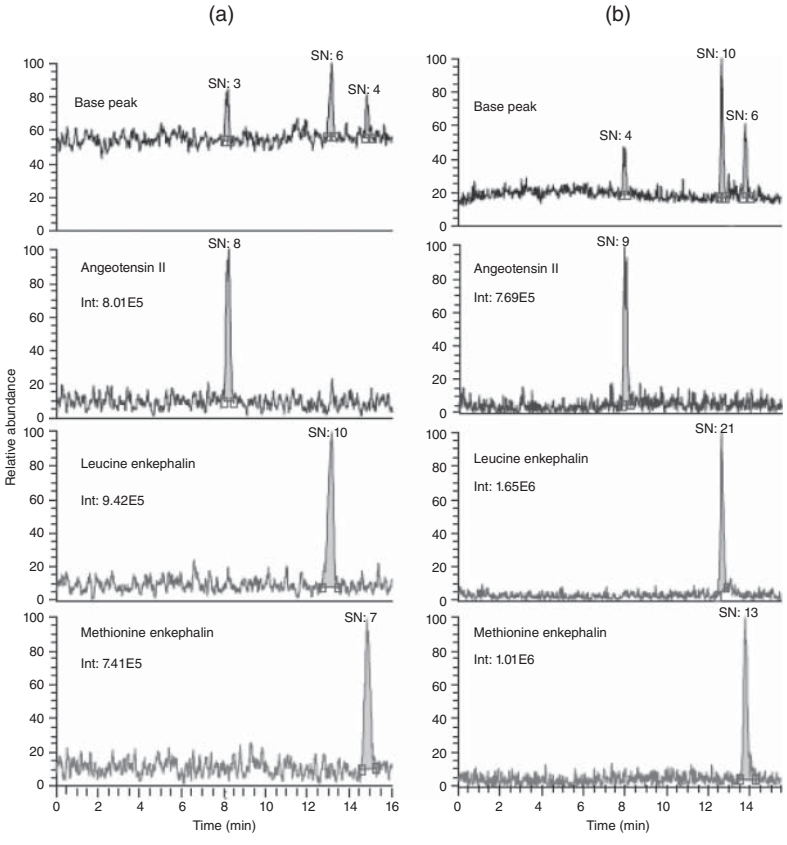


**Figure 5.10** Electropherograms of CE-MS and SPE-CE-MS analysis of a peptide mixture. (a) CE-MS analysis of a peptide mixture at a concentration of 0.25 mg ml$^{-1}$. (b) SPE-CE-MS analysis of a peptide mixture at a concentration of 5 ng ml$^{-1}$, loading sample volume 50 µl. The CE condition: 10 mM, pH 3.80 ammonium formate as the running buffer; the separation capillary: 50 cm, 50 µm i.d. A 400 V cm$^{-1}$ electric field was applied for SPE-CE-MS and CE-MS experiments. Reproduced from Lee and Her [58] with permission of Wiley.

on the development of in-line systems coming from the research groups of Aguilar, De Jong, and Sanz-Nebot, and their coworkers. Considered in Figure 5.11 is the work of Medina-Casanellas and coworkers in the preparation of an SPE microcartridge that was attached in-line to an analytical capillary [60]. The preparation of microcartridges was normally guided by using a microscope. In Figure 5.11a, a piece of fused-silica capillary (150 µm i.d. and 365 µm o.d.) was introduced for approximately 2 mm into a 10-mm PTFE sleeve of 350 µm i.d.. The use of a PTFE sleeve provided tight connections, and thus, adhesive sealing was not required. This was then cut to a total length of 4 mm. The 50 µm i.d. inlet capillary (7.5 cm) was connected to the microcartridge via the other end of the PTFE sleeve. In Figure 5.11b, the microcartridge was dry-packed with sieved C18 sorbent particles

by applying vacuum through the inlet capillary. In Figure 5.11c, the packed microcartridge was carefully pushed toward the middle of the PTFE sleeve using the outlet capillary (50 μm i.d. and 365 μm o.d.). In Figure 5.11d, in order to minimize the dead volume and leaking at the joints, all the capillary ends were cleanly cut and straight. The excellent performance of this SPE microcartridge for the preconcentration of opioid peptides spiked in cerebrospinal fluid (CSF) is shown in Figure 5.12.

The selectivity of the extraction or concentration can be modified by changing the adsorbent used in the SPE microcartridge for CE-MS. Two reports from Botello and coworkers and Medina-Casanellas and coworkers used reversed-phase SPE material for peptides [61, 62]. In the latter work [62], the implementation of t-ITP to the SPE eluate provided an additional 10-fold improvement in sensitivity with LODs of 0.01 in standards and 0.1 ng $ml^{-1}$ in plasma samples. Medina-Casanellas and coworkers reached LODs in the pg $ml^{-1}$ range by using a sheathless CE-MS for the analysis of opioid peptides [63]. Jooß and coworkers used mixed mode (reversed phase and weak anion exchange) for aromatic sulfonic acids and 8-aminopyrene-1,3,6-trisulfonic acid–labeled glycans and reported >800-fold improvements [64]. Maijó and coworkers evaluated different Waters brands, Oasis HLB, Oasis MCX, and Oasis MAX, which were hydrophilic/lipophilic, mixed cation exchanger, and mixed anion exchanger adsorbents, respectively [65]. Three to four orders of concentration magnitude improvements for UV filters (mainly phenones) were achieved. Tak and coworkers also used the Waters HLB to trap different-polarity compounds (i.e., 2-ethylidene-1,5-dimethyl-3,3-diphenylpirrolidine, dihydrocodeine, and codeine) and reached LODs as low as in the sub- picogram per milliliter range [66].

Monolithic-type SPE adsorbents have been developed. Ramautar and coworkers used a monolithic sol–gel and afforded 40-fold concentration of methionine enkephalin [67]. Later, an open-tubular format of a monolithic, reversed-phase adsorbent was used by Ortiz-Villanueva for the analysis of neuropeptides dynorphin A (1–7), substance P (7–11), endomorphin 1, methionine enkephalin, and [Ala]-methionine enkephalin) [68]. Selectivity was found for endomorphin 1 and substance P (7–11), and the 50-fold improvement achieved for these peptides allowed detection in the 10–50 ng $ml^{-1}$ range. Guzman and coworkers have demonstrated and highlighted the use of affinity (e.g., immunoaffinity)-type adsorbents to concentrate specific molecules in a complex mixture before CE-MS [69, 70]. The affinity ligands can be bound to a solid support such as glass beads and then the support is embedded inside the capillary. More recently, several affinity-type adsorbents have also been developed. Medina-Casanellas and coworkers used immunoaffinity adsorbent with antibody fragments for opioid peptides and reached 100-fold improvements [71, 72]. The same group evaluated several commercial metal-affinity sorbents and afforded up to 25-fold improvement for two small peptide fragments of the amyloid protein (A) (A(1–15) and A(10–20) peptides) [73].

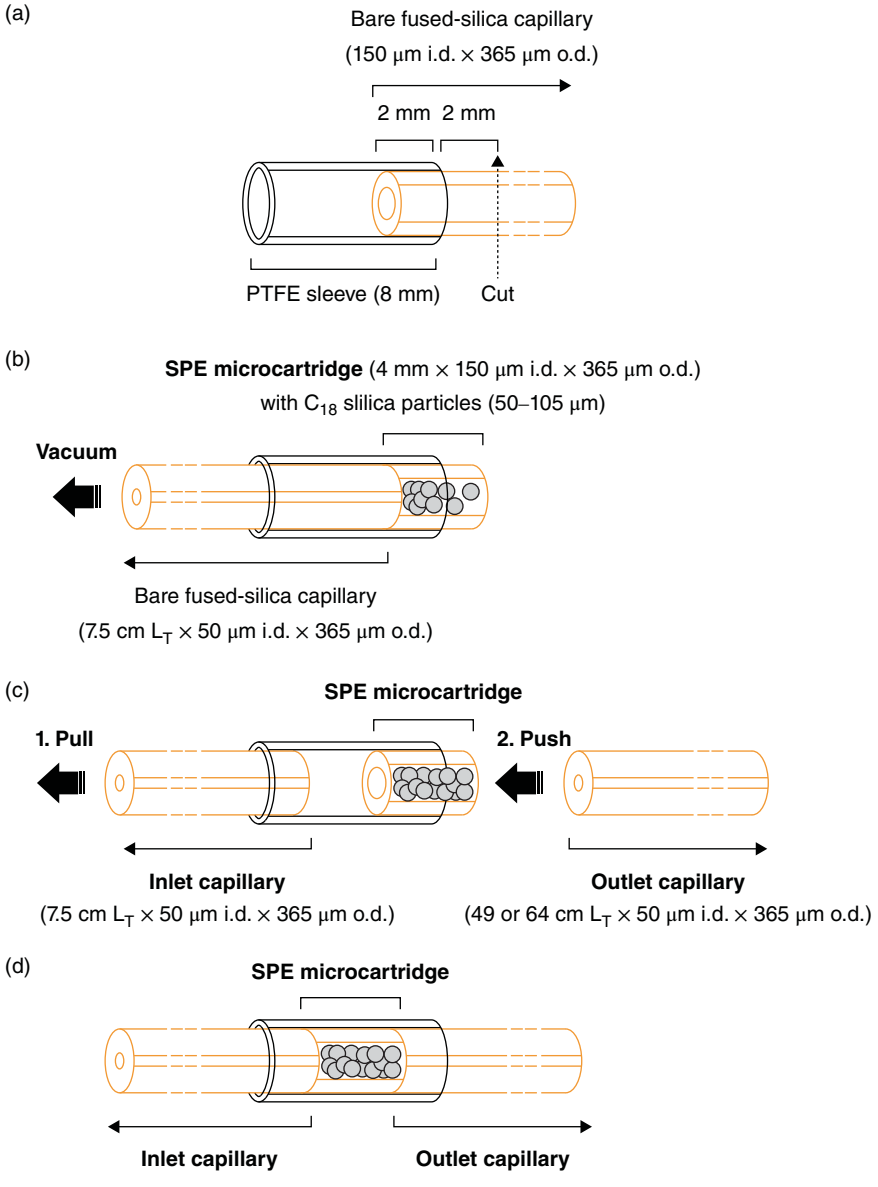


**Figure 5.11** Construction of a fritless microcartridge for in-line SPE CE-MS. Reproduced from Medina-Casanellas *et al*. [60] with permission of Wiley.

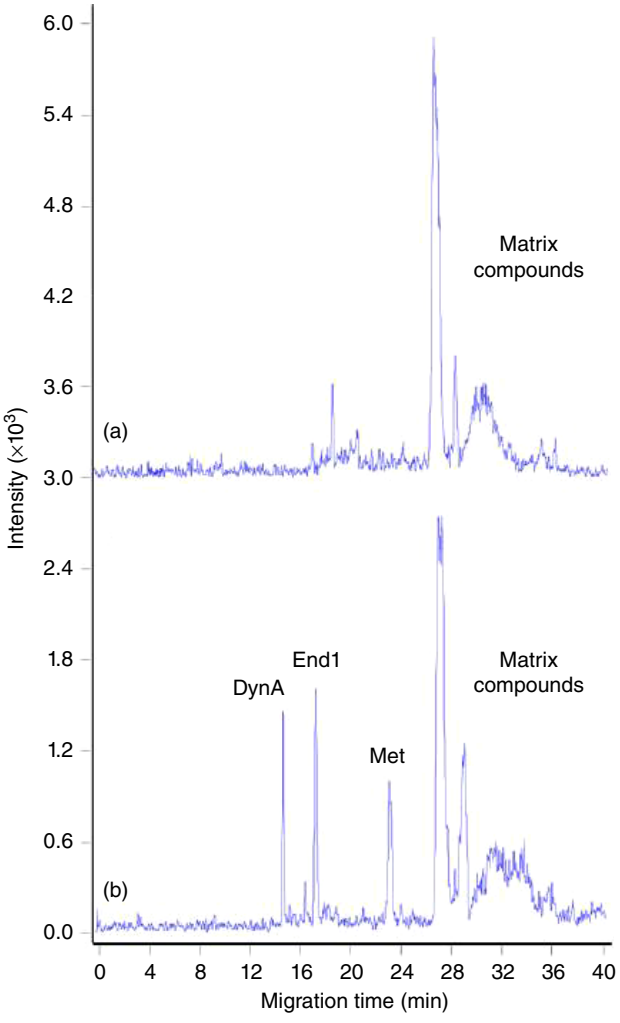


**Figure 5.12** Extracted ion electropherograms obtained by SPE-CEMS of (a) blank CSF and (b) CSF spiked with the opioid peptides (5 ng ml$^{-1}$ each). Experimental conditions: sample loading, 10 min at 930 mbar; elution, 63 s at 50 mbar; separation voltage, 25 kV; extracted ions: 434.7 (DynA), 611.2 (End1), and 574.2 (Met). Reproduced from Medina-Casanellas *et al*. [60] with permission of Wiley.

## 5.4 Conclusion

The stacking techniques currently amenable for use in CE-MS are briefly introduced in this chapter. Stacking techniques expanded the application areas of CE whereby the detection of low-concentration analytes was made possible with concentration factors from 10 to >$10^3$. Sample cleanup is still a requirement, but the

additional concentration effect provided during CE-MS analysis by stacking is still very attractive. This could improve the reliability in detecting trace levels of analytes in various real samples by improving the signal-to-noise ratio. The most popular stacking techniques used by far were t-ITP, field enhancement, and, to some extent, the dynamic pH junction. The use of additives to impart stacking (sweeping, AFMC, and MSS) in CE-MS is an area less explored most probably due to the notion that additives will actually decrease the ionization efficiency in ESI-MS. The use of these latter techniques will hopefully find more use, for example, by the use of ESI-MS-friendly additives such as those synthesized by Shamsi's group [74, 75] or by simple manipulation of injection, stacking, and separation conditions to prevent the entry of the additive into the source during detection. Partial filling of the capillary with additive can provide both stacking and separation of analytes, and this procedure (by pressure injection of additive solution) can easily be performed once the right solution chemistry is identified.

On-line and in-line integration of SPE to CE-MS is another powerful way to improve sensitivity. The availability of different adsorbents to tune selectivity in SPE is attractive, although the fabrication of the SPE devices would require some technical skill. Very high concentration factors have been obtained by simply increasing the sample load, which was normally several times larger than the total capillary volume of the analytical capillary.

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# 6
# CE-MS in Drug Analysis and Bioanalysis

*Julie Schappler, Víctor González-Ruiz, and Serge Rudaz*

## 6.1
## Introduction

The on-line combination of high-efficiency capillary electrophoresis (CE) separations with mass spectrometry (MS) presents major benefits; has generated a wealth of recent data in pharmaceutical [1–3], biomedical (including toxicology [4], metabolomic [5–9], proteomic [10–14]), and environmental [15, 16] research; and has been the subject of comprehensive reviews and book chapters [17–20]. This chapter focuses on recent examples of the on-line coupling of CE and related electrophoresis-based techniques with MS in pharmaceutical and biomedical analyses. The main difference between pharmaceutical and biomedical analysis involves the sample that is analyzed by the analytical system and its complexity. As described in Figure 6.1, pharmaceutical analysis (also termed *drug analysis*) consists of the chemical and/or biological characterization of a pharmaceutical drug, from the raw material to the end product, as well as its analysis during the drug discovery process. Alternatively, biomedical analysis comprises different fields such as (i) drug development (i.e., preclinical and clinical research, PK/PD studies), (ii) forensic toxicology (i.e., analytical toxicology for medicolegal outcomes), (iii) clinical toxicology (i.e., diagnosis and treatment of poisoned or drug-affected patients), (iv) clinical biochemistry (i.e., analysis of body fluids), and (v) omics (i.e., collective characterization and quantification of pools of biological molecules that translate into the structure, function, and dynamics of patients). Biomedical analysis thus addresses the analysis of complex biological matrices, and the analytes of interest can be either exogenous compounds (e.g., pharmaceutical drugs in the context of drug development, forensics, and clinical toxicology) or endogenous compounds (e.g., metabolites or biomarkers in the context of clinical biochemistry and omics). The focus of this chapter is on the former (termed "bioanalysis") because CE-MS in

*Capillary Electrophoresis–Mass Spectrometry (CE-MS): Principles and Applications,*
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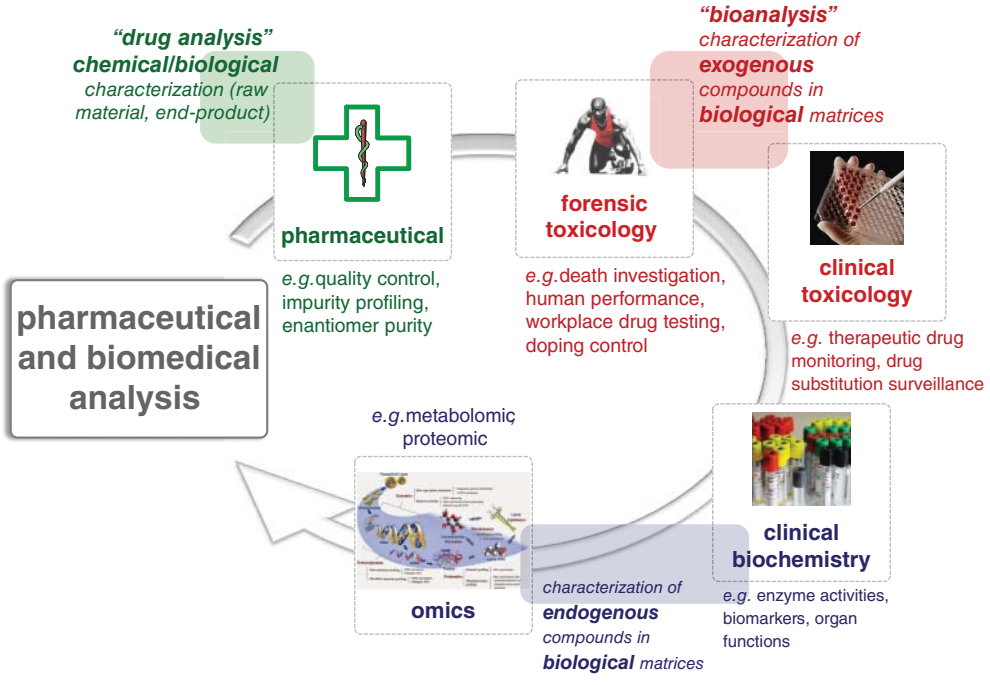


**Figure 6.1** Pharmaceutical and biomedical analysis: this chapter covers the fields of CE-MS in "drug analysis" and "bioanalysis."

the context of "biomarker analysis" is covered elsewhere (see Chapters 10 and 11). Finally, only low-molecular-weight (LMW) molecules (<1000 Da) are considered in this chapter because the CE-MS analysis of high-molecular-weight compounds, such as intact proteins and biotherapeutics, can be found elsewhere (see Chapter 7).

Because CE-MS fulfills key requirements, such as sensitivity, selectivity, and peak assignment certainty, it can be used by pharmaceutical companies to determine major drugs and their by-products in pharmaceutical preparations, as well as active components and their metabolites in biological fluids. In this chapter, applications concerning quality control and impurity profiling, as well as developments that have been recently made for the determination of drugs' physicochemical properties, are presented. CE-MS in the context of bioanalytical assays, as well as its outcomes in drug metabolism studies, is then discussed. Finally, quantitative CE-MS is presented, and the various methodologies used to achieve sensitive and repeatable analysis are discussed.

In the context of bio- and drug analysis applications, volatile background electrolyte (BGE) buffers that are compatible with the chosen ionization source must be used in CE-MS to avoid ion suppression and contamination of the ionization chamber. Electrolytes of low ionic strength, such as acetic and formic

acids, ammonium, and combinations thereof, are preferred constituents for BGEs [21]. Nonetheless, good separations achieved with nonvolatile BGE buffers can sometimes overcome the drawbacks of their use in CE-MS [22].

Among the available CE modes, capillary zone electrophoresis (CZE) is the most commonly employed because it fits the characteristics of many LMW therapeutic substances, most of which are water-soluble molecules with charge-bearing groups under physiological conditions (basic compounds represent more than 85% of ionizable drugs in the pharmaceutical domain). For cases in which a different selectivity is required or the analytes present poor solubility in water, nonaqueous capillary electrophoresis (NACE) is an attractive alternative [23, 24]. This mode is particularly well suited for MS interfacing because the BGE employed in NACE can be more easily transferred to the gas phase, often improving sensitivity. NACE presents additional features, including enhanced efficiency, reduced analysis time, and lower Joule heating, as illustrated in Figure 6.2 with the CZE-ESI-MS versus NACE-ESI-MS analysis of nonsteroidal anti-inflammatory drugs. Better stability of some compounds in organic solvents compared to water can also be advantageous. On the contrary, other modes such as micellar or microemulsion electrokinetic chromatography (MEKC and MEEKC, respectively) are less amenable to hyphenation with MS because of the

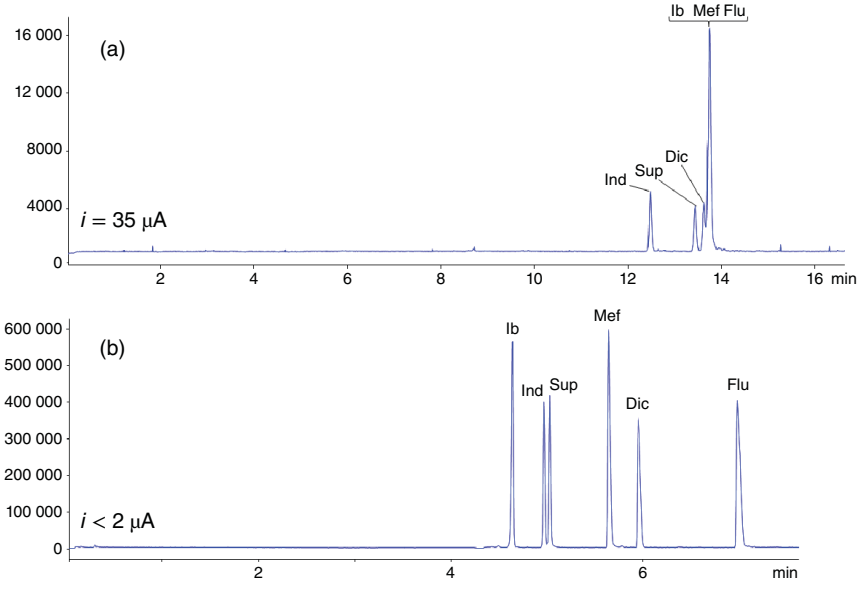


**Figure 6.2** CE-MS electropherograms in negative ESI obtained for selected NSAIDs at 1 μg ml$^{-1}$. (a) Conventional CZE mode with a sheath-liquid interface; BGE: ammonium acetate 50 mM, pH 8.5, and (b) NACE mode with a sheathless interface; BGE, ammonium acetate 5 mM in ACN–MeOH 80 : 20 (v/v). (Reproduced from [24] with permission from Elsevier.)

characteristics of the required modifiers, although some alternatives are available to improve their MS compatibility, such as the use of (i) volatile surfactants [25, 26], (ii) low-molecular-weight (unpolymerized) surfactants either at low concentrations [27] or with a partial-filling approach [28], (iii) high-molecular-weight surfactants, and (iv) atmospheric-pressure chemical ionization or photoionization (APCI and APPI, respectively) sources instead of electrospray ionization (ESI) [29].

Due to its versatility, robustness, and commercial availability for many years, the most widely employed interface in CE-MS for drug and biological samples is the pneumatically-assisted sheath-flow ESI interface. One of its most interesting features is the possibility to tune the sheath-liquid composition to improve the ionization of the analytes, without hampering CE selectivity. However, its presence may cause a dilution effect that leads to a loss in sensitivity. Many LMW drugs and peptides present protonatable nitrogens on their structure; therefore, positive ionization mode is usually chosen because of its better overall performance and decreased likeliness to produce issues such as electrical gas discharges [30]. The recent commercialization of sheathless sources has made them increasingly popular, particularly in peptide analysis, although no validation with quality control has been described to date.

Regarding the ionization source, ESI is the most suitable source for charged analytes (conventionally separated by CZE or NACE), whereas APCI and APPI are more convenient for low-polarity molecules [31]. Matrix-assisted laser desorption/ionization (MALDI) is a valuable tool when a poorly ionizable matrix or BGE is present [32]. In the cases where the quantitation of metal ions in the drugs is of interest, inductively coupled plasma ionization (ICP) is the preferred option [33].

There are no limitations on the type of mass analyzer that can be coupled to CE in the context of bio- and drug analysis. Tandem MS systems afford more structural information and enhance sensitivity, and they play a key role in peptide sequencing. Single- or triple-quadrupole instruments and ion traps are the detectors most frequently found in the literature. Time-of-flight (TOF) is more common in biopharmaceutical analysis due to its accuracy in mass determination – useful for detecting posttranslational modifications in proteins – and broad mass and dynamic range, but it is also of interest in omic fields and untargeted drug analysis.

## 6.2 CE-MS in Drug Analysis

According to the latest estimates, the cost of developing a new drug that successfully reaches the market has increased to $2.6 billion [34]. This reflects the growing complexity of the drug research and development pipeline, taking advantage of new strategies that demand inexpensive and high-throughput analytical approaches. Quality control (QC) is a keystone in pharmaceutical development, product manufacturing, and market surveillance processes. As one of the main pillars in maintaining health and in therapeutics, accurate knowledge of a drug's

composition is critical to ensuring safety and effectiveness. Similarly, many other products from the food and pharmaceutical industries (e.g., dietary complements, nutraceuticals, infant formulas, cosmetics) also require tight control of their compositions to avoid risks and guarantee their value. On the other side, market surveillance is progressively gaining relevance as the presence of counterfeit medicines and parallel distribution channels are growing. Currently, between 10% and 30% of the drugs sold in developing countries, approximately 1% in developed countries, and more than 50% sold on the Internet are counterfeit drugs [35]. Taken together, these facts mean that QC technology needs to roll out more inexpensive and faster analytical strategies that are able to deliver complete information on samples with the greatest automation capabilities, a challenge suited to the promising features of the newest CE-MS techniques. The presence of an active ingredient is subject to several issues – either deliberate or not. In the worst case, the active ingredient is not present at all, or it has been replaced by another product. If the substance is detected, its actual content may differ from that listed, or degradation products or impurities such as synthesis reagents or intermediates may also be present. Bearing this in mind, the goal of analytical chemistry in QC is not only to unequivocally assess the presence of a component and its amount but also to identify and quantify possible impurities.

Currently, QC in pharmaceutical analysis largely relies on liquid chromatography (LC) due to its versatility, robustness, accuracy, and scalability potential. Nevertheless, CE may present several advantages over LC, such as (i) lower exploitation costs, (ii) simpler method development, (iii) lower sample and reagent consumption, (iv) better environmental compatibility, (v) faster separations, and (vi) higher efficiencies. Additional advantages include the ability to set up high-throughput analytical stations by means of arrays of capillaries and a better compatibility with biomolecules such as intact proteins, as detailed in Chapter 7. Although CE-UV has been present in drug QC for years now, most CE-MS strategies are still in proof-of-concept or early development stages. The published literature on CE-MS for pharmaceutical analysis has mainly focused on adapting existing CE-UV methods for MS detection and on comparing the advantages provided by each of the different CE modes and types of MS detectors. Very few complete QC methodologies have been developed and even less have been successfully transferred to routine analyses because interfacing CE to MS is currently subject to several complications compared to on-capillary UV detection. CE-UV methods adapted for MS detection need to be reoptimized, and the results may not always be as good as the previous ones; this can, despite the advantages afforded by MS, be considered a drawback by many analysts in highly demanding production environments. Thanks to the intrinsic selectivity and specificity of MS detection, CE-MS combinations can simultaneously quantify several target analytes in finished pharmaceutical products and nutritional formulas [36–38]. Commonly, only very simple sample cleanup is required to reach limits of detection (LOD) in the ng $ml^{-1}$ or low μg $ml^{-1}$ ranges, without needing complete peak separation. Nevertheless, in the absence of appropriate deuterated standards, quantitation can become problematic when the stability of

the ionization process has not enough consistency over time and large peak area variation can hamper the application to quality control methodologies [39, 40]. This section focuses on the main aspects of drug analysis in which CE-MS has made recent breakthroughs, namely in impurity profiling and chiral analysis, as well as in the determination of drugs' physicochemical properties.

### 6.2.1 Impurity Profiling

Impurity profiling of pharmaceutical products is an important aspect of pharmaceutical analysis. The speed and reliability of analytical data on impurities, which can be both synthesis and degradation products, have a direct impact on the eventual success or failure of a promising drug. A key factor of CE-MS in drug impurity profiling is the orthogonality with LC. Most LC separations for QC in pharmaceutical analysis rely on RP-LC methods in which selectivity is based on the difference in lipophilicity of the compounds. Instead, selectivity in CE is primarily conditioned by the electric charge of the analytes and provides a complementary separation mechanism. Therefore, poor or no correlation exists between the peak separation data obtained from both techniques [41]. This phenomenon allows separating and distinguishing many impurities that would have remained undisclosed using only one technique because compounds coeluting in one dimension can now be separated by the second separation mechanism. Vassort *et al.* [42] developed an interesting approach based on the orthogonality between different CE-MS methods, several LC-MS separations, and both CE and LC analyses. This strategy helped establish a generic methodology that combines the results from independent LC-MS and CE-MS methods to routinely search for drug impurities with very different physicochemical properties with no or minimal method development. Other CE-MS separations have been described as orthogonal and complementary to LC-UV and chiral CE-UV methods. For example, CE-MS equipped with an ESI source and a quadrupole-ion trap was implemented by Visky *et al.* in the method development for impurity profiling of galantamine formulations under harsh conditions. Two degradation products were detected at concentrations lower than 0.05% and further identified by MS/MS [43].

CE-MS has found widespread application in the analysis of biopharmaceuticals, including complete proteins such as monoclonal antibodies. Because such analyses are described elsewhere in this book, herein, we only cover QC assays involving small peptides, and, in this field, CE and LC are also complementary techniques. Whereas CE can readily differentiate incomplete synthesis intermediates lacking part of the amino acid sequence, RP-LC better separates peptides containing additional amino acids or protective groups due to the different degrees of hydrophobicity they impose [44]. CE-MS and LC-MS reveal completely different peptide impurity profiles not appearing when one or the other technique is employed alone [45]. When working with samples of a peptidic nature, convenient deuterated standards will not always be available to correct the

possible variations in ionization efficiency and injection volumes. Strategies such as the multiple injection technique have been developed to successfully address this problem and are discussed in Section 6.5 [46]. Proteomic-like approaches have taken advantage of the high-throughput capabilities of CE-MS to utilize thousands of peptides to characterize several batches of a product consisting of the lysate of probiotic bacteria [47]. Using an adequate data processing workflow, it was possible to distinguish the different batches and assign the precedence of the identified peptides. Peptides derived from protein hydrolysates employed as additives in cosmetic preparations were also analyzed by CE-MS, and their acidity and polarity were predicted based on their sequencing [48]. Peptides are not always the target analytes themselves, but are sometimes a marker obtained by hydrolyzing the relevant protein of interest. This approach has been used to quantify proteins from host cells employed in the production of monoclonal antibodies [49].

### 6.2.2 Chiral Analysis

One of the most important characteristics of drug substances is the determination of their optical purity. Because they are intended to bind to very particular chiral biological targets, the wrong stereochemistry will lead either to molecules exerting completely different and unexpected effects or to inactive substances needlessly overloading the metabolic and clearance routes of the patients. Thus, stereochemical determination remains an issue of utmost importance in drug QC.

CE is a powerful tool in chiral separation. The most frequently applied technique for chiral separations in CE remains the so-called dynamic mode in which the resolution of enantiomers is carried out by adding a chiral selector directly into the BGE for the *in situ* formation of diastereomeric derivatives. Various additives, such as cyclodextrins (CDs), chiral crown ethers, proteins, antibiotics, bile salts, chiral micelles, and ergot alkaloids, have been reported as chiral selectors in the literature, but CDs are by far the most widely used selectors in chiral CE [50, 51]. Because traditional methods using UV detection lack the sensitivity to detect impurities in very low amounts compared to the main isomer, MS detection has been implemented to circumvent this issue and afford additional advantages in terms of selectivity and structural information from unknown chiral impurities [19]. From the standpoint of MS, CE enables the separation of stereoisomers with identical molecular weights, otherwise indistinguishable by MS alone. The main drawback to using chiral selectors in CE-MS is that they are not volatile, thus potentially causing source contamination and ion suppression problems. For chiral selectors with a high affinity for the analyte, a very small amount of the compound is required in the BGE, and it can be allowed to reach the MS without causing deleterious effects on detection [52]. If this is not the case, two strategies can be employed to avoid this issue, the partial-filling technique (PFT) and the chiral selector counter-migration, or a combination thereof, as reviewed in detail by Simó *et al.* [53]. The PFT involves the filling of a discrete portion of

the CE capillary with BGE containing a chiral selector (i.e., partial filling). Neutral CDs were used in earlier works, but charged chiral selectors are currently preferentially employed. When using PFT with a neutral CD, it is quite difficult to avoid any entrance of the chiral selector into the ionization source, particularly at high pH where electroosmotic flow (EOF) is important. The use of a BGE at low pH or a coated capillary to minimize EOF is therefore mandatory. However, the coaxial sheath gas, which generally assists the ionization process, leads to an aspirating phenomenon of the chiral selector in the MS direction. The use of a charged chiral selector is thus the best solution to improve the classical PFT when CE is hyphenated with MS. This approach presents major advantages such as (i) better solubility of the CDs, (ii) additional electrostatic interactions, and (iii) improved stereoselective separation power afforded by the self-mobility of the chiral additives into the BGE. When the electromigration of the chiral species and the analytes are opposite (PFT-countercurrent approach), the mobility difference between free and complexed analytes is increased, leading to a higher resolution than with a neutral CD. In optimized countercurrent conditions, analytes reach the detector while the charged chiral selector migrates toward the opposite side of the MS. Hence, the stability of the ionization is improved by minimal contamination of the source and detector during the electrophoretic run. Because the use of PFT-countercurrent requires the presence of charged chiral selectors at relatively low concentrations, which induces a low conductivity in the BGE, the generated current generally does not exceed the instrumental limitation, even when a high voltage is applied, leading to high efficiencies.

One of the most remarkable advantages of coupling CE to MS for the detection of stereoisomers is the large sensitivity improvement achieved [54–56], reaching values as low as 0.02% of the enantiomeric impurity with regard to the main component as reported in Figure 6.3 [56]; this exceeds the 0.1% requirement of the International Conference on Harmonization (ICH) guidelines [57]. The same strategy was also applied to the chiral separation of amphetamine derivatives at very low levels (sub-nanogram per milliliter) using an electrokinetic injection [58]. A concentration of 1 ppb of each analyte, corresponding to an enantiomeric concentration of 0.5 ppb, could be detected and was validated according to ICH guidelines [59]. Nonchiral CE-MS can also be applied to elucidate the structures of impurities previously detected by means of a chiral CE-UV separation [40]. Although the usefulness of CE-MS in performing chiral separations of short peptides has been demonstrated [60], to the best of our knowledge, no complete analytical procedure applied to pharmaceutical QC has been fully validated to date.

### 6.2.3 Determination of Drugs' Physicochemical Properties

Measurement of the physicochemical properties of new chemical entities (NCEs) at an early phase of drug discovery and development is crucial to reduce attrition rates and represents one of the big challenges for the pharmaceutical industry.

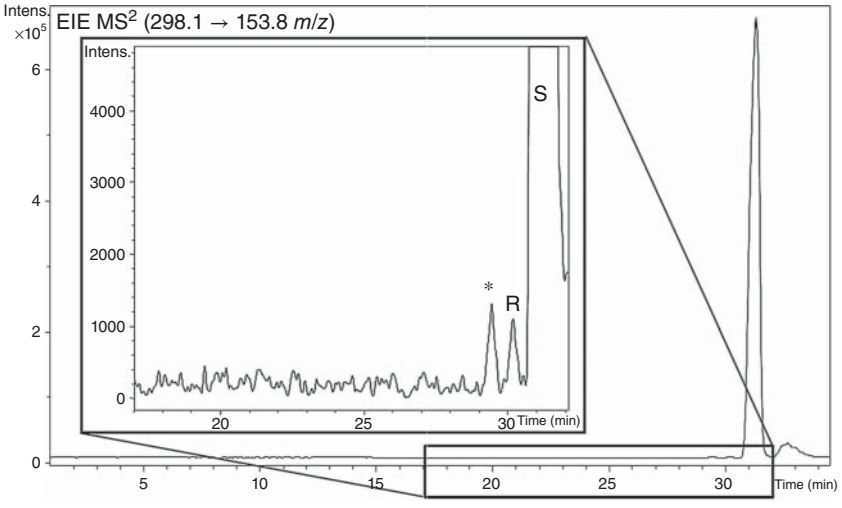


**Figure 6.3** CE-MS$^2$ electropherogram of 0.02 µg ml$^{-1}$ *R*-duloxetine in the presence of 100 µg ml$^{-1}$ *S*-duloxetine achieved by the PFT countercurrent approach. (Reproduced from [56] with permission from Elsevier.)

Moreover, due to the high number of NCEs, the analytical tools used to measure these properties must be automated and adapted to high-throughput screening (HTS). *In silico* methods are widely used to estimate the physicochemical properties of NCE in early ADMET (absorption, distribution, metabolism, elimination, and toxicity) studies. However, they are associated with well-described limitations, such as problems with complex molecular fragments or poor descriptions of intramolecular effects associated with the 3D molecular structure. Therefore, it remains necessary to determine these values experimentally [61]. CE and CE-MS assays are particularly involved in the determination of three major physicochemical properties: acid/base properties (p$K_a$), lipophilicity (log *P*), and plasma protein binding (PPB) and present good features compared to conventional approaches, for which amounts and concentrations of compounds, determination of throughput, and purity needs are often unfavorable. (Table 6.1).

#### 6.2.3.1 p$K_a$ and log *P*

p$K_a$ and log *P* are important parameters because both the ionization state and lipophilicity of a drug affect its ADMET properties, particularly in terms of membrane permeation. They also significantly contribute to the understanding and modeling of ligand–target interactions underlying the pharmacodynamic phase.

Determining the p$K_a$ and log *P* with conventional methods has several drawbacks, such as high sample consumption, low throughput, incompatibility with samples of low purity, or lack of sensitivity. In this context, CE has emerged as an attractive technique because of (i) small solvent and sample consumption, (ii) compatibility with low-purity samples, (iii) easy automation, and (iv) low cost. The CE-based alternative to the commonly used potentiometric titration or

**Table 6.1** Main characteristics of conventional approaches versus CE-based methods to assess acid/base properties ($pK_a$), lipophilicity (log *P*), and protein binding (PPB) of a new chemical entity (NCE).

| | $pK_a$ [61] | | | log *P* [61] | | | PPB [62] | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Potentiometry** | **Spectrophotometry** | **CZE** | **Shake-flask** | **RP-LC** | **MEEKC** | **ED** | **HPAC** | **CE/FA** |
| Range | 2–12 | 1–14 | 1–14 | −3 to 4 | −1 to 8 | −1 to 7 | Low-to-medium interactions | Medium-to-moderately strong interactions | Low-medium-to-strong interactions |
| Usual volumes of drug | µl | µl | nl | µl | µl | nl | µl | µl | nl |
| Usual concentrations of drug | 200–5000 µM | 10–50 µM | 10–500 µM | 10–30 mM | 10–50 µM | 50–100 µM | 50–800 µM | 10–50 µM | 50–800 µM with UV 1–5 µM with MS |
| Purity | Necessary | Necessary | Not necessary | Necessary | Not necessary | Not necessary | Necessary | Not necessary | Not necessary |
| Throughput | Low-medium | High | High | High | High | High | Low | High | Medium |
| Miscellaneous | n.a. | For compounds with a chromophore dependent on the pH | n.a. | Tedious | Secondary interactions possible | Microemulsion instability | Nonspecific adsorption possible | Protein immobilization | n.a. |

CZE, capillary zone electrophoresis; RP-LC, reversed-phase liquid chromatography; MEEKC, microemulsion electrokinetic chromatography; ED, equilibrium dialysis; HPAC, high-performance affinity chromatography; CE/FA, capillary electrophoresis in frontal analysis.

UV spectral shift methods of p$K_a$ measurement is performed by CZE with the measurement of the effective mobility of the investigated compound at several pH values, which are set by the preparation of suitable buffers at constant ionic strength in different pH ranges. CE is also an alternative method to conventional *in vitro* approaches (e.g., shake-flask technique, chromatographic methods) to measure lipophilicity. The determination of log *P* values is based on the measurement of the retention factor *k* of the investigated compound analyzed with a BGE, allowing for the separation of neutral compounds. Thus, CE modes such as MEKC and MEEKC (micellar and microemulsion electrokinetic chromatography, respectively) are currently used.

In CE, several strategies can be applied to reduce the analysis time for p$K_a$ and log *P* determination, including (i) pressure-assisted CE, (ii) short-end injection, (iii) multiplexed CE, (iv) coated capillaries, and, recently, (v) internal standard (IS) CE for p$K_a$. Short-end injection and especially pressure-assisted strategies reduce the apparent peak efficiency, which can decrease the precision of the migration time determination. All of these approaches are generally used separately, but some of them may be combined to save additional time. The additional selectivity offered by MS allows a greater pooling of compounds per analysis, providing an increased throughput that is particularly important in the drug discovery stage. The use of MS detectors also extends the application range to non-UV-absorbing and poorly soluble analytes. This coupling has been rarely reported because the coupling is not straightforward due to the low compatibility between the nonvolatile BGE (entire pH-range electrolytes in CZE and additives used in MEKC or MEEKC) and the ESI source, resulting in increased baseline noise and reduced sensitivity. Despite this limitation, Wan *et al.* developed a method for the simultaneous p$K_a$ measurement of more than 50 compounds in less than 150 min by CE hyphenated with an ion-trap mass analyzer [63]. The developed CE-MS p$K_a$ method required minimal sample preparation and provided higher sensitivity and selectivity over the most commonly used potentiometric titration and UV spectral shift methods, making it suitable for screening newly synthesized compounds including poorly soluble and impure compounds. A good correlation was obtained between measured values with CE-MS and literature data or predicted values, with *R* higher than 0.99 and 0.88, respectively, as illustrated in Figure 6.4. The lower correlation observed with predicted values was mainly attributed to the *in silico* model, which suffered from higher variances for the structures containing conjugated double bonds, for instance. Consequently, differences between predicted and measured values of more than 5 p$K_a$ units were observed in some cases, indicating that measured values are indeed required. This study emphasized that CE-MS method offers not only reliable p$K_a$ measurements but also high-throughput capacity by pooling numerous compounds into a single sample. The same authors also successfully used lauric-acid-based microemulsion systems for log *P* determination by MEEKC-ESI-MS without apparent ion suppression and/or ion source contamination [64]. Another alternative is the use of APCI or APPI sources [65] as these ionization processes are less affected by nonvolatile salts

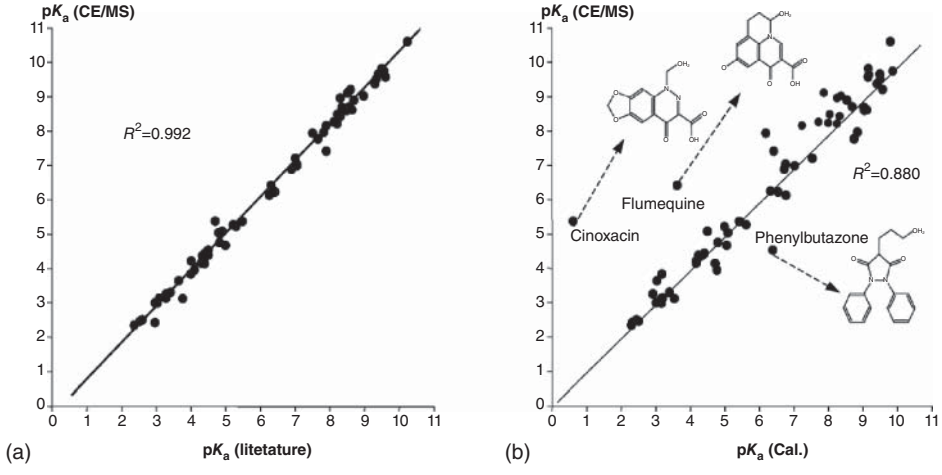


**Figure 6.4** Correlation between p$K_a$ values obtained for 50 drugs by CE-MS and (a) literature data and (b) values predicted by ACD/Labs. (Reproduced from [63] with permission from Wiley.)

or additives, providing interesting approaches to reduce background noise and source contamination.

#### 6.2.3.2 Plasma Protein Binding

Drug–plasma protein interaction is a process that drugs encounter after they have reached the blood. It is commonly accepted that only free molecules are able to cross membrane barriers to distribute into the tissues and that only the free drug fraction is able to exert pharmacological and/or toxicological effects. Drug–PPB thus plays a key role in pharmacokinetics through drug distribution and in pharmacodynamics because it controls the amounts of drugs that are freely available to the receptors. Consequently, the determination of affinity constants that describe the interaction strengths between drugs and plasma proteins is very important during the drug discovery phase when screening NCE.

Several approaches have been used to measure drug–protein interactions, such as equilibrium dialysis, calorimetric and spectroscopic methods, affinity chromatography, and biosensor-based assays [62]. These methods nevertheless present the same drawbacks mentioned for p$K_a$ and log $P$ determination, in addition to specific ones including protein and/or drug adsorption and immobilization of the protein onto a support, which can alter the binding properties of the protein of interest. CE is an attractive alternative method for drug–protein interaction measurements because it can be performed at near-physiological conditions (in addition to the features mentioned for p$K_a$ and log $P$ assessment). Different modes of CE can be used to characterize drug–protein interactions [66], and they are primarily coupled with UV detection. However, the low sensitivity of UV detection may limit the range of accessible binding constants, particularly for the characterization of strong interactions. To enhance sensitivity, CE-MS can be used in

PPB assays. Some studies have used an affinity capillary electrophoresis–mobility shift assay with ESI-MS (ACE-ESI-MS) to screen libraries of related compounds, from which an initial screening generally provides a ranking score by which to select the compounds with the highest affinity for a given target (i.e., the greatest mobility change). Then, a complete binding study can be performed only on the compounds of interest [67]. Frontal analysis continuous capillary electrophoresis –ESI–MS (FACCE-ESI-MS) can also be used to characterize the complexes [68], which can be verified by the MS spectrum, but no binding affinity has been calculated with this technique thus far. CE/frontal analysis (CE/FA) appears to be the most accurate and easiest to perform, where sample volumes of typically 10–20% of the effective capillary length, that is, a few nl, are injected into the capillary. Due to the volumes injected, plateaus are obtained instead of thin peaks. The height of the plateau is then used for quantification purposes. CE/FA-MS is mainly used to measure strong binding interactions, and good performance is achieved if the factors that are commonly associated with ESI-MS signal instability (sheath-liquid composition and ESI parameters) are carefully optimized to obtain reliable signals [69]. Drug–plasma protein interactions can be assessed using either CE/FA-UV for low affinities or CE/FA-MS for strong interactions that are difficult to characterize by UV.

## 6.3 CE-MS in Bioanalysis

The routine analysis of common drugs in human biological fluids (e.g., plasma, urine) is a major concern in preclinical and clinical research (e.g., PK/PD studies), forensic toxicology (e.g., doping control, death investigation, workplace drug testing), and clinical toxicology (e.g., therapeutic drug monitoring, surveillance of drug substitution), as summarized in Figure 6.1 [70]. Method sensitivity is often an issue because many drugs possess a high volume of distribution, resulting in low concentration levels. Thus, CE-MS can be a useful tool for the determination of such substances in bodily fluids, especially when low sample amounts are available. Because biological samples (e.g., saliva, urine, whole blood, plasma, serum, cerebrospinal fluid, and tissue) may contain water, salts, proteins, lipids, anticoagulants, stabilizers, and other compounds that can interact with the capillary and the operating conditions (e.g., injection, ionization), the analytical performance can be drastically reduced, thus hampering the quantitative determination of compounds in such matrices. Therefore, appropriate sample pretreatment and preparation procedures, such as dilution, filtration, protein precipitation, solid-phase extraction (SPE), liquid–liquid extraction (LLE), and solid–liquid extraction (SLE), have been implemented to avoid the degradation of analytical performance. The choice of which procedure to use largely depends on the desired selectivity and sensitivity, as well as the throughput constraints. In addition, there is a constant demand for methods to quantify a lower concentration of a given drug molecule or candidate, the primary objective of which is

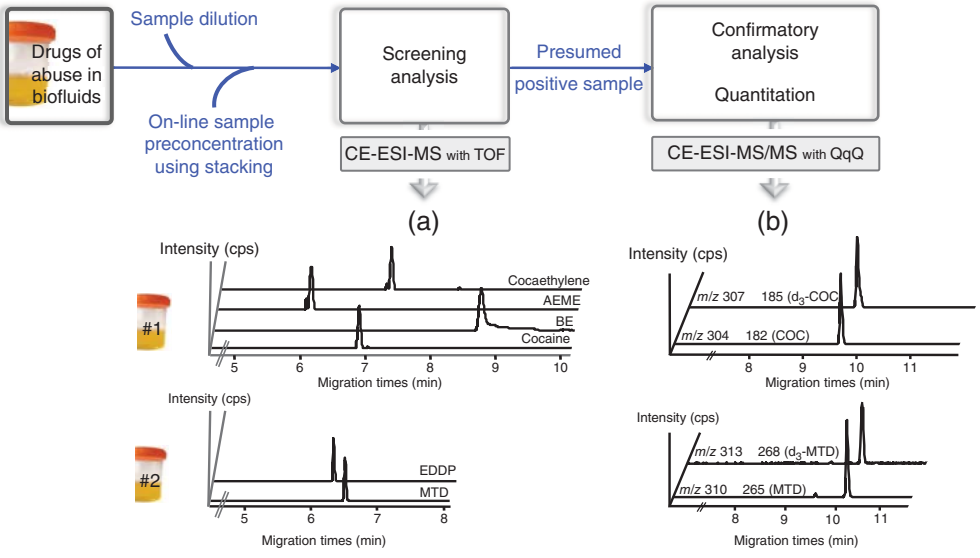


**Figure 6.5** CE-MS two-step workflow used in bioanalysis. (a) Screening with CE-TOF/MS and (b) Quantitation by CE-MS/MS of two urine samples containing cocaine and methadone, respectively. (Reproduced from [72] with permission from Elsevier.)

developing a bioanalytical method that meets the selectivity, accuracy (trueness and precision), and linearity requirements set by authorities [71].

In bioanalysis, a two-step methodology is generally used for the determination of drugs in biological samples, particularly in the forensic toxicology field. First, a rapid, sensitive, and generic screening is performed, followed by an independent confirmatory procedure prior to quantitation in cases of positive results. The combination of CE with a time-of-flight mass spectrometer (TOF/MS) is particularly well adapted to screening due to its relatively high data acquisition rate, mass resolution, and accuracy, which allow identification of unknown compounds. In a second stage, CE is hyphenated to highly selective analyzers such as triple quadrupole (QqQ) in selected reaction monitoring (SRM) for sensitive and selective quantitation. An example of this two-step analytical workflow is presented in Figure 6.5 for the analysis of drugs of abuse in urine after a simple dilution ("dilute-and-shoot" approach) prior to injection in CE-MS, enhanced with a pH-mediated stacking approach [72].

### 6.3.1 Selectivity Issues and Matrix Effects

An important issue regarding selectivity in bioanalysis concerns the assessment and quantitation of matrix effects (MEs), although the potential comigration and ion suppression are reduced because CE yields narrow peaks, improving the sensitivity and reliability of the MS detection. MEs have been described by several

authors, particularly for LC-ESI–MS analysis of biological samples, and can be assessed using qualitative and/or quantitative approaches.

The qualitative evaluation of ME usually consists of a post column infusion of the analyte mixture while different matrices are injected into the separation system, leading to the visualization of ionization suppression or enhancement. Adapted from the configuration proposed by Bonfiglio *et al.* [73], a postcapillary infusion system can be used with the sheath-liquid interface as an analyte-delivering device, as presented in Figure 6.6 [58]. A drug mixture solution is continuously infused with the sheath liquid and the effects associated with the migration of interfering compounds are assessed by measuring the MS responses with the injection of blank or spiked matrices. With this setup, the exact time window in which the MEs occur can be revealed and associated to the type of sample preparation (i.e., selective vs. nonselective) performed prior to injection. The quantitative determination of ME follows a method previously described by Matuszewski *et al.* [74]. Two types of samples are required. Sample A consists of a mixture of all compounds in the sample injection solvent as a standard. Sample B consists of the blank biological fluid prepared with a dedicated sample preparation and then spiked with a mixture of all compounds at a concentration equivalent to that found in sample A prior to injection. The MEs are estimated by comparing the peak areas of sample B versus sample A and are expressed as a recovery (%). Because the mobility is different between compounds in CE, the peak area can be influenced by ME upon ionization (related to the peak height) and in CE (related to the peak width). Therefore, the peak areas must be normalized to the migration times for adequate calculation of ME. Bonvin *et al.* applied both approaches to evaluate the ME in the analysis of phase II metabolites in urine by NACE-MS in negative ESI mode using two different CE-MS interfaces (i.e., sheath flow and sheathless) [24]. Although a simple

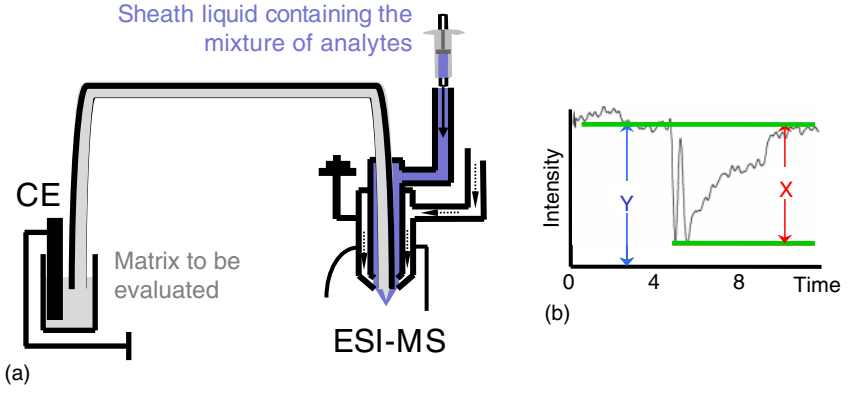


**Figure 6.6** Qualitative evaluation of matrix effects by CE-MS. (a) Schematic representation of the coaxial sheath-flow interface used as a postcapillary infusion device and (b) Typical ESI-MS response measured.

dilution of the sample prior to the CE-MS analysis ("dilute-and-shoot" approach) was applied to the urine samples, a systematic evaluation of the ME revealed the absence of ionization interference in the analytes' detection windows.

### 6.3.2 Sample Preparation

Achieving the desired sensitivity and resolution in a short analysis time is still challenging, particularly in high-throughput bioanalysis methods. In this context, the conventional sample preparation techniques, which remain highly labor-intensive and time-consuming, may become the limiting step in terms of the total analysis time.

Regular LLE procedures are primarily implemented prior to CE-MS and present several benefits in terms of sample cleanup and enrichment. This technique is well adapted to CE-MS analysis because proteins and salts are extensively excluded, which minimizes ME and levels off the sample conductivity, enabling electrokinetic injection and sample preconcentration. Both aspects allow for very low detection limits with good recovery and precision [59]. Nevertheless, LLE usually includes lengthy handling steps and can be advantageously replaced by SLE with the same extraction performance, while potentially automated. Other extraction procedures such as SPE can be used and feature high selectivities when used with an appropriate retention mechanism and high sensitivities because of the small recovered volumes that can be directly injected into CE [75]. In addition, less organic solvent is needed than for LLE, and adverse effects such as foaming are avoided. For these reasons, SPE is currently the leading sample preparation method used in routine bioanalytical assays, even though it may be time-consuming and relatively expensive and can suffer from poor batch-to-batch reproducibility. To address the requirements for high-throughput, 96-well plate SPE methods are being developed, but only a few studies have been reported so far using CE-MS.

The selectivity and sensitivity afforded by extraction procedures such as SPE, LLE, and SLE have become less advantageous since highly sensitive and selective MS analyzers, such as TOF/MS and QqTOF/MS, have been coupled to CE. A dilute-and-shoot procedure can be sufficient to circumvent compound discrimination while maintaining very short delivery times. Such an approach can be considered a general strategy to perform a single-run analysis for a wide range of compounds in a complex but protein-free matrix (e.g. urine). Another advantage is the absence of drug degradation that may occur during sample preparation and cause the drug concentration to be underestimated. However, the dilute-and-shoot procedure is nonselective and does not remove the interferents responsible for ME, although the quantitative determination of drugs of abuse in urine was demonstrated using this simple and fast approach [72].

The large quantities of proteins present in plasma and serum samples remain a major problem for separation techniques coupled to MS. The fastest way to remove most proteins in biological fluids, such as plasma or serum, consists

of precipitating them by adding an organic solvent, salt, or acid (i.e., protein precipitation, PP). This procedure is rapid, easy to perform, and able to be automated and can be applied to a wide range of samples, resulting in a good compromise between throughput and suitable sample cleanup. When PP is performed prior to CE, conventional hydrodynamic injection should be applied because interferents, which are not removed from the sample, may cause variability in conductivity, circumventing the use of electrokinetic injection. However, the use of acetonitrile in PP can improve sensitivity even with hydrodynamic injection according to a stacking phenomenon. For example, a method using CE-MS was developed and validated for the chiral analysis of drugs in plasma prepared by PP with acetonitrile [59]. This method featured high throughput, according to the simple PP step, and an analytical run time of less than 6 min with reduced ME, which enabled the accurate quantitation of each compound in the plasma.

In addition to conventional sample preparation procedures, microextraction techniques represent a field of great development for sample preparation prior to CE because the eluted volumes are perfectly adapted to the capillary format [76]. Microextractions are defined as nonexhaustive procedures that use very small volumes of the extracting phase and for which the volume of sample is relatively large compared with that of the extracting phase [77]. Microextractions feature a reduction of (i) solvent consumption ("green analytical chemistry"), (ii) sample volume, (iii) analysis time, and (iv) operating costs [78]. They are thus perfectly adapted to scarce biological fluids (e.g., sweat, saliva, tears, cerebrospinal fluid) where minute amounts of sample are available. They are also relevant for conventional biological fluids (e.g., urine, blood) for which high preconcentration factors can be attained. Such an approach was evaluated by Kohler *et al.* [79]. In this study, dispersive liquid–liquid microextraction (DLLME) was combined with CE-TOF/MS for the toxicological screening of urine samples. A methodology based on the design of experiments (DOE) was implemented to increase the extraction efficiency. With a preconcentration factor of more than 130, the highly sensitive DLLME-CE-ESI-TOF/MS method was used to detect 30 toxicological compounds in urine with LODs at the sub-ng $ml^{-1}$ level and to analyze real toxicological samples. The overall combination of DLLME and CE was particularly attractive because of the small amount of organic solvents required (less than 500 $\mu l\ ml^{-1}$ of urine analyzed).

## 6.4
## CE-MS in Drug Metabolism Studies

Metabolism converts drugs into metabolites, which are typically more hydrophilic compounds that can be efficiently eliminated from the body. These biotransformation processes include oxidative (phase I) and conjugative (phase II) reactions. The latter generally adds a larger hydrophilic moiety (e.g., glucuronic acid, sulfate, or glutathione) with a polar functional group, while the former inserts or exposes a minor polar functional group (e.g., hydroxyl or amine) into the parent

molecule. The cytochrome P450 (CYP450) subfamily mainly catalyzes the phase I reactions, while the conjugative reactions are catalyzed by various enzymes, including UDP-glucuronosyltransferases, sulfotransferases, and glutathione *S*-transferases. In most cases, metabolism reduces or alleviates drug activities, but some metabolites from phase I metabolism may present higher therapeutic activities or particular toxicity. Determination of the metabolic properties of NCE during the drug discovery process is therefore essential.

In recent years, the combination of ultrahigh-pressure liquid chromatography (UHPLC) with columns filled with sub-2-μm fully porous particles or superficially porous particles (also named *core–shell* or *fused-core particles*) and MS has proven its value as a powerful analytical tool in drug metabolism studies. Compared to LC configurations, microfluidics-based assays (i.e., nano-LC and CE-based approaches) can use nanospray emitters for optimized coupling with MS. This is particularly attractive considering that the sensitivity can be dramatically improved by the small elution volumes generated by the small mobile-phase flow rates; moreover, this coupling should afford improved ionization efficiencies and better long-term ionization source performance. In addition to increased sensitivity, microfluidics-based assays also feature low sample consumption and reduced environmental impact, which greatly benefit metabolism experiments such as *in vivo* and *in vitro* approaches. CE-based assays are used in metabolism studies with two main strategies: (i) electrophoretically mediated microanalysis (EMMA) proposed by Bao and Regnier [80] and (ii) coupled MS detection for the determination of the parent drug and/or the metabolites. Regardless of the strategy, *in vitro* metabolism assays encompass two distinct categories, namely untargeted and targeted analyses. Among the untargeted studies, metabolite profiling and metabolite identification have to be carried out with high-end MS devices for investigating the numerous unknown metabolites derived from metabolism. Targeted analysis consists of quantifying known metabolites, and recent improvements in quadrupole-based instruments have made them more sensitive, selective, and user-friendly.

### 6.4.1 Electrophoretically Mediated Microanalysis

In enzymatic assays involving CYP450 with only a few microliters of volume, EMMA is coupled with subsequent electrophoretic separation, and analyte detection is achieved within the same capillary. In EMMA, the mixing of the reaction's components is achieved by means of differences in their electrophoretic mobilities, and it thus remains relatively complex to simultaneously obtain satisfactory interpenetration of the reactive reagent system and good separation [81]. The main constraint concerns the BGE composition and pH, which must be compatible with both the enzymatic reaction and the analytical conditions. This represents a crucial issue for MS detection because the choice of BGE is restricted to volatile buffers. Therefore, this approach is often modified to use the capillary only as a reaction vessel, and the metabolite analysis is performed off-line

using dedicated analytical techniques (including LC- and CE-MS methods). This concept allows the direct transfer of classical incubation conditions to in-capillary assays and can be applied to any compound or enzyme. More recently, the principle of reactant mixing inside the capillary, based on successive hydrodynamic injections as a series of repeated consecutive plugs with parabolic profiles resulting from the laminar nature of the flow inside the capillary, allows the reactants to be rapidly mixed by transverse diffusion. Moreover, it guarantees a more generic method and facilitates the use of MS detection [82]. Whereas EMMA assays primarily involve UV detection, MS detection has been barely used for the analysis of metabolism with enzymatic reaction inside the capillary, except in the context of stereoselective determinations. For this purpose, the PFT is a suitable and efficient approach to avoid MS source contamination, as well as signal suppression due to nonvolatile additives (see Section 6.2.2).

### 6.4.2 Targeted *in vitro* Metabolism Assays

CE-MS can be used as the off-line analytical technique for the analysis of the parent drug and/or metabolites in the context of targeted analysis. Although phase I metabolism results in minor structural modifications of the parent compound, it enables the presence of quaternary amine nitrogens, polyhydroxyl groups, and carboxylic acid functionalities, which add considerable hydrophilic character to the drugs. These metabolites are generally very polar and may be barely separated from their respective parent drugs using hydrophobicity-based separation techniques such as RP-LC. Therefore, in many cases, CE offers the unique possibility of simultaneously analyzing the parent drugs and phase I metabolites [83]. Important phase II metabolites such as glucuronides are not easily analyzed by aqueous CZE due to their amphoteric properties. For example, opioid glucuronides, which represent one of the major applications of electrodriven methods, are ampholytes with pI values of approximately 5. Thus, the neutral zone is important in CZE, leading to a more complex method development to achieve sufficient selectivity between the parent compounds and their respective metabolites [84]. In the case of tramadol, the glucuronides are mostly zwitterions in the pH range of 3–10, and with a net charge near zero, they are unable to migrate under CZE conditions over this large pH range [85]. In this context, the supplementary interactions (e.g., ion pairing and heteroassociation) that can be provided by organic solvents in NACE can help improve the selectivity. Furthermore, when glucuronides are separated as negatively charged species migrating after the EOF, the interferences from positive endogenous ions (e.g., salts) and compounds (e.g., amino acids and urea) in the biological matrix are attenuated because positive ions are primarily detected before the EOF. In addition, NACE is a tailored mode for coupling CE with MS because the use of highly volatile organic BGEs with low surface tensions improves the formation of easily evaporable droplets, increasing the ionization efficiency while ensuring a stable spray over a wide range of voltages. Moreover, the absence of water reduces the number of electrochemical side reactions, thus

stabilizing the ESI current. All these properties are particularly well adapted to the negative ionization mode used for the detection of glucuronides and other phase II metabolites. Additionally, the deprotonation process in negative ESI can be further improved by using BGEs composed of high gas-phase basicity solvents such as acetonitrile, methanol, or a mixture thereof [24]. Other phase II metabolites of biological relevance such as sulfates, sulfonates, and phosphates can be analyzed with acidic ($pH \leq 2$) or basic aqueous BGEs ($pH > 7$). In the former case, negative ESI is generally used, and only anions of strong acids such as sulfates are determined [86]. In the latter case, strong anionic species are detected after the EOF, as in the direct determination of estriol conjugates in amniotic fluid, for example [87].

## 6.5 Quantitative Aspects in CE-MS

Quantitative aspects are of prime importance, particularly for the pharmaceutical industry where the reliability of analytical data is essential. Method development is generally followed by an evaluation of quantitative performance using an appropriate validation procedure performed in agreement with the criteria established by the ICH, FDA, or SFSTP commissions. However, only a few quantitative CE-MS studies have been reported, which may explain why CE-MS has been rarely used in the QC environment thus far.

### 6.5.1 Instrumental Aspects

A major difficulty in terms of quantitative CE-MS studies can be linked to the sheath-flow interface geometry, such as an improper positioning of the capillary inside the interface, leading to an unstable spray that, in turn, yields inaccurate ionization performance and low sensitivity. A second possible source of error is generally attributed to the electrophoretic process itself and to the impossibility of obtaining uniform EOF, particularly in the presence of capillary laminar flow due to both the nebulizing gas and the sheath liquid. These effects are also detrimental in repeatable sample injections. Finally, it is difficult to correctly thermostat the final part of the capillary in the CE-MS configuration, and this can lead to shifts in migration times and/or peak broadening. Some of these issues can be avoided using a sheathless interface because several operating parameters are absent in this configuration, including the nebulizing gas, the sheath liquid, and the positioning of the capillary inside the needle [6, 88, 89].

Because most of the uncertainty in MS does not refer to the analyzer, but rather to the ionization process, all types of analyzers can be used for CE-MS quantitation. However, because CE enables highly efficient separations, practical issues may arise because acquiring sufficient data points (>15 points per peak) is essential to ensuring reliable quantitation. Consequently, instruments with

high acquisition rates and low dwell times should be preferentially selected for quantitative determinations. For targeted analysis, the sensitivity, and thus the accuracy, is best with quadrupole instruments. Generally, the time resolution should be adapted to the small peak widths of CE; therefore, the selected ion monitoring (SIM) mode is preferred, which also improves sensitivity. The use of tandem mass spectrometry can greatly enhance selectivity using the SRM mode, ensuring more accurate measurements and lower limits of quantitation (LOQ) by reducing the chemical noise.

### 6.5.2 Methodological Aspects

To eliminate problems associated with quantitation in CE-MS and to reduce the impact of system variability on method accuracy, it is mandatory to use an internal standard (IS). Structural analogs and stable isotope-labeled (SIL) compounds remain the gold standards of ISs. With closely migrating ISs, the analyte and IS reach the ionization source at different times. Consequently, short-term variations in the ionization process and ME may be of concern.

In contrast to structural analogs, ionization changes can be efficiently corrected using SIL compounds possessing similar ionization responses and fragmentation patterns. Therefore, deuterated ISs can be used to correct both the overall method variability (i.e., sample preparation, injection, electrophoretic process) and the ME because the amount of suppression from interferents is expected to be similar. The total concentration of the analyte and SIL compound should be below the ionization process's saturation point. This SIL standardization approach was used for the analysis of psychoactive drugs in hair and serum extracted by microwave-assisted extraction prior to CE-TOF/MS [90]. Good quantitative performance was achieved in terms of intermediate precision, trueness, extraction recovery, and matrix effect. The criterion of acceptance was set to 15.0%, ±15.0%, 100 ± 15.0%, and 100 ± 15.0%, respectively [74]. The developed method was eventually applied to the analysis of serum reference materials, and hair samples from patients treated with benzodiazepines confirmed the reliability of the analytical approach, with concentrations as low as 1.2 ng $mg^{-1}$ per hair segment for tetrazepam, for instance.

For numerous candidates (e.g., original compounds, metabolites, naturals products, peptides), SIL compounds are not commonly available and can be very expensive. For this reason, some researchers produce their own SIL-ISs by means of protium–deuterium exchange. An alternative way to compensate for ionization variability and ME is through the so-called multiple injection approach. The latter, inspired by the ECHO technique [91], consists of using the nonlabeled targeted compound as the IS injected into the CE-MS system prior to the injection of the unknown sample. This strategy was applied in the analysis of recombinant human insulin by CE-UV-TOF/MS [46]. In this study, two injections are performed in the same analytical run, the first one with a standard of insulin at a known concentration and the second one with the sample

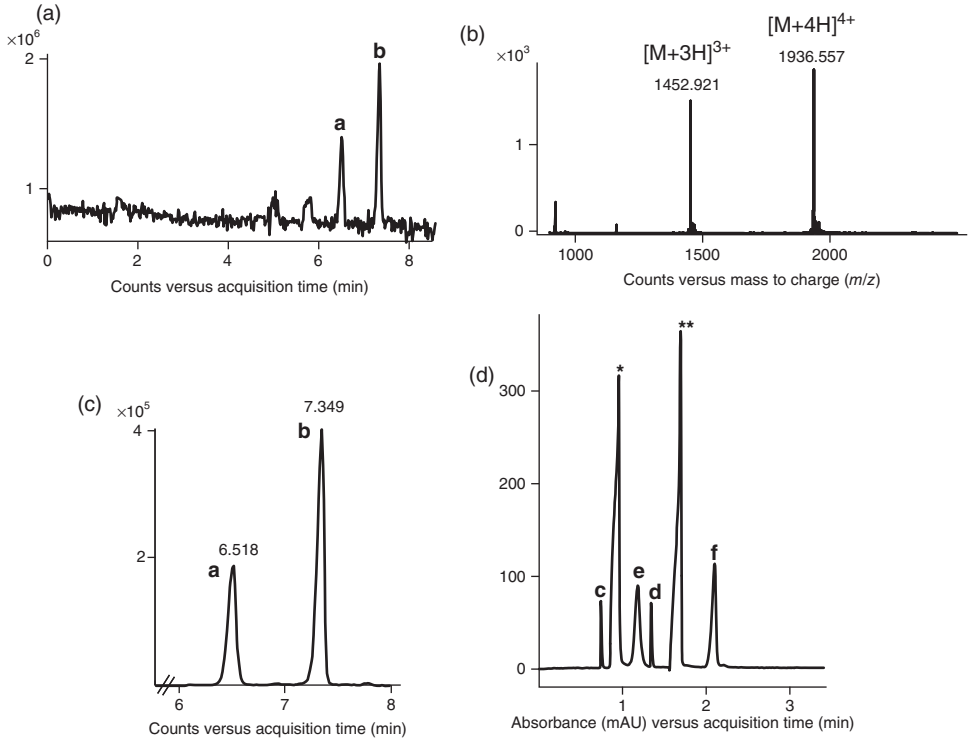


**Figure 6.7** Analysis of insulin by CE-UV-TOF/MS. (a) MS detection, total ion electropherogram (TIE); (b) MS detection, extracted mass spectrum; (c) MS detection, extracted ion electropherogram (XIE); and (d) UV detection. a. and e. insulin (calibration standard) from the first injection, b. and f. insulin (sample to be identified and quantified) from the second injection, c. procaine (UV-IS) from the first injection, d. procaine (UV-IS) from the second injection, * neutral excipients from the first injection, and ** neutral excipients from the second injection. (Reproduced from [46] with permission from Elsevier.)

to be identified and quantified. Figure 6.7A shows the total ion electropherogram (TIE) obtained with this multiple injection approach. The $[M+3H]^{3+}$ and $[M+4H]^{4+}$ multicharged ions are detected as the major extracted ions (1937 and 1453 $m/z$, respectively, Figure 6.7B). The XIE is reconstructed using both ions and integration achieved for quantitation purpose (Figure 6.7C). On-line UV detection is recorded to correct for injection variability between both injections (Figure 6.7D). An appropriate BGE plug is injected between both injections to ensure sufficient resolution between all peaks, namely the UV internal standard (i.e., procaine migrating before the EOF, peaks c. and d.), excipients (i.e., metacresol and glycerol detected in the EOF, peaks * and **), and the compound of interest (i.e., insulin migrating after the EOF, peaks e. and f.). This methodology offers simultaneous identification and quantitation of insulin in a single run due to MS spectral comparison and an external calibration approach, respectively. The complete methodology was fully validated according to ICH guidelines, and

the obtained accuracy profile demonstrated the ability of the CE-UV-TOF/MS method to quantify insulin in pharmaceutical formulations within a ±5% acceptance range. The method was further applied to pharmaceutical formulations obtained from drugstores and on the Internet without a formal prescription, as is often the case for patients suffering from diabetes mellitus due of the potentially lower cost.

## 6.6 Conclusions

The use of electromigration-based separation techniques hyphenated with mass spectrometry has evolved in modern pharmaceutical and biomedical analysis. Given its potential use with several commercially available instruments, including various interfaces, CE-MS coupling can now be easily achieved and implemented in labs as an orthogonal strategy to chromatographic-based assays. CE-MS is a good alternative for trace analysis in QC (i.e., degradation substances, impurities), for HTS in drug discovery (i.e., physicochemical properties), and for samples at low concentration and/or small volume in bioanalysis. MS detection enhances selectivity and sensitivity and expands the potential for quantitation using CE. While CE-MS is widely used in qualitative analyses, few quantitative applications have been published for biological matrices or pharmaceutical formulations analysis, although high quantitative performance can now be achieved with optimized and robust CE-MS methods.

### Abbreviations

| | |
|---|---|
| ACE | affinity capillary electrophoresis |
| ADMET | absorption, distribution, metabolism, elimination, and toxicity |
| APCI | atmospheric-pressure chemical ionization |
| APPI | atmospheric-pressure photoionization |
| BGE | background electrolyte |
| CD | cyclodextrin |
| CE | capillary electrophoresis |
| CE/FA | capillary electrophoresis frontal analysis |
| CYP450 | cytochrome P450 |
| CZE | capillary zone electrophoresis |
| DLLME | dispersive liquid–liquid microextraction |
| DOE | design of experiments |
| EMMA | electrophoretically mediated microanalysis |
| EOF | electro-osmotic flow |
| ESI | electrospray ionization |
| FACCE | continuous capillary electrophoresis frontal analysis |
| HTS | high-throughput screening |

| | |
|---|---|
| ICH | International Conference on Harmonization |
| ICP | inductively coupled plasma ionization |
| IS | internal standard |
| LC | liquid chromatography |
| LLE | liquid–liquid extraction |
| LOD | limit of detection |
| LOQ | limit of quantitation |
| LWM | low molecular weight |
| MALDI | matrix-assisted laser desorption/ionization |
| ME | matrix effect |
| MEEKC | microemulsion electrokinetic chromatography |
| MEKC | micellar electrokinetic chromatography |
| MS | mass spectrometry |
| NACE | nonaqueous capillary electrophoresis |
| NCE | new chemical entity |
| PFT | partial-filling technique |
| PP | protein precipitation |
| PPB | plasma protein binding |
| QC | quality control |
| QqQ | triple quadrupole |
| SIL | stable isotopically labeled |
| SIM | selected ion monitoring |
| SLE | solid–liquid extraction |
| SPE | solid-phase extraction |
| SRM | selected reaction monitoring |
| UHPLC | ultrahigh-pressure liquid chromatography |
| TIE | total ion electropherogram |
| TOF | time-of-flight |
| XIE | extracted ion electropherogram |

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# 7
# CE-MS for the analysis of intact proteins

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## 7.1
## Introduction

Over the past decades, advances in biotechnology and protein chemistry have stimulated a growing interest in improved analytical tools for the study and characterization of proteins [1–4]. This characterization is crucial, since the activity and function of proteins rely on their molecular composition and the presence (or absence) of specific posttranslational modifications (PTMs). For example, pharmaceutical proteins may undergo various modifications during their production, downstream processing, and storage [5]. These can have a severe impact on the bioactivity, efficacy, and toxicity (e.g., immunogenicity), thereby compromising their therapeutic properties.

In order to characterize proteins, generally, an enzymatic digestion is performed after which the formed peptides are separated with liquid chromatography (LC) and detected with mass spectrometry (MS) [6]. With the aid of the gained masses of peptides and their fragments as well as databases, the proteins can then be identified. If identification of unknown proteins in mixtures is the main issue, this so-called proteomics approach is the most suited and often gives excellent results. However, in some instances, the analysis of the intact (i.e., nondigested) protein(s) can be necessary in order to gain the proper information. For example, when it comes to purity determination, the main protein should be separated from its impurities while gaining information about the identity of the components. The same holds for stability analysis of proteins, where protein degradation is monitored in time and structural modifications have to be determined. Besides degradation and impurity, profiling the naturally present isoforms is important when it comes to, for example, evaluating the production process over time. For affinity and bioactivity assessment, proteins obviously should be analyzed in their intact, native form. In addition, analysis of intact proteins can yield information about protein conformation and folding and complexation. Another advantage of analyzing the intact protein is that sample handling is limited to a minimum, preventing introduction of modifications not present in the initial sample [7, 8].

*Capillary Electrophoresis–Mass Spectrometry (CE-MS): Principles and Applications,*
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There is a clear need for techniques that allow intact protein analysis in order to obtain relevant biological, biochemical, and pharmaceutical information. To date, two-dimensional slab-gel electrophoresis is the most powerful separation approach to resolve proteins [9, 10]. However, it is limited by relatively long analysis times, it is rather labor-intensive, it uses off-line detection, and it often lacks precise (and automated) quantitation. Moreover, when it comes to subtle protein modifications, it often does not provide the separation efficiency required. LC is advantageous due to its separation power, ease of automation, and routine coupling with various detection principles, similarly to MS [10, 11]. On the other hand, the stationary phase can cause adverse interactions with and denaturation of proteins seriously hindering LC analyses and decreasing separation efficiency and resolution. Capillary electrophoresis (CE) offers attractive features for the analysis of proteins [10, 12, 13]. CE analyses are normally performed in fused-silica capillaries under aqueous conditions avoiding conformational changes due to organic modifiers and/or a chromatographic sorbents. Furthermore, separation conditions in CE can be chosen in such a way that separations can be performed under (near-)physiological conditions circumventing protein degradation during analysis. CE separation is a function of charge, size, and shape of a compound. Consequently, small differences in the size or charge of proteins may be sufficient for separation, especially with the high efficiencies normally obtained in CE.

MS has developed into one of the most popular and useful detection techniques in separation science because of its sensitivity and selectivity. Furthermore, MS detection with high mass accuracy and resolution can considerably enhance the utility of CE by providing information about the identity of the separated compounds [14]. In addition, recent MS technologies enable fragmentation of intact proteins and obtain primary structure information without prior enzymatic digestion [15]. Therefore, coupling CE to MS creates a powerful analytical tool for the in-depth characterization of intact proteins. Hyphenation of CE with MS is less straightforward than LC-MS coupling. Nevertheless, since the introduction of CE-MS in the late 1980s [16, 17], MS has gained importance as a selective detection technique for CE.

As both CE and MS are increasingly important tools for protein analysis, it is not surprising that a significant number of papers on the development and application of CE-MS for intact protein analysis have appeared over the last three decades. This chapter provides an outline of these CE-MS systems. The chapter is limited to CE-MS studies in which entire ("intact") protein molecules are analyzed, that is, in which protein chains are not deliberately broken down to peptide fragments prior to analysis. In the first section, the most frequently used CE modes and some general aspects concerning CE-MS analysis of intact proteins are described. Subsequently, ways to prevent protein adsorption in CE will be shortly discussed. The most commonly applied ionization techniques and mass analyzers will be treated from the viewpoint of intact protein analysis. Ultimately, intact protein analysis using CE-MS will be systematically discussed, supported by illustrative examples.

## 7.2 CE of Intact Proteins

### 7.2.1 CE Modes

The separation of proteins can be performed using various CE modes, of which the most common are such as capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF), and capillary gel electrophoresis (CGE) [10]. For combining CE with MS, CGE is less suitable though due to the presence of nonvolatile separation media (i.e., polymers and surfactants) [18, 19]. Consequently, CZE and CIEF are most commonly used in combination with MS detection.

CZE is most frequently used in CE-MS of proteins. In CZE, the capillary is filled with a background electrolyte (BGE), and the sample is applied as a narrow zone surrounded by BGE. As the electric field is applied, each component in the sample zone starts to migrate according its electrophoretic mobility. Ideally, the sample components will fully separate to form individual zones prior to (MS) detection. In principle, high separation efficiencies can be obtained in CZE, as longitudinal diffusion is ideally the only source of band broadening. Since protein diffusion coefficients are relatively low, efficiencies in terms of theoretical plates can easily reach up to 1 million or more. Simple buffers can be used as BGE and electrophoretic mobilities – and thus the separation – can be manipulated by changing the pH and/or the ionic strength of the BGE.

In CIEF, amphoteric compounds such as proteins are separated according to their isoelectric point (pI). In order to achieve that, the sample is premixed with a mixture of ampholytes of different pI spanning the desired pH range. This mixture is loaded into the capillary. Under influence of an electric field, a pH gradient is formed by the ampholytes along the capillary, while the proteins migrate according to their electrophoretic mobility. When the proteins reach the point in the capillary in which the pH equals their pI, they become net uncharged and stop migrating. Subsequently, the analytes are led into the mass spectrometer (i.e., the mobilization step) by either a pressure on the inlet or changing the pH at the capillary outlet. An advantage of CIEF is that protein zones will be constantly focused, as a protein molecule that enters a zone of different pH will become charged and migrates back. Furthermore, as the entire capillary can be filled with sample, concentration limits of detection are often lower compared to other electrophoretic methods.

### 7.2.2 Preventing Protein Adsorption

Although high separation efficiencies should theoretically be obtainable in CE of proteins, in practice, this is not always observed. This is often due to protein adsorption to the fused-silica capillary. Adsorption is generally considered to be the result of a combination of electrostatic interactions and hydrogen bonding.

Adsorption can lead to reduced protein recoveries, alterations in peak shape, reduction in separation efficiency and resolution, and changes in electro-osmotic flow (EOF) [20, 21]. This consequently results in irreproducible migration times and erroneous peak area determinations.

To prevent protein adsorption, several approaches can be considered. The easiest is to use BGEs with an extremely low or high pH, which ensures that the silica is not charged (low pH) or that both the silica and protein are fully negatively charged (high pH) [22]. However, proteins tend to be unstable at these pH values. Furthermore, in CZE, it is desirable to be able to tune the pH of the BGE so that separations can be optimized. In CIEF, obviously, the pH must be varied along the capillary to enable the separation. Furthermore, in CIEF, it is desirable to suppress or minimize the EOF to allow complete focusing before the proteins are mobilized. Another option is to increase the ionic strength (i.e., concentration) of the BGE or ampholytes [23]. However, when using MS detection, this may lead to reduced detection sensitivity due to ionization suppression.

Another approach to prevent protein–wall interactions is the addition of organic solvents to the BGE [24]. Often, it is assumed that only electrostatic interactions cause protein adsorption, but hydrophobic interactions and/or protein conformational changes can also be involved in this process. At alkaline pH, when both the capillary wall and proteins are negatively charged, the addition of organic solvent might not lead to an increase of protein peak area, indicating that irreversible protein adsorption is effectively prevented by electrostatic repulsion. However, it still might lead to an improvement in separation efficiency (Figure 7.1a) by reducing reversible protein adsorption. At low pH, the effect of the organic solvent can be much more pronounced (Figure 7.1b), significantly increasing the observed peak area and the separation efficiency. It is difficult to develop a generic approach, so the effect of the organic solvent should be investigated on a case-by-case basis. However, it appears that rigid proteins (insulin and human growth hormone in this specific case) in general benefit

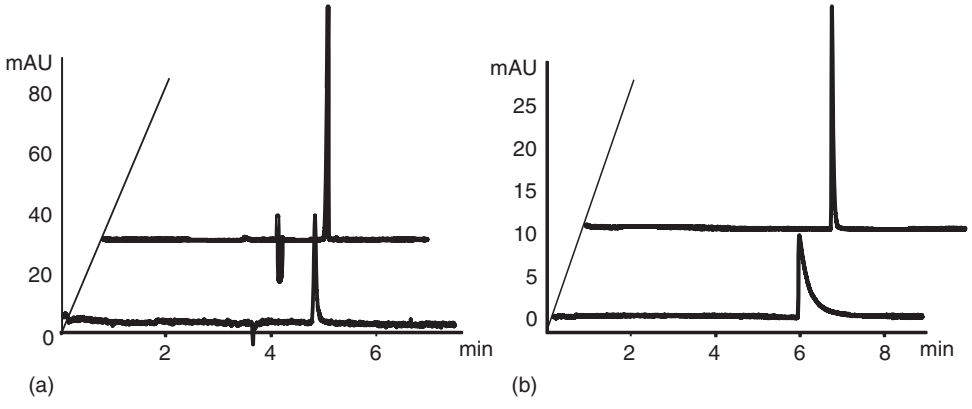



**Figure 7.1** CE-UV electropherogram of insulin obtained with (a) alkaline BGE and (b) acidic BGE. Upper trace: BGE containing 10% ACN. Lower trace: BGE without the addition of ACN. (Reproduced from [24] with permission from Wiley.)

from the addition of organic solvent to the BGE, whereas for flexible proteins (hemoglobin), it negatively affects the results. In several other studies, this positive effect of using organic solvents in the BGE on the separation efficiency of intact proteins has also been shown [25–27].

The most successful strategy to prevent protein adsorption in CE-MS is the use of capillary coatings. Excellent reviews describing and discussing these coatings are available, and the reader is referred to these for more in-depth information [20, 21, 28, 29]. One of the simplest methods of coating is through the use of BGE additives [30]. The additive changes the charge on the capillary wall, the protein, or, in some cases, even both. In all cases, the interaction between the protein and the silica wall is minimized and efficient protein separations can be obtained. Unfortunately, commonly used additives in protein CE are not compatible with MS detection and can cause severe ionization suppression and contamination of the mass spectrometer. Therefore, for CE-MS of proteins, coatings should preferably be covalently attached or physically adsorbed onto to the capillary wall, so that the coating agent does not migrate into the mass spectrometer [28]. Covalent coatings often make use of a reactive silane to attach the coating agent onto the capillary wall. The advantage of covalent attachment is the high stability and lifetime of the coating, but the reaction schemes involved often are complex and labor-intensive. Coating methods are not always easily applicable and reproducible, and differences between individually coated capillaries are not an exception. Noncovalent coatings make use of physical adsorption caused by electrostatic interactions between the coating agent and the capillary wall. Noncovalent coatings are commonly applied by flushing the capillary with a solution of the coating agent. Residual coating solution is easily removed via a rinsing step. The effect of a capillary coating on protein separation is clearly illustrated in Figure 7.2. With a bare fused-silica capillary using an ammonium acetate buffer (pH 5.5), only one of the three proteins can be discerned. However, coating the capillary enables baseline separation of the three proteins under the same BGE conditions and allowing their MS detection and identification [31].

There is not one capillary coating suitable for all proteins. This is not surprising, as proteins may exhibit substantial differences in charge density and hydrophobicity. Consequently, various coatings have been applied in protein analysis by CE-MS [12, 32, 33]. One can distinguish between neutral and charged coatings. For neutral coatings, covering the silanol surface is the most important aspect to avoid analyte adsorption. Neutral coatings have no restrictions regarding the charge of the analyte; however, the usable pH range is often limited to acidic and medium pH conditions owing to coating instability at higher pH (pH >8). Ionic coatings (positive or negative) can be applied to introduce electrostatic repulsion between the capillary wall and the analyte. The overall charge of both the protein and the coated surface should be either positive or negative if adsorption has to be prevented. This means that for cationic coatings, the pH of the BGE should normally be below the pI of the analyzed proteins, whereas for anionic coatings, the pH should be above the pI. In general, charged coatings result in an appreciable and constant EOF toward the capillary outlet, leading to adequate and reproducible interfacing conditions. The disadvantage of a high EOF is that

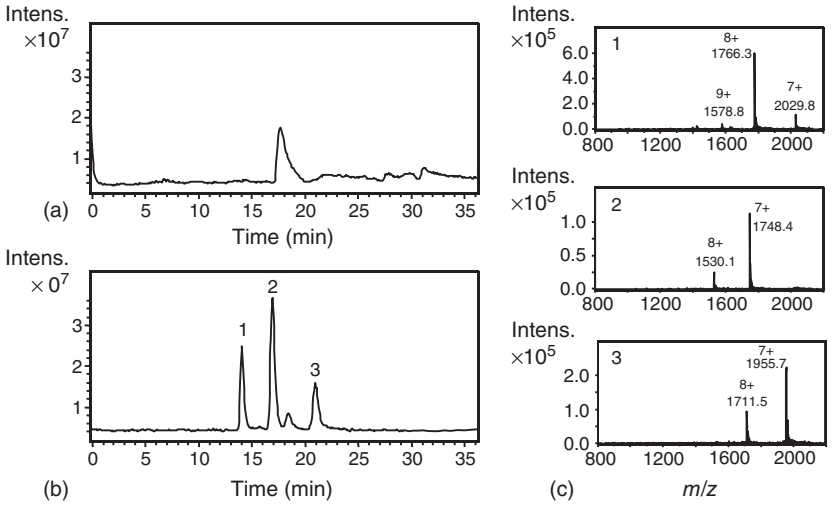



**Figure 7.2** Total ion electropherograms obtained using CZE-ESI-MS of a mixture of the basic proteins lysozyme (1), cytochrome *c* (2), and ribonuclease A (3) using a BGE of ammonium acetate buffer at pH 5.5 and (a) a bare fused-silica capillary or (b) an coated capillary. (c) Mass spectra of the main peaks obtained with the coated capillary. (Reproduced from [31] with permission from Wiley.)

the protein resolution obtained in CE can be limited, since this is inversely proportional to the sum of the electro-osmotic and analyte mobilities [28]. For the highest separation resolution, the electro-osmotic mobility actually should be only slightly higher and opposite to the electrophoretic mobility of the analyte. However, this approach will lead to very long analysis times and tuning of the EOF with respect to protein mobility is often not feasible. Therefore, neutral coatings with virtually zero EOF provide a good alternative. With this approach, the separation is merely dominated by the analyte mobility providing a good separation resolution within a reasonable time frame [34].

## 7.3 MS Detection of Intact Proteins

### 7.3.1 Ionization Modes

As described in much detail in Chapter 2, in CE-MS, the outlet of the separation capillary has to be connected to the ion source of the mass spectrometer. In this case, obviously, no outlet buffer vial can be used. Still, CE requires a closed electrical circuit, and therefore, a CE-MS interface should provide a means to apply voltage to the capillary outlet. Over time, a large variety of interfaces has been developed. For CE-MS of proteins, electrospray ionization (ESI), matrix-assisted

laser desorption ionization (MALDI), and inductively coupled plasma ionization (ICP) have been used.

ESI is the predominant ionization technique used for CE-MS of proteins. Due to the high charge density in the droplets during ESI, proteins tend to yield multiply charged molecular species. This results in the so-called charge envelop in the protein mass spectrum consisting of a number of peaks with mass-over-charge ($m/z$) values typically between 500 and 3000. An advantage of this multiple charging is that it enables detecting proteins in $m/z$ ranges much lower than their actual molecular mass. Therefore, ESI-MS detection of proteins in principle can be done with most mass analyzers. Protein molecular masses are subsequently obtained by deconvolution of ESI mass spectra. Interfacing CE with MS via an ESI source can roughly be performed in two different ways; using a sheath liquid or a sheathless approach [35]. The most important parameters when using the sheath-liquid interface for protein analysis are the sheath-liquid composition and its flow rate [36–39]. These parameters have shown to influence the signal intensity as well as the shape and position of the charge envelop. Therefore, pH, electrolyte concentration, and type and concentration of organic modifier used for the sheath liquid are essential variables. In sheathless approaches, this is obviously not an issue. However, as the overall flow rate is much lower, ESI interfacing parameters, such as spray voltage and gas flows and temperatures, can have a significant influence on the signal obtained with sheathless CE-MS [40]. Using optimized sheath-liquid and sheathless approaches in CE-MS for intact proteins, the latter typically provide 10- to 100-fold improved concentration sensitivity [40, 41]. This gain in sensitivity can be attributed to the dilution of the CE effluent that takes place in sheath-liquid interface on the one hand and the increased ionization efficiencies and decreased ionization suppression as observed for sheathless interfaces on the other hand. Independent of the type of interface, the constituents of the BGE are an important aspect in CE-ESI-MS. In CZE, preferably, volatile components such as formic acid, acetic acid, and ammonium hydroxide are used in low concentrations to enable sensitive ESI-MS (e.g., see studies reported in Table 7.1). These BGEs might not always provide the desired CE selectivity and a compromise between the achievable separation and signal intensity should be made. Nonvolatile BGEs have been occasionally used for CZE-ESI-MS [65–67], but only in one case for intact proteins [65]. Phosphate buffers resulted in a reduction in the MS signal intensity, but with borate buffers, this effect was less severe and the obtained MS information was still adequate for exact mass determinations of intact proteins. The addition of organic solvents, such as acetonitrile, to the BGE can improve the separation and MS detection performance of proteins as discussed previously; however, care should be taken when noncovalent protein complexes are analyzed, as they could easily dissociate [37]. In CIEF-ESI-MS, things are a bit different when it comes to compatibility of the separation medium. The ampholytes required to create a pH gradient will inevitably give rise to MS background signals and protein ionization suppression. The most commonly applied approach is to compromise between separation (favored by high ampholyte concentration) and detection (favored by low ampholyte concentration) [68–71].

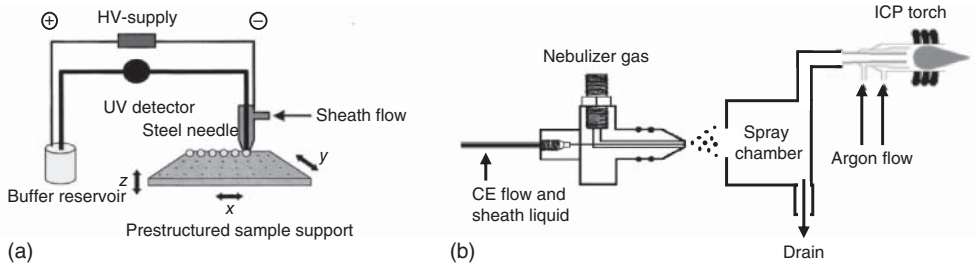



**Figure 7.3** Schematic representation for the most commonly used interfaces for (a) CE-MALDI-MS and (b) CE-ICP-MS.

Approaches that prevent the ampholytes from entering the ion source, such as microdialysis or combining CIEF with reversed-phase LC, have been used as an alternative strategy [72]. However, these have not been extensively applied as they lead to complicated systems that often cannot be handled routinely.

MALDI-MS is another widely applied ionization method for the analysis of intact proteins. For MALDI, the sample and a UV absorbing matrix are mixed, and the mixture is deposited onto a probe. With evaporation of the solvent, the analyte molecules cocrystallize with the matrix. After placing the probe into the vacuum of the MS, the protein spot is hit by a laser beam, which excites and partly vaporizes the matrix, also bringing the analyte molecules into the gas phase. Coupling of CE and MALDI-MS requires protein spotting. The sheath-flow fraction collection interface has proven to be the most simple and straightforward approach to interface CE with MALDI (Figure 7.3a). A droplet of CE effluent is mixed with the matrix solution at the capillary tip and deposited on the MALDI target. After all fractions are collected, the MALDI plate is transferred to the mass spectrometer and analyzed. From MALDI, often only singly or doubly charged proteins will result, which makes the mass spectra relatively simple to interpret. Interestingly, MALDI-MS is able to measure proteins with masses up to hundreds of thousands of Daltons, which can be problematic with ESI. Although MALDI is more tolerable toward buffers and salt than ESI, adduct formation is very common with MALDI [73]. Consequently, MALDI spectra give not as much information about proteins, merely providing a good indication of the molecular mass. Furthermore, the choice of matrix and matrix additives has been shown to significantly influence the MALDI-MS response of proteins [74].

ICP is another ionization method used for the analysis of intact proteins by CE-MS. In ICP-MS, the sample is introduced as an aerosol into the heart of an argon plasma [75, 76], operating at temperatures of 5000–9000 K. As the analytes pass through the plasma, they are completely broken down into charged elements, usually in the $M^+$ state. Subsequently, in the mass spectrometer, the specific elemental masses are monitored. In the basic CE-ICP-MS interface design, the capillary is placed in a nebulizer, through which the CE effluent is sprayed with the aid of a nebulizer gas (argon) into a spray chamber (Figure 7.3b), where the droplets are then transferred to the ICP torch [77, 78]. A sheath liquid is often used to complete

the electrical circuit for CE and to stabilize the formation of the aerosol. CE-ICP-MS allows the use of nonvolatile BGEs, such as TRIS, resembling physiological conditions during separation, while still obtaining good sensitivity. Notably, such conditions might not feasible in CE-ESI-MS. Elements such as carbon, oxygen, and nitrogen are not favorable for protein detection as analyte signals are completely suppressed by the excess of these elements in, for example, the buffer or ampholytes used in CE. Therefore, ICP-MS is most useful for the analysis of proteins containing metals and/or other heavy atoms such as sulfur or phosphorus. As the sample components are fully degraded into atoms, no information on the molecular mass of a protein is obtained with ICP-MS. Consequently, separating proteins prior to ICP ionization is essential in order to distinguish different proteins in one sample.

### 7.3.2 Mass Analyzers

In order to obtain suitable mass spectrometric information from proteins, the choice of mass analyzer can be critical. Initially in CE-ESI-MS, quadrupole and ion trap (IT) mass analyzers were used. These mass analyzers typically can cover a mass range of up to $m/z$ 4000, allowing detection of multiple charged proteins as obtained from ESI. Disadvantage of these two mass analyzers is their relatively low resolution (about 0.5–1 mass unit) in the 100–2000 $m/z$ range. This means that deconvolution of the mass spectra does not lead to a highly accurate mass of the protein and that it is impossible to distinguish between proteins that differ in molecular weight only by a few mass units. Over the last decade, one can observe an increasing use of high-resolution mass analyzers in CE-ESI-MS. Currently, time-of-flight (TOF) MS is the most commonly applied for the analysis of intact proteins [14]. Modern TOF MS systems can achieve resolutions between 30 000 and 80 000. Combined with high mass accuracies (low ppm) provided by these instruments, this means that 30–80 kDa proteins can be analyzed with unit mass resolution allowing modifications as small as a deamidation (leading to a mass difference of 1 Da) to be confidently characterized. Fourier-transform ion cyclotron resonance (FTICR) and Orbitrap mass analyzers have also been used occasionally for intact protein analysis by CE-ESI-MS. These mass analyzers can provide extremely high resolutions (even above 500 000 or more), however, at the expense of long duty cycles. This might compromise the achievable sensitivity of the system and does not allow adequate sampling of narrow CE peaks. For CE-MALDI-MS, only TOF mass analyzers are used. As mass determination with these analyzers is based on ion flight time, the upper mass limit is virtually unlimited, making it highly suited for detection of singly charged high-molecular-weight compounds as resulting from MALDI. In CE-ICP-MS for proteins, quadrupole-based mass analyzers are still the most frequently employed. As specific elemental masses have to be monitored, the mass resolution and accuracy of a quadrupole are sufficient.

## 7.4 Applications of Intact Protein CE-MS

Quickly after Smith *et al*. showed that CE-MS coupling was possible [16, 17], they also demonstrated that proteins could be analyzed [79]. Bovine insulin and two types of myoglobin were separated using CZE and molecular masses were determined with a quadrupole mass analyzer. Their research was the starting point for the exploration and application of CE with MS detection for the analysis of intact proteins. An overview of protein CE-MS is given next, making a subdivision in different fields of application. Throughout the text, the used CE (CZE or CIEF) and ionization mode (ESI, MALDI, or ICP) is indicated. The provided examples give an overview of the possibilities of CE-MS in protein analysis. For a more comprehensive overview of reported applications of intact protein CE-MS, the reader is referred to reviews [12, 13, 32, 33].

### 7.4.1 Biopharmaceuticals

Over the past years, biopharmaceuticals have gained increasing attention as a way to improve therapy for serious diseases. Quite a large number of studies have focused on biopharmaceutical characterization by various CE-MS approaches. Notably, a significant part of the biopharmaceuticals currently on the market are glycoproteins. Their analysis by CE-MS is discussed in more detail in Section 7.4.2. An overview of the CE-MS methods developed for the characterization of (glycosylated) biopharmaceuticals is given in Table 7.1.

Several studies deal with the characterization of recombinant human growth hormone (hGH). Since hGH is a relatively small protein with a pI around 5, it can be analyzed both on negatively charged coated capillaries using a medium BGE [52, 54–56] and on bare fused-silica capillaries utilizing a low-pH BGE [53] and high-pH BGE [48]. Catai *et al*. focused on the repeatable and highly efficient separation of hGH and its degradation products [52, 55]. After careful optimization of CE and MS parameters, stressed standard solutions and expired pharmaceutical formulations were analyzed. In heat-stressed samples, potentially deamidated and dideamidated forms of the protein were observed. Expired formulations also showed the presence of possible deamidation products. However, the resolution of IT mass spectrometer was not sufficient to unambiguously assign the deamidated species. On the other hand, CZE-ESI-IT-MS allowed the identification of oxidation products in the pharmaceutical formulation. In two other studies, high-resolution TOF MS was used to characterize degradation products of hGH formed upon heat exposure [48, 56]. Using a bare fused-silica capillary in combination with a high pH BGE, various degradation products could be separated (Figure 7.4a) [48]. The TOF MS was operated at a resolving power of nearly 30 000. Using these settings, it was possible to resolve the isotopes of rhGH (Figure 7.4b). Based on the shift in migration time and the clear mass shift of 1 Da, the observed three peaks could be ascribed to intact,

**Table 7.1** Overview of CE-MS methods for the analysis of (glycosylated) biopharmaceuticals.

| Analytes | BGE | Capillary coating | Mass analyzer[a)] | Remarks | References |
|---|---|---|---|---|---|
| Erythropoietin | 0.5–2 M Acetic acid | Several | TOF | Coating comparison and optimization | [34] |
| Erythropoietin | 1 M Acetic acid (pH 2.4), 20% methanol, and 2 M acetic acid | Polybrene-, polyacrylamide-based coating | TOF | Glycoform characterization; glycan release and analysis | [42] |
| Erythropoietin | 1 M Acetic acid (pH 2.4), 20% methanol | Polybrene | TOF | Glycoform characterization | [43] |
| Erythropoietin | 1 M Acetic acid (pH 2.4), 20% methanol, and 2 M acetic acid | Polybrene, polyacrylamide-based coating | TOF | Glycoform characterization; glycan release and analysis | [44] |
| Erythropoietin | 1 M Acetic acid (pH 2.4), 20% methanol | Polybrene | TOF | Glycoform characterization | [45] |
| Erythropoietin | 1 M Acetic acid | Linear polyacrylamide | TOF | Statistical evaluation of glycoprofiles | [46] |
| Erythropoietin | 2 M Acetic acid | Acryl amide | IT | Glycoform profiling | [47] |
| Erythropoietin, human growth hormone | 1 M Acetic acid and 40 mM ammonium bicarbonate (pH 8.5) | No coating and linear polyacrylamide | TOF | Product characterization | [48] |
| Erythropoietin, interferon-β-1a | 0.05–2 M Acetic acid | Polyacrylamide-based coating | TOF | Glycoform profiling | [40] |
| Glucagon | 1% Pharmalyte (pI 3–10) | Commercial neutral coating | TOF | CIEF-MALDI-TOF analysis | [49] |
| Growth hormone, insulin, hemoglobin | 75 mM Ammonium formate (pH 2.5 and 9.0) with can | No coating | TOF | Use of organic solvent to prevent protein adsorption | [24] |
| Hemoglobin | 75 mM Ammonium formate (pH 9.5) | No coating | TOF | Determination of hemoglobin-based oxygen carriers in plasma | [50] |

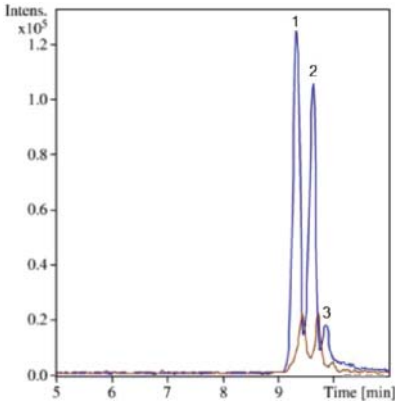
(*continued overleaf*)

**Table 7.1** (*Continued*).

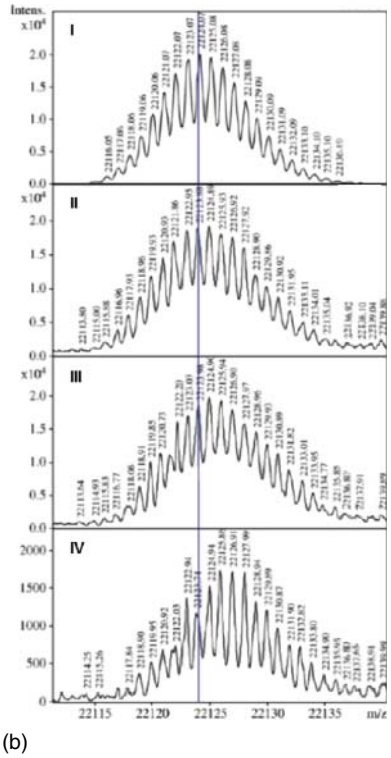
| Analytes | BGE | Capillary coating | Mass analyzer[a)] | Remarks | References |
|---|---|---|---|---|---|
| Human chorionic gonadotropin | 2% Acetic acid (pH 2.5) | Poly(vinyl alcohol) | FTICR | Glycoform profiling | [51] |
| Human growth hormone | 75 mM Ammonium formate (pH 8.5) | Polybrene-poly(vinyl sulfonic acid) | IT | Characterizing degradation products | [52] |
| Human growth hormone | 75 mM Ammonium formate (pH 2.5), 20% acetonitrile | No coating | TOF | Impurity profiling | [53] |
| Human growth hormone conjugate | 75 mM Ammonium formate (pH 8.5) | Polybrene-poly(vinyl sulfonic acid) | TOF | Determination conjugation degree | [54] |
| Human growth hormone, insulin | 75 mM Ammonium formate (pH 8.5) | Polybrene-poly(vinyl sulfonic acid) | IT | Optimizing CE-MS conditions and characterizing protein degradation products | [55] |
| Human growth hormone, insulin, | 75 mM Ammonium formate (pH 8.5) | Polybrene-poly(vinyl sulfonic acid) | IT | Characterization protein degradation products | [55] |
| Human growth hormone, interferon-β-1a, oxytocin | Acetic acid (pH 3.0) and ammonium formate (pH 8.5) | Polybrene-poly(vinyl sulfonic acid) and polybrene–dextran sulfate–polybrene | TOF | Characterization protein degradation products and glycoform profiling | [56] |
| Human serum albumin | 100 mM Ammonium bicarbonate (pH 7.8). | Hydroxypropyl cellulose | TOF | Product characterization | [57] |
| Insulin | 75 mM Ammonium formate (pH 9.0) with 10% ACN | No coating | TOF | Product characterization | [58] |
| Monoclonal antibody | 400 mM ε-Aminocaproic acid (pH 5.7) | Hydroxypropyl cellulose | TOF | CZE separation with off-line MALDI-MS | [59] |

| | | | | | |
|---|---|---|---|---|---|
| Monoclonal antibody | 200 mM ε-Aminocaproic acid (pH 5.7) | Hydroxypropyl cellulose | TOF | CZE separation with fraction collection. Subsequent direct ESI infusion in TOF-MS | [60] |
| Protein-drug conjugates | 100 mM Acetic acid (pH 3.1) | Polyethyleneimine | TOF | Determination conjugation degree | [61] |
| Protein-linker conjugates | 100 mM Acetic acid (pH 3.1) | Polyethyleneimine | TOF | Determination conjugation degree | [62] |
| Protein-linker conjugates | 100 mM Acetic acid (pH 2.8) | Polybrene–dextran sulfate–polybrene | TOF | Determination conjugation degree | [63] |
| Vascular endothelial growth factor 165 | 60 mM Formic acid | poly-LA 313 | TOF | Glycoform profiling | [64] |

a) FTICR, Fourier-transform ion cyclotron resonance; IT, ion trap; TOF, time-of-flight.




(a)




(b)

**Figure 7.4** (a) CZE-ESI-MS analysis of stressed hGH. The blue trace represents the EIE of hGH (1), deamidated hGH (2), and dideamidated hGH (3). The brown trace represents the EIE of peaks 1–3 after spontaneous elimination of PhePro. (B) Simulated isotope pattern of intact rhGH (I) and charge deconvoluted mass spectra of peaks 1 (II), 2 (III), and 3 (IV) of stressed rhGH, respectively. The blue line is to clarify the shift of the maximum of the isotopic distribution by 1 Da at a time. (Reproduced from [48] with permission from Elsevier.)

singly, and doubly deamidated rhGH, respectively. Interestingly, additional peaks were observed that had a slightly longer migration time; however, these were not fully separated from the main peaks (Figure 7.4a). After deconvolution, a mass difference of 244 Da was observed. Masses and relative migration times led to the assumption that these peaks corresponded to proteins originating from non-, mono-, dideamidated rhGH and spontaneous removal of the two *N*-terminal

amino-acid phenylalanine and proline due to the stress conditions. Similar analyses performed at high pH applying a negatively charged coating showed that, upon heat exposure, oxidation, sulfonate formation, and deamidation take place [56]. To distinguish natural hGH from recombinant hGH, a CZE method that uses a low-pH BGE containing acetonitrile in combination with a bare fused-silica capillary was developed [53]. The method was successfully applied to distinguish hGH and recombinant hGH in samples of unknown origin. Besides identifying degradation products, CZE-ESI-MS was used to characterize intentional modifications of hGH [54]. A chelating agent was covalently coupled to hGH to enable the protein to be used for diagnostic and therapeutic purposes. CZE-ESI-MS showed that the reaction mixtures were heterogeneous between 0 and 5 chelating molecules attached per molecule hGH.

CZE-ESI-MS was also used for the characterization of other protein conjugates [61–63]. For example, conjugates of a kinase inhibitor coupled to lysozyme via a noncovalent platinum(II)-based linker were analyzed by CE-MS. Narrow symmetrical peaks for the various reaction products were obtained demonstrating that conjugates remained stable during the CE analysis and subsequent ESI. Components observed in the drug–protein products were assigned based on their relative migration times and the molecular mass obtained by TOF MS, revealing the presence of unmodified protein and protein with up to two drug molecules. The same approach was also used to characterize llama antibodies that were modified with *N*-succinimidyl-*S*-acetylthioacetate [62, 63].

Blood doping involves the use of products that enhance the uptake, transport, or delivery of oxygen to the blood. One approach uses artificial oxygen carriers, known as *hemoglobin-based oxygen carriers* (HBOCs). CZE-ESI-MS was successfully applied to measure these components in plasma samples [50]. To prevent adsorption of the proteins to the fused-silica capillary wall, a high-pH BGE was used. Hemoglobin and a commercial HBOC were baseline separated under these CE conditions, while with ESI-TOF MS, the proteins were identified. In the gas phase, hemoglobin and the HBOC dissociated into their α- and β-subunits. As the HBOC subunits had a different mass with respect to those of hemoglobin, both proteins could be discriminated.

Human serum albumin (HSA) [57] and insulin [58] have been analyzed with CZE-ESI-MS as well. In the case of albumin, up to nine different isoforms could be separated by CZE, whereas ESI-MS allowed unambiguous identification of five of these, including truncated and glycated products [57]. Furthermore, applying the developed methodology for the analysis of albumin preparations marketed by five pharmaceutical industries revealed significant differences in isoform distribution between the products. A generic quantitation CE-MS method was developed for insulin [58]. The quantitation was based on a multiple injection strategy, where a plug of reference (insulin) standard is injected followed by a large plug of BGE. Subsequently, the (insulin) sample of interest is injected and the separation is started. Two peaks, both corresponding to insulin, are obtained, of which the first can be used to quantify the second. The methodology was validated according to international guidelines and was successfully applied to the analysis of insulin formulations obtained from regular and parallel markets.

The analysis of the biopharmaceutical glucagon has been performed using CIEF and MALDI-TOF MS via a spotting device [49]. MS electropherograms were constructed by plotting the intensities of the $m/z$ values corresponding to the proteins versus migration time (related to spot number). Focusing and spotting times were optimized using the constructed MS as well as the UV electropherograms. The applicability of the system was demonstrated by the analysis of the biopharmaceutical glucagon that was incubated with hydrochloric acid for 2 weeks. Both glucagon and a single degradation product were baseline separated. Based on the observed shift in pI and mass difference of 1 Da, the degradation product was ascribed to a single deamidation.

### 7.4.2 Glycoproteins

Glycosylation is an important parameter in defining biological and biophysical properties of a broad range of proteins. Oligosaccharides are attached to proteins posttranslationally, and therewith, glycosylation is a major source of protein heterogeneity. The carbohydrate structure plays an important role in determining biological activity, and quite some efforts have been made to characterize these proteins using CE-MS. The first protein with a complex glycosylation pattern, which was extensively analyzed with CZE-ESI-MS, is erythropoietin (EPO) [34, 40, 42–46, 48]. By using a suppressed EOF, very high separation efficiencies of closely related recombinant human EPO isoforms can be obtained (Figure 7.5a)

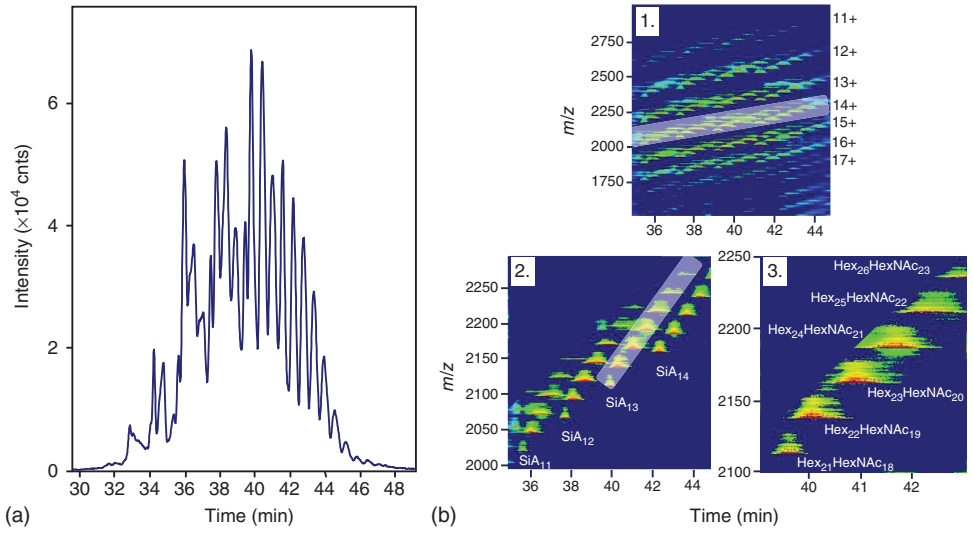



**Figure 7.5** CZE-ESI-MS of recombinant human erythropoietin employing a sheathless interface in combination with a neutrally coated capillary. (a) Base peak electropherogram; (b1) contour plot with zooms of (b2) the $14^+$ charge state of the glycoforms and (b3) the $SiA_{13}$ sialoforms of the $14^+$ glycoforms. (Reproduced from [40] with permission from American Chemical Society.)

[40]. The separation is mainly due to differences in amount of sialic acid residues, leading – in this specific case – to almost a 2-min migration time shift per sialic acid unit (Figure 7.5b). Differences in the hexose-*N*-acetyl-hexosamine content also led to small differences in electrophoretic mobility and, thus, partial separation. Overall, more than 250 different isoforms, including glycosylation, oxidation, and acetylation products, could be distinguished in one CE-MS run of EPO. As this study was performed using a sheathless interface and a capillary flow of about 5 nl $min^{-1}$, nanoESI conditions were achieved. This significantly improved ionization efficiencies and decreased ionization suppression for intact proteins, leading to limits of detection of isoforms in the picomolar range [40].

More and more biopharmaceuticals come off patent, and as a result, significant amounts of the so-called biosimilars and biogenerics become available. In order to compare these products to the original one, especially when they are complex glycoproteins, statistical methods are key. A statistical approach to differentiate between various EPO preparations on the basis of CE-MS data was developed [46]. As input, relative peak areas of selected intact EPO isoforms were used as variables in both principal component analysis and hierarchical agglomerative clustering. Both approaches enabled clear differentiation of EPO preparations, differing in manufacturer, production cell line, and/or batch number. Even closely related preparations were distinguished on the basis of the combined information on the antennarity, sialoform, and acetylation of the observed isoforms.

CZE-ESI-MS has been used for the analysis of the glycosylation of various other proteins [47, 51, 56, 64, 80–87]. Neutral, negatively charged, and positively charged coatings have been used to (partially) separate the different protein isoforms. With the negatively charged coating, high-pH BGEs are required to prevent protein adsorption to the wall, while with the other coatings, a low-pH BGE is often used. An interesting example of glycoprotein analysis by CZE-ESI-MS is the analysis of vascular endothelial growth factor 165 ($VEGF_{165}$) [64]. $VEGF_{165}$ is a putative biomarker and is considered as a promising therapeutic drug. An in-house developed polycationic polymer coating in combination with a low-pH formic acid BGE was found suitable for the separation of isoforms of recombinant human $VEGF_{165}$ expressed in *Spodoptera frugiperda*. At least seven partially separated protein species were detected. However, protein mass spectra were complex, showing multiple series of protein charge envelopes within a single electrophoretic peak. After deconvolution of the mass spectra, 16 glycoforms of intact $VEGF_{165}$ were identified. The glycosylation of this protein is mainly based on biantennary structures consisting of hexose and *N*-acetylhexosamine units. In some glycoforms, deoxyhexose units were also attached to the biantennary structures. Besides glycoforms, protein variants with a loss of amino acids at the *C*- and *N*-terminus were identified, giving rise to a total number of 19 different variants of intact $VEGF_{165}$.

α-1-Acid glycoprotein was analyzed by CZE-ESI-MS in biological samples [82]. A positively charged coating in combination with a BGE of low pH was used to separate the protein glycoforms. Upon CE-MS analysis, a large number of isoforms differing in attached glycans were found. However, due to the high number of possible sugar substitutions and other minor modifications, no unequivocal

carbohydrate composition could be elucidated directly from the CE-MS data. Therefore, the deglycosylated protein and released glycans were also analyzed with CE-MS. Combination of the recorded data allowed identification of more than 150 isoforms, differing both in amino acid sequence and in attached glycans. Interestingly, this approach was used to analyze samples from 16 individuals (8 healthy, 8 bladder cancer) [83]. Upon data analysis employing different statistical techniques, significant differences between the two groups were observed. These differences between the groups were assigned to a higher abundance of tri- and tetra-antennary fucosylated glycoforms in cancer patients.

Recently, two CZE-MS studies have focused on the characterization of monoclonal antibody charge variants and glycosylation. As monoclonal antibodies are

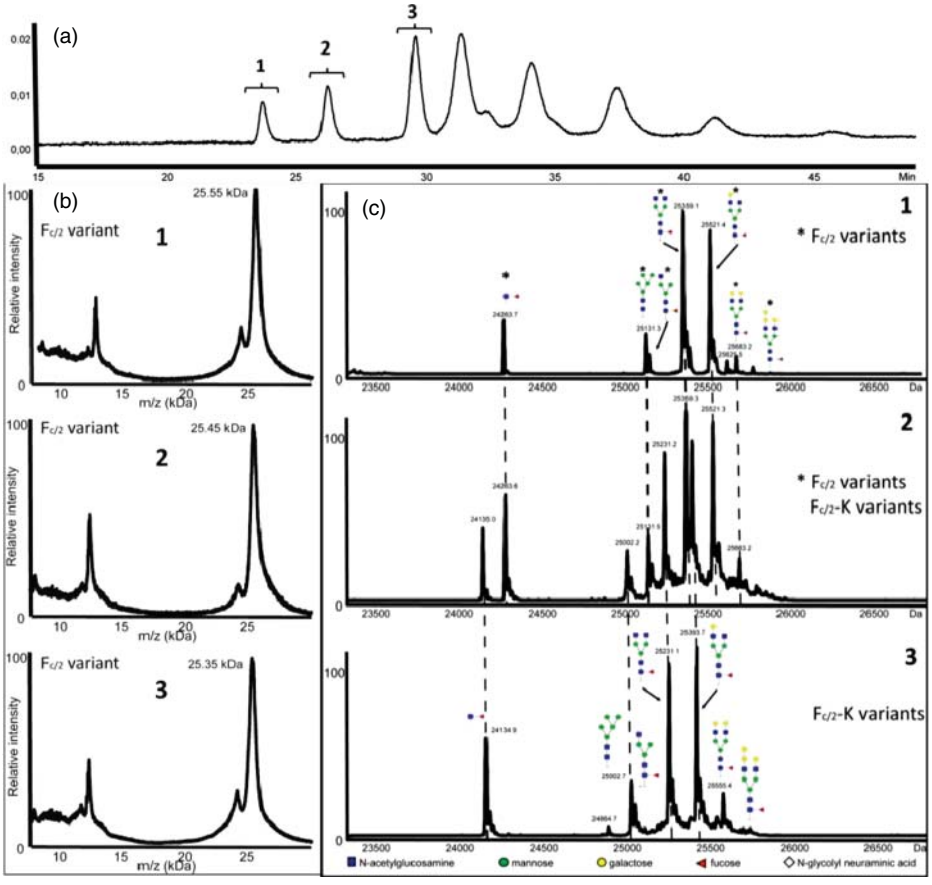



**Figure 7.6** CZE analysis with off-line MS detection of partially digested antibody. (a) CZE-UV electropherogram showing the charge variants of the Fc/2 (peak 1–3) and $F(ab')_2$ (peaks between 30 and 45 min) fragments. (b) MALDI-TOF-MS and (c) ESI-TOF-MS mass spectra obtained for the three separated and off-line collected Fc/2 charge variants. Identified glycoforms are annotated in panel (c). (Reproduced from [60] with permission from American Chemical Society.)

currently the most important therapeutic protein class, their analysis also gained a lot of interest. Separation of intact antibody charge variants could be achieved using a BGE containing high concentrations of nonvolatile salts [59]. The use of this BGE made direct coupling with ESI-MS impossible. Consequently, the CE effluent was spotted on a plate to allow off-line MALDI-TOF MS analysis. Unfortunately, the accuracy and resolution of their mass spectrometer were insufficient to provide any detailed information about the presence of isoforms. In a next study, the approach was slightly changed. The antibody was partially digested using the immunoglobulin degrading enzyme of *Streptococcus pyogenes*, resulting in the formation of $F(ab')_2$ (100 kDa) and Fc/2 (25 kDa) fragments [60]. These fragments could not only be separated from each other, but charge variants of both fragments could be discriminated as well (Figure 7.6a). Subsequent MALDI-TOF MS analysis of the collected peaks again did not yield accurate mass data (Figure 7.6b). However, when the collected fractions were infused using nanoESI, accurate and detailed mass data was obtained (Figure 7.6c). This provided detailed information about the presence of charge variants as well as glycoforms in different parts of the antibody.

### 7.4.3 Protein–Ligand Interactions

The bioactivity of proteins is based on their capacity to bind to their targets. CE-MS has proven to be very useful in studying these processes. One way to approach this is to analyze the protein and add the ligand to the BGE. In a recent study, the binding of the protease inhibitor aprotinin to trypsinogen was studied by CZE-ESI-MS [88]. A trypsinogen sample comprising several modifications was analyzed using a BGE of 25 mM ammonium acetate (pH 8.0) containing increasing concentrations of aprotinin. A capillary coating of polybrene–dextran sulfate–polybrene was employed to prevent adsorption of the proteins to the capillary wall. The trypsinogen variants were separated and could be assigned based on detected molecular masses and relative migration (Figure 7.7). For most trypsinogen variants, shifts in electrophoretic mobility were observed upon increasing the aprotinin concentration (Figure 7.7a and b), allowing determination of their dissociation constants. All the interacting trypsinogen variants showed similar low-micromolar affinities toward aprotinin. In presence of aprotinin, both free and aprotinin-bound trypsinogen were detected, revealing a 1 : 1 binding stoichiometry (Figure 7.7c). Their ratio was also explored as a way to determine dissociation constants. The obtained dissociation constants were higher and less precise as compared to values based on the mobility shifts. However, they did show high similarity with results obtained in direct infusion MS. This indicated that the higher values and spread were caused by MS detection and not by the ACE analysis.

CZE-ESI-MS was used to get insight into the dimerization process of stromal cell-derived factor-1 [89]. Using a neutrally coated capillary in combination with a BGE of physiological pH, it was demonstrated that the native protein and a site-directed (nonbinding) mutant are monomeric in solution. The addition

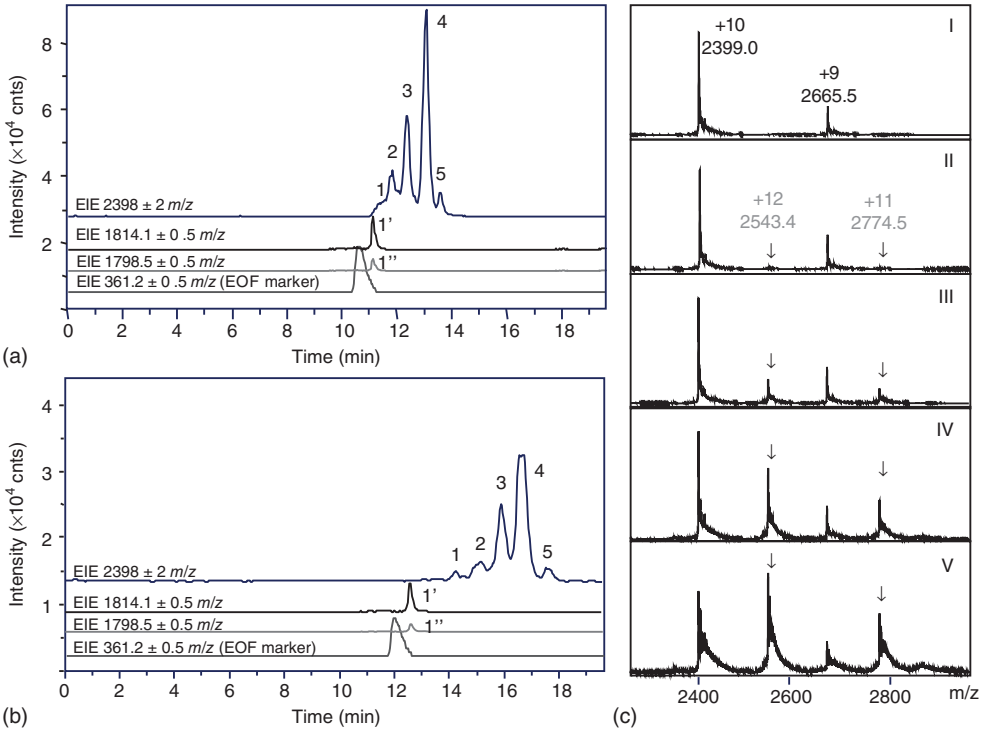



**Figure 7.7** CZE-ESI-MS analysis of trypsinogen sample using a positively charged coated capillary and a BGE of 25 mM ammonium acetate (pH 8.0) containing no (a) or 25 μM (b) aprotinin. Extracted-ion electropherograms of trypsinogen (2398 *m/z*), trypsinogen variants (2397–2399, 1814.1, and 1798.5 *m/z*) and cortisone (EOF marker) (371.2 *m/z*) are depicted. (c) Mass spectra obtained for peak 4 during CZE-ESI-MS of trypsinogen using a BGE of 25 mM ammonium acetate (pH 8.0) containing no (I), 2 (II), 15 (III), 50 (IV), or 150 μM (V) aprotinin. Arrows indicate trypsinogen–aprotinin complex ions. (Reproduced from [88] with permission from American Chemical Society.)

of sulfated disaccharides to the BGE showed that, under these conditions, primarily a 1 : 1 saccharide–protein complex is formed. When subsequently longer oligosaccharides (up to six repeating units) were added to the BGE, mainly the 1 : 2 saccharide–protein complex was observed. Performing the same experiments using the mutant demonstrated that this protein did not interact with any of the used oligosaccharides.

Besides adding the ligand to the BGE, the protein and ligand can be incubated prior to analysis. When a small sample plug is injected, this is referred to as *the direct separation approach*. This approach has been most commonly used to study the binding of metallodrugs to HSA and transferrin (Tf) using ICP-MS detection [90–99]. The binding of ligands to these proteins is very important as, in general, HSA and Tf are the main binding partners of various drugs. In these studies, consecutive measurements with increasing ligand concentrations allow the determination of the binding constant, whereas measuring in time under one

set of conditions allows determination of the kinetics of the interaction. Affinities of, for example, anticancer drugs [90–94, 99] and antidiabetic complexes [95] toward both proteins were monitored. Interestingly, the CZE analysis of HSA and Tf can be carried out using a bare fused-silica capillary with a buffer at physiological pH (7.4), although the use of a coating and a low-pH BGE could improve the separation between the two proteins [93]. BGE conditions can severely affect the observed affinity, even though the sample is preincubated prior to analysis. Sometimes, low-pH conditions will lead to satisfactory results [93], whereas in other cases, protein–ligand binding can only be probed at physiological pH [91]. Even when working at physiological pH, changes in the composition of the BGE can lead to different outcomes of the same affinity experiments [91, 95].

Other studies using the direct separation approach have used ESI-MS detection. Lyubarskaya *et al.* studied the interaction between the src homology 2 domain and tyrosine-phosphorylated peptides [100]. The authors used CIEF coupled with ESI-MS to study this interaction. Stable complexes were formed by preequilibration of the compounds in solution. These complexes could be separated from the free ligand and receptor on the basis of differences of isoelectric points. $MS^3$ fragmentation allowed release of the peptide from the complex and subsequent sequencing of the free ligand, enabling peptide identification. Hoffmann and Martin developed a direct separation CE-MS method that allowed the analysis of α-chymotrypsin and a chymotrypsin–chymostatin complex under ESI-MS-compatible conditions [101]. A neutral coating in combination with an ammonium formate BGE (pH 4.0) allowed the most efficient separation of chymotrypsin and the chymotrypsin–chymostatin complex. A sheath liquid containing water, isopropanol, and formic acid was found as a compromise between signal intensity and maintaining the complex intact in the gas phase. In this case, MS excitation voltages could be used to selectively release the ligand from the enzyme complex. The excitation voltage in which 50% of the complex dissociated was defined as the binding strength of the complex. Other protein–ligand complexes were analyzed at this excitation voltage, and a relative comparison of binding strengths could be made based on MS/MS signal areas.

A pre-equilibrated sample can also be introduced in a relatively large volume. In this so-called frontal analysis (FA) approach, it is assumed that two of the three components in the sample (commonly receptor and complex) have approximately the same mobility and that the mobility of the third (the ligand) differs significantly. Upon application of the electrical field, the free ligand starts to separate from the mixture and two plateaus will be detected. FA-MS has, until now, been exclusively used to study the interaction between proteins and lower-molecular-weight ligands. In two instances, the interaction between proteins and drugs was studied [102, 103]. Both studies were mainly focused on optimizing separation and detection conditions and eventually were able to determine affinity constants for selected protein–drug interactions in aqueous solutions [102] as well as in diluted human plasma [103]. Fermas *et al.* used a slightly different approach for the affinity analysis of antithrombin and a sulfated pentasaccharide [104]. In their study, they employed frontal analysis continuous CE (FACCE), where the sample is

introduced continuously rather as a discrete plug. Hence, sample introduction and separation are integrated and separated analytes appear as progressive plateaus in the electropherogram. In their case, as the pentasaccharide is strongly anionic, the complex was more negatively charged than the free protein, thereby allowing the separation of both species. The FACCE-ESI-MS method was compared with direct analysis of the pre-equilibrated sample. It was demonstrated that a higher sensitivity was obtained with FACCE-MS, most likely due to the continuous injection. In this study, ammonium acetate (pH 6.5) was required as sheath liquid to prevent complex dissociation.

One study focused on the comparison between adding the ligand to the BGE and pre-equilibrating the protein and ligand. This study used CE-ICP-MS to determine the binding constants of organotin compounds toward HSA [98]. In the former case, measured dissociation constants were in close agreement with literature values. On the other hand, when analyzing the pre-equilibrated sample, the dissociation constants were about one order of magnitude higher. Although the reason for the difference was not fully investigated, protein adsorption to the inner wall of the capillary was indicated as the cause. Resulting loss of compound and extra peak tailing would affect accurate determination of the peak areas needed to calculate the dissociation constant.

### 7.4.4 Metalloproteins

Metallothioneins (MTs) are proteins that have a relatively low molecular weight and a high cysteine and metal content. MTs are thus frequently analyzed with CZE-ICP-MS. The analysis of MTs is very straightforward, as in most cases, a bare fused-silica capillary with a buffer around physiological pH (6.8–7.8) gives sufficient separation [78, 105–120]. The identity of the MTs is determined by analyzing the traces of copper, zinc, cadmium, and/or sulfur. This can provide stoichiometric information on the proteins if more than one element is monitored. MTs have been analyzed by CZE-ICP-MS in a variety of matrices including human brain cytosols [113], mussel hepatopancreas cytosols [115], rabbit liver [109, 110, 121], eel liver [108], rat liver [111, 122], roe deer liver [106], bream liver [106, 107], and cyanobacterium *Synechococcus* [123]. Due to the element-specific detection, matrix components do not interfere with ICP-MS, which makes the analyses rather straightforward. As a typical example, Wang *et al.* used a covalent 2-acrylamido-2-methyl-1-propanesulfonic acid coating in combination with TRIS (pH 7.4) as BGE for CZE-ICP-MS [109]. Nine different MTs could be resolved in a rabbit liver sample, while monitoring the traces of sulfur, copper, zinc, and cadmium allowed for the stoichiometry of the different MT isoforms to be estimated (Figure 7.8). The MTs, coming from a commercial rabbit liver MT solution, were found to be isoforms of MT1 and MT2, and one unknown MT. CZE-ESI-MS was also used to identify this type of proteins in rat [122, 124], rabbit [125–127], sheep [127], mice [128], and baker's yeast [127] samples. As ICP and ESI give complementary information, CZE-ICP-MS and CZE-ESI-MS have been used together

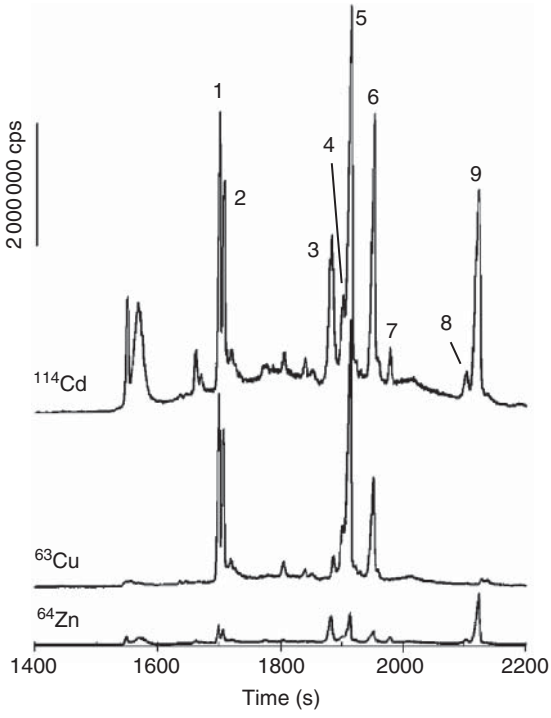



**Figure 7.8** CZE-ICP-MS analysis of a rabbit liver MT sample using a 2-acrylamido-2-methyl-1-propanesulfonic acid coated capillary. The proteins are detected by their cadmium, copper, and zinc content as monitored with ICP-MS. Numbers indicate the MT isoforms that were identified. (Reproduced from [109] with permission from American Chemical Society.)

for MT analysis in order to unambiguously determine which isoforms are present in a sample [111, 121, 122].

Other clinically relevant metal-binding proteins have also been examined by CE-MS. The analysis of hemoglobin variants, for example, is often hindered by the fact that the variants are very similar and, therefore, difficult to separate. With CIEF, it is possible to separate hemoglobin variants, although their pI differs only as little as 0.05 pI units [71, 129–131]. Hemoglobin variants A, C, F, and S were separated after focusing in a polyacrylamide-coated capillary containing a pH gradient ranging from 5 to 8 [71]. Hemoglobin consists of four noncovalently bound chains: two α-chains, which are present in each variant, and two β- or γ-chains, which differ per variant. In the ESI interface, hemoglobin can dissociate, and by scanning specific single mass traces, corresponding to the different chains, the hemoglobin variant can be identified. Concentration limits of detections were calculated to be in the range of $10^{-8}$ M and were attributed to the focusing during mobilization and the selective detection of the hemoglobin chains, which minimizes interferences of other constituents. It is even possible

to determine hemoglobin in whole human blood with CIEF-ESI-MS without any sample preparation [129]. One of the major problems to be circumvented is the presence of high concentrations of physiological salts. A voltage gradient can be applied before focusing the proteins in order to migrate the salts out of the capillary [129, 132]. Although complex electropherograms will be obtained, the relatively high concentration of hemoglobin and the selection of specific mass traces for the hemoglobin chains will make it rather straightforward to analyze this protein. The CZE-ESI-MS analysis of hemoglobin [133–136] in human red blood cells has also been reported. Aminopropylsilane-coated capillaries were used in combination with acetic acid BGEs in order to minimize interactions with the capillary wall and to create a stable EOF. The analysis of hemoglobin in a single red blood cell (~90 fl) with CE and high-resolution mass spectrometry was described [135]. An FTICR mass analyzer was used in combination with a 20 μm ID CE capillary, and the α- and β-chains of hemoglobin were detected in the cell lysate by scanning a small mass range to improve sensitivity. Cao and Moini used a similar methodology, with a 30 μm ID capillary in combination with a TOF mass analyzer [134]. No baseline separation of the cellular content was obtained, but monitoring of specific mass traces allowed detection of the α- and β-chain of hemoglobin.

### 7.4.5 Top-Down Protein Analysis

In classical proteomics, protein-derived peptides are separated and subsequently fragmented in MS/MS approaches to reveal the amino acid sequence. Based on these peptide sequences, proteins can be identified. Intact protein fragmentation, however, has long been a challenge. Over the last years, techniques that enable efficient intact protein fragmentation – such as electron-transfer dissociation (ETD) and higher-energy collisional dissociation (HCD) – have been introduced. Over the last few years, a few studies have demonstrated that efficient CE separations can be combined with a top-down proteomics approach [137–142]. Besides evaluating this approach on protein standards, top-down proteomics has been applied to characterize unknown proteins in cell lysates [137, 140], cell secretome [142], and culture filtrates [138], as well as to determine protein phosphorylation sites [141]. Whereas the top-down approach does allow identification of several dozens [137, 138, 141, 142] up to hundreds of proteins [140], unambiguously pinpointing site-specific modifications still proves troublesome [141]. It should be noted that the time to generate good-quality fragmentation spectrum is limited to the protein peak width (often around 0.5 min). Currently, with these top-down techniques, long acquisition times are still required (up to several tens of minutes), so complete sequence coverage is often not obtained. Low-molecular-weight proteins are most confidently identified, although identifications of proteins with a molecular weight up to 80 kDa have been reported [137]. To improve sequence coverage, a combination of several top-down fragmentation approaches might be used. For example, Zhao *et al*.

used a combination of ETD and HCD for intact protein identification [142]. As a proof of concept, they were able to separate four model proteins efficiently with CZE (Figure 7.9a) and used the two fragmentation methods to generate MS/MS spectra (Figure 7.9b). Although ETD and HCD separately enabled intact protein identification, combining the data increased the sequence coverage of the proteins on average with a factor of 2 (Figure 7.9c). This combined ETD/HCD

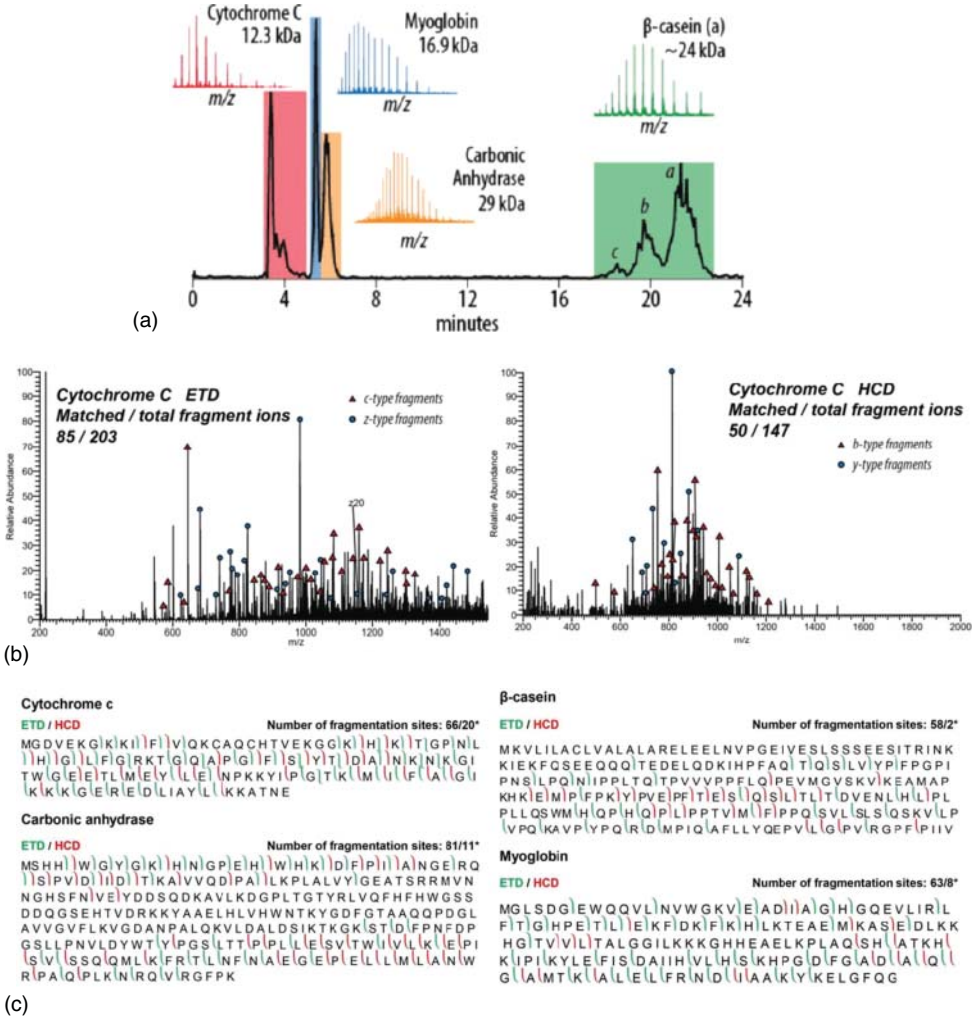



**Figure 7.9** CZE-ESI-MS analysis of four standard proteins. (a) Base peak electropherogram. Colored MS spectra correspond to migration profiles of the designated proteins. (b) ETD and HCD fragmentation spectra of cytochrome *c* with the identified fragment ions. (c) Sequence maps show combined fragmentation patterns of ETD and HCD for the four standard proteins. *Number of fragmentation sites: total fragmentation sites/overlapped fragmentation sites. (Reproduced from [142] with permission from American Chemical Society.)

approach was subsequently used to identify proteins in a cell secretome. Similar to the model proteins, an increase in sequence coverage was obtained and a larger set of proteins was identified compared to separate ETD and HCD experiments.

### 7.4.6 Other Selected Applications

A good example of the separation efficiency of CZE is the analysis of reduced and oxidized forms of cytochrome c [143]. Cytochrome *c* containing $Fe^{3+}$ can be reduced by thiol-containing compounds to the $Fe^{2+}$-containing protein. As a consequence, the proteins will differ by one positive charge and can be separated by CZE (Figure 7.10). Using a BGE of pH 6 in combination with a neutral coating, the positively charged proteins (pI 9.6) migrate toward the detector. Simultaneously, the excess of the negatively charged reducing agent (pI 3.9) migrates toward the inlet of the capillary and, therefore, does not interfere with MS detection. As the only difference in the two protein forms is an electron, the mass spectra did not show any difference between the reduced and oxidized cytochromes (Figure 7.10).

CGE is a well-established technique in biopharmaceutical industry, especially for the characterization of monoclonal antibodies. Consequently, its coupling to MS detection has an added value for protein identification. Recently, a novel strategy to couple CGE with MS detection was developed [19]. CGE-separated proteins were collected on a poly(tetrafluoroethylene) membrane placed in a grounded outlet reservoir that is moved during the separation. The SDS that is commonly used in CGE could be washed off from the proteins deposited on the

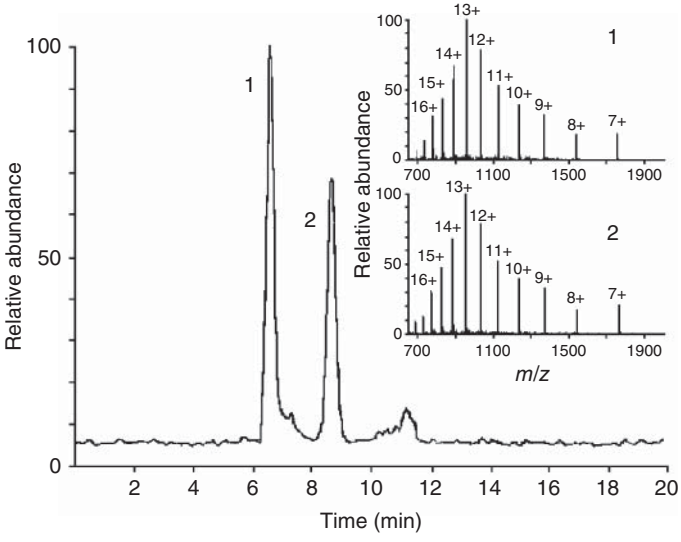



**Figure 7.10** CZE-ESI-MS analysis of cytochrome *c* incubated with Bcl-$X_L$ peptide, showing both the oxidized (1) and reduced cytochrome *c* (2). Insets show the ESI mass spectra of the peaks. (Reproduced from [143] with permission from Elsevier.)

membrane. Subsequent MALDI-TOF-MS analysis enabled protein identification. Although just demonstrated for model compounds, proteins that differ significantly in molecular weight could be efficiently separated and detected. The main limiting factor was actually the limited separation efficiency of CGE for proteins of similar molecular weight.

Some proteins have a quaternary structure, which means that they are a complex of multiple (different) protein molecules. Martinovic *et al*. showed that noncovalent protein complexes can be characterized using CIEF separation and subsequent ESI-MS detection [144]. The advantage of using CIEF is that the entire capillary can be filled with sample, enabling low concentration limits of detection. Moreover, in this case, only ampholytes are used for separation, ensuring that the complexes stay intact during the focusing step. The separation of two protein complexes was accomplished with a neutral polyacrylamide-coated capillary containing a pH gradient ranging from 3 to 10. After mobilization, the acidic sheath liquid and high voltages of the electrospray interface caused dissociation of the complexes. This resulted in the detection of monomeric species using an FTICR mass analyzer (Figure 7.11a). By using a medium-pH sheath liquid and lower ESI

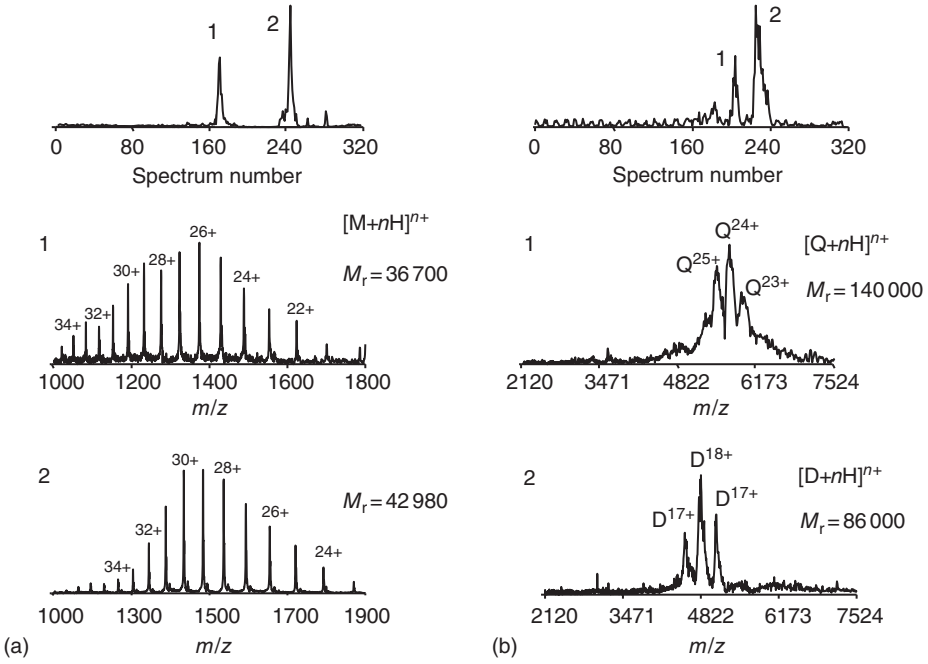



**Figure 7.11** CIEF-ESI-MS analysis of a mixture of the noncovalent protein complexes GADH (1) and CPK (2). (a) Mass spectra of monomeric, dissociated species are obtained upon addition of an acidic sheath flow and high ESI voltages. (b) Mass spectra of GAPDH tetrameric and CPK dimeric complexes are obtained with a medium-pH sheath liquid and lower ESI voltages. (Reproduced from [144] with permission from American Chemical Society.)

voltages, complexes could be preserved (Figure 7.11b). From the molecular masses, the stoichiometry (quatenary or dimeric complex) could be derived.

As indicated previously, the sheath liquid can lead to some loss of sensitivity. However, it can also be tuned to modify the ionization without affecting CE selectivity and efficiency. For example, the addition of the so-called supercharggers to the sheath liquid can modulate the charge state distribution of intact proteins [145]. Four supercharging reagents – 2-nitrobenzyl alcohol, 4-nitrobenzyl alcohol, 3-nitrobenzyl alcohol, and sulfolane – were tested and their impact on protein ionization, conformation, and detection sensitivity was evaluated. With two of the selected superchargers (3-nitrobenzyl alcohol and sulfolane), effects on the protein charge state distribution were observed, and in some cases, even an improvement in sensitivity was achieved. It should be noted that these observations were most pronounced when low-pH BGEs were used. It was postulated that proteins that are negatively charged in solution (i.e., when using high-pH BGEs) are not easily affected by superchargers in the gas phase. Importantly, it was shown that, independently of the pH of the BGE, the CE performance did not deteriorate by the presence of supercharging reagents in the sheath liquid.

## 7.5 Conclusions

This chapter provides an overview on the current status of the analysis of intact proteins when combining the high separation efficiency of CE with selective and sensitive MS detection. Over the last three decades, many combinations of CE modes and ionization techniques have been used to tackle the analytical question at hand. From the number of presented examples, it is obvious that CE-ESI-MS is the most widely used and mature combination. Currently, most studies using this combination are application-driven; however, there are still methodological – more fundamental – studies being performed to push the technique forward. Although used in a much lesser extent, both CE-MALDI-MS and CE-ICP-MS also have reached a state of maturity, and there is now a more stronger focus on the applicability of these combinations.

From the presented examples, it can be concluded that CE is especially suitable for the separation of protein modifications leading to charge differences. For example, degradation products as result of deamidation can be efficiently resolved from the parent compound. Also, protein glycoforms, which differ in the number of sialic acid groups, can be separated. Protein modifications that do not lead to a charge difference are more difficult to separate. Still, when the modification leads to a considerable size difference, a partial separation can often be obtained. Moreover, the partially resolved components can still be assigned when mass spectrometers of sufficiently high resolution and accuracy are applied.

From an application point of view, it seems that CE-MS is best established in the characterization of (glycosylated) biopharmaceuticals. This is not surprising, since this application area has gained increasing attention since the

pharmaceutical industry is moving more and more in this direction. Since the main biopharmaceuticals on the market are antibody-based, it is foreseen that research will be more directed to this field. However, due to their high molecular weight and significant heterogeneity, the analysis of intact antibodies will not be straightforward. Modifications of antibodies (e.g., deamidations or glycosylation) will have a relatively small effect on the overall charge and size of the 150 kDa protein. Creating gas-phase ions by ESI or MALDI from such high-molecular-weight compounds after a CE separation will require thorough optimization and characterization of separation and ionization parameters. Approaches using mild reduction or partial enzymatic cleavage of the antibody seem a good middle road for characterization at this stage.

Other main fields of application are the measurement of protein–ligand interaction and the analysis of metalloproteins. CE-ICP-MS is well established in both fields to look at the stoichiometry of metals present in proteins, but also to investigate the interaction between metal-containing components and proteins. Over the past years, an increasing interest has been observed for the CZE-ESI-MS characterization of protein–ligand interactions. This is again triggered by the biopharmaceutical industry, where the efficacy of a drug can depend on the protein modifications present. Affinity determinations with CZE-ESI-MS theoretically can provide assignment of the molecular weight of protein sample components (including modified forms, other variants, and impurities), as well as protein-variant-selective determination of multiple dissociation constants and establishment of protein-complex stoichiometries. This clearly is a very strong combination when characterizing proteins.

A drawback of analyzing intact proteins with CE-MS might be that an unambiguous assignment of protein modifications is not always possible. To facilitate the identification and position of modifications, bottom-up approaches (i.e., proteolytic digestion of the protein) can be performed. However, with the recent advent of advanced fragmentation tools for intact proteins, more opportunities for in-depth intact protein characterization have appeared. Although these top-down approaches are still in the exploratory phase, the first results look promising and will surely find more widespread use.

Overall, it is concluded that CE-MS in all of its facets is a valuable technique with high potential for intact protein analysis, providing useful information on protein identity and purity, including modifications and degradation products. Especially, compared to other current methodologies on the market, CE-MS is strong in the cases where separation of highly similar protein species or complex mixtures of proteins is paramount.

#### Abbreviations

| | |
|---|---|
| BGE | background electrolyte |
| CE | capillary electrophoresis |
| CGE | capillary gel electrophoresis |

| | |
|---|---|
| CIEF | capillary isoelectric focusing |
| CZE | capillary zone electrophoresis |
| EOF | electro-osmotic flow |
| EPO | erythropoietin |
| ESI | electrospray ionization |
| ETD | electron-transfer dissociation |
| FA | frontal analysis |
| FACCE | frontal analysis continuous capillary electrophoresis |
| FTICR | Fourier-transform ion cyclotron resonance |
| HBOC | hemoglobin-based oxygen carrier |
| HCD | higher-energy collisional dissociation |
| hGH | human growth hormone |
| HSA | human serum albumin |
| ICP | inductively coupled plasma |
| IT | ion trap |
| kDa | kilodalton |
| LC | liquid chromatography |
| MALDI | matrix-assisted laser desorption ionization |
| MS | mass spectrometry |
| MT | metallothionein |
| pI | isoelectric point |
| ppm | part per million |
| PTM | posttranslational modification |
| SGE | slab-gel electrophoresis |
| Tf | transferrin |
| TOF | time-of-flight |
| TRIS | tris(hydroxymethyl)aminomethane |

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# 8
# CE-MS in Food Analysis and Foodomics

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## 8.1
## Introduction: CE-MS, Food Analysis, and Foodomics

As a result of globalization in food trade, the distance between the producers of food and food ingredients and the consumers has increased enormously. Consequently, maintaining safety and quality along the food chain has become a difficult challenge nowadays. Moreover, food safety and quality are crucial not only due to their impact on consumers' health but also because food industry is a mainstay of the economy of many countries. To ensure consumption of safe and high-quality foodstuffs, it is essential to improve traceability. The EU defines traceability as the ability to trace and follow a food, feed, food-producing animal or substance intended to be or expected to be incorporated into a food or feed, through all stages of production, processing, and distribution [1]. Effective traceability systems are useful for the prevention of fraudulent practices, which, in most cases, focus on illicit economic gain. Such practices may include adulteration, mislabeling, false use of geographical indications, and deliberate fraudulent practices (production, processing, and distribution), among others. Not only is traceability a way to improve food safety, but it can also be seen as a strategic tool to improve the quality of foods and a way to increase the trust of costumers, and therefore, companies can use traceability as a source of competitive advantages [2].

Foodomics has been defined as a discipline that studies the food and nutrition domains through the application and integration of advanced omics technologies to improve consumer's well-being, health, and confidence [3, 4]. Thus, Foodomics is intended to be a global discipline that includes all of the emerging working areas in which food (including nutrition), advanced analytical techniques (mainly omics tools), and bioinformatics are combined [3]. For both conventional food analysis and Foodomics, advanced analytical strategies are applied [4], and among them, mass spectrometry (MS) plays a crucial role [5]. However, due to the complexity of the food matrices, the use of MS is often not enough to unravel their composition. To overcome this important limitation, hyphenated mass spectrometry techniques such as gas chromatography–mass spectrometry (GC-MS), liquid chromatography–mass spectrometry (LC-MS), and capillary

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electrophoresis–mass spectrometry (CE-MS) have achieved great success in food analysis applications [6]. LC-MS is applicable to the analysis of a wide range of semipolar compounds with a wide range of molecule sizes. GC-MS is particularly appropriate for the analysis of volatile organic compounds. CE-MS, on the other hand, is particularly suited for the rapid separation of ionic, weakly ionic, and/or highly polar compounds with very high resolution. Main advantages include minimum sample and reagent consumption and fast separation speed. It is also environmentally friendly due to its low solvent consumption. On the other hand, poor sensitivity is one of the main drawbacks of CE, which can be improved by combining CE with MS detection [7]. Moreover, the use of preconcentration strategies can give further sensitivity gain. It is worth mentioning that CE-MS is not as robust and stable as GC-MS or LC-MS, due to the need to complete the CE electrical circuit for separation and simultaneously provide an electrical potential to the spray tip. Typically, this is accomplished with a sheath-liquid ESI interface due to its higher robustness compared to other ESI interface approaches. Sheath liquid compromises sensitivity, and thus, continuous efforts are still being made for the development of robust sheathless interfaces with the aim to improve stability and sensitivity. The use of high-resolution mass spectrometry also greatly improves the analytical performance of CE-MS and offers a good combination of selectivity and sensitivity. When working with CE-MS, the combination of migration time, accurately measured mass, and, when possible, MS/MS fragmentation spectra can support compound identification. Within this context, the use of CE-MS in food analysis and Foodomics offers numerous opportunities to obtain valuable information that can directly be correlated to food quality, safety, bioactivity, and other features related to food processing, storage, authenticity assessment, and so on.

This chapter presents an overview on the advantages and drawbacks of the application of CE-MS in food safety and quality, as well as in other aspects related to food traceability and bioactivity following classical food analysis as well as novel Foodomics approaches.

### 8.1.1 CE-MS and Food Safety

Food safety is still a global health objective. Although the safety of food has dramatically improved, foodborne diseases from microbial contamination, chemicals, and toxins are a major cause of illness and death worldwide. Food safety assessment involves the examination of food for the presence of hazards induced by pathogen agents such as bacteria, viruses, parasites, toxigenic molds, microalgae and also for the presence of noxious chemical compounds such as agrochemicals, toxins, industrial/environmental contaminants, veterinary drugs, and allergens. On the other hand, adulteration and other deliberate fraudulent practices (production, processing, packing, distribution) might also have implications in food safety. Analytical information, including the following data for both recognized and new hazardous compounds, is therefore essential to advance in this important area of research.

Economically motivated food adulteration is an emerging health risk in a growing globalized food trade. In most cases, fraudsters are focused on economic gain and do not have the resources/knowledge to assess whether such a manipulation poses any toxicological or hygienic risk to the purchaser or the consumer [8]. A clear example is the adulteration of milk and milk products with melamine to falsely increase the apparent protein levels [9]. This dangerous fact promoted analytical method development worldwide focused on the detection of that contaminant [10]. LC and GC coupled with MS have demonstrated to be powerful techniques to analyze melamine and related compounds [11]. CE/capillary electrochromatography (CEC)-MS have also been shown to be attractive alternative methods for fast and minimum sample and reagent consumption approaches [12, 13]. As an example, a CEC-MS method was proposed by Huang *et al*. as an alternative to LC-MS, employing poly(divinyl benzene-alkene-vinylbenzyl trimethylammonium chloride) monoliths as stationary phase for the analysis of melamine and its three by-products (ammeline, ammelide, and cyanuric acid) [13]. MS detection permitted the reduction of the LODs by three orders of magnitude relative to UV detection reaching values of $2.2-19.4\,\mu g\,l^{-1}$.

Determination of veterinary drug residues in food products from treated animals is an issue of major concern on food analysis due to its implications on human health. Animals may be treated with drugs for prevention or cure of diseases or to promote their growth. As a result, undesirable antibiotic residues can remain on meat, milk, eggs, and so on. The presence of these drug residues in food is strictly regulated by specific legislation in many countries through the imposition of maximum residue limit (MRL) values. Thus, an efficient analysis of veterinary drugs residues in foodstuffs, and also in environmental samples, is a constant challenge for researchers. Excellent review works on CE analysis of antibiotics have been published in which interested readers on this issue can find more detailed information [14, 15]. The problem of the low sensitivity of CE for drug residue analysis has been addressed by a variety of preconcentration techniques. For example, field-amplified sample injection (FASI)-CE permitted the quantification of amprolium in eggs at concentrations down to $75\,\mu g\,kg^{-1}$ [16]. In that work, CE-MS permitted the identification of one interference from the matrix (thiamine) eluting at the same migration time as amprolium and the subsequent method optimization. Domínguez-Álvarez *et al*. developed and validated a combination of QuEChERS (quick, easy, cheap, effective, rugged, and safe) methodology with CE-MS for the determination of trace levels of benzimidazoles (2-aminobenzimidazole, carbendazim, albendazole-2-aminosulfone, 5-hydroxy-thiabendazole, oxibendazole, albendazole, fenbendazole, oxfendazole, albendazole-sulfone, fenbendazole-sulfone) in eggs [17]. Following this approach, the LODs for these residues in egg samples were between 3 and $51\,\mu g\,l^{-1}$. Molecularly imprinted polymers (MIPs) have been evaluated as sorbents for in-line solid-phase extraction (SPE) and sample cleanup in CE-MS for antibacterial drugs [18]. This in-line MISPE-CE-MS system permitted direct milk injection for the determination of eight veterinary quinolones and related metabolites (danofloxacin, sarafloxacin, difloxacin, enrofloxacin, ciprofloxacin, flumequine,

marbofloxacin, and oxolinic acid) with LOD values from 1.0 to 1.4 $\mu g\,kg^{-1}$, which are below the MRLs established by the EU regulation. A group of fluoroquinolones were analyzed by CE-UV and off-line combination of CE and matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF) [19]. Although the advantages of this off-line approach are not clear, it permitted the separation and identification of ciprofloxacin, norfloxacin, and ofloxacin in bovine milk samples.

Analysis of pesticide residues is of the outmost importance in environmental and food samples. Within this field, a comprehensive overview of the current CE developments for herbicide analysis was published in 2014 [20]. There are also examples of the application of novel CE developments for the determination of fungicides and insecticides in food samples [21, 22]. Regarding the use of CE-MS, Daniel *et al*. showed the applicability of this technique for the determination of halosulfuron-methyl herbicide residues in samples of sugarcane juice and tomato [23]. The samples were submitted to a QuEChERS extraction procedure prior to CE-MS/MS analysis to accomplish with the sensitivity and specificity values indicated by the international legislation about halosulfuron-methyl in sugarcane and tomato. The reported recovery values for spiked samples ranged from 96% to 105%, and the LOD for the herbicide in juice and tomato was 2 ppb.

In addition to the analysis of drug/pesticide residues, there is a wide range of **contaminants** that can be found in food/feed. According to the *Codex Alimentarius* [24], a contaminant is "any substance not intentionally added to food, which is present in such food as a result of the production, manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food or as a result of environmental contamination." A variety of industrial chemicals have been reported as persistent organic pollutants or environmental contaminants, which may enter at several stages of food/feed production. Food-packaging-derived chemicals may also contaminate foodstuffs, and some of them can alter functions of the endocrine system and consequently cause adverse health effects. The feasibility of CE-MS for the analysis of several endocrine disruptors has also been demonstrated [25]. CE-MS was applied to the analysis of 2,4-dichlorophenol, 2,4,5-trichlorophenol, pentachlorophenol, bisphenol-A, 4-*tert*-butyl-phenol, and 4-*tert*-butyl benzoic acid in spiked honey samples. Prior to CE-MS, a liquid–liquid extraction procedure was applied and LODs were in the 1–4 $ng\,g^{-1}$ range. Naturally occurring toxins (produced by algae, fungi, and plants), which are not intentionally added to food and feed, can also get into the food chain [26]. Phycotoxin-producing algae can be accumulated in edible shellfish, crustaceans, and fish, causing possible poisoning when inadvertently consumed. Poisoning due to marine toxins occurs worldwide and can produce acute effects, or chronic health effects, but also economic damages to shellfish farming. Sassolas *et al*. have reviewed current capillary electromigration approaches, among others, for the analysis of okadaic acid, a toxin that contaminates bivalves and causes severe public health problems and economic damages to shellfish farming [27]. Keyon *et al*. compared four CE methods with different detection methods, for the analysis of paralytic shellfish toxins (PSTs) in terms

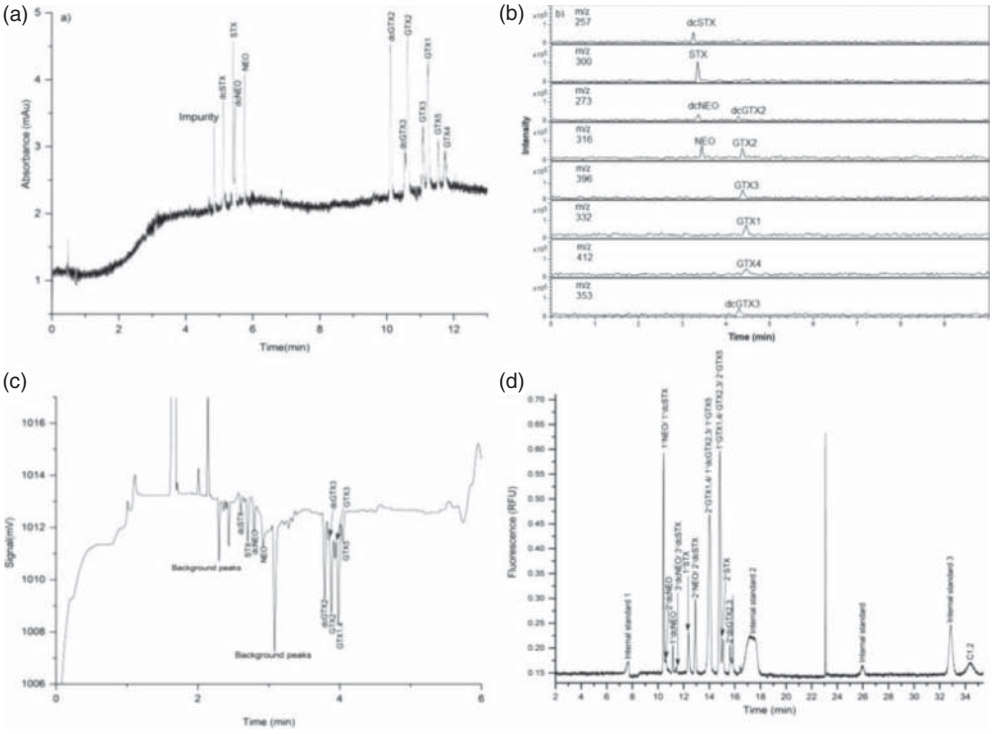


**Figure 8.1** Separation of PST mixture using four different CE methods. (a) Electropherogram of CZE-UV using 30 mM phosphate buffer pH 2.5, sample injected at 5 kV for 10 s. (b) Extracted ion electropherogram) of CZE-MS at selected *m/z* using 35 mM morpholine pH 5.0, sample injected at 5 kV for 10 s. (c) Electropherogram of CZE-C4D using 25 mM sodium acetate buffer pH 4.22, sample injected at 5 kV for 10 s. (d) Electropherogram of MEKC-FLD using 30 mM phosphate buffer at pH 8.5 containing 80 mM SDS, sample injected at 0.7 psi for 10 s. (Reproduced from [28] with permission from Wiley.)

of resolution, selectivity, separation efficiency, and sensitivity [28]. A standard mixture of PSTs (saxitoxin, decarbamoylsaxitoxin, neosaxitoxin, decarbamoylneosaxitoxin, gonyautoxin 1 to gonyautoxin 5, decarbamoylgonyautoxin 2, decarbamoylgonyautoxin 3, and C toxins) was used for that purpose (Figure 8.1). The developed capillary zone electrophoresis–ultraviolet detection (CZE-UV) and CZE capacitively coupled contactless conductivity detection (C4D) methods provide overall better resolution, selectivity, and separation efficiency compared to CZE-MS and micellar electrokinetic chromatography–fluorescence detection (MEKC-FLD). On the other hand, the sensitivity of the CZE-C4D and MEKC-FLD methods was superior to that of UV and MS. Although the CZE-MS method only separates saxitoxin, decarbamoylsaxitoxin, decarbamoylneosaxitoxin, and neosaxitoxin from the gonyautoxins (1–5), differences in mass and fragmentation pattern allow for specific quantification of at least 10 of the 11 charged PSTs. When applied to real shellfish samples, the CZE-C4D method suffered from

significant interferences from matrix, while MEKC-FLD was successfully used for PST screening. Mycotoxins can appear in the food chain because of fungal infection of crops, either by being consumed directly by humans or by being used as livestock feed [29]. The last developments in mycotoxin analysis, including those based on capillary electromigration methods, were recently reviewed by Berthiller *et al*. [30]. Acute poisoning is also seen to be related to the consumption of wild mushrooms. In this regard, Ginterová *et al*. proposed a CE-MS/MS method for the screening of mushroom intoxication [31]. For that purpose, mushroom toxins, ibotenic acid, muscimol and muscarine were analyzed in a spiked urine sample. The LOD values were in the range of 0.05–0.73 ng ml$^{-1}$. The presence of biogenic amines (BAs) above a certain level can be considered as indicative of undesired microbial activity. Biogenic amines (BAs) are nitrogen compounds (e.g., putrescine, cadaverine, spermine, spermidine, tyramine, phenylethylamine, histamine, tryptamine), which are present in a wide range of food products [32], especially in fish products and fermented foods [33]. They can cause severe health problems, particularly to sensitive individuals. CE is considered as a good alternative to high-performance liquid chromatography (HPLC) for the analysis of BAs in food samples [34]. Putrescine, cadaverine, histamine, phenylethylamine, tyramine, and other less common BAs (ethanolamine, isoamylamine, tryptamine, spermine, and spermidine) are BAs frequently found in wines; they have been analyzed in less than 8 min by CE-MS [35].

During food processing, certain substances not present or present at much lower concentrations in the raw material might be produced. Some of these substances might be undesirable either because they have an adverse effect on product quality or because they are potentially harmful. Evaluation of chemical changes during food processing is a difficult task, considering the complexity of food matrices and the large number of parameters that can contribute to those changes during processing, such as temperature, pH of the system, pressure, oxygen availability [36]. Particular attention has been given to detect known contaminants and suspected carcinogenic food processing contaminants such as acrylamide, furan, 5-hydroxymethylfurfural (HMF), furosine, heterocyclic amines, and polycyclic aromatic hydrocarbons [37–39]. In this field, a new CE-MS/MS method was developed for the analysis of furosine in foods. The formation of furosine is directly related to the heat applied to many foods. Flour samples of different origin (wheat, chestnut, lupin, einkorn, chickpeas), as well as processed foods such as pasta, milk, and bread, were analyzed. LOD and LOQ values determined by CE-MS/MS were 0.07 and 0.25 mg l$^{-1}$, respectively. CE-MS/MS results were compared with those obtained by reversed-phase HPLC-UV, achieving a good agreement between the results provided by both techniques. A similar CE-MS/MS approach was applied for the quantification of hydroxy-methyl-furfural (HMF) in food products [40]. Although it is not present in fresh or untreated foods, it rapidly accumulates during the heat treatment. Thus, HMF is widely recognized as a marker of food deterioration due to excessive heating or inadequate storage conditions. The CE-MS/MS method was applied to the analysis of cereal-based baby foods, coffee, soft beverages, and

vinegars. When compared to RP-HPLC-UV, good agreement between the two methodologies was also observed [41].

A variety of hazardous compounds used as additives in food products in some countries are currently illegal and prohibited for use as food additives by other countries, because of their carcinogenicity to humans. As an example, Sudan dyes have been extensively used in some countries as additives in foods (i.e., chilli powder, paprika, sauces, etc.), and they are prohibited in the EU. In order to give response to this issue, a MEKC-MS/MS method was developed for the detection of Sudan dyes I–IV in chilli powder samples [42]. Since UV detection was not suitable for the determination of these dyes, MS detection in the positive ion was employed and MS/MS spectra of each dye permitted their identification.

Foodstuffs may also be a major source of toxic metals causing health hazards to the consumers. The toxicity, environmental mobility, and accumulation of certain elements in living organisms depend on the form in which they occur. Thus, elemental speciation is an important discipline within food safety. In the recent years, CE with inductively coupled plasma MS (ICP-MS) has become one of the preferred techniques for speciation analysis [43], and the number of reports on its use in food safety has continued to grow. An interesting example is the arsenic speciation. As occurred with other elements such as mercury [44]. The effects of arsenic in certain foods are less dependent on its total concentration, but more on the concentration of its various species [45]. Marine food contains a great variety and relatively high concentrations of arsenic compounds. This is because in seawater, arsenic occurs predominantly as inorganic species, namely, arsenate and arsenite. Marine organisms exhibit mechanisms of biotransformation and detoxification, and they produce a large variety of organoarsenic species with very different toxic effects. Thus, there is a clear interest to separate, identify, and quantify every arsenic species individually. CE-ICP-MS has been applied to identify and quantify the arsenic species arsenobetaine (AsB), arsenite ($As^{III}$), arsenate ($As^{V}$), and dimethylarsinic acid (DMA) in fish samples. LODs obtained were $1 \times 10^{-7}$ M for AsB, $5 \times 10^{-7}$ M for DMA, and $1 \times 10^{-6}$ M for $As^{III}$ and $As^{V}$ [46]. Rice products are also of particular interest since rice plants have higher accumulation rates of arsenic when compared to other crops [47]. Quantification of common arsenic species in rice and rice cereal using CE-ICP-MS has also been reported [48]. In that work, an enzyme (i.e., α-amylase)-assisted water-phase microwave extraction procedure was used to extract four common arsenic species, including dimethylarsinic acid (DMA), monomethylarsonic acid (MMA), arsenite ($As^{III}$), and arsenate ($As^{V}$), from the rice matrices (Figure 8.2). Nanoparticles are currently used as additives to food and drinks, since they can prevent caking, help to deliver nutrients, and prevent bacterial growth. But as nanoparticles increase in use, so do concerns over their health and environmental effects. CE-ICP-MS has also been applied for rapid and high-resolution speciation and characterization of metals (e.g., gold, platinum, and palladium) [49]. In this sense, it was observed that the diameters of the nanoparticles followed a linear relationship with their relative electrophoretic

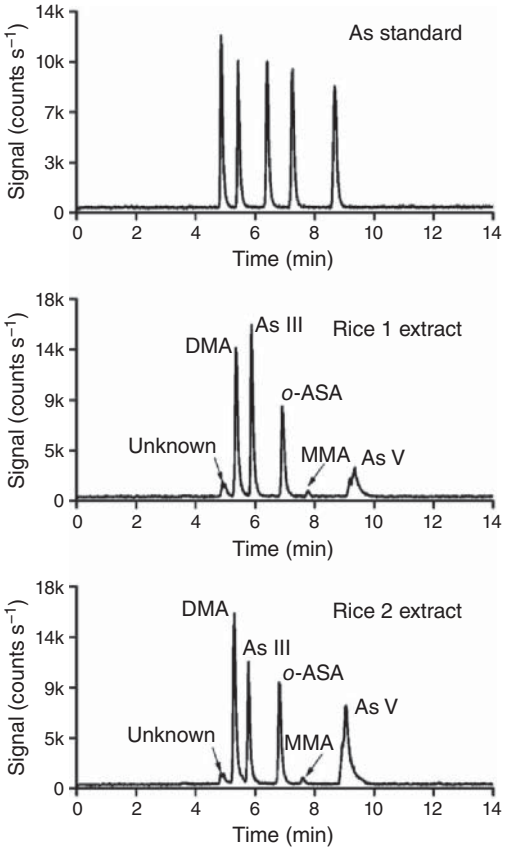


**Figure 8.2** CE-MS electropherograms of arsenic standards and rice extracts with post-extraction addition of the internal standard *o*-arsanilic acid (*o*-ASA). DMA, dimethylarsinic acid; As III, arsenite; MMA, monomethylarsonic acid; As V, arsenate. CE conditions: sodium carbonate at pH 11 as separation buffer. Injection: 15 mbar for 8 s. Running voltage: +30 kV. (Reproduced from [48] with permission from American Chemical Society.)

mobility, and size information on unknown nanoparticle samples could be extrapolated from a standard curve. The CE-ICP-MS method was successfully applied to the analysis of commercially available metallic nanoparticle-based dietary supplements.

Food allergies represent an important health problem [50]. Most cases of food allergy are associated with a limited range of products, such as molluscs, eggs, fish, peanuts, tree nuts, soybeans, milk, celery, mustard, sesame, cereals containing gluten, crustaceans, lupin, and sulfur dioxide [51]. The extent to which allergy is associated with particular foods varies with dietary habits and preferences, as well as the introduction of new foods, but also in the way in which foods are processed and prepared [52]. Sensitive and selective determination methods are therefore in continuous development to guarantee food safety for sensitive

individuals. Milk whey proteins are among the main allergens and can cause allergy even at a very low concentration. Among them, β-lactoglobulin and α-lactalbumin are the main allergens in milk. They have been successfully detected by immunoaffinity capillary electrophoresis (IACE) off-line coupled to MALDI-TOF MS. Sensitive quantitative analysis of β-lactoglobulin and α-lactalbumin was achieved in different milk samples including fortified soy milk [53] as well as total IgE quantification in blood serum of milk-allergic patients [54]. Lysozyme is another potential allergenic agent, and thus, it has to be declared according to the labeling requirements in each country. CE-MS method has been applied for preservative lysozyme in cheese [55, 56]. To prevent protein adsorption onto the fused-silica capillary inner surface, a novel copolymer carrying pendant dendronic triamines has recently been synthesized and used as effective capillary coating for the determination of the basic protein lysozyme in complex food samples [57]. The coating showed full compatibility with MS detection. As can be seen in Figure 8.3, the method was demonstrated to be effective for the analysis of lysozyme in commercial cheese samples, as well as some remaining whey proteins as α-lactalbumin and β-lactoglobulins were identified in the cheese. Moreover, a variety of peaks with lower molecular masses (550 – 1200 Da) were detected in the same analysis, most probably from polypeptides derived from the proteolysis that occurred during the ripening process.

### 8.1.2 CE-MS in Food Quality and Authenticity

As mentioned earlier, food chain integrity includes not only safety concerns but also origin fraud and quality concern. Food quality is intrinsically linked to food safety and is determined to a large extent by the biological and genetic variability of the raw constituents and also by the treatment of food during production, processing, and storage [58]. Food traceability can be employed to know the composition and origin of a particular food product during the whole manufacturing process [59]. In this context, the profiling of free amino acids (AAs) has been frequently used to discriminate the origin of a substance and its shelf life [60]. Apart from the nutritional properties of amino acids in foodstuffs, they have important impact on the quality. CE-MS offers a selective, sensitive approach for amino acid analysis in complex samples [61]. CE-MS/MS has been employed for the analysis of AAs to assess the quality of several commercial royal jelly products as well as of honey [62]. Thus, 16 AAs (Ala, Arg, Asp, GABA, Glu, Gly, His, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, and Val) were analyzed in less than 20 min with LODs lower than 10.5 $\mu g\, g^{-1}$. The CE-MS/MS method permitted discrimination between the different royal jelly products (tablet, liquid drink, and raw material) and among the royal jelly raw materials and honey. Chemical characterization of different inactive dry yeast preparations (normal and glutathione-enriched) typically used within the wine industry has been recently presented [63]. The capabilities of CE-MS permitted the detection, for the first time, of 14 sulfur-containing compounds in the glutathione-enriched dry yeast (g-IDY) that could have direct effect on the

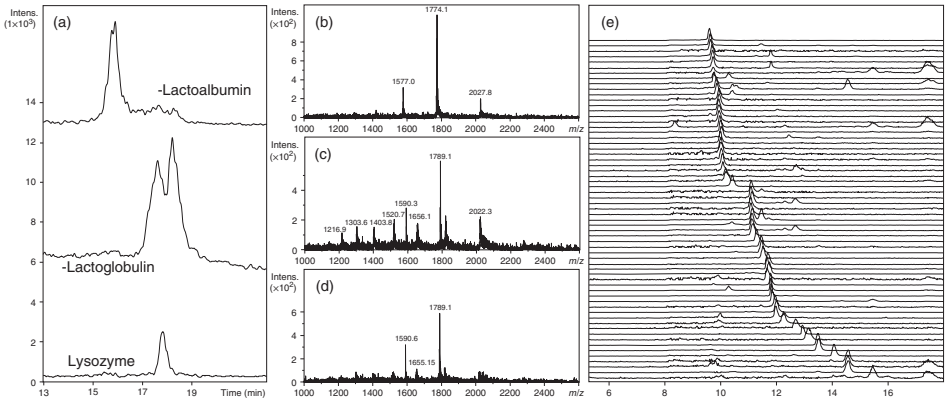


**Figure 8.3** (a) CE-MS extracted ion electropherograms of detected proteins in a cheese extract. Mass spectra from (b) α-lactoalbumin, (c) β-lactoglobulin, and (d) lysozyme. (e) CE-MS extracted ion electropherograms of low-molecular-mass compounds. Capillary coated with poly-(TEDETAMA-co-HPMA) 50 : 50 copolymer. CE conditions: 35 mM ammonium acetate at pH 4.8 as separation buffer. Injection: 0.5 psi for 10 s. Running voltage: −30 kV. (Reproduced from [57] with permission from Wiley.)

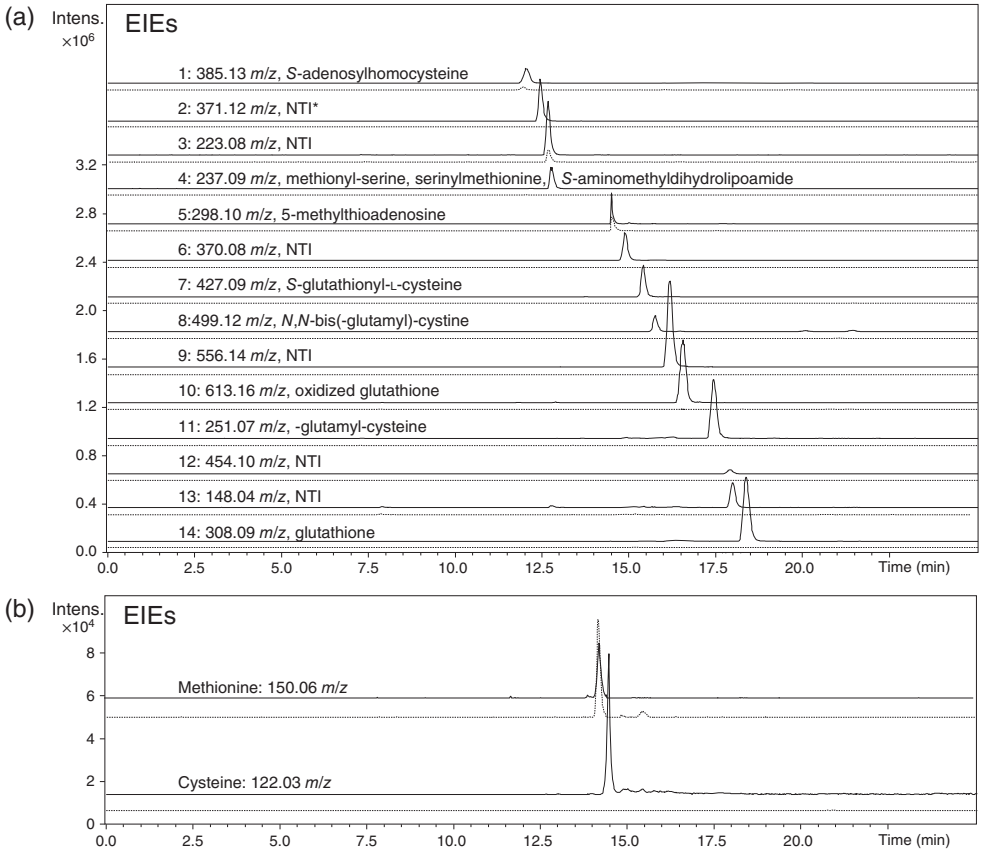


**Figure 8.4** CE-TOF MS extracted ion electropherograms (EIEs) of (a) the 14 most abundant sulfur-containing compounds and (b) methionine and cysteine from inactive dry yeast (n-IDY) and GSH-enriched inactive dry yeast (g-IDY). A continuous line for g-IDY permeate and a dotted line for n-IDY permeate are used. CE conditions: 3 M formic acid as separation buffer. Running voltage: +25 kV. Injection: 0.5 psi for 80 s. (Reproduced from [63] with permission from American Chemical Society.)

better properties of this yeast preparation (Figure 8.4a). Moreover, other interesting sulfur-containing amino acids (methionine and cysteine) that might have useful properties for wine preservation were also detected (Figure 8.4b). Enantioselective separations of amino acid by CE-MS have been successfully applied for identifying adulterated foods and beverages and for controlling and monitoring the fermentation processes and products [64, 65]. CE-MS has also been employed by [66] for the analysis of vitamins B. The group of vitamins B involves thiamine (B1), riboflavin (B2), nicotinic acid/nicotinamide (B3), pantothenic acid (B5), pyridoxine (B6), biotin (B7), folic acid (B9), and cyanocobalamine (B12), and they are water-soluble and provide (individually or simultaneously) many important biological actions in organism. The simultaneous and highly reliable determination

and identification of vitamins B are very important for the quality control of vitamin-fortified food products, food supplements, and pharmaceuticals. On the other hand, their determination is problematic due to the very different chemical structures and low stability of these vitamins [66]. CE-MS is also a promising technique to guarantee authentication and origin of agro-products, through the study of their chemical composition. Analysis of amino acids [65], phenolic compounds [67], proteins [68], peptides [69], and so on by CE-MS can be applied as a valid procedure to obtain information about the origin of foods. As an example, CE has scarcely been employed to analyze phospholipids due to the low hydrosolubility of these compounds; in this sense, the electrophoretic modes more frequently used have been MEKC and nonaqueous capillary electrophoresis (NACE). A NACE-MS method was developed by Montealegre *et al*. for the determination of glycerophospholipid profiles in olive oil and olive fruit of different varieties and geographical origins [70]. Namely, three different olives varieties (i.e., Arbequina (collected in two Spanish regions), Empeltre, and Lechín) and a monovarietal extra virgin olive oil from Arbequina variety were analyzed in that work. Lysophosphatidic acid (lyso-PA), phosphatidylcholine (PC), lysophosphatidylethanolamine (lyso-PE), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylglycerol (PG) were detected in olive fruit (Figure 8.5). Apparently, differences in the relative abundance of the glycerophospholipid profiles were observed as a function of the different geographical origins and variety of the olive fruits analyzed.

### 8.1.3 CE-MS and Foodomics

The progress of novel, high-throughput omics technologies has opened new frontiers in the research of food safety, food quality, food traceability, and dietary components and their effects on health. Among omics technologies, Genomics, Transcriptomics, Proteomics, Peptidomics, and Metabolomics aim to detect the complete set of genes, transcripts, proteins, peptides, and metabolites in a biological system, respectively. These technologies allow for generating and integrating huge amounts of information. Recent advances in omics technologies present the potential to expand the coverage of analytes that can be simultaneously determined in a single analysis. Thus, omics technologies significantly improve the simplistic and reductionist experimental models used by classical target approaches that offer only a temporal snapshot of the huge complexity and dynamic nature of biological networks. Omics development and progress have led to an increasing demand on the knowledge about the possible effects and interactions between diet and individual gene expression, as well as the different individual responses to nutrients depending on gene polymorphisms. However, health and disease status are affected not only by genetic elements but also by behavioral and environmental factors, among others. In this scenario, there is a need for expanding our knowledge about the processes occurring at molecular level due to dietary components and their consequences on gene, protein, and metabolite expression addressed by

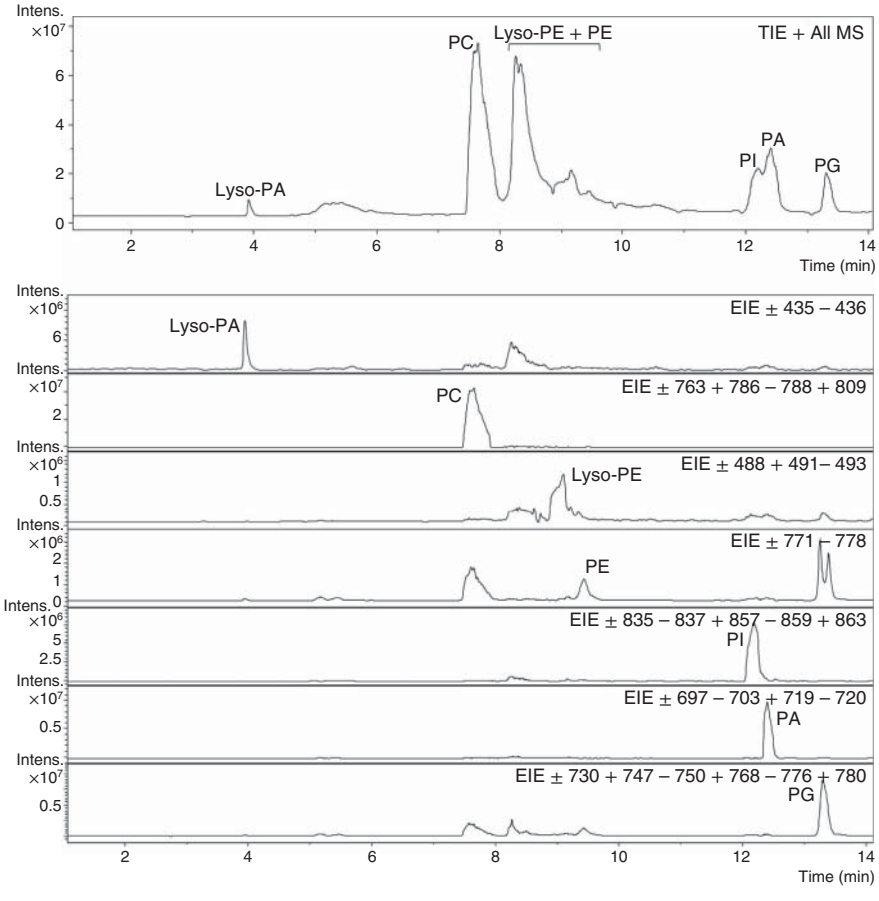


**Figure 8.5** Total ion electropherograms (TIE) of glycerophospholipid profile and EIEs for each phospholipid from Arbequina olive oil samples analyzed. Conditions were as shown in Figure 8.2. Peaks: lyso-PA, lysophosphatidic acid; PC, phosphatidylcholine; lyso-PE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; PG, phosphatidylglycerol. CE conditions: 100 mM ammonium acetate in 60 : 40 (v/v) methanol/ACN with 0.5% acetic acid as separation buffer. Running voltage: +25 kV. Injection: 50 mbar during 5 s. (Reproduced from [70] with permission from American Chemical Society.)

omics technologies. As a long-term goal, different individual responses to dietary compounds could be determined, leading to a personalized diet and/or tailored dietary preventive interventions. In this complex framework, Foodomics discipline has emerged to integrate information from omics technologies concerning food science and nutrition in order to improve human nutrition and consumers' well-being and confidence [3, 71]. CE-MS has proven to be a powerful analytical technique in Foodomics, allowing for the analysis of a wide range of analytes, from ions to macromolecules, with high efficiency and selectivity. Within

the global Foodomics field, CE-MS has been shown to be especially suitable for metabolomics and peptidomics applications.

Metabolomics is conceived as the ultimate response of a biological system to genetic, physiological, environmental, and dietary factors and aims for the analysis of the maximal possible coverage of low-molecular weight compounds in a biological system. However, a single biological system is complex because it contains a great variety of metabolites with a very heterogeneous nature (lipids, carbohydrates, and many other small compounds such as amino acids, organic acids, nucleic acids, fatty acids, phytochemicals, minerals) and in a wide range of concentrations (from pmol or lower to mmol) [72]. This fact hampers the analysis of the global metabolome using a single analytical methodology, which is firstly limited by the selected sample treatment. The selection of the most suitable metabolite purification protocol for metabolomics of adherent mammalian cell culture by CE-MS has been investigated by comparing different metabolite extraction strategies [73], and by using different extraction solvents [74].

Elucidation of composition and characterization of foods and food ingredients is the starting point for the study of their potential beneficial effects on health. Going further, foods are generally complex matrices that can be modified through, for example, alteration of environmental conditions, food manipulation, transport, storage, or fermentation, which alter the composition and thus can have impact on human health. In this regard, CE–time-of-flight mass spectrometry (CE-TOF MS) has been combined with LC-MS/MS to evaluate the effects of time and temperature of storage on pasteurized and unpasteurized Japanese sake [75]. Thus, pasteurization and storage modified both metabolic and peptide profiles of sake. For instance, amino acid content was significantly altered with storage time and pasteurization and peptides increased during storage. However, it was observed that the type of sake and storage temperature did not meaningfully modify sake composition. In another work, the effect of absence of light during plant development on tea leaves has been characterized by a metabolomics multiplatform including CE-MS, GC-MS, and LC-MS analysis [76]. Cationic and anionic metabolites were acquired in the positive and negative MS ionization modes, respectively. As a result, more than 200 metabolites could be detected, among which altered concentrations of volatile compounds and amino acids were highlighted. More recently, the same metabolomics multiplatform has been employed for the investigation of mesocarp metabolites in different ripening stages of high- and low-yielding oil palm populations [77]. Lipid biosynthesis was the metabolic pathway most severely affected when high- and low-yielding populations were compared. Other important metabolic features included changes in lipids, glycolysis, citric cycle organic acids, amino acids, and nucleosides at different ripening stages of fruit [77]. Ripening time has also been demonstrated to change the metabolic profiles of avocado fruits [78]. In that work, quantification of 10 compounds including flavonoids and phenolic acids in 18 avocado samples at different ripening states by CE-MS was carried out using two MS modes, multiple reaction monitoring (MRM) and full scan [78]. After comparison of the levels of the compounds in the function of ripening

time, perseitol, quinic acid, chlorogenic acid, *trans*-cinnamic acid, pantothenic acid, abscisic acid, epicatechin, and catechin decreased throughout the fruit development, while ferulic and *p*-coumaric acids increased in the same period of ripening.

Metabolomics has also been employed for the investigation of soybean fermentation for the production of a traditional Korean food (cheonggukjang) whose health benefits are mainly attributed to its content in vitamins, minerals, isoflavonoids, and saponins [79]. For that purpose, CE-TOF MS and GC-TOF MS analytical platforms were used for the evaluation of the metabolic profiles using different fermentation microorganisms and fermentation times. After multivariate data analysis, it was observed that samples were mainly differentiated as a function of fermentation time. Some of the most relevant metabolites altered during fermentation time were hypoxanthine, guanine, and xanthine. Apart from bacterial fermentation processes, pathogen bacteria are included among the main factors that led to an alteration of foodstuff. For instance, bacteria producing bovine mastitis affect milk quality and quantity of production, leading to important economic consequences. To elucidate the causes of those effects at a molecular level, Mansor *et al*. [80] compared milk peptidic profiles obtained from cows diagnosed with mastitis versus healthy cows. Data analysis and statistical results led to a diagnosis method with 100% sensitivity and specificity enabling differentiation of cows. Furthermore, cows under study suffering mastitis caused by either of the two pathological agents (i.e., *Staphylococcus aureus* and *Escherichia coli*) could be differentiated with 75% sensitivity with a set of 47 peptides.

The role of bioactive peptides from milk is being increasingly investigated. To going further into this topic, a peptidomic approach was conducted to evaluate three hypoallergenic infant milk formulas by CE-TOF MS [81]. To purify the peptidic fraction, two different SPE cartridges (i.e., C18 and StrataX™ (STX)) were used in parallel in order to widen the analytes coverage. After CE-MS analysis, a number of bioactive peptides ranging from 64 to 116 could be identified in milk formulas depending on the sample. This CE-MS method was substantially faster than the more spread method based on LC-MS offering complementary information on peptide composition. Another CE-MS method was developed for the analysis of phosphopeptides in tryptic digests of bovine milk, which showed unprecedented sensitivity for those analytes [82]. To achieve this good sensitivity, a transient-isotachophoresis (t-ITP) step as the sole sample concentration stage followed by a sheathless CE-MS method was applied to milk samples using a neutrally coated capillary.

Foodomics discipline is especially concerned with diet ingredients evaluation and their effect on humans health, including the prevention of chronic diseases such as cancer. A number of investigations have been devoted to this topic using CE-MS-based metabolomics strategies to study cancer cell cultures following a Foodomics approach [74, 83–89]. Investigations have been carried out, given the potential antiproliferative effects of certain food ingredients such as olive oil [83], dietary polyphenols [89], or rosemary [85] on colon-cancer cells. Thus, differences at the molecular level were determined to explain the reduction on

colon-cancer cell proliferation macroscopically observed after the treatment with polyphenol-rich extracts obtained from rosemary [85]. Metabolic fingerprints from the cytosolic content of HT29 colon-cancer cells treated and nontreated with the rosemary extract were achieved using three complementary analytical platforms. Thus, information from CE-TOF MS, HILIC/UPLC-TOF MS, and RP/UPLC-TOF MS was integrated. As a result, an increase in the ratio between reduced and oxidized glutathione in the treated cells was observed together with a significant alteration in the polyamine content and its catabolites. In order to clarify those effects at a cellular level, other omics technologies were applied to the same samples [86]. Integration of the results obtained from Metabolomics with Transcriptomics and Proteomics information was performed following a global Foodomics approach for the first time [86] (Figure 8.6). Thus, 1308 genes, 17 proteins, and 65 metabolites were observed to be significantly altered. Using the "Ingenuity Pathway Analysis" software, differences observed in the metabolomic and transcriptomic data could be integrated, revealing alteration in nitrogen metabolism, glutamate, glutathione, arginine, and proline and in the urea cycle and metabolism of amino groups. The same software was used for the integration of Metabolomic and Transcriptomic information for the investigation of rosemary extract effects on two lines of K562 leukemia cell

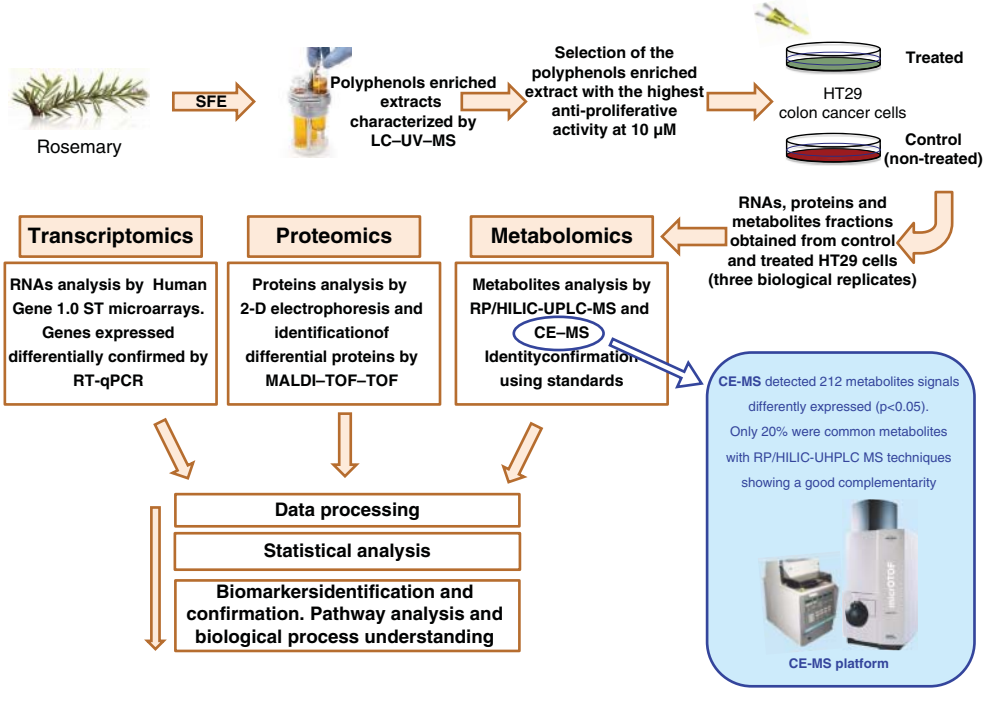


**Figure 8.6** Global Foodomics strategy used by Ibáñez *et al.* [85] to investigate the activity of rosemary polyphenols against colon-cancer HT29 cells at molecular level. Contribution of CE-MS on this workflow is highlighted. (Reproduced from [85] with permission from Wiley.)

proliferation [87]. Namely, a human erythroleukemia K562 and daunomycin (DNM)-resistant K562/R cell lines were under scrutiny. Metabolic extracts obtained after ultrafiltration were analyzed via CE-MS and RP/UHPLC-MS. After integration with Transcriptomics, the behaviors of both cell lines after rosemary extract treatment were observed to be slightly different (e.g., glutathione metabolism, urea cycle, and metabolism of amino groups). The special relevance is also the investigation of cell lines from intestinal epithelium in order to unravel physiological mechanisms of absorption and metabolism of dietary ingredients before they are absorbed. Shimoda *et al*. evaluated the effects of *Cistus monspeliensis* leaf extract, mainly focusing on the energy metabolism effects in Caco-2 cells by CE-TOF MS [88]. The extract under study was shown to induce ATP production in human intestinal cells and was postulated as a possible antiaging agent probably associated to its phenolic compounds content (e.g., quercetin, kaempferol, aesculin, myricetin, and flavan-3-ols).

## 8.2 Concluding Remarks

CE-MS has shown to play a relevant role in food analysis and Foodomics. Representative applications of CE-MS in the field of food safety, food quality, food authenticity, and food bioactivity as discussed in this book chapter clearly demonstrate its value. CE-MS can help in different applications, including monitoring of food fermentation, detection of the undesirable compounds formed during food processing/cooking, determination of industrial/environmental contaminants, detection of unexpected natural toxins, quantification of veterinary drugs and pesticide residues, allergen detection, assessment of the quality of foods through components determination. Moreover, the capabilities of CE-MS in Foodomics have already demonstrated the full potential of this technique in this new field of research.

CE-MS complies with essential requirements in food analysis such as short analysis time, high efficiency and resolution and environmental friendliness. However, there is a variety of challenges that still need to be addressed, including sensitivity and reproducibility of this technique, and that will remain to be investigated. In spite of these needed improvements, the global outlook that CE-MS proposes is a good response to solve complex issues and challenges in the current and future food science and Foodomics.

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# 9
# CE-MS in Forensic Sciences with Focus on Forensic Toxicology

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## 9.1
## Introduction

Forensic science is a unique scientific discipline that includes all those scientific technologies and applications that can be used to support the forensic investigation and/or to bring scientific evidence in court. On many occasions, forensic science also provides the information needed for drafting new laws and rules requiring scientific knowledge [1–3]. The main areas of this discipline are forensic toxicology, analysis of explosives, gunshot residues and chemical warfare, trace analysis (fibers, hairs, inks, dyes, glass, etc.), forensic genetics, and so on. For forensic genetics, which is based on DNA fragment analysis and sequencing, the development of CE technology has been a fundamental step forward [4], being the standard technique used worldwide today.

In almost all its fields of application, the development of analytical forensic sciences finds two milestones: (i) introduction of chromatography and electrophoresis and (ii), more recently, introduction of mass spectrometric techniques. In both chromatography and electrophoresis, the miniaturization of the separation compartment in a capillary format has greatly increased the analytical efficiency and improved the possibility of coupling with mass spectrometry.

Capillary electrophoresis–mass spectrometry (CE-MS), as discussed widely in different sections of this book, couples two analytical techniques based on different chemical–physical principles (for this reason called *orthogonal*), being particularly suitable in forensic analysis, where unequivocal identification and accurate measurement are strictly required.

Among the advantages of CE-MS, it is worth mentioning its ability to deal with samples often in poor quantity, nevertheless requiring preservation for further cross-analysis in disputed cases. In addition, in many cases, crime scene evidence is highly contaminated and/or degraded, hence requiring accurate separation to get rid of all possible interfering compounds prior to the MS identification and quantitation of the substance(s) of interest. Also, forensic samples often show high complexity, because of their biological origin, the presence of metabolites and/or degradation products, and possible contaminants.

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First Edition. Edited by Gerhardus de Jong.

All of these critical conditions can be tackled and, in most cases, overcome by using CE-MS, which, differently from gas and liquid chromatography, mostly works in open microtubular compartments (capillaries) without any packing material.

Last but not least, CE separations are based on physical–chemical principles different from those operating in liquid chromatography, thus being "orthogonal" to this more traditional separation technique. This means that the results from the two methodologies, if in agreement, are mutually confirmatory, thus meeting a fundamental requirement of forensic analysis (i.e., confirmation of a result by a different method, analytically orthogonal to the previous one).

Interestingly enough, admissibility of CE in court was the subject of a specific article by Kuffner *et al*., which already appeared in Analytical Chemistry in 1996 [5]. In this article, the authors used CE to explain how new science may meet the requirements of the federal rules of evidence and those defined by the so-called Daubert Standard, still adopted in the US courts.

Since the first pioneering years of CE-MS, from the late 1980s until the mid-1990s, one could observe a steady increase in the number of publications [4]. From about 30 research papers per year in the last decade of the twentieth century, the publications in the field reached numbers over 60 per year in the last decade.

This chapter reviews the literature on CE-MS applications in the main analytical fields of forensic science from the end of 1980s to the present time. However, because of the number of papers and the variety of subjects of potential forensic interest, no review can be comprehensive. Moreover, any ambition of completeness would be overwhelmed in the near future by the tide of publications of new research. Hence, the present work aims mainly at showing and discussing the different strategies adopted in the development and application of forensic CE-MS, with particular attention devoted to the peculiarities of this new technology.

Before engaging directly with the applications of CE-MS in forensics, a few technical aspects will be discussed, in order to show how this peculiar technique may offer important advantages over other separation technologies. In particular, given the variety of sample matrices occurring in the forensic scene and their frequent poor quantity (and quality), special attention should be devoted to this point. Also, the most important separation modes and detection techniques will be illustrated, focusing on their suitability for the forensic analysis. Last but not least, a comparison between CE-MS and other available hyphenated techniques will be presented.

## 9.2 Sample Preparation of Forensically Relevant Matrices

Sample preparation is a crucial part of the analytical process, particularly in CE in which the coexistence of ions with highly different mobilities and concentrations

may lead to disturbances of the separation process, for example, peak defocusing. The purpose of any sample preparation is cleanup of the sample to extract and/or preconcentrate the substances of interest with the final aim of submitting to the separation process only a selected fraction of the sample containing all the molecules of interest and excluding all possible interferents. Sometimes, it may also be necessary to transform the analytes into different chemical forms, which can facilitate their separation and/or detection [6]. With these general considerations, when the focus of discussion is on CE, another aspect is worth mentioning. CE deals with minute amounts of samples, and therefore, it is particularly suitable for the application of the so-called microextraction techniques, which have been developed in different forms (single drops, fibers, hollow fibers, packed sectors of the capillary, etc.) [7–11]. Unfortunately, very few of these methods have so far been used, in practice, in conjunction with CE-MS and particularly in forensics.

Moreover, it has to be taken into account that the choice of any sample pretreatment will depend on the sample nature, the analyte characteristics, and the subsequent analytical separation technique to be employed, sometimes requiring a case-by-case development. Therefore, no universal sample preparation is available.

Ideally, sample preparation steps should be kept to a minimum not only because this would reduce the analytical time and complexity but also because the greater the number of steps, the higher the probability of introducing errors and loss of analytes [12].

Differently from liquid and gas chromatography, in CE, sample preparation also involves removal of salts from the biological samples, which cause peak defocusing. In addition, inorganic salts hinder the application of sample injection techniques based on electrostatic force (i.e., electrokinetic (EK) injection, field-amplified sample stacking (FASS), etc.), which, when properly applied, may increase the analytical sensitivity by factors up to 1000 (and more).

Forensic samples are often found in low amounts and/or in the form of highly complex matrices, sometimes degraded (e.g., biological specimens in the crime scene), making crucial the sample collection as well. Due to the aforementioned reasons, the choice of sample preparation in forensic analysis may eventually be even more difficult than in clinical analysis, where samples are, in general, highly standardized and well preserved.

Since forensic analysis also deals with nonbiological materials (clandestine preparations of drugs, explosives, pharmaceuticals, etc.), forms of sample pretreatment (from simple dilution to SPE, etc.) may be required, although less usually, in these cases and should be carefully selected.

### 9.2.1 Blood

Blood, the most important transport fluid in the human body, is the specimen of choice in forensic toxicology when a direct correlation of drug concentrations and observed biological effect is being studied. For this reason, toxicological blood

analysis is required by clinical toxicologists and emergency-room personnel in the cases of acute intoxications. Under these circumstances, often the current immunoassay-based targeted determinations are not sufficient, since the cause of poisoning/intoxication is unclear and/or the suspected drug is not comprised in the classes usually screened by immunoassays (often the so-called NIDA five: opiates, cannabinoids, cocaine, amphetamines, phencyclidine). Therefore, a broad-spectrum analysis is required, which is being usually performed by HPLC-MS or GC-MS, after careful sample pretreatment, mostly based on SPE.

For blood analysis, CE suffers typically from a very low sensitivity, which is related to the minimum amount of sample being injected in the capillary. This limit can be circumvented by using field-driven injection methods, such as electrokinetic injection, FASS, which, however, require preliminary sample desalting.

For example, Gottardo *et al*. proposed a new method for the determination of 10 drugs of abuse (cocaine, methadone, morphine, codeine, benzoylecgonine, 6-monoacetylmorphine (6-MAM), amphetamine and its metabolites) in the blood by CZE-ESI-TOF MS, achieving a sensitivity of 2–10 ng $ml^{-1}$ by using FASS [12]. Postmortem blood samples, collected from the femoral veins, were saturated with ammonium sulfate solution and centrifuged. The supernatant was then mixed with $Na_2HPO_4$ and liquid–liquid extracted with chloroform–isopropanol mixture (9 : 1). The organic phase, free from most of the small ions of the biological sample, was then evaporated and the residue dissolved in distilled water containing pholcodine (IS). Also, Schappler *et al*. worked on blood sample pretreatment by comparing two strategies of sample preparation and injection, that is, protein precipitation (PP) prior to hydrodynamic injection (HD) and liquid–liquid extraction (LLE) followed by electrokinetic (EK) injection [13, 14]. The former was a very valuable tool for highly concentrated samples (>1 μg $ml^{-1}$) and allowed a high throughput, but, in order to achieve sensitivity levels comparable to HPLC with LODs in the low ng $ml^{-1}$ range, the latter approach had to be used. Under optimized conditions, EK injection proved to be suitable also for quantitative analysis.

Notwithstanding the aforementioned problems related to the presence of inorganic salts in the injected sample, simple phosphoric acid/ACN protein precipitation, which cannot remove sample salts, proved to be suitable for achieving sensitivities in the range of few tens of nanograms per milliliter with hydrodynamic injection without any appreciable peak defocusing. An example can be found in a paper by Boatto *et al.* on the CZE-ESI-MS (single quadrupole) analysis of phenethylamine designer drugs in the human plasma [15]. However, quite surprisingly, the same research group needed SPE with $C_{18}$ cartridges for extracting plasma samples containing four 2,5-methylenedioxy derivatives of 4-thioamphetamine (ALEPH series) preliminarily to their determination with a similar CZE-ESI-MS (single quadrupole) method achieving comparable LODs [16].

In addition, a class of antidepressants, including imipramine and desipramine, were extracted from the human plasma via a single one-step LLE with hexane. The dried organic phase was reconstituted with a solution of 5% acetic acid, 10 mmol $l^{-1}$ ammonium acetate in methanol/water (20 : 80). In this study, the

compounds were quantitatively determined by a chip-based CZE-ESI-QqQ system [17].

SPE, using a $C_8$ cartridge, was also the sample preparation method of choice for determining lidocaine and its metabolites in the human plasma, in a paper by Anderson *et al*. comparing the UV detection against single-quadrupole ESI-MS after nonaqueous capillary electrophoresis (NACE) separation [18]. An analytical sensitivity in the range of approximately $100-150\,ng\,ml^{-1}$ was achieved when using UV detection, compared to MS with LODs in the range of $20-100\,ng\,ml^{-1}$.

For the determination of paracetamol and its metabolites in serum, three sample preparation procedures were investigated: (i) direct injection, which required micellar electrokinetic chromatography (MEKC) separation conditions; (ii) protein precipitation with ACN followed by CZE determination; and (iii) ultrafiltration through a 30 kDa ultrafiltration membrane followed by CZE. Detection was either by UV absorption (recording the UV spectra) or by ESI-ion trap MS. The ability of CE to deal also with polar and ionized compounds allowed for a simultaneous determination of paracetamol and its two main metabolites (paracetamol glucuronide and paracetamol sulfate), which are hard to separate by traditional chromatographic techniques. To achieve this aim, relatively unselective sample preparation procedures, as the three methods described earlier, were needed to avoid loss of analytes [19].

In conclusion, today, it is agreed that complex biological matrices, such as blood, plasma, and serum, need procedures of sample preparation, if the best performances of separation and MS detection are to be accomplished. In fact, small organic and inorganic ions, proteins and other blood constituents may adsorb onto the capillary wall, affecting separation [20]. On the other hand, the same components may also interfere in ionization of analytes, giving rise to unpredictable ion suppression or enhancement. Without the contribution of the sample preparation, all the burden of "cleaning" the analytes of interest before they enter the ionization chamber of the MS is on separation step. In this respect, CE may contribute to the reduction of these phenomena, thanks to the miniaturization of the separation compartment and therefore of the amount of the injected material reaching the ion source.

### 9.2.2
### Urine

Urine is a body fluid containing metabolic wastes and a multitude of other substances including drugs, xenobiotics, and their metabolites that the kidney withdraws from the circulatory fluids and expels from the body. From an analytical point of view, among the other body fluids, urine appears to have distinct advantages. First, urine is easily accessible, sample collection does not require any trained medical personnel [20], urine composition reflects the functioning of several organs. Second point, by collecting the "24 hour urine," the analyte concentration can be converted into the mass of the analyte excreted per day. On

the other hand, the analyte concentration in urine is influenced by the kidney function and perfusion and by the hydration status of the body, which makes difficult the interpretation of the quantitative data in terms of concentration in a urine sample. In the "history" of analytical toxicology, urine analysis has been for decades the standard tool for drug screening in the most varied contexts, such as penal cases involving illicit drug abuse, driving license issuing, workplace drug testing, assessment of the fitness-to-job, anti-doping.

Kohler *et al*. analyzed a mix of 30 compounds (i.e., amphetamines and derivatives, opiates, cocaine and metabolites, metoprolol, ketamine, procaine, and trimipramine) in urine, with an integrated CE-MS procedure, providing both qualitative screening (CZE-ESI-TOF MS) and further quantitation of the identified compounds (CZE-ESI-QqQ MS) [21]. As for the sample preparation, the urine samples were centrifuged, filtered through a 0.45-μm nylon filter, diluted with BGE and water (1 : 1 : 8, v/v/v), and injected by adopting a large volume injection, corresponding to about 20% of the capillary length. This was possible without sacrificing the separation efficiency, because injection was carried out in an isotachophoretic-like mode. Under these conditions, sensitivities in the range of 2 – 50 ng $ml^{-1}$ could be achieved for a panel of more than 30 drugs, without any extraction procedures. The advantage of large-volume injection in terms of sensitivity can easily be understood if we compare these data with the LODs reported by Ramseier *et al*. for amphetamines (amphetamine, methamphetamine, MDA, and MDMA) [22]. In this case, the extraction method used a commercial LLE kit (Toxi-Tube A, from Agilent Technologies, Santa Clara, CA, USA) followed by hydrodynamic injection (50 or 100 mbar for 6 or 12 s) after solvent evaporation and residue reconstitution in small volume (1 to 20 concentration factor). The CZE analysis based on ESI-ion trap MS detection provided sensitivities in the range of 50 – 200 ng $ml^{-1}$. In short, even omitting sample extraction, the sensitivity of the large volume injection exceeded by a factor of about 10 that of the traditional hydrodynamic injection of a 20-fold concentrated LLE.

A different extraction procedure was carried out by the same group for the analysis of methadone and EDDP. These compounds were extracted by a two-step SPE method on a copolymeric resin (Bond Elut Certify cartridges, Varian, Harbor City, CA, USA). The eluant in the first step was $CH_2Cl_2$, whereas a mixture of $CH_2Cl_2$:isopropyl alcohol (8 : 2 v/v) containing 5% v/v concentrated ammonium hydroxide was used for the second elution. A washing step with methanol was carried out between the two. The eluates were evaporated and the residues dissolved in water before injecting in CZE-ESI-QqQ MS [23].

A NACE-ESI-MS (single quadrupole) method for amphetamine and derivatives was described by Geiser *et al*. In this study, the urine samples were prepared by an LLE with chlorobutane. In this case, the dried residues were reconstituted with methanol:buffer (90 : 10), the buffer being a mixture of MeCN-MeOH 80 : 20 v/v, ammonium formate, and formic acid [24].

In addition, 10 benzodiazepines were extracted from urine samples after LLE with ethyl acetate followed by CEC-TOF MS on a hexyl-acrylate-based porous

monolith. The research group of Blas *et al*. reported LODs as low as 1 ng $ml^{-1}$, using a preconcentration step at the head of the column [25].

Furthermore, urine (as well as other biological fluids) was subjected to a cleanup procedure using a technology known as *membrane preconcentration* (*mPC*) followed by CE. Briefly, a disc of polymeric membrane impregnated with a chromatographic stationary phase is installed as the adsorptive phase of a cartridge. The membrane is placed inside a Teflon tubing where it completely fills the cross section of the tube. As a final step, two fused-silica capillaries are inserted onto each end of the Teflon tube [26]. In particular, the neuroleptic drug haloperidol was assayed in urine via mPC-CZE-ESI-MS (QqQ and EB, electrostatic – magnetic analyzer): the presence of its main metabolite, reduced haloperidol (4-(4-chlorophenyl)-1-[4-(4-fluorophenyl-4-hydroxybutyl]-4-piperidinol), was determined by direct analysis. However, other two pyridinium species (4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl]-pyridinium and 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-hydroxybutyl]-1-pyridinium) needed a further preanalytical step in which urine was directly loaded and concentrated onto a $C_{18}$-impregnated membrane. The latter was subsequently submitted to on-line washing and elution either with a mixture of methanol and water or only with methanol [27].

### 9.2.3 Hair

Hair is a skin annex of humans and most mammals. It is not homogeneous but consists of keratinized cells glued by the cell membrane complex. Hair originates from the hair follicle, located few millimeters under the skin surface and surrounded by a rich capillary system, which provides the growing hair with the necessary metabolic material through the blood (Figure 9.1). Together with nutrients, other molecules, such as xenobiotics, present in the blood reach the hair follicle, where they are trapped into the growing hair structure. Thanks to these peculiar features, such substances embedded in the hair matrix may last from months to years since they are protected from external environment and from the activity of the metabolic reactions. The use of hair in forensic toxicology was introduced in the late 1970s by Baumgartner *et al*., as this peculiar specimen proved to be suitable for investigating retrospectively drug abuse histories [29]. Since then, notwithstanding fierce criticisms among forensic toxicologists, hair analysis has become a well-established tool in analytical toxicology to investigate chronic abuses of drugs in forensic, clinical, and administrative contexts [30].

Only a limited number of research groups applied CE, and particularly CE-MS, to hair analysis, despite often with excellent results. For example, Gottardo *et al*. reported the quantitative determination of morphine, cocaine, benzoylecgonine, MDMA, MDA, 6-MAM, amphetamine, methamphetamine, ephedrine in the human hair by CZE-ESI-ion trap MS [31]. Hair samples (~100 mg) were washed twice with an aqueous solution of 0.3% Tween-20 (20 ml), in order to remove contaminants possibly present on the surface, then cut into small fragments,

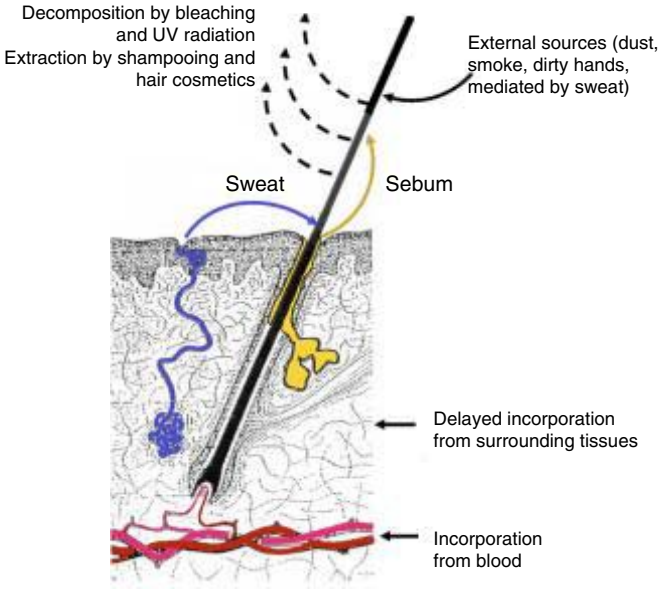


**Figure 9.1** Incorporation in and loss of compounds from hair. (Reproduced from [28] with permission from Elsevier.)

and incubated overnight in 0.1 mol $l^{-1}$ HCl (1 ml) at 45 °C. The incubation mixture was then neutralized with equimolar amounts of NaOH and extracted into organic phase with a commercial LLE device, that is, Toxi-Tubes A. The organic layer was collected and evaporated under a stream of air, and finally, the dried residue was reconstituted with 500 µl of bidistilled water for injection. The same research group also detected the same major drugs of abuse and codeine in hair samples via CZE-TOF MS, by using the same sample preparation procedure [32].

### 9.2.4 Saliva

Saliva is a readily available biofluid, which consists of secretions from the salivary and oral mucous glands. Analysis of saliva components is useful for noninvasive determination of both pharmaceuticals and controlled drugs in body fluids [33].

Jhang *et al*. developed a novel drug-screening system, consisting of paper spray-MS (PS-MS) followed by a CZE-ESI-ion trap MS confirmation [34]. This system can be easily switched either to PS-MS, for rapidly screening samples, or to the traditional CZE-ESI-MS, for separation before obtaining detailed mass spectral information. The sample preparation procedure was alternatively LLE or solid-phase microextraction (SPME). This very intriguing approach was tested for the detection of 4-chloro- and 4-fluoroamphetamines in oral fluid [35, 36].

## 9.3
## Separation Modes and Analytical Conditions

### 9.3.1
### Capillary Zone Electrophoresis

Capillary zone electrophoresis (CZE), usually performed in aqueous phase, is the original and still most common CE separation technique, particularly when coupled with mass spectrometry.

However, NACE is, in principle, even more compatible with mass spectrometry, because of the utilization of volatile solvents, hence being particularly suitable for the analysis of compounds poorly soluble in water [37]. For this reason, in some instances, it can be considered a valid alternative to CZE in aqueous buffers.

Jussila *et al*. used NACE-ESI-ion trap MS for the detection of methadone, pentazocine, levorphanol, dihydrocodeine, and morphine [38]. The study introduced a modified type of interface, where the ESI spray capillary is extremely thin so that its inlet end is inserted into the CE capillary.

Also, methamphetamine, alprenolol, and levorphanol were detected by NACE by means of a sheathless nano-ESI interface based on liquid junction. Again, the interface was modified with a thin spraying needle to allow for the coupling of a wide-bore CE capillary with ion trap MS [39].

NACE-ESI-ion trap MS also proved to be suitable for chiral separations. For example, salbutamol enantiomers were separated and quantified in urine samples, using derivatized beta-cyclodextrins (CDs) as chiral selectors (see also in the specific section "Chiral Separation", Section 9.3.6) [40].

Capillary wall coating is a usual procedure in CZE, particularly when dealing with macromolecules, in order to avoid analyte adsorption onto the inner surface of the capillary, having a detrimental effect on separation. Despite the almost universal use of physically adsorbed coatings in CZE with optical detection (since they can easily be applied, removed, or replaced), chemically bonded coatings are preferred in CE-MS, to avoid leaking of the coating material into the ion source, which may produce background noise, suppression of analyte signal, and/or contamination of the ion source and MS optics [41].

Notwithstanding this problem, Vanhoenacker *et al*. analyzed six benzodiazepines in urine samples with CZE-ESI-ion trap MS and CZE-MS/MS by using a buffer system that dynamically coated the inner wall of fused-silica capillaries (CEofix™, Analis, Suarlée, Belgium) [42]. According to the procedure, a buffer containing a polycation ("initiator") is flushed through the capillary. The polycations adsorb strongly to the capillary wall due to charge interactions. The capillary is then flushed with the running buffer containing a polyanionic additive (called *accelerator*). These polyanions interact with the lipophilic part of polycations forming a double layer. The polyanion layer contains sulfate groups and is rather insensitive to pH variations. Therefore, a stable and pH-independent EOF is ensured, when an electric field is applied across the capillary. After the analysis, the coating is stripped from the wall by a short rinse with NaOH

followed by water. The use of such dynamic coating produced a high, constant, and pH-independent EOF, without appreciable negative side effects on the MS side.

A similar dynamic coating was employed by Kohler *et al*. for the screening procedure of a two-step forensic toxicology analysis [21]. CZE-ESI-TOF MS was used for the screening step, requiring only urine dilution and on-line sample preconcentration with pH-mediated stacking. Subsequently, quantitation of positive samples was performed by CZE-ESI-QqQ MS. The commercial dynamic coating CEofix™ was employed only for the screening experiments, proving to be compatible with MS detection. Instead, an uncoated, bare fused-capillary was used for quantitative experiments.

An advantage of CE is that the core of instrumentation can be miniaturized in a microchip format. In this configuration, the consumption of sample and reagents, analysis time, and costs may be reduced to the minimum. Also, analytical speed and instrument portability are also increased [43, 44]. The chips can be made up of various materials, among which are glass, silicon [43], and polymer such as poly(dimethylsiloxane) [45]. The epoxy-based negative photoresist SU-8 has shown to be a very suitable material for microchip production. Sensitive and efficient drug screening of urine samples was reported by using the SU-8 CZE-ESI-QqQ MS microchips. The microchip method including rapid CZE separation followed by MS detection was applied to two parent drugs, paracetamol and tramadol, as well as to their metabolites (glutathione-, glucuronide-, and mercapturate-paracetamol; *O*-desmethyl-, and *N*-desmethyl-tramadol) [46].

### 9.3.2
### Capillary Isotachophoresis

Capillary isotachophoresis (CITP) uses a discontinuous buffer system composed of a leading electrolyte (LE) and a terminating electrolyte (TE). The LE and TE have electrophoretic mobilities that are higher and lower, respectively, than the analytes of interest in the sample. The sample is located between the two electrolyte solutions and the analytes stack according to their electrophoretic mobilities (from the highest to the lowest) after the LE zone. Once the zones reach their steady state, the entire system moves at constant velocity, hence the name, "iso" implies same and "tacho" speed [47].

So far, fairly scarce literature has been published on CITP coupled to MS, particularly in forensics. ITP-TOF MS and ITP-CZE-TOF MS were used for the analysis of angiotensin peptides even at trace concentrations ($0.3\,\mu mol\,l^{-1}$). The sample loading capacity in both ITP and ITP-CE was impressively high (up to 10 µl), if compared with the typical nanoliter-sized injection volumes of CE. It was concluded that ITP-TOF MS alone was adequate for the separation and detection of high concentration samples. The outcome was different in lower analyte concentrations at which formation of mixed zones occurred, producing ion suppression and miss-assignment of masses. This problem was overcome by

adding a CZE capillary between the ITP and TOF MS. In such an arrangement, samples were preconcentrated in the high load ITP capillary and then transferred into the CZE capillary, where they were separated into nonoverlapping peaks prior to their detection by TOF MS. The advantage of this integrated arrangement is that there is no need to discard portions of the sample in order to avoid overloading of the CE capillary. The authors concluded that this approach can be valuable for the analysis of complex samples with wide ranges of component concentrations [47].

CITP, indeed, is most often used as a sample focusing technique in several CE separation modes. Just to mention an example, on-line CITP sample focusing allowed for loadability enhancement in capillary electrochromatography (CEC) fitted with UV or mass spectrometry (single quadrupole) detection. Its application to the analysis of small drugs such as salbutamol, fenoterol, neostigmine, and scopolamine in the plasma and urine samples provided sensitivities in the low nmol $l^{-1}$ range [48].

### 9.3.3
### Micellar Electrokinetic Chromatography

In MEKC, two distinct phases, an aqueous and a micellar phase, are used, the latter acting as a pseudostationary phase. This phase is established by adding surfactants, such as SDS (sodium dodecyl sulfate), to an aqueous buffer above their critical micellar concentration (CMC) [49].

Being based on both electrophoretic and "chromatographic" principles, MEKC is suitable for the separation of charged compounds as well as neutrals, and therefore, it allows for the analysis of a wide range of compounds of different nature, as it often occurs in forensic analysis. Clearly, the coupling of MEKC with MS seems extremely attractive and advantageous, as it would combine a highly versatile separation technique with mass-selective and structure-elucidative detection. However, the presence of high concentrations of surfactants in the separation electrolyte *per se* may hinder the MEKC-MS coupling, because of possible poisoning of the ion source [50].

A strategy to overcome the problem is to use a "partial filling" technique, in which the micellar phase is present only in the first part of the capillary, thus producing analyte separation without introduction of surfactant into the ion source. Stubberud *et al*. employed this approach for the analysis of ibuprofen, codeine, and their relative degradation products and impurities [51]. Specifically, by employing partial filling (PF)-MEKC-MS (single quadrupole), the separation and identification of all the compounds of interest were achieved, preventing the ion source contamination. First, the analytes migrated through the micelle zone where they were separated; subsequently, the migration continued through the electrophoretic BGE without micelles, until the analytes reached the ion source. Then, the potential was turned off, thus hindering the contamination of the ion source by the micelles.

### 9.3.4 Capillary Electrochromatography

CEC stems from the combination of two separation techniques, namely liquid chromatography (LC) and CZE. In fact, a capillary column containing an LC stationary phase is eluted by a mobile phase, which, rather than by pressure, is pushed forward by the electric field, producing an effective EOF at the silica surface. CEC offers advantages such as the mixed mode separation, higher sample capacity, and greater "peak capacity" over CE. However, the CEC column reproducibility is still questionable [52].

Mixtures of benzodiazepines, corticosteroids, and thiazide diuretic drugs were analyzed by Taylor *et al*. using UV absorbance and ESI-MS detection [53]. Separations were performed on fused-silica capillary columns packed with CEC Hypersil ODS (Thermo Fisher Scientific, Waltham, MA, USA) or Apex ODS particles (Jones Chromatography, Hengoed, UK).

### 9.3.5 Capillary Gel Electrophoresis

CGE is the method of choice to separate polymers formed by monomers with the same charge. Given a constant charge-to-mass ratio, these molecules migrate at the same velocity under standard electrophoretic conditions. The addition of a sieving network (chemically or physically bound) to the separation buffer introduces a further separation dimension, which enables the separation based on molecular size. Large biomolecules such as DNA, RNA, and proteins are typically analyzed by using CGE. The major drawback of on-line CGE-MS is the need for highly concentrated buffer solutions, which dramatically decreases the sensitivity of MS detection [52].

### 9.3.6 Chiral Separation

The separation of enantiomers in capillary electrophoresis by means of chiral selectors can be performed by two methods: the chiral selector is introduced into the running buffer as an additive or the chiral selector is employed as a stationary phase [54]. In the first option, the chiral selector is therefore a constituent of the buffer itself. Notwithstanding its popularity in CE with optical detection, this could be a disadvantage when the CE separation is to be coupled with MS, because the chiral selector could contaminate the ion source, causing loss of sensitivity and increase of the background noise [37].

CDs added to the BGE are by far the most popular chiral selectors used in CE [55]. Among all, alpha-, beta-, and gamma-CDs as well as several of their derivatives have been and still are applied in forensic analyses that require chiral separation.

Enantiomeric separation of two local anesthetics (bupivacaine and ropivacaine) was achieved in PF-CZE coupled on-line to microelectrospray QqQ MS, avoiding the poisoning of the ion source with the chiral selector methyl-beta-CD. This was achieved by coating the capillary with polyacrylamide in order to minimize the EOF. Furthermore, prior to sample injection, the capillary was partially filled with CDs dissolved in acetate buffer. Hence, when the voltage was applied, the positively charged enantiomers migrated toward the mass spectrometer through the zone containing the neutral chiral selector, which did not move forward in the absence of EOF. Under such conditions, a racemic mixture of bupivacaine and ropivacaine was successfully separated and an impurity of 0.25% of *R*-ropivacaine in *S*-ropivacaine could be detected [56].

The use of counter-migration of the chiral selector is another well-known alternative approach to avoid, in a simple way, the CD entrance to the ion source of the MS. Schulte *et al*. adopted such a strategy for the enantioselective separation of etilefrine, mianserin, dimethindene, chlorpheniramine, and tropic acid in CZE-ESI-ion trap MS [57]. The charged CDs employed were carboxymethyl ether of beta-CD (CM-beta-CD), sulfobutyl ether of beta-CD (SBE-beta-CD), and 2-hydroxy propyltrimethylammonium salt of beta-CD (TMA-beta-CD) (Figure 9.2).

As mentioned earlier, the chiral selector may also be incorporated in the stationary phase of a CEC column. Von Brocke *et al*. reported on the on-line coupling of pressure-supported CEC using ESI-MS and coordination ion spray (CIS)-QqQ MS for the chiral analysis of barbiturates and chlorinated alkyl phenoxypropanoate enantiomers [58]. Capillaries were packed with

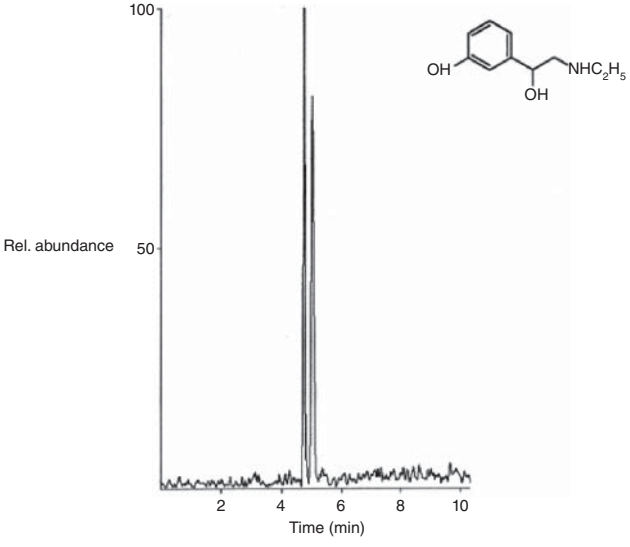


**Figure 9.2** CE-ESI-MS electropherogram of (6)-etilefrine plotted in the single ion track (*m/z* 5181.7–182.7) mode with 3 mg ml$^{-1}$ CM-beta-CD as a chiral selector (pH 4.3). (Reproduced from [57] with permission from Elsevier.)

permethylated-beta-CD-modified silica (Chira-Dex-silica, Polymicro Technologies, Phoenix, AZ, USA). Silver(I), cobalt(II), copper(II), and lithium(I) ions were used for the postcolumn conversion of the analytes into charged coordination compounds, which were detected in the CIS-MS mode. In this study, the high separation efficiency of CEC combined with the high selectivity and sensitivity of CIS-MS offered the possibility of detecting and identifying enantiomers.

In addition to CDs, other chiral selectors have been used, although less frequently. Crown ethers are synthetic macrocyclic polyethers able to form stable inclusion complexes with various inorganic and organic cations, which have been used as chiral selectors in forensic CE-MS.

Amino acid racemization (AAR) is a posttranslational modification, which is based on the intrinsic instability of certain amino acid residues, which leads to their racemization, a phenomenon of high relevance in the investigation of the age of biological samples. Proteins are synthesized from L-amino acids (L-AAs); however, after incorporation into proteins, L-AAs start slowly to racemize to their D-form under a reversible first-order kinetic reaction until equilibrium is reached. Each amino acid has a different intrinsic racemization rate, which is a function of the physical, chemical, and biological (protein sequence and its secondary, tertiary, and quaternary structures) properties of the molecule, acting as a kind of biological clock. A CZE-ESI-ion trap MS technique using (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid as BGE was reported by Moini *et al*. for age estimation of silk textiles based on AAR rates [59]. With an L to D conversion half-life of ~2500 years for silk (*Bombyx mori*) aspartic acid, the technique was capable of dating silk textiles spanning in age from several decades to a few 1000 years. The analysis required only ~100 μg or less of silk fiber. Except for a 2 h acid hydrolysis at 110 °C, no other sample preparation was required. The CE-MS analysis took ~20 min, consumed only nanoliters of the amino acid mixture, and provided both amino acid composition profiles and D/L ratios for ~11 amino acids.

In the past years, several types of polymeric surfactants (also known as *micelle polymers* or *molecular micelles*) have been synthesized and widely applied to enantioseparations in MEKC-UV. For the analysis of chiral compounds, the feasibility of direct coupling of MEKC to MS using polymeric surfactant was studied by Hou *et al*. [60–62]. Six amino-acid-based polymeric surfactants, including three carbamate-type polymers and three amide-type polymers, were synthesized and evaluated for MEKC enantioseparation and ESI-MS (single quadrupole) detection of two benzodiazepines, ((±)-oxazepam (OXA) and (±)-lorazepam (LOR)), and one benzoxazocine, ((±)-nefopam (NEF)). The use of poly(sodium *N*-undecanoyl-L-leucinate) (poly-L-SUL) in combination with organic modifiers (acetonitrile, methanol, and isopropanol) provided the simultaneous enantioresolution of (±)-OXA, (±)-LOR, and (±)-NEF in a single run. As for the two benzodiazepines, chiral MEKC (CMEKC)-ESI-MS (single quadrupole) method was also developed by using a dipeptide polymeric surfactant, poly (sodium *N*-undecenoxy carbonyl-L,L-leucyl-valinate) (poly-L,L-SUCLV) [60].

Moreover, the simultaneous separation of eight stereoisomers of ephedrine and related compounds ((±)-ephedrine, (±)-pseudoephedrine, (±)-norephedrine, and (±)-*N*-methylephedrine) was achieved by CMEKC-ESI-MS (single quadrupole). The polymeric chiral surfactant selected was the poly(sodium *N*-undecenoxycarbonyl-L-leucinate) (poly-L-SUCL) [62]. The same research group used the same procedure, but with (poly-L-SUCL) with ACN/$H_2O$ (30/70 v/v), for separation, identification, and quantitation of 10 enantiomers of ephedrine and related compounds. The method was then validated in terms of linearity, LOD (0.125 – 0.0625 μg $ml^{-1}$), LOQ (0.25 – 0.50 μg $ml^{-1}$) precision, and robustness [61].

Also, the simultaneous enantioseparation of three barbiturates (mephobarbital, pentobarbital, and secobarbital) was optimized using MEKC-ESI-MS (single quadrupole), enabling the development of a high-throughput method with high efficiency and sensitivity. Polysodium *N*-undecenoxy-L-isoleucinate (poly-L-SUCIL) was used as the chiral selector. The method was validated, proving to be suitable for the quantitative analysis of the three compounds in serum samples [63].

(−)-DIKGA ((−)-2,3:4,6-di-*O*-isopropylidene-2-keto-L-gulonic acid) is a chiral selector with a rigid structure, which possesses hydrogen-accepting ether functions in the vicinity of the carboxylic group. Thus, (−)-DIKGA enables multipoint interactions with enantiomeric amines. This compound was chosen by Lodén *et al*. to separate the enantiomers of pronethalol and phi-ephedrine by PF-NACE-ESI-QTOF MS [64].

One of the most powerful chiral selectors for the separation of compounds containing free carboxylic groups is vancomycin. This was tested by Fanali *et al*. for enantioseparation of nonsteroidal anti-inflammatory drugs (NSAIDs) by CZE-ESI-ion trap MS [65].

Enantiomer separation may be performed in an open-tubular format, in which the chiral selectors may coat and subsequently be immobilized onto the internal surface of the capillary. The method is called *open-capillary electrochromatography* (*o-CEC*). While the analyte is drawn along the column under an electric field, the chiral separation of the enantiomers is accomplished by diastereomeric interactions at the wall surface.

Schurig *et al*. have reviewed o-CEC-ESI-MS (single quadrupole) and Chirasil–Dex as chiral stationary phase (CSP) for the enantioseparation of numerous chiral compounds [54].

### 9.3.7
### Analytical Conditions

Since the analytical conditions, with the limitations imposed by the technology of the CE-MS coupling, are strictly dependent on the needs of the specific analytical problems, this subject will be dealt with in detail in the following sections.

In this brief chapter, the attention will be focused on two general problems: (i) the injection (ii) and the restrictions on the composition of the separation buffer required by MS.

As previously mentioned, achieving high sensitivity is, in fact, of the highest importance in forensic analysis. With the MS detection being substantially dependent on the amount of analyte entering the ion source (and there efficiently ionized), more than on analyte concentration (as it occurs in UV detection), the method sensitivity will depend greatly on the mass of analyte that can be injected into the CE system. Achieving this goal on-line in a narrow-bore capillary is not an easy task and requires special attention. The simplest approach is just by applying electrokinetic injection, which, by electrostatic force, draws the analytes with a suitable charge from the sample solution into the capillary inlet. Wey *et al.* reported the use of electrokinetic injection for CZE-FASS-ESI-ion trap MS of opioids (dihydrocodeine (DHC), nordihydrocodeine (NDHC), dihydromorphine (DHM), nordihydromorphine (NDHM), codeine (COD), normorphine (NMOR), norcodeine (NCOD), and morphine (MOR) in urine and urinary extracts [66]. Electrokinetic injection was found to provide higher sensitivity than hydrodynamic injection by a factor of over 1000.

As it is well known, electrokinetic injection is heavily affected by the composition of the sample solution and particularly by its content of small ions. Because of that, analyte desalting is crucial in this procedure, as it is usual in protein and polynucleotide analysis by using CE.

A sophisticated improvement is represented by head-column FASS, which, based on an electrolyte binary system, proved to be a robust approach to enhance the sensitivity. After replenishing the capillary with the running buffer, a short low-conductivity zone (water plug) is introduced at the inlet side prior to electrokinetic injection from a sample solution of low conductivity. During electrokinetic injection, charged solutes migrate rapidly through the water zone, because of a very high local electric field. Upon reaching the interface with the running buffer, their migration velocity is drastically decreased because of the drop in the electric field due to a local higher conductivity. As a result, charged solutes are effectively concentrated at this interface, prior to their electrophoretic separation [66].

Another approach that can be adopted to increase sensitivity in CE, which also stems from electrokinetic injection, is the so-called large-volume sample injection. In this scheme, an important part of the capillary is filled with the sample dissolved in a low-conductivity buffer. After voltage application, because of a higher electric field in the sample plug than in the separation capillary, electrolytes with the suitable charge run faster until the interface with the separation buffer, where, because of a lower electric field, they stack in a small band, and the separation starts.

A further electrokinetic method to dramatically increase the sample load is the so-called sweeping, which also applies to nonionic species. The application of the on-line sample concentration sweeping technique to MEKC-APCI-MS

(single quadrupole) was investigated by Isoo *et al*. to enhance the concentration sensitivity of environmental pollutants, such as several organic amines and alkyl phthalates [67]. In this study, both neutral (in the presence of EOF) and acidic (with suppressed EOF) conditions were applied and their sensitivities compared. Under the neutral conditions, a 40- to 100-fold gain in the concentration sensitivity was attained in the sweeping MEKC-APCI-MS system compared to the conventional MEKC-APCI-MS system, whereas under the acidic conditions, a 100- to 600-fold sensitivity enhancement was achieved.

As mentioned elsewhere in this chapter, one of the major points of weakness of the coupling of separation techniques in liquid phase with MS comes from the possibility that the ionization process may be affected by nature, and concentration of the compounds entering the ion source. In short, substances entering the ion source might cause suppression of the analytes' ionization as well as contamination of the source itself and optics of the mass spectrometer. This typically poses high restrictions on the composition and concentration of the buffers used in the separation process. In LC-MS, particularly, in order to reduce these disturbances, the traditional mobile phases containing inorganic salts are mandatorily replaced with organic buffers. However, in comparison with liquid chromatography, CE deals with much smaller amounts of sample and much lower separation buffer flow rates and consequently seems inherently suitable for reducing to a minimum the aforementioned disturbances of ionization [68]. Also, when CE is hyphenated to MS through a sheath liquid interface, the dilution (1 : 20 – 1 : 30) exerted by the sheath flow and the liquid sheath effect (BGE inorganic anions migrate toward the injection end) may further reduce these problems [69]. Since often CE efficiency and resolution are not maintained when using volatile buffers, with consequent peak defocusing, the possibility of using inorganic buffers in CE-MS seems very promising for the success of this technique.

On the basis of these considerations, a few research groups have published contributions on the possibility of using nonvolatile buffers in CE-MS in different fields. In particular, Somsen *et al*. reported the feasibility of introducing nonvolatile buffers and surfactants into ESI by testing the direct infusion of mebeverine [50]. Also, the research group of Nieddu proposed the separation of amphetamines congeners in urine or plasma extracts by CE-ESI-MS using a nonvolatile buffer electrolytes without sacrificing analytical sensitivity, with LODs ranging from 3.98 to 4.64 ng ml$^{-1}$ in urine and from 11 to 23 ng ml$^{-1}$ in plasma [31, 32, 70]. Moreover, Gottardo *et al*. reported the effect on the separation and mass spectrometry response of a mixture of MDA, MDMA, methadone, cocaine, morphine, codeine, and 6-MAM by comparison of three nonvolatile BGEs (phosphate, borate, and Tris buffers) versus a typical volatile buffer (ammonium formate) [68]. Inorganic nonvolatile borate and tris buffers proved to be hardly suitable for CE-MS analysis, but quite unexpectedly ammonium phosphate buffers showed good separation and ionization performances for all the analytes tested.

## 9.4 Applications

### 9.4.1 Forensic Toxicology

The term "forensic toxicology" covers those applications of the biomedical and analytical sciences aimed at the elucidation of the toxicological problems occurring in the criminal investigation, in the penal trials (e.g., poisoning), as well as in the civil litigation (e.g., child custody, divorce) and in different administrative fields (e.g., fitness to drive or to work) [44].

In forensic toxicology, according to a well-established international agreement, a two-step approach is used in the analysis of biological (and complex nonbiological) samples (Figure 9.3). The first step usually consists of rapid screening, generally using high-throughput qualitative (or semiquantitative) methods, for example, immunoassays. In case of a presumptively positive result (i.e., including the possibility of a false-positive), it is necessary to perform a second analytical step of confirmation. For that purpose, a highly accurate quantitative procedure is required, based most often onto physical–chemical technologies. Many analytical methods have been used for the confirmation of targeted compounds as well as for the so-called generic screening (i.e., broad range search of any possible drug or toxicant), but those currently adopted include GC or HPLC with MS or $MS^n$ detection or, more recently, with high-resolution MS. In this context, since the 1990s, CE has started to be considered as an alternative technique, finding a quite huge success in the academic research, but so far only limited application in the "real world" [71]. The reasons underlying this apparently inexplicable phenomenon are tentatively discussed in Section 9.5.

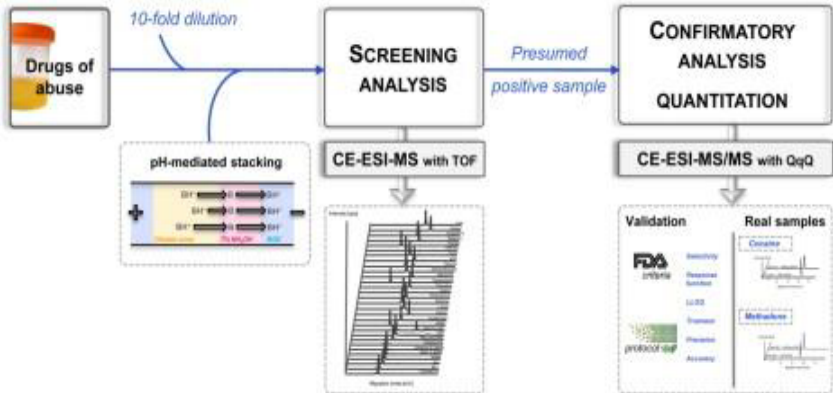


**Figure 9.3** Scheme of two-step forensic toxicology analysis. (Reproduced from [21] with permission from Elsevier.)

#### 9.4.1.1 Drugs of Abuse

A drug of abuse may be defined as "any substance whose possession or supply is restricted by law because of its potential harmful effect on the user."

At national and international levels, laws and regulations exist to govern the control of such substances, which are scheduled according to their harmful effects on abusers and on their involvement in criminal activities.

Different analytical approaches based on CE-MS for determination and quantitation of drugs of abuse for forensic purposes have been reported, but only a few of them are reviewed and discussed. The most popular separation method, by far, was CZE. For example, confirmation testing of urinary amphetamine, methamphetamine, MDMA, MDA by CE combined with atmospheric-pressure ESI and ion trap MS was reported by Ramseier *et al*. [22]. CZE-MS and CZE-$MS^2$ provided data that permitted the unambiguous determination of these drugs together with other basic compounds, such as methadone, EDDP, morphine, and nicotine, in external quality control urines. The organic phase of urine samples was extracted by LLE and then reconstituted with water. A standard coaxial interface was used providing an additional sheath liquid flow to stabilize the electrospray. $MS^2$ proved capable of discriminating co-migrating substances, provided that they produced different fragments in the collision cell.

For the analysis of matrices simpler than biological fluids, such as seized drug samples, Lazar *et al*. described the use of continuous infusion into a ESI-TOF MS through a CZE instrument [72]. The investigated molecules were tetracaine, procaine, methamphetamine, cocaine, amphetamine, heroin, and impurity. Since the main objective was to identify the drug present in the confiscated material, seized samples were directly analyzed by TOF MS via infusion through a microelectrospray interface. Thus, the analysis time depended only on the sample preparation, taking only about five minutes. The spectra of the seized samples displayed mainly the protonated molecular ion of the drug. Weak signals corresponding to polymeric contaminants were also present in each of the spectra. Other components, possibly present, did not give a measurable signal in the positive ion electrospray mode.

Despite this interesting possibility, analyte separation before MS is generally preferred for the analysis of confiscated drug preparations, also when CE is coupled to highly selective MS.

The synthetic opioid fentanyl and its derivatives have the highest analgesic potency, which can be up to 8000 times greater than that of morphine. Apart from their therapeutical applications, there is an abuse of fentanyl and congeners in the drug market as heroin substitutes. A NACE-ESI-ion trap $MS^n$ method, in positive-ion mode, was developed and validated by Rittgen *et al*. for the separation and identification of six fentanyl derivatives including fentanyl, *cis*- and *trans*-methylfentanyl, sufentanil, alfentanil, and carfentanil [73]. This method was successfully used for the analysis of real samples from seizures of illegal fentanyl laboratories.

Confirmation testing of urinary amphetamine, methamphetamine, MDMA, MDA by CZE combined with atmospheric pressure ESI and ion trap MS was

reported by Ramseier *et al*. [22]. CZE-ESI-ion trap MS (in the CZE-MS and CZE-$MS^2$ formats) provided data that permitted the unambiguous confirmation of these drugs as well as other basic compounds, such as methadone, EDDP, morphine and nicotine in external quality control urine samples. Urine samples were extracted by LLE and then reconstituted with water. A coaxial interface was used with an additional sheath liquid flow to stabilize the electrospray.

More than 25 alkaloids exist in the different varieties of opium, but the pharmaceutically important molecules are subdivided into two chemical groups: the isoquinoline derivatives, such as papaverine and narcotine, and the phenanthrene derivatives, such as morphine and codeine. The major alkaloids in opium and their abundances are as follows: morphine (10–16%), noscapine (4–8%), codeine (0.8–2.5%), papaverine (0.5–2.5%), thebaine (0.5–2%), narcotine (0.75–10%), and narceine (0.1–0.5%). Lazar *et al*. proposed a peculiar CZE-ESI (microspray)-TOF MS method for opium powder rough extracts, achieving low fmol detection [74]. Standard samples were prepared by dissolving the appropriate amount of analyte in mixtures of $CH_3OH/H_2O/CH_3COOH$, or $CH_3CH_2OH/H_2O/CH_3COOH$, which were then filtered through 0.2 μm filters. A syringe pump was used to perform continuous infusion experiments.

Roscher *et al*. reported a NACE-ion trap MS method as a tool for unraveling complex alkaloid mixtures present in the samples of *Sceletium tortuosum* and in drug products called Kanna made thereof, containing a large number of isobaric compounds [75]. Analysis of Kanna alkaloids is particularly challenging, because of the complexity of the alkaloid mixture including a large number of diastereomers, their poor solubility in water, and the lack of several alkaloid standards. In the mentioned paper, NACE-ion trap MS was successfully used for the relative quantification of these analytes showing a large variation of relative alkaloid concentrations in *Sceletium* plants and in Kanna formulations.

A further application of CE to herbal extracts was reported by Wahby *et al*., who developed a CZE-ESI-ion trap MS method for the identification and determination of choline and atropine compounds in hairy root extracts from *Cannabis sativa* L. [76]. Atropine (a tropane alkaloid) and choline (quaternary base) have considerable pharmacological implications. Atropine is normally used as a parasympatholytic, anticholinergic, spasmolytic, and antiemetic drug. Choline is a precursor of essential molecules such as the membrane lipids phosphatidylcholine and sphingomyelin, the neurotransmitter acetylcholine, and the signaling lipids platelet-activating factor and sphingosylphosphorylcholine. The method proved to be able to resolve both choline and atropine, showing very high sensitivity (LODs of 18 μg $ml^{-1}$ and 320 ng $ml^{-}$, respectively), using standard uncoated fused-silica capillaries and a simple volatile alkaline buffer (ammonium acetate/ammonium hydroxide). The method was also tested on real samples of hairy root clones.

Cheng *et al*. studied 16 synthetic drugs in adulterated Chinese medicines, such as acetaminophen, flufenamic acid, oxyphenbutazone, bucetin, caffeine, indomethacin, phenylbutazone, diazepam, ketoprofen, prednisolone, ethoxybenzamide, mefenamic acid, salicylamide, fenbufen, niflumic acid, and sulindac, by

CZE and MEKC coupled to ESI-Q ion trap MS [77]. Chinese medicine proposes a natural-drug-based therapy, in which it is not uncommon to find synthetic chemical drugs, which are illegally added to medicines to improve illicitly the drug efficacy. CZE was initially chosen because its on-line coupling with ESI-MS is more straightforward. However, since its separation power was insufficient, MEKC was then preferred as the separation method. However, in practice, the direct coupling of MEKC with ESI-MS appeared much more difficult, as the signals were less stable than in CZE-MS, and the electrical continuity was relatively difficult to be maintained. It was observed that the interference of SDS on ESI was less severe in positive ion mode than in negative ion mode. Indeed, up to 20 mmol $l^{-1}$ SDS could be used in direct coupling of MEKC with ESI-MS if the mass spectrometer was operated in positive ion mode. By infusion experiments, it was found that SDS concentrations greater than 20 mmol $l^{-1}$ produced signal reduction to less than 30% for most analytes, in comparison with those observed without SDS. Accordingly, the 16 analytes were separated using SDS concentrations lower than 20 mmol $l^{-1}$. Notwithstanding these limitations, the synthetic drugs could be analyzed and the resolution appeared to be significantly better than that obtained with CZE.

Chen *et al*. analyzed a Chinese drug preparation, named wuyoufun-13, reported to contain three potentially active alkaloids namely coptisine, berberine, and palmatine, by using CZE-ESI-ion trap MS [78]. These molecules were identified by their migration times, molecular ions, and specific fragment ions produced from collision-induced dissociation (CID). The concentrations of coptisine, berberine, and palmatine found in the sample were 14, 50, and 17 mg $l^{-1}$, respectively. So they were present at too low concentrations for producing a significant medical effect.

Merola *et al*. used beta-CD and HS-gamma-CD for achieving the chiral separation of 12 cathinone analogs, belonging to the benzoylethanamine class and sold illegally as "bath salts," by means of PF-CZE-ESI-TOF MS [79]. These compounds differ from one another by the presence of substituents that may include halogen, dioxol, and alkyl groups; hence, a highly efficient and selective method for the discrimination of such closely related compounds is needed. The procedure used beta-CD for CZE-UV analysis and HS-gamma-CD for CZE-MS. The method provided chiral resolution and identification of individual analytes present in the mixture by determination of the exact mass by TOF MS. The method was tested using a small set of commercial "bath salts" and finally applied to seized drug sample.

HS-gamma-CDs were also the chiral selector of choice for the simultaneous chiral separation of nine amphetamine-type stimulants by reverse polarity (RP) CZE with electrospray positive ionization QTOF MS. This method was also applied to the analysis of trace precursors in illicit methamphetamine seizures [80].

Sulfated (S-beta-CD) CE was the most effective for the chiral separation of anesthetic drugs including bupivacaine, mepivacaine, prilocaine, and ketamine (Figure 9.4), according to Cherkaoui *et al*., who reported the on-line coupling of CZE with ESI-MS (single quadrupole), using the countercurrent PF technique

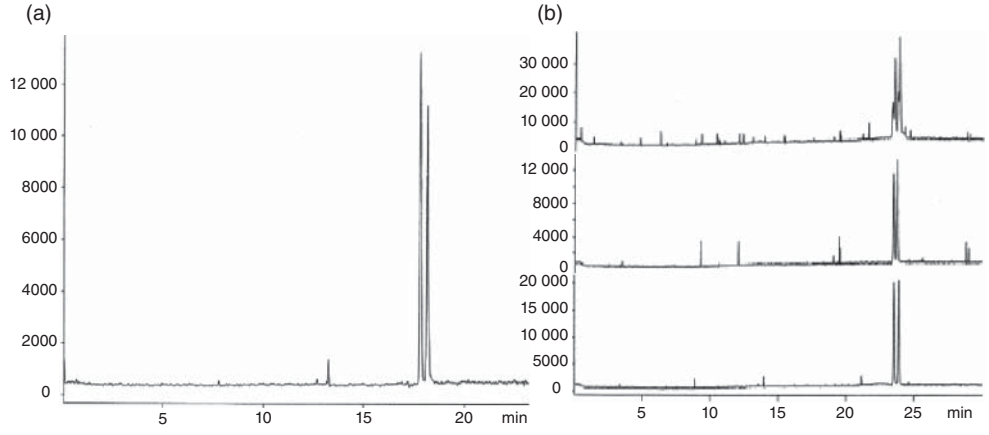


**Figure 9.4** CE-ESI-MS enantioseparation of ketamine, prilocaine, and mepivacaine. CE conditions, running buffer, 20 mM ammonium formate at pH 3 in the presence of *S*-beta-CD (a) ketamine and (b) top: reconstructed ion electropherogram, middle: ion 221, bottom: ion 247, prilocaine and mepivacaine. (Reproduced from [81] with permission from Elsevier.)

[81]. The optimized method was successfully applied to the enantioseparation of mepivacaine in injectable solutions.

The separation of four basic drugs (methadone, fluoxetine, venlafaxine, and tramadol) in standard solutions was obtained using low concentration of highly sulfated-gamma-CD (HS-gamma-CD) in normal polarity using PF-CZE-ESI-MS (single quadrupole) [82]. The same research group used the same chiral selector for the analysis of plasma samples containing seven amphetamines (Figure 9.5) [83].

Lorazepam (3-hydroxy-1,4-benzodiazepine) is a benzodiazepine drug commonly used and often abused. Furthermore, it is a chiral molecule, which undergoes enantiomerization at room temperature. In humans, lorazepam (LOR) is mainly excreted in urine as its 3*O*-glucuronide. MEKC-ion trap MS with negative ESI, in the presence of HP-beta-CD as chiral selector, was used to determine the two diastereoisomers of LOR-3*O*-glucuronide in urine samples by Baldacci *et al*. [84].

HS-gamma-CDs were employed for the chiral analysis of MDMA and methadone from plasma samples. Two different method settings were employed prior to CZE-ESI-MS (single quadrupole): protein precipitation (PP) with hydrodynamic injection (HD) and liquid–liquid extraction (LLE) followed by electrokinetic (EK) injection. The use of PP prior to a HD injection was suitable for high-concentration samples ($>1\ \mu g\ ml^{-1}$) allowing high throughput, because of the ease of sample pretreatment. In contrast, the combination of LLE and EK injection required a longer preanalytical time but permitted to achieve sensitivities at the ppb level [14].

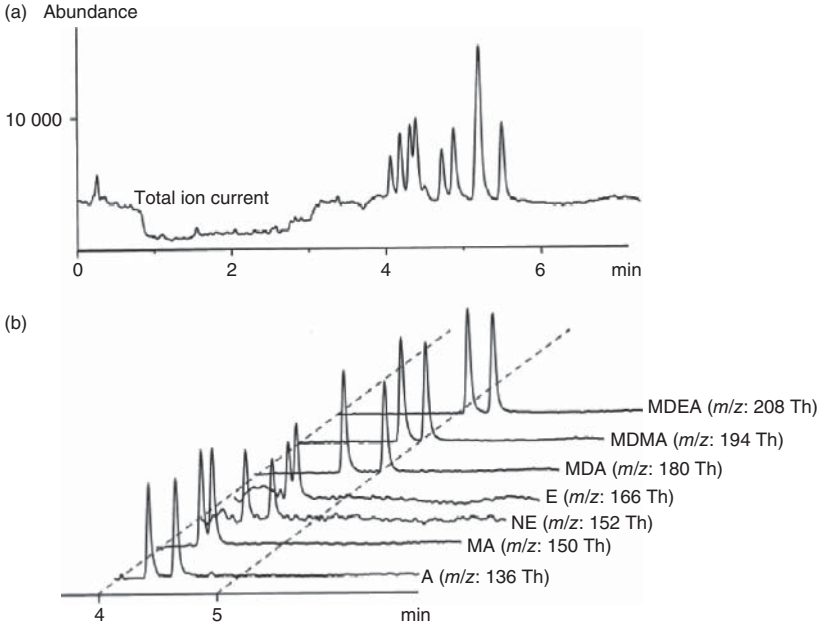


**Figure 9.5** Chiral analysis of amphetamine, ephedrine, norephedrine, methamphetamine, MDA, MDMA, and MDEA enantiomers with low HS-gamma-CD concentration and MS detection. (a) Standard solution. (b) Spiked plasma after LLE extraction. (Reproduced from [83] with permission from Wiley.)

Lu *et al*. investigated two beta-CD derivatives (DM-beta-CD and HP-beta-CD) as additives to the running buffer in the presence of different organic modifiers using CZE-ESI-MS [85]. The separation of the enantiomers of the chiral drugs ketamine, propranolol, and terbutaline was reported.

Also, DM-beta-CDs were used in a CZE with ion spray-QqQ MS system to analyze standard solutions of ephedrine and terbutaline as well as spiked urine samples of the latter [86].

Rudaz *et al*. described a successful application of CZE-ESI-MS (single quadrupole) to the simultaneous enantioseparation of tramadol and five of its phase I metabolites [87]. Various negatively charged CDs (sulfobutyl ether beta-CD (SBE-beta-CD-I), (SBE-beta-CD-II), gamma-CD phosphate sodium salt and carboxymethyl beta-CD (CMB-beta-CD), sulfated beta-CD (S-beta-CD)) were tested using a PF technique. The best result in terms of chiral separation was achieved with SBE-beta-CD, which not only allowed enantioseparation of the investigated drugs, but also improved the selectivity toward metabolites possessing the same molecular mass, such as *O*-demethyltramadol and *N*-demethyltramadol, as well as *N*-bis-demethyl tramadol and *O*-demethyl-*N*-demethyl tramadol. The described method was applied to the stereoselective analysis of tramadol and its main metabolites in a plasma sample.

DAS-beta-CDs were used by Iio *et al*. for the quantitative chiral analysis of seven methamphetamine-related compounds, namely methamphetamine, amphetamine, a methamphetamine metabolite, p-hydroxymethamphetamine (pOHMA), dimethylamphetamine (DMA), ephedrine, norephedrine, and methylephedrine, by using CZE-ESI-MS (single quadrupole) [88]. The developed chiral method enabled the discrimination of methamphetamine users from DMA users and from patients undergoing selegiline pharmacotherapy. Such methods also allowed the identification of *d*-pseudoephedrine, one of the isomers of ephedrine, which is available as an OTC (over-the-counter) drug in some countries. The method was applied to urine matrix, just using filtration prior to direct injection in CE.

Also, Dieckmann *et al*. developed a CZE-ESI-ion trap MS method that allowed the chiral separation and the identification of a wide range of drugs and adulterants including amphetamine, methamphetamine, ephedrine, norephedrine, *N*-methylephedrine, synephrine, methadone, tetramisole, and MDMA within 30 min [89]. Enantiomeric resolution was achieved using a running buffer containing HP-beta-CDs and heptakis (2,3-di-*O*-acetyl-6-*O*-sulfo)-beta-CD (HDAS-beta-CDs) as chiral selectors at low concentrations to avoid contamination of the mass spectrometer. Several forensic applications were tested with the selected method, that is, (i) analysis of illicit methamphetamine to discriminate between different batches by comparing their chiral impurities; (ii) enantioselective investigation of a blood sample extract containing methadone and spiked with methadone-$d_9$, with the discrimination between labeled and unlabelled methadone only by employing MS detection; (iii) identification of an unusual and potentially toxic cutting agent, Levamisole (the levo enantiomer of tetramisole), in a cocaine sample.

Varesio *et al*. developed a method for the identification of ecstasy and derivatives (amphetamine, methamphetamine, MDA, MDMA, MDEA, MBDB) in urine by CZE-ESI-MS (single quadrupole) after LL extraction [90]. It is worth mentioning that MDEA and MBDB have identical molecular masses but have different molecular structures. Unfortunately, migration times of the two compounds were very similar and peaks not baseline resolved. Since electrospray is a mild ionization technique, it generally results in mass spectra dominated by the protonated molecular ion, with little or virtually no fragmentation. However, it was possible to enhance the fragmentation by working at an elevated skimmer voltage. This high voltage enhanced the kinetic energy of the ions and permitted the fragmentation of a specified parent ion, according to a technique called *up-front CID*. Using this method, different daughter ions, at $m/z$ 177 and 163, were produced from MBDB and MDEA, respectively, by the loss of amine groups. Urine samples spiked with six amphetamines were analyzed without interference from endogenous components.

Iwamuro *et al*. validated a simple and rapid method for the determination of $\Delta^9$-tetrahydrocannabinol (THC), the primary psychoactive component of cannabis, one of the most commonly used illicit drugs in the world, in urine by CZE-ESI-QqQ MS [91]. The major metabolites of THC,

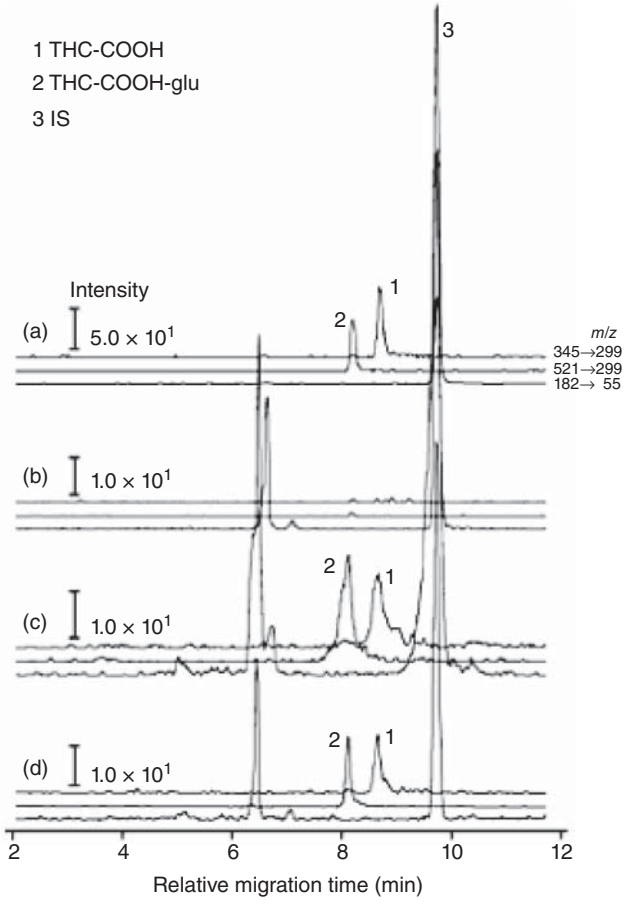


**Figure 9.6** MRM pherograms of (a) a standard mixture of THC-COOH (0.1 μg ml$^{-1}$), THC-COOH-glu (0.1 μg ml$^{-1}$), and IS (0.5 μg ml$^{-1}$), (b) a control urine sample, (c) a control urine sample spiked with THC-COOH and THC-COOH-glu (each 0.5 μg ml$^{-1}$ in urine), (d) sample (e) diluted fourfold with methanol. (Reproduced from [91] with permission from Wiley.)

11-nor-$\Delta^9$-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) and its glucuronide, were also determined (Figure 9.6). The only pretreatment needed for urine samples collected from cannabis users was just dilution with methanol containing an internal standard and centrifugation. Three product ions for THC-COOH (345→327, 345→299, 345→193) and four product ions for THC-COOH-glu (521→345, 521→327, 521→299, 521→193) were detected. No interfering signals were observed. Clearly, the MRM detection improved the selectivity and specificity of the method. In this method, it is worth noting that CZE is capable of analyzing charged glucuronides, which most often are problematic in HPLC and, obviously, impossible in GC.

Tricyclic and tetracyclic antidepressants have a well-known toxicological relevance, mainly because of their cardiologic side effects. In addition, their misuse, occurring in depressed patients undergoing pharmacological therapy, can be directly or indirectly related to suicide. Sasajima *et al*. developed a NACE-ESI-TOF MS method for the simultaneous determination of 20 antidepressants in plasma samples after SPE [92]. NACE was chosen as a powerful technique for the separation of poorly water soluble compounds, such as most antidepressants. In fact, when compared with aqueous CE-MS, NACE-MS showed better resolution and peak shape as well as higher detection sensitivity ($0.5-1\ ng\ ml^{-1}$) for the highly hydrophobic antidepressants.

Also, on-line CZE-ESI-MS was reported by Lu *et al*. for the determination of some tricyclic antidepressants (imipramine, doxepin, and amitriptyline) as well as beta-adrenergic blockers (propranolol and alprenolol) [93]. Optimized EKC-ESI-MS (double-focusing sector) conditions were obtained using low concentration of a polymerized surfactant poly(sodium undecylenic sulfate) (poly-SUS) as buffer additive. It was found that even at low concentration (i.e., below the CMC of the monomeric surfactant), the polymeric surfactant was still effective, enhancing the analyte mobility differences and thus improving selectivity and resolution. The use of low concentrations of additive was also beneficial to ESI-MS detection, by decreasing the surface tension of the electrospray droplets and thus enhancing the electrospray efficiency. These results suggest that the use of polymeric surfactants may improve ESI-MS detectability, in contrast to the monomeric surfactants used in MEKC. Furthermore, it is worth mentioning that the use of polymerized surfactants (chiral and achiral) in EKC-UV has in general shown excellent separation power for structurally similar compounds including isomers, enantiomers, and diastereomers.

A relatively simple and straightforward application on a biological sample was reported by Wey *et al*., who determined codeinoids by CZE and hydrodynamic injection after extraction with SPE mixed-mode polymer cartridges (Bond Elut Certify, Varian, Harbor City, CA, USA) in urine [94]. Detection was by an ion trap MS instrument equipped with an ESI interface operating in the positive ion mode. Codeine, dihydrocodeine, and, importantly, their glucuronides could be detected down to $100-200\ ng\ ml^{-1}$. It is worth noting that this sensitivity, although in general fairly modest, is sufficient to meet the cutoff levels of immunoassays used in real practice of routine analytical toxicology.

The same research group reported the analysis of morphine, morphine-3-glucuronide, 6-monoacetylmorphine, codeine, codeine-6-glucuronide, dihydrocodeine, methadone, and EDDP in a toxicological quality control urine by the use of a similar CZE-ESI-ion trap MS instrument. The study included the comparison of different sample preparation modes, that is, dilution, LLE, and SPE [95]. Moreover, direct MS analysis was compared with CZE-ESI-ion trap MS: detection limits for urinary free opioids were determined to be lower than $50\ ng\ ml^{-1}$. At that drug level, the presence of DHC, NDHC, DHM, and NDHM could still be confirmed via MS-MS and MS spectra. It was found that, when the analyte concentrations were high, the opioids could be detected

directly by MS, $MS^2$, or $MS^3$, but, below 2–5 μg $ml^{-1}$, analyte extraction and concentration were required. With the same instrumental configuration, the authors analyzed oxycodone, a strong semisynthetic opioid, and its metabolites, including oxymorphone, noroxycodone, and noroxymorphone. A peculiarity of these molecules is the presence of the –OH group in position 14, which loses $H_2O$ in $MS^2$ level, a phenomenon that is not observed with other opioids such as codeine, dihydrocodeine, morphine, and analogs [96]. Later, Baldacci *et al*. identified and characterized phase I and phase II metabolites of oxycodone in human urine, after SPE extraction using CZE-ESI-ion trap MS in positive mode, using computer simulations of fragmentation [97]. The CZE analysis was performed either by introducing hydrodynamically the sample by applying a positive pressure or by electrokinetically applying a positive voltage.

McClean *et al*. applied CZE-ESI-Q ion trap to the determination of diazepam and its metabolites, *N′*-desmethyldiazepam, oxazepam, and temazepam, in hair [98]. In a first attempt, $MS^n$ characterization and detection of the drug compounds was carried out by utilizing an ESI interface. For MS determinations, the capillary length from injection point to UV detection window was 34 cm after which the capillary traveled a further 61 cm to its point of entry into the mass spectrometric interface at the end of the electrospray needle. The variability in migration times was quite relevant, and LODs were in the range of 2.2–3 μg $ml^{-1}$, that is, not low enough for practical use in hair trace analysis. Subsequently, however, the same research group carried out further studies in order to improve sensitivity and area and migration time reproducibility. Optimization of sheath and auxiliary gas flows, capillary tip tapering, capillary tip positioning, sheath liquid composition and flow rate, and pressure application during separation step improved the ionization efficiency, lowering the LOD down to 0.1–1 ng $ml^{-1}$ with a stable ESI performance. The variability of the peak areas and migration times was reduced as well as linearity [99].

CEC, because of the mixed mode of separation (electrophoretic and chromatographic), may offer additional selectivity. Moreover, its peak capacity is higher than CZE. Both these features may be advantageous when dealing with complex matrices, as it occurs in forensic analysis. The simultaneous separation and determination of nine drugs of abuse (i.e., amphetamine, methamphetamine, MDA, MDMA, MDEA, cocaine, codeine, heroin, and morphine) in human urine (Figure 9.7) by CEC-ESI-ion trap MS in positive ion mode were carried out by Aturki *et al*. [100]. The CEC experiments were performed in fused-silica capillaries packed in house either with a 3-μm cyano-derivatized silica stationary phase (Varian, Darmstadt, Germany) or with LiChrospher 100 $RP_{18}$ 5 μm particles (Merck, Darmstadt, Germany) [101]. After SPE on a strong cation-exchange cartridge, good separation of almost all studied compounds was achieved, except for cocaine and heroin, which, however, could be easily resolved by MS detection. Good sensitivity was also achieved with LODs ranging between 0.78 and 3.12 ng $ml^{-1}$. This method offered good precision in terms of migration time and peak areas and accuracy. It was also successfully applied to real urine samples spiked with the investigated drugs of abuse.

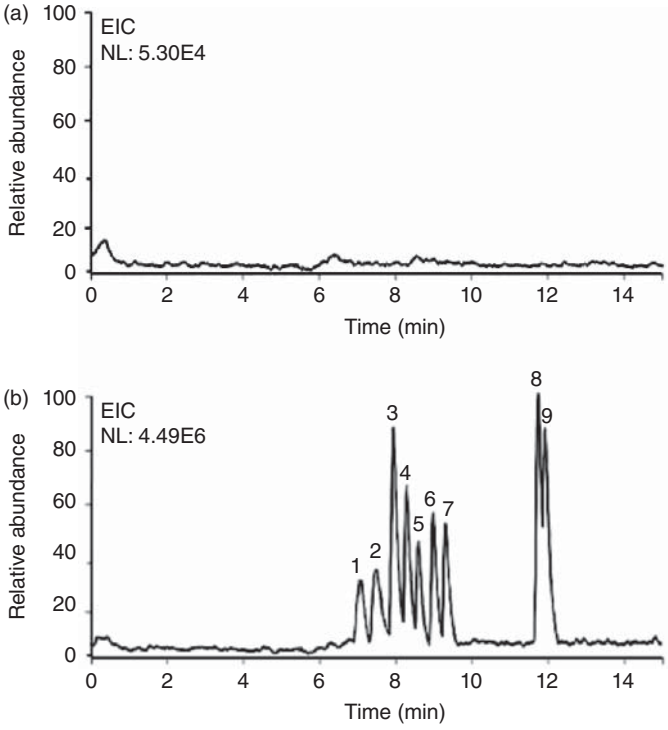


**Figure 9.7** Extracted ion electrochromatograms of (a) blank urine sample and (b) blank urine sample spiked with the drugs of abuse mixture, after SPE procedure. Samples were electrokinetically injected (12 kV, 10 s). (Reproduced from [100] with permission from Wiley.)

The methylenedioxy derivatives of amphetamine represent one of the first "designer drugs" appeared in the market. Nieddu *et al*. reported two methods for simultaneous screening and quantification of these analytes in urine samples after SPE extraction, using CZE-ESI-ion trap MS [70, 102]. Interestingly, in the first of the two CZE methods, the authors used a separation electrolyte containing a relatively high concentration of inorganic ions (i.e., 10 mmol $l^{-1}$ sodium phosphate monobasic), whereas in the second method, they used a more traditional volatile buffer composition (i.e., 50 mmol $l^{-1}$ ammonium acetate). As a matter of fact, LOD values of the first method ranged from 3.98 to 4.64 ng $ml^{-1}$ and those of the second method from 0.31 to 4.29 ng $ml^{-1}$. One could infer from these data that, with some limitations, inorganic buffers apparently do not preclude ESI efficiency. In conclusion, not only did the coupling of CZE-MS allow for the quantitative determination of amphetamine derivatives in biosamples but it also proved to be comparable in terms of sensitivity, accuracy, and precision to the current GC-MS and LC-MS.

Vanhoenacker *et al*. [103] used the previously described CEofix $^{TM}$ buffer system for the analysis of five basic drugs (amphetamine, ephedrine, codeine, salbutamol, trazodone) in urine samples, with runs lasting only about 10 min

(with accelerator) instead of more than 20 min (without accelerator). Notwithstanding the use of formic acid as a volatile electrolyte, the presence of the CEofix$^{TM}$ component "accelerator" resulted in fast and reproducible CE analyses even at a very low pH. In an effort to adapt the method to coupling with MS, which could suffer from the introduction of the "accelerator" into the ion source, the authors introduced a rinsing procedure of the dynamic double coating. This procedure allowed for a compatibility of the dynamic double coating with CZE-ESI-ion trap MS, offering high throughput and reproducibility without sacrificing sensitivity and ion source "cleanliness." Under these conditions, CZE-ESI-ion trap MS in positive ion mode provided LODs of less than 50 ng ml$^{-1}$. The method was successfully applied to the analysis of a spiked urine sample.

Heitmeier *et al*. proposed a CZE-ESI-ion trap MS method in negative ion mode for analyzing directly nonopioid analgesics (paracetamol, acetylsalicylic acid, antipyrine, ibuprofen, naproxen, ketoprofen, and propyphenazone) and their metabolites in urine [104]. These substances are frequently used for the treatment of fever and minor pain and are available without prescription. However, they are also susceptible to chronic abuse and accidental intoxications, and particularly paracetamol and acetylsalicylic acid, they recur in suicidal contexts. In the mentioned study, CZE-ESI-ion trap MS, compared with CZE-UV, showed clear advantages in terms of selectivity, allowing for the possibility of direct analysis of urine samples.

The broad window of drug identification of high-resolution mass spectrometry (HRMS) was exploited by Gottardo *et al*. in a forensic toxicology context using CZE-ESI-TOF MS for the determination of therapeutic and abused drugs and their metabolites in the hair of subjects undergoing detoxication treatments, in order to monitor their compliance to therapy [105]. Thanks to the sensitivity and selectivity of TOF MS in full scan, it was possible to screen for a broad spectrum of data in a single run, under fairly "generic" analytical conditions (CZE with FASS injection followed by mass analysis in ESI TOF in positive ion mode) with the possibility of postexperiment data reevaluation. Subjects with different addiction histories, different pharmacological treatments, and different durations of treatment were intentionally chosen in order to build up a study group as heterogeneous as possible (Table 9.1). Hair samples were cut at an average length of 2.0–2.5 cm from the scalp and collected, in order to investigate retrospectively a period of about 2 months prior to hair collection for each individual.

A peculiar variant of CE, named pressure-assisted CEC (pCEC) with ESI-MS (single quadrupole) using a silica-based monolithic column, was successfully applied by Lu *et al*. and for rapid analysis of five typical narcotics such as methadone, pethidine, fentanyl, morphine, and diamorphine (i.e., medicinal heroin) in real urine samples [106]. The proposed method showed distinct advantageous features, such as high sensitivity, speed, high efficiency, low sample and organic solvent consumption. On this basis, this pCEC-MS was proposed as a simple, robust, rapid method in doping control and forensic analyses with a sensitivity of 0.6 to 22.8 ng ml$^{-1}$.

**Table 9.1** Summary of the cases studied.

| Subject | Drug use history (as reported) | Time since admission to the program | Therapy | Identified compounds |
|---|---|---|---|---|
| Case 1 | Psychotic drugs | 5 months | Valproic acid, Trazodone, Levomepromazine, Paroxetine, Lorazepam, methadone | EDDP, Methadone des-Methyl Levomepromazinesulfoxide Levomepromazinesulfoxide m-Cl-phenylpiperazine Nicotine |
| Case 2 | Cocaine | 7 weeks | Trazodone, promazine, Estazolam, Escitalopram | des-Methylcitalopram, escitalopram, Cocaethylene, Cocaine m-Cl-phenylpiperazine |
| Case 3 | Heroin + cocaine | 2 months | Escitalopram | Escitalopram Cocaethylene, Cocaine, Benzoylecgonine, Nicotine |
| Case 4 | Heroin | 1 month | Methadone | EDDP, Methadone, Codeine, 6-MAM, Morphine |
| Case 5 | Alcohol | 2 years | Rivastigmine, Piracetam, Escitalopram, Nebivolol | des-Methylcitalopram, Escitalopram, Rivastigmine, Rivastigmine metabolite |

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Gamma-hydroxybutyric acid (GHB), a small organic acid, has a limited therapeutic use in the treatment of narcolepsy and in the control of the alcohol withdrawal syndrome, but recently it gained popularity as a "rape drug." Low concentrations (few $\mu g\,ml^{-1}$) of GHB were reported in urine of healthy subjects, as this compound is also an endogenous metabolite. Direct CZE-ESI-ion trap MS analysis of GHB in urine (requiring only sample dilution) was developed and validated by Gottardo *et al*. MS detection was carried out in selected ion monitoring mode of negative molecular ions, and the quantification was carried out on the

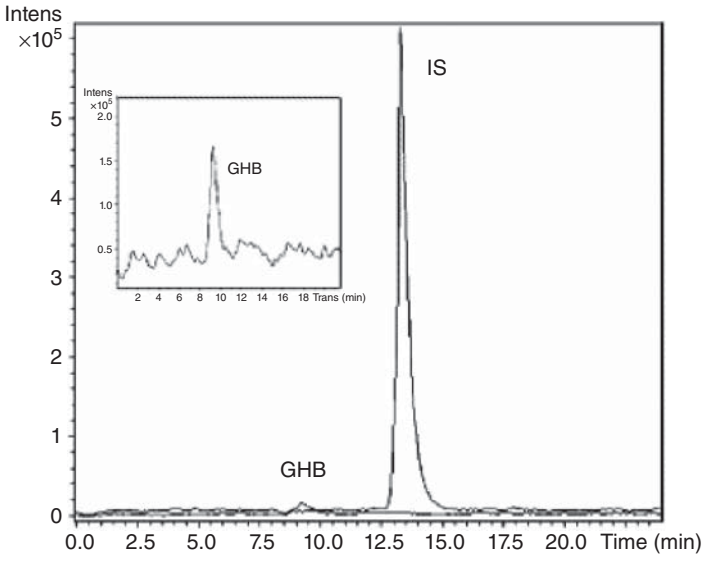



**Figure 9.8** CE-ESI-ion trap MS analysis of a urine sample from a subject under therapeutic treatment with GHB. GHB concentration: 47 μg ml$^{-1}$. Inset: expanded view of electropherogram at the migration time of GHB. (Reproduced from [107] with permission from Elsevier.)

basis of peak areas by using the internal standard method. The method validation included a comparison of ESI-MS response with GHB dissolved in water and in urine, showing a negligible matrix effect. Although this method was directly applicable to acute intoxications (Figure 9.8), with a LOD of 20 μg ml$^{-1}$, it was not sensitive enough for determining the endogenous GHB concentrations [107].

#### 9.4.1.2 **Alcohol Abuse Biomarkers**

The objective diagnosis of alcohol abuse has a high relevance in different areas of clinical and forensic medicine, including occupational and traffic medicine and forensic pathology. In addition to the traditional diagnostic approach based on anamnestic information, clinical examination, questionnaires, and a few nonspecific clinical biochemistry parameters (GGT, AST, MCV), in the recent years, new specific biomarkers have been introduced, which have revolutionized the diagnosis of this medical condition.

**Carbohydrate-Deficient Transferrin** Among the new markers, CDT is more prevalent in the diagnostics of chronic alcohol abuse. CDT represents a group of minor glycoforms of human transferrin (Tf), the major serum iron-transporting protein. The major glycoform of Tf contains two biantennary *N*-glycans with a total number of four sialic acid residues (tetrasialo-Tf, p*I* 5.4), but other isoforms with two (disialo-Tf, p*I* 5.7), three (trisialo-Tf, p*I* 5.6), five (pentasialo-Tf, p*I* 5.2), and six (hexasialo-Tf, p*I* 5.0) sialic residues (Figure 9.9) have been identified in normal human serum. In addition, traces of asialo-Tf and monosialo-Tf can

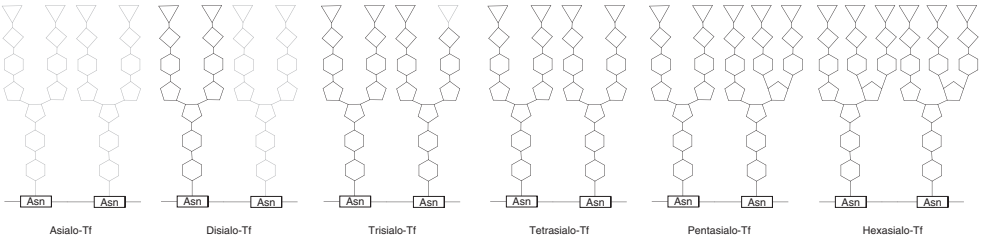



**Figure 9.9** Schematic representation of the clinically relevant Tf isoforms. Asn, asparagine residue; hexagon, *N*-acetylglucosamine; pentagon, mannose; diamond galactose; and triangle, sialic acid. Trisialo-Tf contains one biantennary di-sialylated *N*-glycan and one biantennary mono-sialylated *N*-glycan. (Reproduced from [108] with permission from Elsevier.)

also be determined under pathological conditions. The transferrin glycoforms, collectively referred to as *CDT*, include the isoforms asialo-Tf and disialo-Tf. According to a vast body of literature, after sustained alcohol intake, there is an increase in "total CDT" (i.e., asialo-Tf + disialo-Tf) serum concentration. However, it is well known that asialo-Tf is typically identified in the sera of alcohol abusers showing high increases of the disialo-Tf [109].

Although current analytical methods are based on HPLC or CZE with optical detection, CZE-TOF MS and capillary liquid chromatography (μLC)-TOF MS were used to detect and characterize human Tf glycopeptides obtained by tryptic digestion. In this process, the use of a commercial surfactant was necessary to increase the digestion yield. Even if the CZE-TOF MS achieved the separation of the glycoforms from the *N*-glycopeptides, the electrophoretic peaks were distorted and analytical reproducibility was poor. This phenomenon was attributed to the interaction of the surfactant with the inner walls of the fused-silica capillary. The problem apparently did not affect the μLC-TOF MS providing excellent separation between both N413 and N611 glycopeptides without peak distortion. Using both methods, the detected glycopeptide glycoforms fitted perfectly with the intact glycoforms present in a nonpathological sample [110].

A peculiar CZE-MS approach to detect CDT was adopted by Rodrìguez *et al*., who, after CZE separation of the transferrin isoforms from a standard solution of human apotransferrin (saturated with iron), used ICP-MS for detection, in comparison with UV detection [111]. The electrophoretic profile obtained by CZE-ICP-MS focused on the detection of the iron bound to Tf showing the trisialo-Tf, tetrasialo-Tf, and pentasialo-Tf peaks, but not the disialo-Tf and hexasialo-Tf forms, probably because of a lack of sensitivity of ICP-MS. On the other hand, the electropherogram from CE-UV, although less selective, showed all the Tf isoforms, proving better sensitivity of this method (in the range of 0.27 – 0.37 μmol $l^{-1}$ for UV versus 14 – 17 μmol $l^{-1}$ of ICP-MS).

**Ethylglucuronate and Ethylsulfate** Ethanol metabolism leads mainly to the formation of acetaldehyde, but, in minor proportion, also to EtS. EtG is formed

by conjugation of ethanol with activated glucuronic acid in the presence of membrane-bound mitochondrial uridine diphosphate glucuronyl transferase, whereas EtS is formed via sulfonation of ethanol in the presence of sulfotransferases. Less than 0.1% of the ingested ethanol is excreted in urine as EtG or EtS, but these metabolites are nonvolatile and water-soluble and have a longer half-life than ethanol and acetaldehyde. They can be detected for an extended time length after complete elimination of ethanol in blood and urine. EtG and EtS indicate recent ethanol consumption (up to 60 h) with high specificity and selectivity and thus cover a clinically and forensically important time window between short-term markers, such as ethanol, and long-term markers, such as CDT [112, 113].

Caslavska *et al.* used CZE-ESI-ion trap $MS^n$ after negative ionization for the analysis of EtG and EtS in human serum and urine [114]. The method required urine and blood SPE selective extractions for EtG (on Clean Screen ETG cartridges, UCT, Bristol, PA, USA) and EtS (Strata X-AW cartridges, Phenomenex, Torrance, CA, USA). Fragmentation of the $[M-H]^-$ ions resulted in known $MS^2$ spectra, the major fragments observed for EtG (*m*/*z* 221) were with *m*/*z* 203 ($-H_2O$), 159, 129, 113, 85, and 75; the transition observed for EtS (*m*/*z* 125) was *m*/*z* 97 ($HSO_4^-$) (Figure 9.10). This fragmentation equates those previously reported with LC-$MS^2$. Sample injection was hydrodynamic, and sheath gas flow was interrupted during the injection time. In order to force the analytes to

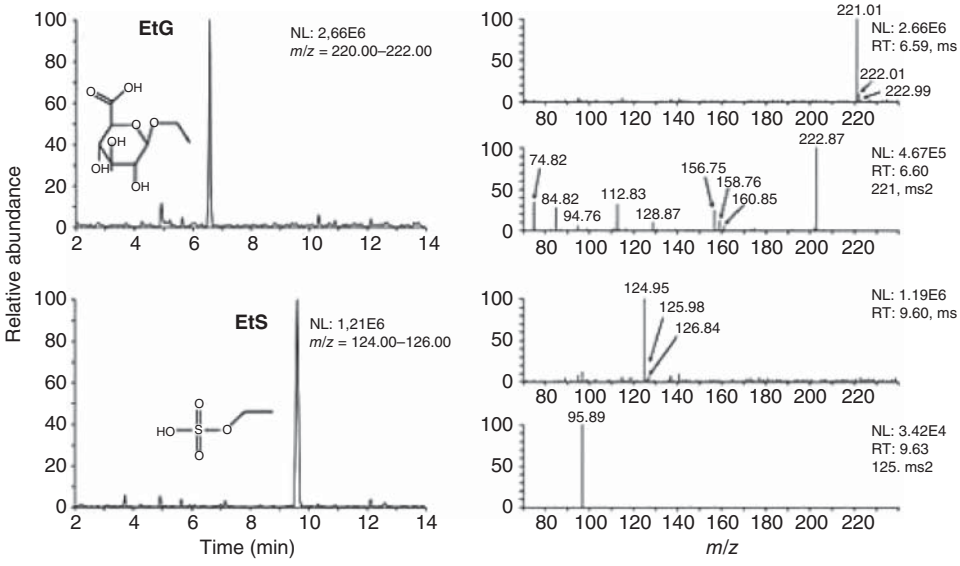



**Figure 9.10** Mass traces for EtG and EtS together with the chemical structures (on the left) and extracted MS spectra (upper graphs on the right) and $MS^2$ spectra (lower graphs on the right) obtained with a standard containing 17.3 µg $ml^{-1}$ EtG and 15.4 µg $ml^{-1}$ EtS dissolved in 10-fold diluted running buffer. (Reproduced from [114] with permission from Wiley.)

reach the detector within a reasonable time, an additional cathodic buffer flow generated via application of constant pressure of 5 mbar at the anodic side was used. The sensitivity achieved was found to be sufficient for confirmation of the presence of these compounds in serum and urine down to about 200 ng $ml^{-1}$.

A selective CZE-ESI-QTOF MS method in negative mode ionization was developed for the analysis of sulfate, sulfonate, and phosphate metabolites in urine by Bunz *et al*., which also proved to be suitable for the detection of EtS [115]. Before injection, it was necessary to perform a cleanup procedure by SPE extraction of EtS and removal of phosphates and other salts and proteins, for which, as in the previous work, Strata X-AW cartridges were chosen. The analytical sensitivity obtained was 50 ng $ml^{-1}$, with further possible improvements by optimizing the sample pretreatment. Moreover, the specificity of this method for sulfated and sulfonated substances was demonstrated by evaluation of the isotope pattern of sulfur in the detected compounds.

**Phosphatidylethanol** Phosphatidylethanol (PEth) is an abnormal phospholipid formed from ethanol and phosphatidylcholine in cell membranes via the transphosphatidylation reaction catalyzed by phospholipase D. PEth is not a single molecule, comprising a group of phospholipids with a common phosphoethanol head onto which two fatty acids of variable carbon chain length and degree of saturation are attached. After a sustained intake of ethanol, PEth is accumulated in the cells and may remain in circulation for more than 2 weeks. Thus, PEth is determined in whole blood and represents a marker for long-term alcohol abuse [112, 113].

A NACE-ESI-ion trap MS method for the detection and quantitation of PEth in blood was developed and validated by Nalesso *et al*. [116]. Indeed, the poor solubility of PEth in aqueous media made NACE the electrophoretic technique of choice for the separation of this compound. The molecular structure of the identified PEth species was confirmed by $MS^2$ analysis. Due to the difficulties in performing multiple MS/MS experiments on coeluting compounds, structural characterization of PEth homologs was carried out stepwise through repeated sample injections, each time monitoring the fragmentation of the following molecular species: $m/z$ 675.6, 701.6, and 727.6. The most abundant product ions observed in $MS^2$ mass spectra were $m/z$ 255.5, 255.5 – 281.5, and 281.5. These fragmentation patterns are in agreement with those expected for PEth 16 : 0/16 : 0, 16 : 0/18 : 1, and 18 : 1/18 : 1. The optimized method, with a LOD of 0.1 µmol $l^{-1}$, was finally applied to a real sample collected from a patient consuming about 180 g ethanol per day for more than 4 months. The MS detector allowed for the identification of three different PEth homologs corresponding to the molecular species at $m/z$ 675.6, 701.6, and 727.6.

**Acetaldehyde Adducts** The protein modification by acetaldehyde, the first metabolite of ethanol, plays an important role in the pathogenesis of tissue damage in alcoholics. Chemical modification of native proteins has been found to occur in the liver of alcoholic patients. Acetaldehyde-derived adducts have been

measured from erythrocytes and plasma proteins, which have been detected up to 3 weeks after the last alcohol intake [113].

De Benedetto *et al*. focused their attention on the development of an efficient CZE-ESI-ion trap MS method in positive ion mode for the detection of Ach adducts of hemoglobin [117]. The method employed in-house coated silica capillaries to hinder protein adsorption and a coaxial sheath liquid interface. The method was applied to characterization of the tryptic digests of Ach-protein adducts produced *in vitro*. The effects of the experimental parameters on the separation efficiency and resolution, including the alignment of the separation capillary and spray tip, were studied. The method permitted the identification of four stable Ach-induced hemoglobin modifications *in vitro* and $MS^2$ experiments confirmed their presence in the erythrocytes of a social drinker.

#### 9.4.1.3 **Doping**

Drug misuse is defined as doping when utilized in an attempt to enhance performance in a sport [44]. At present, by the World Anti-Doping Code (Code), the document harmonizing anti-doping policies in all sports worldwide, doping is defined as "the use of an expedient (substance or method) that is potentially harmful to athletes' health and/or capable of enhancing their performance, or the presence in the athlete's body of a prohibited substance or evidence of the use thereof or evidence of the use of a prohibited method." A list of prohibited substances in and out of competition is published and regularly updated, including anabolic agents, peptide hormones, growth factors and related substances and mimetics, $beta_2$-agonists, hormone antagonists and metabolic modulators, diuretics and other masking agents, stimulants, narcotics, cannabinoids, glucocorticosteroids, alcohol, and beta-blockers (banned in particular sports). In this list, the parent drugs are present, but the screening procedure must also be extended to their metabolites. The sample matrix types to be analyzed are urine and blood.

**Anabolic Agents** The use of anabolic androgenic steroids in sports is prohibited by WADA at all times in and out of competition. Owing to their lipophilic characteristics, they are excreted mainly in human urine after conversion into more water-soluble molecules mainly by glucuronidation.

Differently from GC and, to some extent, HPLC, CE is particularly suitable to deal with ionized molecules such as glucuronides. Cho *et al*. reported a CZE-ESI-ion trap MS method in negative ion mode (Figure 9.11) for quantitating in urine 3-glucuronides of androsterone (An-3G), 11-ketoandrosterone (11-ketoAn-3G), 11beta-hydroxyandrosterone (11beta-OHAn-3G), dehydroepiandrosterone (DHEA-3G) and 17-glucuronides of testosterone (T-17G), epitestosterone (Epi-17G), and dihydrotestosterone (DHT-17G)) with LODs around 5–10 ng $ml^{-1}$ [118].

Also, CEC-ESI-ion trap MS was reported by Que *et al*. as a valuable tool in steroid multicomponent analysis [119]. Groups of steroids, including both the neutral forms and their conjugates in urine (androsterone, 11beta-Hydroxyandrosterone, dehydroisoandrosterone, equiline, estrone, progesterone,

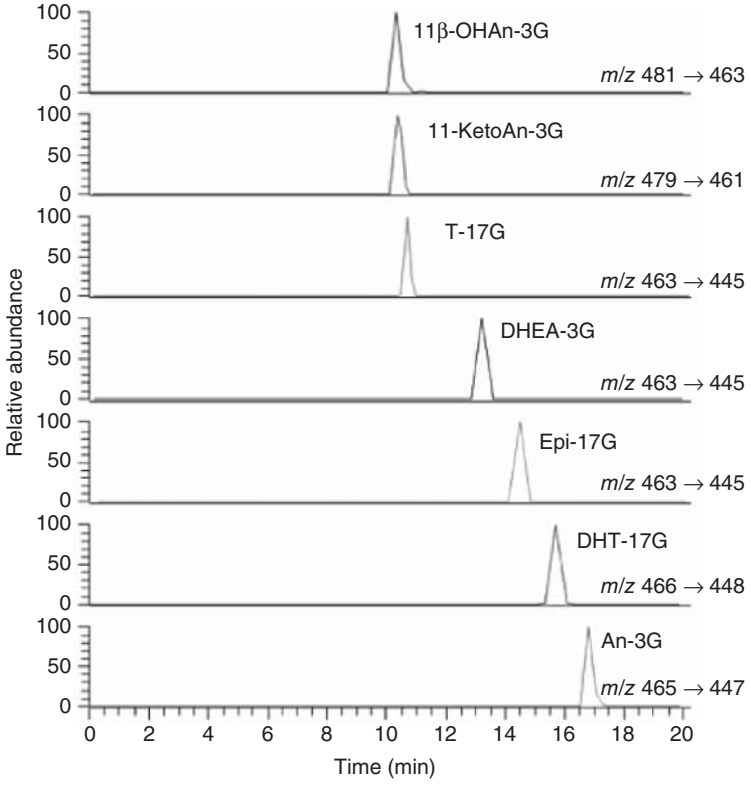



**Figure 9.11** CE-ESI/MS/MS electropherograms for seven androgen glucuronides in the negative ionization mode. (Reproduced from [118] with permission from Wiley.)

5-alpha-androstan-17-one, 19-hydroxy-4-androstene-3,17-dione, dehydroisoandrosterone), were analyzed. After hydrolysis, Sep-Pak $C_{18}$ cartridge (Waters, Milford, MA, USA) was used to purify the liberated neutral steroids, followed by successive washing with 5 ml water. The neutral steroids were further purified through an anion-exchange column with QAE Sephadex resin. This step was necessary to remove the naturally fluorescent pigments, which, being negatively charged, interfered with the fluorescence detection. These conditions allowed for the detection of neutral steroids down to 10 ng $ml^{-1}$ and for the conjugates down to about 500 ng $ml^{-1}$.

**Peptide Hormones, Growth Factors and Related Substances, and Mimetics** Recombinant human growth hormone (rhGH; somatropin) was one of the first biotechnologically produced proteins in *Escherichia coli*. The protein comprises 191 amino acids and has a p*I* of about 5.1. Although its primary use is in the treatment of human growth disorders, it is abused in sport competitions to improve muscle mass.

Electrophoresis and related methods have since long established themselves as the most important tools in protein and peptide analysis, and consequently, the possible use of CE was extensively investigated in this context.

Catai *et al*. used CZE in a capillary coated with a bilayer of polybrene (PB) and poly(vinyl sulfonate) (PVS) to achieve stable and relatively fast separations of rhGH [120]. PB-VS-coated capillaries were used to characterize thermally stressed and aged samples of the biopharmaceutical rhGH. Bilayer-coated capillaries yielded faster and more efficient and reproducible separations than did bare fused-silica capillaries and than those suggested in the European Pharmacopoeia method for rhGH. This advantage was attributed to minimized interaction between the proteins and the capillary wall. The bilayer coating proved to be compatible with MS detection, and consequently, CZE-ESI-ion trap MS in positive ion mode was applied, providing further information on the nature of the degradation products in rhGH samples.

**Erythropoietin** Erythropoietin (EPO) is a sialylated glycoprotein hormone produced by the kidneys in humans. It stimulates the production of red blood cells, by promoting the proliferation and the differentiation of erythroid progenitor cells. Human EPO consists of a 166-amino-acid polypeptide chain with disulfide bonds and four heavy polysaccharide chains. The molecular mass of EPO is 30 – 34 kDa, but carbohydrate chains amount to about 40% of the molecular mass. The carbohydrate moiety is of great importance for the biological activity of EPO. Recombinant human erythropoietin (rHuEPO) has been extensively used as pharmaceutical product for anemia treatment. This hormone quickly became misused as a doping agent for endurance athletes to improve aerobic performances, and the International Olympic Committee (IOC) officially prohibited its use in 1990. Because endogenous EPO and rHuEPO possess nearly identical biochemical and immunological properties, the identification of illegal use of rHuEPO was soon realized as quite difficult [121].

Different pharmaceutical preparations of rHuEPO are available for clinical use: two commercial ready-to-use pharmaceuticals of rHuEPO (epoetin-alpha and epoetin-beta) were separated and characterized by Balaguer *et al*. [122]. The main differences between the two epoetins were investigated by using CZE-ESI-TOF MS. This approach provided a detailed and fast characterization of the carbohydrate heterogeneity, finding the existence of two additional basic sialic acid isoforms for epoetin-beta and the higher degree of *O*-acetylations for epoetin-alpha.

An improved method for intact rHuEPO glycoform characterization by CZE-ESI-TOF MS was developed by the same group using a novel capillary coating. The novel dynamic coating provided electrophoresis at an EOF close to zero, enabling better separation, which resulted in improved mass spectrometric detection of the minor isoforms [123].

Sanz-Nebot *et al*. also reported an application of CZE-ESI-MS (single quadrupole) for the separation and characterization of rHuEPO glycoforms using polybrene (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide or hexadimethrine bromide, a cationic polymer) as permanent coating of the

internal walls of the capillaries [124]. This polymer containing quaternary amines strongly adsorbs onto the fused-silica inner surface. Since the net positively charged amine layer formed on the capillary wall reversed the EOF, the voltage polarity used for the separation also needed to be reversed in order to ensure that the positively charged analytes migrated toward the detector. By this procedure, a separation of five major peaks, although partially resolved, of rHuEPO glycoforms was achieved in 15 min.

On the basis of the literature, it is clear that hindering the spurious interactions between protein analytes and capillary wall is a crucial point for successful CE analysis. In fact, Yu *et al*. tested a series of ionene polymers synthesized in order to coat fused-silica capillaries for the separation of recombinant and urinary human erythropoietin (rHuEPO and uEPO) by CZE-ESI-MS [125]. These molecules are polycationic polymers containing ammonium function that strongly adsorbs onto the fused-silica inner surface, hindering rHuEPO adsorption while reversing the EOF toward the anode. Among these polymers, the separation efficiencies of 6,6-ionene and PB-coated capillary for rHuEPO glycoforms were similar and better than other ionene-coated capillaries.

A method for the accurate mass determination of integral glycoforms of rHuEPO based on CZE-ESI-TOF MS was developed by Neusüß *et al*. [126]. The different glycosylation forms were separated based on differences in both the number of negatively charged sialic acid residues and the size of the glycans. Two different rHuEPO samples and two batches from the same source were analyzed and compared. Using the accurate masses, an overall composition of each single glycoform was proposed.

A hyperglycosylated analog of rHuEPO, referred to as *novel erythropoiesis-stimulating protein* (NESP), was analyzed by CZE-ESI-ion trap MS in positive ion mode by Giménez *et al*. [127]. NESP differs from rHuEPO in five amino acid residues, and the presence of two new Asn residues enables the additional attachment of two extra *N*-glycans containing up to four terminal sialic acid residues each. The higher sialic acid content of NESP results in a longer circulating half-life and extended biological activity. Two physically adsorbed capillary coatings from UltraTol™ Pre-Coats [low normal (LN) and high reverse (HR)] were evaluated for the separation of rHuEPO and NESP. The results obtained with the neutral LN coating provided an acceptable glycoform resolution for rHuEPO, whereas the separation obtained for NESP was less good due to the higher microheterogeneity of this glycoprotein. Reproducibility studies confirmed the lack of stability and some bleeding of the LN coating, causing problems on the MS side, such as a dramatic loss of sensitivity and the presence of peaks in the mass spectra corresponding to molecular ions in the coating. On the other hand, the cationic HR coating yielded faster but poorer glycoform separations due to the presence of an anodal EOF. However, the positive charge of the coating provided enhanced hydrolytic stability, making it more suitable than the LN coating for the on-line MS coupling. The HR is another coating of the UltraTrol™ family where the polyacrylamide is derivatized with cationic substituents. The adsorption of the neutral LN polymer to the inner surface of

the fused-silica capillaries through hydrophobic interactions seemed to be insufficient to ensure the integrity of the coating using a BGE of 2 mol $l^{-1}$ acetic acid. HR-coated capillaries were assumed to present enhanced hydrolytic stability, as the cationic polymer could interact in a more intensive way through electrostatic interactions.

**Antihistaminic and Beta$_2$-Agonist Drugs** Antihistamines are drugs used to treat the symptoms of allergies and allergic rhinitis by blocking the action of histamine, a molecule released by the mast cells in their response to allergens. Some of these drugs, such as carbinoxamine, pheniramine, chlorpheniramine, brompheniramine, and doxylamine, were analyzed by Chien *et al*. by CZE-ESI-ion trap MS in positive ion mode [128]. Phosphate buffer was used for the CE separation of antihistamines. In the coupling of CE with ESI-MS, several approaches were adopted to mitigate the associated ion suppression in ESI and, at the same time, to maintain the separation integrity. These approaches included the substitution of the sodium ion by ammonium ion, the use of an acidic separation condition, the use of a low-flow sheath liquid interface, and the use of a sheath liquid containing a low concentration of phosphoric acid. With these arrangements, the separation integrity was preserved and the ion suppression was significantly alleviated. Unlike sodium ions, ammonium ions can be converted into ammonia and evaporated from the droplet. The signal obtained using ammonium phosphate appeared to be significantly better ($S/N = 36$) than the corresponding signal obtained using sodium phosphate ($S/N = 3$), thanks to an increase in analyte signal and a decrease in noise.

Sympathomimetic drugs have beta$_2$-adrenoceptor-stimulating properties and are widely used in the treatment of respiratory diseases. They are also well-known doping drugs. Since these drugs are usually administrated as racemates, each enantiomer needs to be monitored separately, since metabolism, excretion, or clearance might be radically different for both enantiomers.

CD-NACE-ESI-ion trap MS in positive ion mode was investigated by Servais *et al*. for the enantioselective detection and quantitation of salbutamol's enantiomers in human urine after SPE sample pretreatment [40]. This approach was successfully applied to the quantitative determination of low concentrations of salbutamol enantiomers in human urine. The used chiral selector was (HDAS-beta-CD). In this study, LOD values were 8 ng $ml^{-1}$ for the first enantiomer and 14 ng $ml^{-1}$ for the second one.

The combined use of the PF technique with neutral chiral selectors and suppressed EOF was the strategy of choice for chiral separation reported by Toussaint *et al*. using CZE-MS/MS (triple quadrupole) in positive ion mode [129]. Allowing the use of high CD (CM-beta-CD) concentrations, which, in the absence of EOF, do not enter the ion source, such strategies permitted to successfully resolve clenbuterol isomers in aqueous solution and in plasma samples. LODs and LOQs could be estimated at 0.8 and 2.5 fg $ml^{-1}$, respectively, for clenbuterol enantiomers and at 1.7 and 6 fg $ml^{-1}$, respectively, for salbutamol.

**Diuretics** Diuretics are widely used in the treatment of congestive heart failure and hypertension. The major indications of diuretics are the enhancement of renal excretion of salt and water. Diuretics were forbidden by the Medical Commission of the IOC since 1988 because athletes may try to reduce their body weight quickly in order to qualify for a lower mass class. Also, they may dilute their urine to avoid a positive doping result for other classes of drugs.

Twelve diuretics (amiloride, triamterene, bumetanide, canrenone, spironolactone, furosemide, indapamide, metolazone, etacrynic acid, bendroflumethiazide, hydrochlorothiazide, and chlortalidone) were analyzed by CZE-ESI-MS (single quadrupole) in positive ionization mode in real urine samples with spectra in both full scan and SIM mode. The sample preparation consisted of simply filtering the urine samples through a membrane filter. Notwithstanding a rough sample pretreatment, the method, using hydrodynamic injection, provided good reproducibility and linearity and excellent identification power. The detection limits of the 12 diuretics were in the range of 30–804 ng $ml^{-1}$ [130].

Furosemide is a potent, acidic (p$K$ values of 3.8 and 7.5) diuretic drug that is widely used in pharmacotherapy. Caslavska *et al*. developed a screening method for urine samples (Figure 9.12), which were analyzed as-received or after 2- to

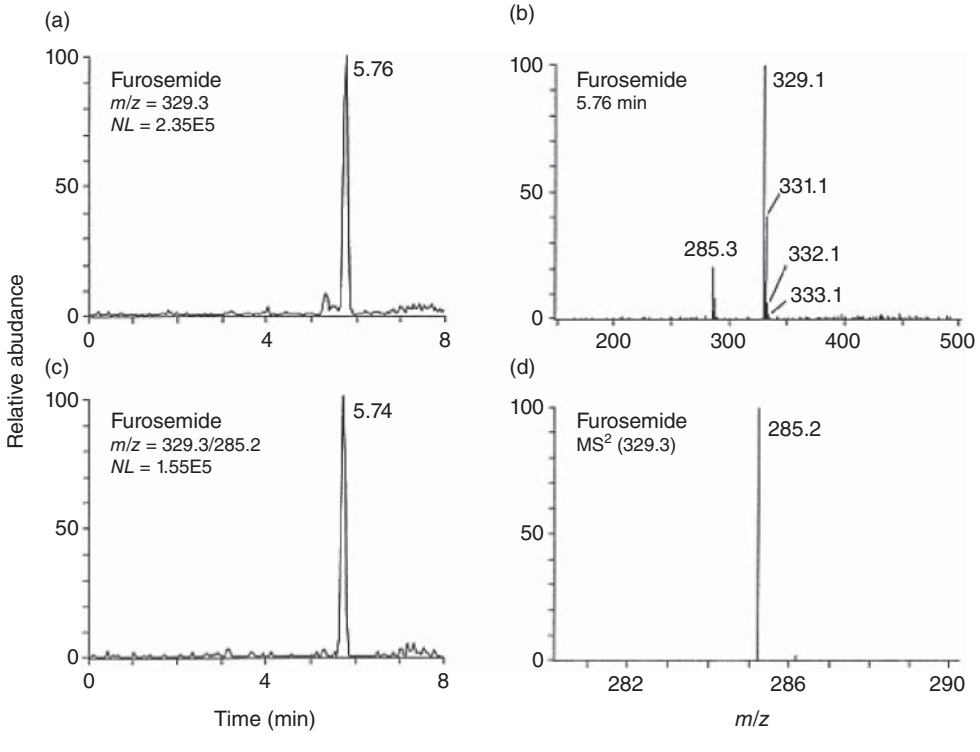



**Figure 9.12** CE-MS data obtained for the SPE extract prepared from 2.5 ml of urine sample derived from a patient under therapy. (a) Mass trace, (b) mass spectrum, (c) SRM mass trace 2, and (d) MS mass spectrum. (Reproduced from [131] with permission from Elsevier.)

10-fold dilution with water by CZE-ESI-ion trap MS in negative ion mode [131]. Analysis of the SPE extract provided increased signals and a mass spectrum comparable to that determined with the standard. Without extraction, SRM was found to permit detection of about 0.1 μg $ml^{-1}$ of urinary furosemide.

**Stimulants** Caffeine is a naturally occurring methylated xanthine alkaloid (1,3,7-trimethylxanthine) that increases the basal metabolic rate and acts as a mild central nervous system (CNS) stimulant, myocardial stimulant, and smooth-muscle relaxant. Caffeine is the most widely consumed psychoactive substance in the world, contained in coffee and tea, but also a ubiquitous ingredient in many soft drinks. Moreover, numerous over-the-counter medications contain caffeine. It has also been reported that caffeine may become a sort of drug of abuse with strong tolerance and dependence. When used in high doses, it may cause insomnia, nausea, trembling, nervousness, and seizures. Caffeine is not prohibited by WADA, but it is included in a monitoring program.

The metabolism of caffeine is complex, with its metabolic profile varying from species to species and even among individuals under different health conditions. Caffeine is metabolized by the liver to form dimethylxanthine and monomethylxanthine, dimethyl uric acid and monomethyl uric acid, trimethylallantoin and dimethylallantoin, and uracil derivatives.

Peri-Okonny *et al*. employed CZE-ESI-MS (single quadrupole) using a simple aqueous BGE (50 mmol $l^{-1}$ carbonate buffer pH 11.0) for the separation and characterization of caffeine and a group of 11 metabolites in urine samples (Table 9.2) [132]. The migration order for caffeine metabolites was mainly determined by their acid dissociation (p$K_a$) values. Different types of SPE cartridges for the cleanup of urine samples were tested. Recoveries greater than 90% were obtained with phenyl packing using acidified urine samples and acidic eluent solvent.

Kartsova *et al*. developed CZE-ESI-MS (single quadrupole) method in positive ion mode for the determination of catecholamines, including adrenaline [133]. Adrenaline is classified by WADA, among specified stimulants. However, adrenaline (also known as *epinephrine*) is not prohibited for local administration, for example, nasal, ophthalmologic, or coadministration with local anesthetic agents. The authors, furthermore, reported a comparative study of CZE-UV and CZE-MS, reaching a detection limit of 0.17 μg $l^{-1}$ for CZE-MS and of 0.99 μg $l^{-1}$ for CZE-UV. CZE-ESI-MS with a single quadrupole was applied on simulated mixtures of catecholamines after purification by SPE on alumina. The electrophoretic condition was 0.05 mol $l^{-1}$ acetate buffer containing $5 \times 10^{-3}$ mol $l^{-1}$ diethylamine hydrochloride (pH 4.5).

### 9.4.2
### Trace Evidence Analysis

At a crime scene, there are often tiny fragments of physical evidence such as fibers from clothing or carpeting, fragments of paints or pieces of glass, fire accelerants, and gunshot residues that can help tell the story of what happened. These are

**Table 9.2** Caffeine and its metabolites.

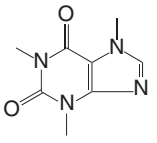
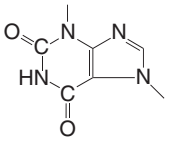
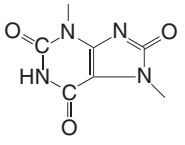
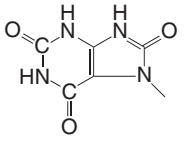
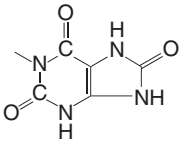
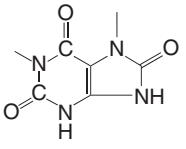
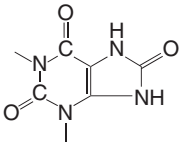
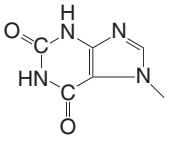
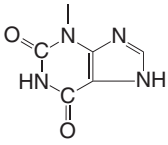
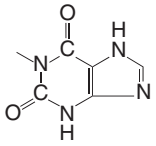
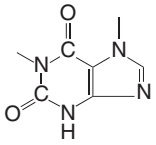
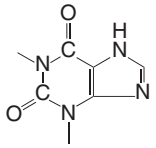
| Analyte | $pK_a$ | MW (g mol$^{-1}$) | Structure |
|---|---|---|---|
| Caffeine | 1.39 | 194.19 |  |
| Theobromine | 9.90, 1.37, 10.04 | 180.16 |  |
| 1,3,7-Trimethyluric acid | 5.89 | 210.19 |  |
| 7-Methyluric acid | 7.22 | 182.14 |  |
| 1-Methyluric acid | 6.04 | 182.14 |  |
| 1,7-Dimethyluric acid | 5.86 | 196.16 |  |
| 1,3-Dimethyluric acid | 5.64 | 196.16 |  |
| 7-Methylxanthine | 7.60, 1.34 | 166.14 |  |

**Table 9.2** (*continued*)

| Analyte | p$K_a$ | MW (g mol$^{-1}$) | Structure |
|---|---|---|---|
| 3-Methylxanthine | 11.15, 1.03 | 166.14 |  |
| 1-Methylxanthine | 7.64, 1.02 | 166.14 |  |
| 1,7-Dimethylxanthine | 8.50, 1.36 | 180.16 |  |
| Theophylline | 8.60, 8.77 | 180.16 |  |

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referred to as *trace evidence* and are typically transferred when two objects touch or when small particles are dispersed by an action or movement. For example, paint can be transferred from one car to another in a collision, or a fiber can be left on an object/person in a physical assault. This evidence can be used to reconstruct an event or indicate that a person or thing was present at the scene. Scientists examine the physical, optical, and chemical properties of trace evidence and use a variety of tools to find and compare samples and look for the sources or common origins of each item. Most of the test methods require magnification and/or chemical analysis. In this section, trace analysis of GSR, weapons, textile fibers, ink, dyes, and glass is overviewed.

#### 9.4.2.1 Gunshot Residues, Explosives, and Chemical Weapons

**Chemical Warfare Agents** Since the implementation of the Chemical Weapons Convention (April 1997), prohibiting the development, production, stockpiling, and use of chemical warfare agents, efficient analytical techniques have been developed for detection and identification of chemical warfare agents and their degradation products in order to verify the compliance with this convention. The

most lethal chemical warfare agents are nerve agents such as sarin, soman, tabun, or VX. Most of these toxic chemicals are generally easily degraded by hydrolysis to first produce alkyl alkylphosphonic acids and, secondly, alkylphosphonic acids [134].

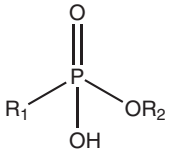
One of the first methods developed in CZE-ion spray-MS for the separation and identification of degradation products of warfare agents was reported by Kostiainen *et al*. using negative ionization mode and volatile electrolytes such as ammonium acetate [135]. The soft ionization mode of the ionspray interface allowed minimal fragmentation with production of very abundant negative ions such as $[M\text{-}H]^-$ corresponding to the loss of one proton of each analyte.

Lagarrigue *et al*. proposed a CZE-ESI-ion trap MS in negative ionization mode for evaluating the separation and identification of chemical warfare agent degradation products (alkylphosphonic acids and alkyl alkylphosphonic acids) [134, 136]. A standard mixture of five alkylphosphonic (di)acids and five alkyl alkylphosphonic (mono)acids containing isomeric compounds was used in order to evaluate CZE selectivity and MS identification capability. The obtained electropherograms revealed that CZE selectivity was very limited in the case of alkyl alkylphosphonic acid positional isomers, whereas isomeric isopropylphosphonic and propylphosphonic acids were baseline-separated. CZE-MS/MS experiments provided an unambiguous identification of each isomeric comigrating alkyl alkylphosphonic acids because of the presence of specific fragment ions. On the other hand, CZE separation was mandatory for the identification of isomeric alkylphosphonic acids, which led to the same fragment ion and could not be differentiated by MS/MS. The developed method was applied to the analysis of soil extracts spiked with the analytes (before or after extraction treatment) and appeared to be very promising since the resolution and sensitivity were similar to those observed in deionized water. The soil samples were subjected to pressure-assisted solvent extraction by water, followed by purification of the soil extract through a cation-exchange cartridge, and the obtained aqueous soil extract was finally concentrated by evaporation. Analytes were detected and identified in soil extract spiked at 5 $\mu g\,ml^{-1}$ with each compound before extraction treatment (Table 9.3).

The identification of alkylphosphonic acids in spiked tap water was investigated by using on-line CZE-ion spray-QqQ MS in negative ion mode by Mercier *et al*. (Table 9.4) [137]. They developed a CZE-MS/MS methodology to identify alkylphosphonic acids (Figure 9.13) in different matrices such as tap water while avoiding the derivatization and preconcentration steps, which are always time-consuming and difficult to manage correctly. After optimization of several parameters (electrolyte pH, makeup chemical composition, and makeup flowrate), a 5 $\mu g\,ml^{-1}$ limit of detection was achieved for these analytes in selected ion monitoring MS detection. MS-MS detection was also investigated to reach a lower detection limit (100 $ng\,ml^{-1}$) for alkyl alkylphosphonic acids in spiked tap water.

**Explosives** An explosive is defined as a substance or a mixture of substances that may undergo a rapid chemical change with the liberation of large quantities of energy, generally accompanied by the generation of hot gasses. An explosive

**Table 9.3** Name of some phosphonic acids.




| Name | Abbreviation | $R_1$ | $R_2$ | Migration order | LOQ in deionized water ($\mu g\ ml^{-1}$) | LOQ in local soil extract ($\mu g\ ml^{-1}$) |
|---|---|---|---|---|---|---|
| Ethyl ethylphosphonic acid | EEPA | Et | Et | 1 | 12.5 | 15 |
| Methyl propylphosphonic acid | MPrPA | Pr | Me | 2 | 12.5 | 12.5 |
| Propyl methylphosphonic acid | PrMPA | Me | Pr | 3 | 10 | 12.5 |
| Methyl ethylphosphonic acid | MEPA | Et | Me | 4 | 15 | 20 |
| Ethyl methylphosphonic acid | EMPA | Me | Et | 5 | 20 | 25 |
| Isopropylphosphonic acid | IPA | iPr | H | 6 | 24 | 24 |
| Propylphosphonic acid | PrPA | Pr | H | 7 | 25 | 25 |
| Ethylphosphonic acid | EPA | Et | H | 8 | 60 | 60 |
| Phenylphosphonic acid | PhPA | Ph | H | 9 | 10 | 15 |
| Methylphosphonic acid | MPA | Me | H | 10 | 300 | 320 |

Limits of quantitation obtained in deionized water and in a blank local soil extract for a signal-to-noise ratios of 10.
Reproduced from [136] with permission from Elsevier.

mixture usually contains an oxidizing agent and a reducing agent. Explosives are often used illegally in terrorism acts and criminal activity. Some explosive compounds (e.g., some nitrate esters) are used in medicine or in the paint industry (cellulose nitrate). Forensic analysis of explosives deals with the identification of explosives both preblast and postblast. The aim of forensic analysis of unexploded explosives is often to prove their illegal possession. Sometimes, it is also important to identify the starting materials for the preparation of explosives (e.g., acetone and hydrogen peroxide for the synthesis of triacetone triperoxide (TATP)). In postblast analysis, because the explosion has already occurred, explosive identification has the highest priority, because information about the type of explosives involved can be of great assistance to the investigation. Sometimes, it is not unequivocally clear whether the explosion was initiated by the ignition of a fuel–air mixture ("vapor explosion") or by other explosives. When an explosive is identified in the debris, it may be assumed that this explosive caused the explosion. On the other hand, if no explosive is identified in the debris, it may suggest a vapor explosion. In addition, the type of the explosive may sometimes direct the investigator as to whether the explosion was carried out by terrorists

**Table 9.4** Phosphonic acids and CID product ion spectra $(M\text{-}H)^-$.

| Name | Abbreviation | Mr ($g\,mol^{-1}$) | Precursor ion ($m/z$) | Product ion spectra $m/z$ (% abundance) |
|---|---|---|---|---|
| *Alkyl methylphosphonic acids* | | | | |
| Methyl methylphosphonic acid | MMPA | 110 | 109 | 109, (100), 95, (5) |
| Ethyl methylphosphonic acid | EMPA | 124 | 123 | 123, (100), 95, (5) |
| Isopropyl methylphosphonic acid | IMPA | 138 | 137 | 137, (100), 95, (10) |
| Cyclopentyl methylphosphonic acid | CPMPA | 164 | 163 | 163, (100), 95, (11) |
| Cyclohexyl methylphosphonic acid | CMPA | 178 | 177 | 177, (100), 95, (6) |
| Pinacolyl methylphosphonic acid | PMPA | 180 | 179 | 179, (100), 95, (7) |
| *Alkyl ethylphosphonic acids* | | | | |
| Ethyl ethylphosphonic acid | EEPA | 138 | 137 | 137, (100), 109, (15) |
| Isopropyl ethylphosphonic acid | IEPA | 164 | 163 | 163, (100), 109, (20) |
| (1,2-Dimethylpropyl) ethylphosphonic acid | DEPA | 180 | 179 | 179, (100), 109, (13) |
| Cyclohexyl ethylphosphonic acid | CEPA | 192 | 191 | 191, (100), 109, (27) |
| *Alkylphosphonic acids* | | | | |
| Methylphosphonic acid | MPA | 96 | 95 | 95, (100), 79, (5) |
| Ethylphosphonic acid | EPA | 110 | 109 | 109, (100), 79, (5) |
| Propylphosphonic acid | PPA | 124 | 123 | 123, (100), 79, (5) |
| Butylphosphonic acid | BPA | 138 | 137 | 137, (100), 79, (5) |

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or by criminals unrelated to terrorist activity. In any case, the analysis typically deals with trace amounts of unreacted explosives mixed with large amounts of interfering materials [138].

The CZE-ESI-ion trap MS system and SB-beta-CD as chiral selector were applied in negative ionization mode by Groom *et al*. for the resolution and detection of nitroaromatic and polar cyclic or caged nitramine energetic materials including TNT, TNB, RDX, HMX, and CL-20 [139]. This CZE-MS system was utilized to analyze environmental samples as contaminated marine sediment and soil obtained from an ammunition manufacturing site. Limits of detection were determined as $25\,\mu g\,l^{-1}$ for all substances, except for CL-20, which could be detected down to $500\,\mu g\,l^{-1}$. The authors reported on the coupling of sulfobutylether-beta-CD (SBE-beta-CD)-assisted CZE method to a quadrupole

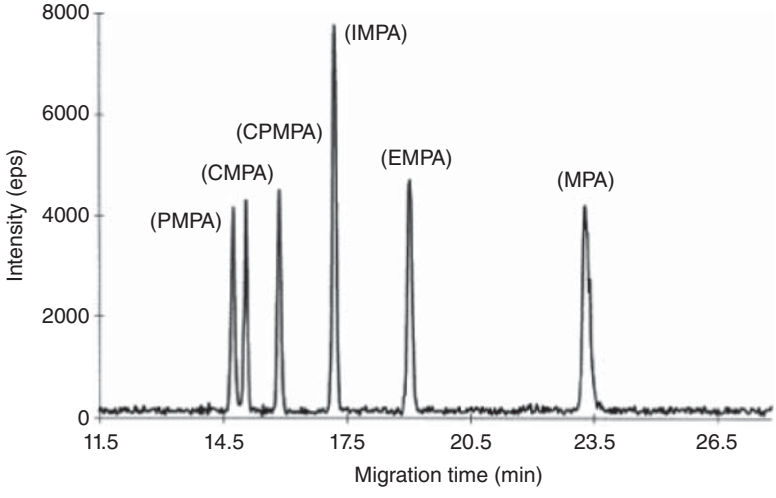


**Figure 9.13** CE-MS-MS analysis of alkyl methylphosphonic acids (5 mg $l^{-1}$). (Reproduced from [137] with permission from Elsevier.)

ion trap MS for the identification of frequently encountered explosives and their degradation products in environmental samples. The CZE-MS system was applied in negative ionization mode for the resolution and detection of nitroaromatic and polar cyclic or caged nitramine energetic materials including TNT (2,4,6-trinitrotoluene), TNB (1,3,5-trinitrobenzene), RDX (hexahydro-1,3,5 trinitro-1,3,5-triazine) HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine), and CL-20 (2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane). A sulfobutylether-beta-CD-assisted capillary electrophoresis system was applied in conjunction with a quadrupole ion trap–mass spectrophotometer for the identification of nitroaromatic and cyclic nitramine explosives and their degradation products. This work demonstrated the use of CD-dependent CZE-MS for the analysis of explosives in environmental samples, offering the potential for the identification of highly polar or charged degradation products. However, the presence of highly charged CDs in the electrospray aerosol might affect the identification of target explosive analytes in at least two respects: (i) the presence of high-molecular-weight, nonvolatile ions in high concentration will inhibit the passage of target analytes to the vapor phase; and (ii) if the analyte in question forms a highly stable inclusion complex with the CD, then the observed mass signal will be that of the noncovalent inclusion complex and not the parent molecular ion.

Smokeless gunpowder is commonly used as a propellant for rifle and handgun ammunition. Because of ease of purchase, smokeless powders can readily be used in improvised explosive devices. Identifying the type and source of the powder can be critical in order to determine the perpetrator of the bombing. De Perre *et al*. developed a method for the separation and identification of 14 organic compounds commonly found as constituents in commercial smokeless powders via

the application of CEC coupled to UV or TOF-MS, using a hexyl-acrylate-based porous monolith (i.e., compatible with MS detection) [140]. The CEC-UV method proved effective and efficient for the detection of all powder additives (i.e., stabilizers, plasticizers, flash suppressants, deterrents, opacifiers, and dyes). However, the TOF-MS procedure provided better sensitivity and selectivity, offering a more sophisticated tool for an additional confirmation. Since the ultimate goal was to identify the source of the smokeless powder found at a bombing scene by detecting the number and type of additives used in the manufacture of the powder, the coupling of the CEC separation with TOF-MS allowed the integration of accurate mass determination with retention times for a more reliable identification of most of the sample components. In addition, the preliminary separation provided the purification of the analyte mixture to be subjected to HRMS analysis, avoiding problems of ion suppression in the ionization phase. However, nitroglycerin and dinitrotoluenes were not detected in the positive ion mode and required the production of negative ion adducts. It could be concluded that CEC-MS may be more useful as a confirmation tool in-line with CEC-UV analysis, which provided more accurate quantification.

#### 9.4.2.2 **Inks**

Ink is a liquid or paste that contains pigments or dyes and is used to color a surface to produce an image, text, or design. From a forensic point of view, ink analysis is mainly requested for ink-source comparisons, commonly conducted in casework involving crimes such as alteration of documents or signatures, insurance fraud, and currency counterfeiting. The ability to distinguish different inks can be useful in criminal cases of document alteration, where two or more inks of the same apparent color, but with different dye compositions, were used in a document [141].

The development of digital printing, especially inkjet printing, has significantly facilitated and reduced the costs of document replication. However, it has also led to proliferation of both amateur and professional forgery – the process of making or adapting and altering documents through overwriting, erasures, or other modifications. If the authenticity of a document is doubtful or its source unknown or questionable, it becomes a questioned document – in fact, this is the main type of case dealt with by prosecution and justice bodies. The great demand for printing instruments has led to a dramatic increase in the variety of ink compositions, hence in the variety of chemical compounds used in printing inks. Furthermore, the possibility of contamination from the carrier (paper) and chemical aging of inks must also be taken into account. Thus, it can be stated that separation techniques are indispensable when analyzing such a complex mixture [142].

CZE-ESI-TOF MS for the analysis of inkjet inks was investigated by Kula *et al.*, who developed and validated a method that allowed reliable and repeatable analysis of black inkjet inks extracted from printouts [143]. Over a dozen inkjet printouts printed on various printer models from different manufacturers were analyzed to determine the variation of chemical composition of inks between different brands and types. For most of the investigated inks, the studies showed

the presence of a characteristic mass spectrum originating from the surfactant or polymer. The mass distribution of the additive was distinctive for some inkjet ink producers and allowed for group identification of inks. The results showed the strength of the CZE-ESI-TOF MS method as an effective technique for forensic purposes, requiring a small amount of ink samples and giving analytical information that was useful in the identification of compounds. Two analytes were considered as the same if the differences between their $m/z$ values and relative migration times were less than or equal to 0.003 and 0.1, respectively. The developed and validated CZE-ESI-TOF MS method was applied to the separation of components of inks extracted from 13 black printouts (printed by printers equipped with original ink cartridges). Each extracted component of the investigated samples was characterized by an $m/z$ value and a relative migration time. The differentiation of inks was based on the number of significant peaks with individual $m/z$ values, relative migration times, and MS spectra. The study revealed the presence of a characteristic mass spectrum originating from a surfactant or polymer, for example, polyethylene glycol with different end groups, for most of the investigated inks. The mass distribution of the additive was distinctive for Hewlett-Packard and Lexmark, and in some cases for Epson and Canon inkjet inks, and allowed for group identification of ink samples. On the other hand, a lack of such an MS profile may indicate that the examined ink is produced by Brother company.

The versatility of the method makes it not only suitable for the analysis of black inkjet inks but also potentially useful for the analysis of other types of inks such as writing inks.

CZE-ESI-TOF MS was utilized by the same research group to investigate violet, red, blue, and green stamp inks on paper surfaces. Electropherograms and mass spectra of inks purchased from 10 different producers were obtained showing that the method could be useful for the objective analysis of stamp inks on questioned documents. Also, in the same study, MEKC-DAD and CZE-MS techniques were used for analysis and discrimination of stamp inks of similar colors. Both the applied methods were developed, optimized, and validated for dye-based inkjet printing inks. The greatest value, equal to 0.004 $m/z$, was found to be the similarity/difference threshold. Thus, two analytes were considered the same if the difference between their $m/z$ values was less than or equal to 0.004 [144].

#### 9.4.2.3 **Dyes**

Dyeing is the process of imparting color to a textile material by interaction with a dye, which is able to absorb and reflect certain visible wavelengths of light. Dyes must have affinity for the substrate on which they are being applied. Dyes can be natural or synthetic. However, the main classification scheme typically used by forensic chemists is based on the method of dye application. The major dye classes in such scheme are acid dyes, basic dyes, azoic dyes, direct dyes, disperse dyes, metallized dyes, reactive dyes, sulfur dyes, and vat dyes.

CZE-ESI-ion trap MS in negative ion mode was used for the identification of anthraquinone color components of cochineal, lac-dye, and madder, natural

red dyestuffs often used by ancient painters. The method developed permitted to identify unequivocally carminic acid and laccaic acids as coloring matters in the examined preparations of cochineal and lac-dye, respectively. In madder, European *Rubia tinctorum*, alizarin and purpurin were found. The method allowed for the rapid, direct, and straightforward identification and quantitation of components of natural products used in art, with detection limits in the range of 0.1 – 0.5 μg $ml^{-1}$. The coupling of CZE with ESI-MS offered two valuable advantages: (i) ESI-MS proved to be selective and sensitive in identifying unequivocally anthraquinone natural coloring matters isolated by CE, even when they were not perfectly separated; (ii) CZE enabled to differentiate laccaic acids, which is almost impossible by using LC [145].

Flavonoids and anthraquinoids are compounds of plant and insect origin that have many applications as drugs and dyes, especially in food. In old times, they were also used for dyeing different objects. Five flavonoids (quercetin, luteolin, apigenin, rhamnetin, morin), five anthraquinoids (carminic acid, alizarin, purpurin, xanthopurpurin, emodin), and gallic acid compounds most often found in natural dyes were analyzed with CZE with both DAD and ESI-ion trap MS in negative ion mode by Surowiec *et al*. [146]. In negative ion mode, only deprotonated ($[M-H]^-$) ions of all compounds were observed. For most of the analytes, no signal in positive ion electrospray was observed, or the signal intensity was very low compared to that obtained in the negative ion mode. The LOD values (range 0.1 – 1.9 μg $ml^{-1}$) were lower than those obtained by CE-DAD (range 0.8 – 7.8 μg $ml^{-1}$). Reportedly, none of the flavonoids investigated in this work had been determined earlier with CE-MS.

The Maillard reaction, also called nonenzymatic browning, is a complex network of reactions involving carbonyl and amino compounds, such as reducing sugars and amino acids or proteins. It is the main reaction responsible for the transformation of precursors into colorants and flavor compounds during food processing. In the course of the Maillard reaction, the sugar molecule is broken down *via* intermediary conjugates into reactive alpha-dicarbonyls that enter further reactions, thus increasing not only the complexity but also the diversity of the reaction mixture. Amadori compounds are *N*-substituted 1-amino-1-deoxyketoses, representing an important class of relatively stable Maillard intermediates. They are formed in the initial phase of the Maillard reaction by the so-called Amadori rearrangement of the corresponding *N*-glycosyl amines, the latter being obtained by condensation of amino acids and aldose sugars. Amadori compounds of several amino acids, such as glycine, valine, isoleucine, methionine, proline, and phenylalanine, as well as a cysteine-derived compound, were separated and/or discriminated using CZE-MS/MS with a triple quadrupole by Hau *et al*. [147]. This technique in CZE-MS and CZE-MS/MS was reported for the first time as a valuable tool for the characterization of Amadori compounds but could also be useful to study the stability and degradation kinetics of other labile charged Maillard intermediates that play an important role in food and medical science.

Anthocyanins belong to a group of plant polyphenols widely studied due to their positive effects on health, showing primarily antioxidative and antimicrobial activity. They are responsible for the color of plant tissues or food products. The anthocyanin profile may serve as efficient marker of wine authenticity. It is also used to distinguish varieties or to estimate the age of wines. Monitoring of changes in the content of anthocyanins together with other phenolics during the process of wine production can contribute to the optimization of winemaking technology. For these reasons, analysis of the anthocyanin profile is much in demand by wine producers as well as by food control authorities. For the monitoring of anthocyanins in wine and wine musts, CZE-ESI-ion trap MS was performed in two electrolytes, an acidic electrolyte (chloroacetate-ammonium, pH 2) and a basic electrolyte, with high selectivity toward derivatives containing vicinal hydroxyl groups (borate-ammonium, pH 9). The setup of MS was optimized and the fragmentation of common anthocyanins was studied in detail. Attention was also focused on the fragmentation of anthocyanidin skeleton. The anthocyanidins were substituted with hydroxy groups fragment *via* a cascade of neutral losses of water and carbon monoxide. Fragmentation of anthocyanidins containing a methoxy group on their B-ring starts with the cleavage of methane and/or methyl radical. The optimized method was utilized for monitoring changes in anthocyanin profile in red wines as well as the process of release of anthocyanins to wine must [148].

CZE-ESI-ion trap MS was used by López-Montes *et al*. for the separation and the identification of 23 synthetic organic dyes, among those used in early twentieth-century color photographs such as autochromes [149]. The autochrome is an early color photograph process on glass, based on additive color mixing theory, which uses three colored lights to produce a full color gamut. It was patented in 1903 by the Lumière brothers, being among the first industrially produced color photographs. In replacement of the glass plate, a film-based photograph called Filmcolor was, then, launched in 1931 albeit it was short-lived, as Kodak and Agfa began to produce multilayer subtractive color films. Synthetic dyes are also used for very different applications, in pharmaceutical, textile, cosmetic, and food industries and were used especially in graphics and fine art in the nineteenth and twentieth centuries. The positive and negative ion modes (positive mode for cathionic dyes and negative mode for anionic dyes) were used alternately on the same sample. The methods proposed allowed for the separation and identification of 14 cationic dyes and 9 anionic dyes, which could be found in early color photographic artifacts such as the autochrome Lumière and the Filmcolor. A CZE-PDA method developed was the first method to enable the analysis of such a large panel of dyes of opposite charge in a single, simple, and short analysis (15 min). Sampling on the Filmcolor artifact was carried out as follows. Ten square millimeters of the surface containing the dyed starch layer and upper varnish layers were scratched with a scalpel and transferred in 3 ml ethyl acetate in order to solubilize the varnish. After 10 min, the mixture was filtered (PTFE, 0.45 μm) using a membrane filter and filter holder. Once dried, the filter, which had retained the dye particles, was introduced in methanol:water (1 : 1) and heated during 30 min at 70 °C in a heating block unit. After removing

the filter, the solution was centrifuged at 9000 rpm for 3 min and evaporated to dryness. The mass of dyes recovered after this step was estimated to 0.5 μg at most. Finally, the dry residue was dissolved in 25 μl of water: methanol (1 : 1). CZE-ESI-MS could be applied to small samples such as those originating from valuable cultural heritage artifacts. This method was successfully applied to a historic sample chosen for identifying synthetic dyes in a real artifact.

#### 9.4.2.4 Textile Fibers

Natural or artificial fibers are the basic unit of matter that form the components of fabrics and textiles. The main constituent of natural fibers is cellulose, while the chemical ones can be artificial if they are prepared from cellulose with different manufacturing processes or synthetic if produced by synthetic polymers. Although a multitude of classifications and subtypes exist, fibers can be broadly classified as either natural or human-made. Natural fibers are further subdivided according to their source (animal, vegetable, or mineral). Human-made fibers are subdivided according to their base material (synthetic polymer, natural polymer, or others). Textile fibers are arguably one of the most important forms of trace evidence given that they have many classifications and subtypes, are physically and chemically differentiable, have various processing procedures, and are transferred easily. One of the most important characteristics for fiber comparisons is color, which reflects the dyes and pigments that were used on the fabric [150].

Experiments based on a simple mixture design were employed to explore the effects of three solvent components (water, formic acid, and aqueous acetic acid), extraction time, and extraction temperature for the automated microextraction of basic (cationic) dyes from acrylic fibers.

Extractions were conducted by an automated liquid handling system, and dye extraction was evaluated using a UV/VIS microplate reader. The highest extraction efficiency for two subclasses of basic dyes (methine and azo) from acrylic fibers was achieved with an extraction solvent containing formic acid. Cationic dyes were analyzed by CZE-ESI-QTOF MS. The advantage of microextraction combined with CZE-MS for analysis of extracts from trace fibers was demonstrated by the detection and characterization of three basic dyes extracted from a 2 mm length of single acrylic fiber. For extraction prior to CZE-MS analysis, 10 μl of aqueous formic acid was added to a single tri-dyed acrylic fiber and heated at 100 °C for 60 min in a sealed vial. After evaporation to dryness, the extract was reconstituted in 5 μl of water. Although the analysis was destructive to the sample, only an extremely small sample was required (~2 mm of a single 15 -μm-diameter fiber) [151].

As already mentioned in the chiral separation section, silks are naturally occurring polymers, which are composed of a filament core protein (fibroin) and a glue-like coating consisting of sericin proteins. The raw silk fiber most commonly used in fabric manufacture is reeled from the cocoons of the silk worm *Bombyx mori* (*B. mori*) and is usually degummed by the removal of the sericin during processing. Because of the existence of a large number of silk artifacts in museums and private collections, the identification of the nature of the degradation state of such

historic masterpieces is often critical to their preservation. For several decades, archeologists and forensic scientists have used amino acid racemization (AAR-D/L ratio) to determine the relative age of biological materials such as bone, shells, and teeth. A CE-ESI-MS technique using (+)-(18-crown-6)- 2,3,11,12-tetracarboxylic acid used as BGE was proposed by Moini *et al*. for age estimation of silk textiles based on amino acid racemization rates [59]. With an L to D conversion half-life of ~2500 years for silk (*B. mori*) aspartic acid, the technique was capable of dating silk textiles ranging in age from several decades to a few thousand years old. Analysis required only ~100 µg or less of silk fiber. Except for a 2 h acid hydrolysis at 110 °C, no other sample preparation is required. The CE-MS analysis took ~20 min, consumed only nanoliters of the amino acid mixture, and provided both amino acid composition profiles and D/L ratios for ~11 amino acids; for details, see Section 9.3.6.

Morgan *et al*. reported microscale extraction protocols for dyes from different fiber types and CZE-UV and MS analytical methods [152]. Protocols were developed for the analysis of unknown dyes from textile fibers using three CE methods compatible with detection by DAD and MS (ESI-QTOF MS). Microextraction and CE methods were developed to span the analysis of nylon, cotton, polyester, and acrylic fibers dyed with six different classes of textile dyes, that is, acid, direct, reactive, vat, cationic, and disperse dyes. The dye extracts from single textile fibers down to 2 mm in length were analyzed by CE-MS. The four types of fibers underwent different treatments for extraction. Separation of acid, direct, and reactive dyes, from cotton and nylon fibers, was performed with an anionic buffer system 15 mmol $l^{-1}$ (ammonium acetate in acetonitrile – water 40 : 60, v/v) at pH 9.3; vat dyes from cotton fibers were separated after the addition of a reducing agent (sodium dithionite) to this same buffer. Extracts from acrylic fibers containing basic cationic dyes were analyzed with a cationic buffer system (45 mmol $l^{-1}$ ammonium acetate buffer in acetonitrile – water 60 : 40, v/v) at pH 4.7. Separation of hydrophobic disperse dyes from polyester was performed by NACE.

### 9.4.3 Forensic DNA

In the most recent decades, CE, in the form of CGE, with fluorescent detection has become the standard technique for DNA sequencing and DNA fragment sizing, particularly in the forensic context, but almost no application of CE-MS was reported in this field. On the other hand, a direct MS analysis of DNA amplicons has been commercially proposed (often outside the forensic field) with the introduction of a dedicated instrument, the Plex-ID® (Abbott Molecular, Abbott Park, IL, USA). The Plex-ID device automatically desalts and purifies the PCR-amplified DNA strand segments, which are then ionized in ESI mode and analyzed with a TOF mass spectrometer (www.omnica.com/ibis-biosciences-plex-id-pathogen-detector/, [153, 154]).

Also, it is known that nucleotide mutations can be induced by a wide variety of chemicals by their covalent binding to the nucleophilic sites of DNA, resulting in the formation of DNA adducts. Therefore, the identification and the structure analysis of such adducts play an important role for risk assessment and for better understanding of the role of cytotoxic agents. A traditional approach for the detection and characterization of modifications in DNA is based on enzymatic digestion of the DNA followed by the determination of the resulting modified nucleosides, nucleotides, or oligonucleotides. In this field, the coupling of CE to MS has emerged as a powerful tool: CE confers rapid analysis and efficient resolution, while MS provides high selectivity and sensitivity with structural characterization of minute amounts of compound [155].

Feng *et al*. used a pressure-assisted electrokinetic injection (PAEKI), an on-line enrichment technique to be extended to the on-line concentration of DNA oligonucleotides, for the identification and measurement of low levels of oligonucleotides and their chemical adducts by using CZE coupled with a single-quadrupole mass spectrometer [156]. The preconcentration of samples enhanced the signals of the analytes, thereby allowing the identification of oligonucleotide adducts at a low micromolar concentration. With optimized PAEKI conditions, an on-line sample concentration power of 300–800 times could be reached for both single-stranded (ss) and double-stranded (ds) oligonucleotides during a 90-s PAEKI injection. The detection limits using single-quadrupole MS in the scan mode were 0.01–0.03 μmol $l^{-1}$ for ss and 0.04–0.08 μmol $l^{-1}$ for ds oligonucleotides, respectively.

Several cytostatic drugs, such as platinum compounds, are designed to react with DNA as the basis of their pharmacological activity, resulting in the formation of covalent cross-links with nucleotides, called *platinum DNA adducts*. Warnke *et al*. investigated the formation of adducts following the reaction of *cis*-diamminedichloroplatinum (II) (cisplatin) with various DNA nucleotides [157]. Baseline separation of unmodified and modified nucleotides (adducts) was achieved through CZE within 11 min in one single run (Figure 9.14). In order to elucidate the observed peak pattern, the system was coupled with CZE-ESI-QqQ MS. After incubation of mononucleotides with cisplatin, monochloro, monoaqua, and bifunctional adduct species were detected. Moreover, the time-dependent conversion from monochloro to monoaqua and subsequently to bifunctional adducts was monitored.

CZE coupled with negative ion ESI-MS (triple quadrupole) was used by Barry *et al*. for the detection and identification of adducts formed from the reaction of DNA with (±)-*anti*-7,8,9,10-tetrahydrobenzo[a]pyrene-7,8-diol 9,10-epoxide (BPDE), an active metabolite of benzo[*a*]pyrene (BaP) [158]. Low nanogram detection limits (<10 ng or <15 pmol) for normal scan spectra (selective reaction monitoring, SRM) and collision-induced dissociation (CID) spectra of the main nucleotide adduct formed from this reaction were demonstrated. Exploitation of SRM produced detection limits in the low picogram range (<85 pg or <130 fmol), whereas the application of sample stacking significantly increased the concentration detection limit to approximately $10^{-8}$ mol $l^{-1}$. These techniques were applied

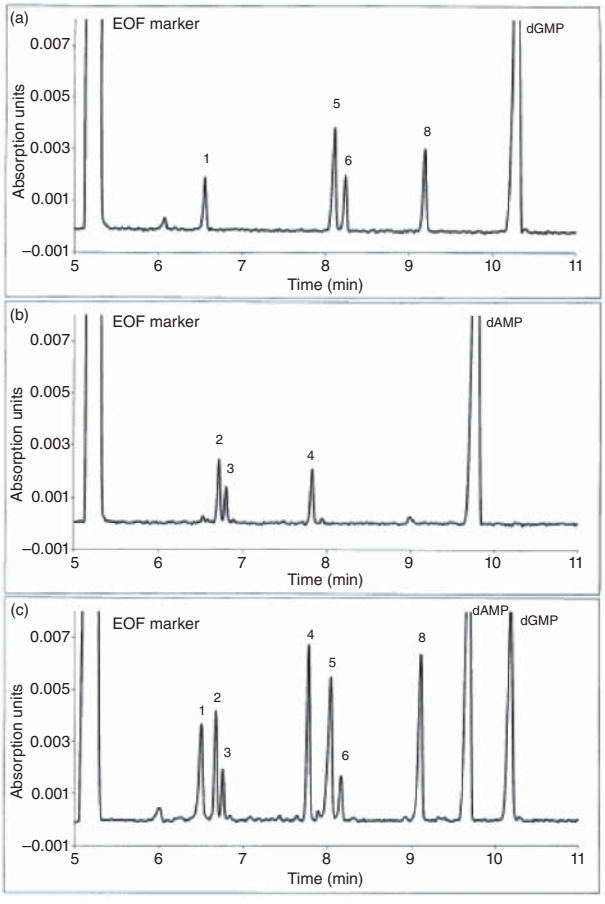


**Figure 9.14** Separate incubation of dGMP (deoxyguanosine monophosphate) and dAMP (deoxyadenosine monophosphate) with cisplatin. (a) Incubated solution of dGMP. (b) Incubated solution of dAMP. (c) Electropherogram after coinjection of both solutions. (Reproduced from [157] with permission from Wiley.)

to the analysis of the adducts formed from the *in vitro* reaction of BPDE with DNA. In addition, it was shown that CZE-ES-MS, combined with solid-phase sample cleanup, could detect adducts at levels of four adducts in 107 unmodified bases or less.

Styrene has widely been studied about carcinogenicity and mutagenicity due to its active metabolite, styrene-7,8-oxide, which shows a tendency to react, among others, with DNA and DNA constituents. Occupational exposure to styrene occurs mostly in the work place by inhalation of vapor during the manufacture of styrene-containing products. Schrader *et al*. developed an enzymatic procedure for the investigation of DNA adducts able to distinguish different reaction products of styrene oxide in DNA [159]. The procedure was designed with the intention to observe sequence-specific reactions of DNA alkylating compounds.

The oligonucleotides were analyzed using CZE interfaced to ESI-MS (double-focusing sector, single quadrupole). It was shown that the alkylation products migrated slightly faster than the corresponding unreacted species, and therefore, DNA adducts could be distinguished from the unreacted oligonucleotides. These results lead to the conclusion that styrene oxide reacts firstly with purine bases within the DNA.

Deforce *et al*. studied the *in vitro* adduct formation with phenyl glycidyl ethers (PGEs) on 2′-deoxynucleotides and DNA [160]. The modified DNA was hydrolyzed enzymatically, and the mixtures consisting of unmodified and modified 2′-deoxynucleotide adducts were analyzed by CZE, CZE-ESI-MS, and CZE-ESI-QqQ MS using sample stacking. The CZE analysis enabled the comparison of the electropherograms of 2′-deoxynucleotides mixtures with those from the DNA hydrolysates, both treated with PGEs, and the assignment of adducted and nonadducted 2′-deoxynucleotide peaks. CZE-ESI-MS provided the necessary structural information and revealed the presence of mono- and dialkylated 2′-deoxynucleotides. Finally, interpretation of the CZE-ES-MS/MS data of the monoalkylated products allowed differentiation between purine or pyrimidine alkylation and alkylation of the 5′-phosphate moiety. A sample stacking technique allowed the detection of adducts in a concentration down to $10^{-8}$ – $10^{-9}$ mol $l^{-1}$.

### 9.4.4 Occupational and Environmental Health

In recent years, applications of CE-MS have been reported for the separation, detection, and determination of environmental pollutants and food contaminants. Several applications of CE-MS for trace analysis of low-molecular-mass amines, nitroaromatics, alkylphosphonic acids, azo dyes, antidepressants, and antibiotic drugs, among others, in air, sediment, and water samples have been published. The analysis of pollutants, mainly in water samples and in ambient air, sediments, and soil, requires very low operating LODs, challenging the sensitivity of CE-MS. For this reason, quite often, preconcentration of the analytes or trace enrichment in the cleanup of the sample is necessary.

Single liquid–liquid extraction and a preconcentration procedure such as SPE or SPME are the most commonly used methods [161].

Low-molecular-weight (LMW) amines such as volatile and semivolatile short-chain odorous and toxic alkyl amines play an important role in nitrogen cycling, nutrient transfer, and atmospheric acidity. Their lifetime in the environment is short due to their highly reactive nature, so they are easily oxidized under atmospheric conditions and highly carcinogenic *N*-nitrosamines can be formed from secondary amines.

Fekete *et al*. developed a method for the determination of these amines with $m/z$ values higher than 50 in indoor and ambient air by CZE–indirect UV and CZE-ESI-ion trap MS detection [162]. In addition, a novel monolithic polymeric material based on poly(methacrylate-acrylate) copolymer was developed for sampling the short-chain amines from gaseous phase. CZE–indirect UV and CZE-MS,

combined with sophisticated air sampling, according to the authors, were useful for the estimation of short-term exposure of the selected biogenic amines. The method was, then, adapted for metalworking fluid (MWF) aerosols indoor. They are oil–water emulsion aerosols found in specific industrial working places, where coolants or cutting oils are used to perform different mechanical operations such as grinding, forming, turning, boring, drilling and hobbing, and band-sawing of metal pieces. Health effects have been connected to MWF aerosol and to some of their individual components. Selected amines (methyl amine, ethylamine, propylamine, butylamine, dimethylamine, diethylamine, cadaverine, putrescine, and spermidine) were separated from interfering metal ions and amino alcohols present in the samples with an imidazole-based buffer with ethanol and EDTA as modifier. The analysis conditions were applied to quantify the selected short-chain amines with detection limits between 1 and 2 $\mu g\,filter^{-1}$ when 40 l air was sampled with 1 $l\,min^{-1}$ velocity. The method looks to be useful for the estimation of short-term exposure of the selected amines listed earlier.

CZE-ion trap MS and CZE-TOF MS were also applied to the analysis of biogenic amines in wine by Simó *et al*. [163]. A group of five amines (putrescine, cadaverine, histamine, phenylethylamine, and tyramine) was selected since they are the biogenic amines most frequently found in wines. They were determined in three red wines and one white wine, showing, as expected, a higher concentration in red wines. Moreover, CZE-ion trap MS and CZE-TOF MS were compared regarding their ability to detect other biogenic amines different from the selected ones in wine samples, with CZE-TOF MS showing a wider detection range. For example, putrescine, cadaverine, histamine, phenylethylamine, tyramine, tryptamine, spermidine, spermine, ethanolamine, and isoamylamine were identified by CZE-TOF MS in a single analysis. Both methods allowed the identification of biogenic amines in wines without any previous treatment except dilution 1 : 1 with water and filtration. However, LODs obtained by the compared methods differed: 0.08–2.81 $\mu g\,ml^{-1}$ with CZE-ion trap MS against 0.01–0.071 $\mu g\,ml^{-1}$ via CZE-TOF MS. The LODs obtained with the CZE-TOF MS were comparable to those obtained using HPLC with fluorescence detection, without any previous derivatization step and with analysis times fivefold faster (40 min by HPLC and 8 min by CZE-TOF MS).

A flow injection (FI)-CZE-ESI-Q ion trap MS method for the determination of nine biogenic amines (histamine, ethanolamine, isopropylamine, 2-pentylamine, isoamylamine, phenylethylamine, cadaverine, heptylamine and tyramine) was proposed by Santos *et al*. [164]. Biogenic amines occur in a variety of samples (particularly foods), and the interest in their determination lies in their biological activity: although some play a natural role in human physiology, high concentrations can have deleterious effects on health. Biogenic amines may also result from the activity of microbes on food proteins in the form of ripening, fermenting, and decomposition processes. Some biogenic amines are employed as quality indicators for raw food materials and for the technological processes to which they are subjected. In most cases, their content is a measure of how well a given food has been preserved. CZE-ESI-MS was successfully used to determine

biogenic amines in red and white wines with limits of detection ranging from 0.018 to 0.09 μg $ml^{-1}$. A flow manifold was readily connected to the CE equipment and afforded the automatic processing of samples and their introduction into the CE-MS system. The method represents a general automated approach that can be extended to sample treatment for CE-MS in other situations. Also, MS detection provided undeniable advantages over UV detection, particularly with regard to the unequivocal identification of analytes with an increased sensitivity.

Heterocyclic aromatic amines (HAs) show strong mutagenic activity in bacterial tests and induce tumors in laboratory animals; hence, they can be carcinogenic for humans. These compounds are mainly formed from protein-rich foods when processed by thermal treatments. As a result, in order to evaluate the exposure to HAs, there is a need for accurate and sensitive methods for the analysis of these compounds not only in cooked foods but also in body fluids. A CZE-ESI-ion trap MS method was developed for the determination of 16 heterocyclic amines, including the most important biomarkers of exposure to HAs such as MeIQx (2-amino-3,8 dimethylimidazo[4,5-f]quinoxaline) and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine). Although a perfect separation was not achieved, the analysis of all the amines was possible on the detection side, since MS allowed the selective determination of comigrating amines by choosing the appropriate *m/z* ratios. With this method, good detection limits and precisions values (run-to-run and day-to-day) were achieved. Moreover, a preconcentration method (FASS with a methanol plug) was also used, achieving a major improvement in sensitivity, with LODs between 0.75 and 20.8 ng $g^{-1}$ with FASS and between 18 and 360 ng $g^{-1}$ of hydrodynamic injection. The developed method using FASS also showed acceptable precision for analytical use. Hence, this procedure was suggested as an alternative to LC-MS [165].

#### 9.4.4.1 Toxins

Toxins and poisons are products of living organism that often have a polypeptide nature, being complex and very unstable molecules [44].

A CZE-ESI-MS/MS (QqQ) method was developed for the identification, separation, and determination of mushroom toxins, namely ibotenic acid, muscimol, and muscarine. The isoxazole derivatives ibotenic acid and muscimol are the major low-molecular-weight toxins in the hallucinogenic mushrooms *Amanita muscaria*, *Amanita pantherina*, and *Amanita gemmata*, together with the other toxins occurring in *Amanita mushrooms*, *A. muscarine*. The intoxication by *A. muscaria*, *A. pantherina*, and *A. gemmata* may be intentional or accidental: accidental poisoning results when these poisonous mushrooms are mistaken for edible mushrooms. On the other hand, hallucinogenic mushrooms have for centuries been used for recreational purposes. This CZE-ESI-MS/MS proved to be sensitive and thus useful for the analysis of real samples omitting a laborious and time-consuming pretreatment step. The method was applied to spiked human urine, achieving LOD values at a nanomolar level (ibotenic acid 0.15 ng $ml^{-1}$, muscimol 0.05 ng $ml^{-1}$, muscarine 0.73 ng $ml^{-1}$) suitable for the analysis of human urine after mushroom intoxication [166].

Rittgen *et al*. applied CZE-ESI-ion trap MS to the analysis of ama- and phallotoxins, which are potent toxic oligopeptides of the highest forensic interest [167]. In fact, over 90% of the lethal cases of fungus poisoning in humans are caused by the species of *Amanita*, which contain these toxins, such as *Amanita phalloides* ("death cap"), *Amanita verna* ("white death cap"), and *Amanita virosa* ("destroying angel"). A CZE method was developed to separate their main toxins, that is, alpha-, beta- and gamma-amanitin, phalloidin, and phallacidin, while the fragmentation patterns in $MS^n$ experiments were investigated in the positive and negative ion modes. With the optimized method (in the negative polarity detection mode), LODs in the range of 13–79 ng $ml^{-1}$ were achieved. The CZE-MS procedure was successfully applied to the identification of ama- and phallotoxins in extracts of air-dried mushroom samples, but the sensitivity was too low to face the analysis of body fluids.

The application of on-column sample preconcentration with CITP and discontinuous buffer systems prior to CZE separation with ESI-MS (QqQ) detection was investigated for the analysis of paralytic shellfish poisoning (PSP) toxins in scallop. PSP is caused by a group of toxins produced by marine dinoflagellates. When shellfish such as scallops and clams feed on these dinoflagellates, PSP toxins accumulate mostly in the digestive glands of the filter-feeding organisms without causing any apparent harm to the shellfish. However, this group of toxins is one of the most potent toxicant to humans ($LD_{50}$, 6–40 μg $kg^{-1}$). Consumption of contaminated shellfish results in a variety of neurological symptoms that can lead to death. The simple preconcentration techniques (CITP) permitted the injection of larger sample volumes of PSP toxin and the detection limits obtained were 16 nmol $l^{-1}$ for STX and NE0 and 30 nmol $l^{-1}$ for GTX toxins [168].

#### 9.4.4.2 **Venoms**

All venoms contain more than one toxic component, which act in combination to produce the toxic action, and the proportion and distribution of the active components vary from species to species. The complex nature of such mixtures present in snake venoms provides a challenging analytical problem [169].

Tetramine (tetramethylammonium (TMA) ion) is an autonomic ganglionic-blocking agent that is found in several marine gastropod species. It is responsible for numerous cases of human intoxication in Japan, Europe, and Canada, due to the consumption of whelks (sea snails). The method developed by Zhao *et al*. was applied to the analysis of two different *Neptunea* whelk species from Canada (*N. decemcostata* and *N. despecta tornata*) and one from Japan (*N. arthritica*) [170]. The analysis of one sample, implicated in a poisoning incident in Labrador, revealed high levels of tetramine (430 μg $g^{-1}$) as well as choline and an unusual choline derivative. CZE-ESI-MS (QqQ) using SRM acquisition mode allowed for the quantitation of TMA present in two different *Neptunea* whelk species from Canada and one from Hokkaido, Japan. Showing a detection limit for TMA of 7 ng $ml^{-1}$ is especially useful for forensic analyses, as it was demonstrated in the case of the Labrador sample, and it should also be useful for controlling other related compounds such as choline and its esters.

The dried venoms from members of the *Elapidae* family of snakes, which include the mambas (*Dendroaspis*) and coral snakes (*Micrurus*), are complex mixtures of 90–95% small-molecular-mass protein components. In their study, Perkins *et al*. developed a method for characterization of the lower-molecular-mass fraction of venoms from *Dendroaspis jamesoni kaimosae* (Jameson's Mamba) and *Micrurus fulvius* (Eastern Coral Snake) using CE-ESI-SIM-MS [169]. The method was focused on the venom of *D. jamesoni kaimosae*, which contains some previously described peptides, and subsequently applied to the completely unknown venom of *M. fulvius*. Such extremely sensitive methodologies indicated the presence of as many as 83 peptides in the venom of *D. jamesoni kaimosae* and 49 peptides in the venom of *M. fulvius* in the molecular-mass range 6000–8500 Da. Moreover, it provided confirmation of the toxins in *D. jamesoni*, which have been previously described in the literature.

#### 9.4.4.3 **Pesticides**

A pesticide is any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest. Pests are living organisms that occur where they are not wanted or that cause damage to harvests or humans or other animals. Examples include insects, mice and other animals, unwanted plants (weeds), fungi, microorganisms such as bacteria and viruses, and prions.

An approach based on ICP-MS (single quadrupole) coupled to CZE to determine organophosphorus pesticides (OPPs) was reported by Wuilloud *et al* [171]. OPPs, despite their toxicity, are still used by farmers and in the formulation of various home pesticide repellent products. An immediate consequence of the acute exposure is their effect on the nervous system by reducing the regulatory effect of cholinesterase on acetylcholine, which may lead to paralysis of muscles. Since the OPPs are employed in many developing countries, there is high potential for human exposure. This may occur through drinking contaminated water or by eating contaminated food and vegetable products. Among the several OPPs employed up to date, *N*-(phosphonomethylglycine) (glyphosate), DL-homoalanine-4-yl-(methyl) phosphonic acid (glufosinate), and aminomethylphosphonic acid (AMPA) are nonselective herbicides used to control a wide variety of grasses and weeds. Element-specific detection of $^{31}P$ with ICP-MS was performed for the detection of OPPs. Three common OPPs (glyphosate, glufosinate, and aminomethylphosphonic acid (AMPA)) were analyzed by CE-ICP-MS to demonstrate its suitability for the analysis of OPPs. Detection limits obtained with this system were in the range of 0.11–0.19 mg $l^{-1}$. The proposed methodology was finally applied for the determination of the aforementioned OPPs in natural river water samples.

Kawai *et al*. reported on a CZE-ESI-TOF MS method for the forensic analysis of phosphorus-containing amino-acid-type herbicides, glyphosate (GLYP), glufosinate (GLUF), and bialaphos (BIAL) [172]. A new sheathless interface, a high-sensitivity porous sprayer (HSPS), was used in this study, which provided with limits of detection of 7.6, 0.61, and 0.57 pg for GLYP, GLUF, and BIAL,

respectively. These values were 4–36 times lower than those obtained by conventional CZE-ESI-MS using a sheath-liquid interface. The developed method was successfully applied to the analysis of three kinds of beverages (green tea, grape juice, and cola) spiked with a mixture of three commercial formulations of herbicides. Clear electropherograms were obtained only by dilution and filtration of the samples. Also, the 20-fold produced a significant stacking effect. The peaks of the three herbicides were clearly detected in 15 min without interference of matrices.

Hernández-Borges *et al*. presented the determination of a group of triazolopyrimidine sulfoanilide herbicides (cloransulam-methyl, metosulam, flumetsulam, florasulam, and diclosulam) in soy milk by CZE-ESI-ion trap MS [173]. In order to increase the sensitivity of the CE-MS method, an off-line sample preconcentration procedure based on SPE was combined with an on-line stacking procedure. The method also allowed the quantitative determination of these triazolopyrimidine pesticides obtaining LODs values down to 74 ng $ml^{-1}$.

The same research group also worked on CZE-UV and CZE-ESI-ion trap MS determination of other pesticides, such as pyrimethanil, pyrifenox, cyprodinil, cyromazine, and pirimicarb. The best limit of detection that could be achieved for these pesticides using CE-UV was 0.6 μg $ml^{-1}$. On the other hand, CZE-MS provided LODs one order of magnitude better (0.09–0.30 μg $ml^{-1}$). Chemometrics with the aim of optimizing the multiple parameters in SPME and CZE-MS was applied to improve the LODs in food sample ions down to 15 ng $ml^{-1}$. The usefulness of this approach was demonstrated by detecting multiple pesticides in different food samples such as grapes and orange juice in a single run [10].

A method to identify and quantify six pesticide residues (dinoseb, pirimicarb, procymidone, pyrifenox, pyrimethanil, and thiabendazole) in peaches and nectarines using CZE-ESI-ion trap MS was described by Juan-García *et al*. [174]. Under optimized CE-MS/MS conditions, the minimum detectable levels for the six pesticides in spiked peach samples were between 0.01 mg $kg^{-1}$ for pirimicarb and 0.05 mg $kg^{-1}$ for procymidone.

A method based on SPME and CZE-ESI-MS (single quadrupole) was described for determining simultaneously five acidic pesticides (*o*-phenylphenol, ioxynil, haloxyfop, acifluorfen, picloram) in fruits. Rodríguez *et al*. validated this procedure by the analysis of spiked apple, grape, orange, and tomato samples [175]. They also compared the CZE-UV and CZE-MS methods obtaining LOD values of 3 μg $ml^{-1}$ for the former and LOD values of 0.1–3 μg $ml^{-1}$ for the latter, confirming the advantage of using CZE-MS.

CZE-ESI-MS (single quadrupole) in negative ion mode was investigated for the determination of chlorinated acid herbicides and several phenols in water. Sixteen analytes (bentazon, dinoseb, 4-nitrophenol, pentachlorophenol, acifluorfen, chloramben, dicamba, picloram, 3,5-dichlorobenzoic acid, 2,3,4-trifluorobenzoic acid, 2,4-D, dichloroprop, 2,4,5-TP, 2,4-DB acid, MCPA acid, MCPP acid, and 2,4,5-T acid) were separated as their anions in less than 40 min. A sample stacking technique performed on 10-fold diluted samples with distilled water was used to provide lower detection limits. The 16 herbicide acids, the related compounds,

and the internal standard TFBA were added to Cincinnati drinking water at a concentration of 5 μg $ml^{-1}$ and analyzed without sample stacking [176].

Safarpour *et al*. applied CZE-ESI-ion trap MS for the quantitative analysis of imazamox pesticide in well water, potable water, and pond water [177]. Imazamox is an imidazolinone herbicide developed by BASF. The relatively high polarity of the compound may be the reason for the weak adsorption of imazamox to soil as it is hydrophilic in the pH range encountered in the environment. A rapid sample preconcentration, using an RP-102 cartridge (Applied Separations, Allentown, PA, USA) combined with CZE-MS, permitted the analysis of water samples at ng $l^{-1}$ levels within 3 h. The CZE-MS quantitative analysis of imazamox at LOQ of 200 ng $l^{-1}$ (LOD of 20 ng $l^{-1}$) in water samples was robust and reproducible. The application of this method for imazamox degradation products in all environmental samples, such as water and soil, was reported to be ideal.

Simultaneous determination of three herbicides paraquat, diquat, and difenzoquat and two plant growth regulators chlormequat and mepiquat by pressure-assisted CZE-ESI-ion trap MS was reported by Núñez *et al*. [178] . This group of herbicides includes two nonselective herbicides, paraquat (PQ) and diquat (DQ), the selective herbicide difenzoquat (DF), and two plant growth regulators, chlormequat (CQ) and mepiquat (MQ). For drinking water, the Office of Water of US Environmental Protection Agency (EPA) has established a maximum contamination level of 20 μg $l^{-1}$ for DQ and a maximum contamination level of 3 μg $l^{-1}$ for PQ. The detection limits accomplished were between 0.5 and 2.5 mg $l^{-1}$ with hydrodynamic injection (10 s) and between 1 and 10 μg $l^{-1}$ with electrokinetic injection (20 s, 10 kV) using standard solutions. Quantitation was carried out using labeled standards. The method has been applied to the analysis of contaminated irrigation water and spiked mineral water samples.

Heptakis (2,3,6-tri-*O*-methyl)-beta-CD (TM-beta-CD) was used for the enantiomeric separation of some herbicides such as 2-(2,4 dichlorophenoxy)propionic acid (dichlorprop), 2-(4-chloro-2-methylphenoxy)propionic acid (mecoprop), and 2-(2,4,5-trichlorophenoxy)propionic acid (fenoprop). These phenoxy acids are optically active, but only the (+)-isomer is active as herbicide. Differently from other research groups, Otsuka *et al*. employed CDs for CZE chiral separation followed by ESI-ion trap MS detection, without any special technique for preventing the introduction of CD into the ESI interface [179]. Although the authors stated that the chiral selector migrated directly into the ESI interface, effects of the presence of CD in the separation buffer on MS sensitivity (i.e., signal intensity and contamination in the ESI interface) would need further and more detailed consideration.

## 9.5 Conclusions

The combination of separation techniques (GC, LC, and CE) with MS had a tremendous impact on analytical chemistry over recent decades, and today, it is the standard approach in many areas of analytical research and application,

including forensic analysis. If the coupling of capillary GC with MS soon proved to be quite straightforward, much more challenging has been the interfacing of liquid-phase separation techniques, such as LC and, to some extent, TLC. To this aim, important efforts were successfully devoted to improve the coupling of the molecular separation step occurring in a liquid mobile phase with an efficient and stable ionization, which is needed by the following mass spectrometric analysis. Although, today, ESI, APCI, and a few other ionization modes have proved their suitability, their implementation in the current instrumentation has undergone a continuous optimization to achieve the excellent results offered by the modern LC-MS instrumentation. LC-MS indeed, after decades of absolute prevalence of GC-MS, has recently become the leading technique adopted in forensic analysis. For example, the great part of the forensic toxicological analysis of biological fluids, excluding a few exceptions (e.g., determination of THC–COOH in hair), is today performed by using UHPLC-QqQ, which often allows the injection of samples after minimal pretreatment.

On the contrary, even if CE has shown an excellent suitability for an efficient coupling with MS, mostly using tricoaxial liquid-sheath interfaces, much less efforts have been spent to develop an optimized, robust, and user-friendly CE-MS interfacing device, suitable for wide use in nonspecialized laboratories. Without a specific industrial effort in this direction, CE-MS might remain confined in a *niche* of analytical research. Only very recently, a reportedly robust, efficient, and "ready-to-use" sheathless CE-MS interface has been marketed, promising excellent performances in terms of sensitivity and separation efficiency, because of minimized "postcolumn" added volumes. The "CE community" is anxiously waiting for the results of the practical application of this important innovation.

On the other hand, the CE coupling with MS has shown to offer distinct advantages in terms of analytical performances, which are mostly related to the plug-like EOF-generated flow in the capillary reducing peak broadening, to the peculiar separation exerted by electrophoresis (and related techniques), to the ease of changing the analytical conditions in the absence of a stationary phase (but for CEC), and, mainly, to the dramatic miniaturization of the separation compartment. This last condition reduces required sample volume to few tens of nanoliters and the total volume of solution entering the ion source to few microliters per run. Both these features contribute to minimizing ion suppression, which probably is one of the major drawbacks of the application of MS in liquid phase. For this reason, CE-MS, differently from LC-MS, has proved its tolerance to the use of inorganic buffers. Last but not least, the use of chiral selectors in solution (mainly CDs), although with some adjustments, can also be compatible with CE-MS. With specific regards to the variety of forensic applications herein reviewed, it is worthy to report some examples of improvement of the analytical performances: (i) analysis time can markedly be reduced in CE, as reported by Simó *et al*., who compared a long-lasting HPLC analysis (40 min) versus a rapid CZE-TOF-MS lasting only 8 min [163]; (ii) sensitivity has proved to be similar, and sometimes higher, when comparing CE-MS to other hyphenated techniques [145, 165]; (iii) CE separation,

moreover, offers the chance to analyze a greater variety of analyte categories, and particularly charged molecules such as glucuronides of drugs and hormones, which are of the highest forensic toxicological interest [91, 118]; (iv) finally, separation buffers that can be used in CE separations are of a greater variety than the ones used in LC-MS, as was reported by several authors [31, 32, 50, 68–70].

However, a general evaluation of the present real situation leads to conclude that the application of CE-MS in forensic analysis and particularly in forensic toxicology is still limited to only few centers worldwide. This difficulty in penetration into this quite conservative area can also be explained with less enthusiasm of the forensic analysts to use a separation technique based on electrophoretic principles, in which they have too often little confidence . On these grounds, the importance of the dissemination of the "electrophoretic knowledge" in the field of forensic analysis should be pointed out, which could be a preliminary to a wider use of CE. In this respect, we should mention that since the year 2006, at the University of New Haven (West Haven, CT), in the Graduate Program in Forensic Science, a specific course on CE and its forensic application has been established (http://catalog.newhaven.edu/preview_course_nopop.php?catoid=3&coid=5996, [180]), which so far has attracted the interest of many students and distinguished researchers. Specific research and teaching in the field of forensic applications of CE-MS have also been carried out at the Florida International University, Miami (FL), and at the George Washington University, Washington (DC). Also, at the University of Verona, Verona, Italy, CE and CE-MS are subjects of teaching and research, particularly in the forensic toxicology context, in the PhD program on "Nanoscience and Advanced Technologies."

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# 10
# CE-MS in Metabolomics

*Akiyoshi Hirayama and Tomoyoshi Soga*

## 10.1
## Introduction

In biological samples (e.g., cells, body fluids), the metabolome is defined as the total set of low-molecular-weight compounds that remain after removal of DNA (genome), RNA (transcriptome), and proteins (proteome). Generally, low molecular weight in this case is defined as compounds with molecular weights of less than 1.5 kDa. Metabolites are the final product in biological systems, and variation in the metabolome is thought to reflect responses caused by numerous metabolic perturbations. There are approximately 3000 metabolites in the human metabolome, which is a small number compared with the number of genes in the human genome (about 22 000), transcripts in the human transcriptome (about 100 000), and proteins in the human proteome (about 1 million).

Metabolomics, or metabolome analysis, is an omics technology and a rapidly growing area of research. It can be applied to comprehensive analysis of metabolites in biological samples. Metabolites are not species specific, and theoretically, a single analytical platform could be used to analyze for a target compound in extracts from multiple species. However, from the point of view of analytical chemistry, it is difficult to analyze all metabolites simultaneously with a single analytical method. This is because the chemical and physical properties of the metabolites are quite varied, and their concentrations can extend over an estimated seven to nine orders of magnitude (pmol to mmol) [1]. Consequently, researchers first define a target metabolite subset and then select an appropriate analytical approach to detect as many target metabolites as possible. Two approaches, targeted analysis and nontargeted analysis, are generally employed for analysis of the metabolome. Targeted analysis involves identification and quantification of preselected metabolites in the sample using a technique that is selected specifically for those metabolites. By contrast, in nontargeted analysis, an analytical technique is selected without a target in mind, and all metabolites that can be detected by this in a given sample are identified.

*Capillary Electrophoresis–Mass Spectrometry (CE-MS): Principles and Applications,*
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To date, metabolomics has been performed with nuclear magnetic resonance (NMR) spectroscopy [2], gas chromatography–mass spectrometry (GC–MS) [3], liquid chromatography–mass spectrometry (LC–MS) [4, 5], and capillary electrophoresis–mass spectrometry (CE-MS) [1, 6, 7]. NMR has several advantages over the other techniques. For example, direct analysis of solid sample without any preparation is possible with NMR, and the total analysis time is relatively short. Furthermore, NMR is rarely affected by ion suppression and matrix effects, which are often problematic in MS methods. However, NMR has relatively low sensitivity compared with MS, and this means a large sample volume is required for analysis.

GC–MS has been used extensively in metabolomics. Electron ionization is frequently used as the ionization method and provides universal, reproducible, and characteristic fragmentation patterns that enable reliable matching to publicly or commercially available databases. For nonvolatile compounds, derivatization steps are required, and these are generally time-consuming and increase the variance in the analysis. In addition, metabolites that are still not volatile after derivatization cannot be analyzed by GC–MS.

Among the aforementioned techniques, LC–MS is the most frequently used in metabolomics because it can be used for a wide range of metabolites without derivatization. There are many different columns with various retention mechanisms available commercially, and among these, reversed-phase columns such as C18 and C8 are the most commonly used in metabolomics. For polar metabolites, ion-pair LC–MS [8] and hydrophilic interaction chromatography–MS [9] are becoming common. However, in the cases where LC is combined with MS, matrix effects can lead to quantification inaccuracy.

CE-MS has emerged as a powerful tool for the analysis of charged metabolites. Many metabolite intermediates in primary metabolism contain amino, hydroxyl, carboxyl, and phosphate groups in their structures and are charged, which makes them suitable for CE-MS analysis. In addition, CE requires only a small sample volume and is suitable for volume-limited samples. A CE-MS-based metabolomics was firstly applied for comprehensive and quantitative analysis of charged metabolites from a metabolic extract of *Bacillus subtilis*, and 1692 peaks were successfully detected [10]. Currently, CE-MS has been used in various research fields, including clinical, biomedical, environmental, and plant metabolomics [6, 7, 11–15].

In this chapter, the application of CE-MS is described with a focus on several samples frequently used in CE-MS-based metabolomics, including blood (serum and plasma), urine and other biofluids, cell cultures, tissue, and plants.

## 10.2 Sample Preparation and MS Systems

Ideal sample preparation in metabolomics results in recovery of as many metabolites as possible with minimal loss. Currently, there is no standard sample

preparation method, and researchers employ several methods according to the purpose of their study.

In nontargeted analysis, it is important to minimize handling steps to prevent unexpected sample losses during preparation. Consequently, deproteinization with an organic solvent is often employed for sample preparation in nontargeted analysis. For instance, Canuto *et al.* deproteinized parasite pellets by addition of 200 µl methanol/ethanol 1 : 1 (v/v) at 4 °C, followed by analysis using a sheathless CE-MS [16]. For three injections of quality control samples, the authors found that relative standard deviations for migration time and peak area were below 6% and 25%, respectively. These results were acceptable for routine metabolome analysis using a sheathless CE-MS. However, this method is often insufficient for many samples, especially serum and plasma, which contain large quantities of proteins. If not all of the protein is removed by the deproteinization step, the remaining protein can adsorb to the inner capillary wall and affect the analysis.

Alternatively, liquid–liquid extraction (LLE) is often used for sample preparation. Lipid species such as fatty acids, triglycerides, and phospholipids in biological samples sometimes hinder CE-MS analysis, and these compounds should be removed in advance. Several pure solvents and solvent mixtures in various ratios have been tested. The most commonly used solvent mixture is a combination of methanol, chloroform, and water, which is used in the Bligh–Dyer method for metabolomics and lipidomics to effectively separate hydrophilic and hydrophobic metabolites [17]. In some cases, water-soluble proteins and large molecules still remain in the aqueous layer after LLE, but these can be easily removed by ultrafiltration.

In targeted analysis, solid-phase extraction (SPE) can be applied to separate metabolites from the sample matrix. In addition, SPE can selectively concentrate the target metabolites and increase the detection sensitivity. Soliman *et al.* applied SPE to the determination of urinary sarcosine, which is a potential prostate cancer biomarker [18]. They selected a Strata-X strong cation mixed-mode cartridge (Phenomenex, Torrance, CA) for this extraction. Under the optimized SPE protocol, urinary sarcosine was concentrated five times and good recovery ($94.4 \pm 7.4\%$) was achieved. Botello *et al.* developed an in-line SPE–CE-MS method and applied it for the determination of four drugs of abuse in human urine samples [19]. For this method, they constructed an analyte concentrator by inserting a small capillary (2 mm) filled with Oasis HLB sorbent into the inlet of the separation capillary. The detection limit range for the tested drugs was 0.013–0.210 ng $ml^{-1}$ and the repeatability was below 7.2%. This system could be used for quantification of trace levels of the target metabolites. Although there many kinds of SPE cartridges available commercially, only a few can be applied to CE-MS metabolomics because the chemical properties of the main target metabolites (highly polar compounds) and matrix (e.g., salts) are so similar and they are difficult to separate by SPE.

Recently, electrically driven sample pretreatment methods have been reported, including electromembrane extraction [20] and electroextraction [21]. For

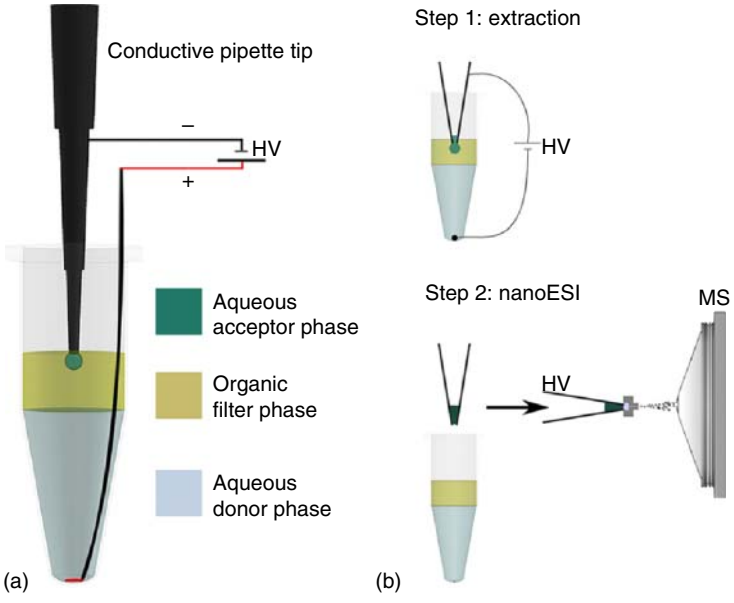


**Figure 10.1** (a) Three-phase electroextraction setup, (b) analytical workflow. (Reproduced from [21] with permission from American Chemical Society.)

example, Raterink *et al.* developed a three-phase electroextraction system that enabled fast migration of analytes from an aqueous donor phase through an immiscible organic filter phase and into an aqueous acceptor phase on application of an electric field between the donor and acceptor phases (Figure 10.1) [21]. They used acylcarnitines as test compounds to spike human plasma as a donor phase and achieved good reproducibility, linearity, and a 10-fold improvement in the limit of detection. This technique is promising for sample preparation in bioanalysis.

Although all commercially available MS can be coupled to CE, single-quadrupole MS and triple-quadrupole MS are most frequently used in targeted analysis. Selected reaction monitoring (SRM), which is carried out on a triple-quadrupole MS, enables highly sensitive and quantitative analysis even for complex matrices. CE–tandem mass spectrometry with SRM has been applied for the determination of γ-glutamyl peptides in clinical samples [22] (Figure 10.2).

In nontargeted analysis, time-of flight (TOF)MS has been widely used. TOFMS can provide high resolution, high mass accuracy (<5 ppm error), and sufficient data points across the peak. In addition, the elemental compositions of compounds can be determined from their isotopic patterns, which is helpful for compound identification. Example electropherograms of anionic metabolites extracted from mouse liver [23] are shown in Figure 10.3.

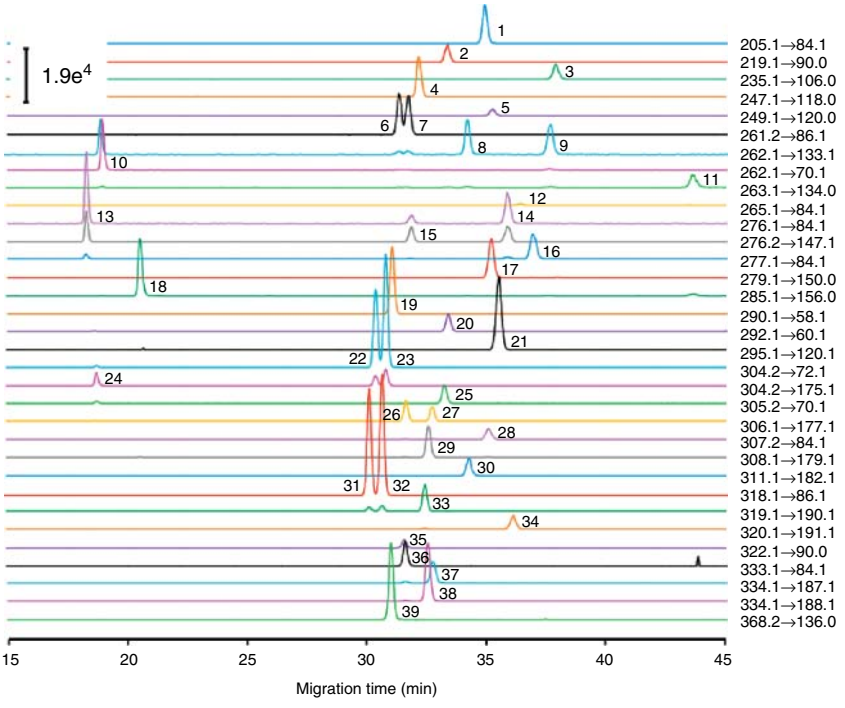


**Figure 10.2** Electropherogram of a standard mixture of γ-glutamyl peptides by CE-MS/MS. Peak identification: 1, γ-Glu-Gly; 2, γ-Glu-Ala; 3, γ-Glu-Ser; 4, γ-Glu-Val; 5, γ-Glu-Thr; 6, γ-Glu-Ile; 7, γ-Glu-Leu; 8, γ-Glu-Gly-Gly; 9, γ-Glu-Asn; 10, γ-Glu-Ornithine; 11, γ-Glu-Asp; 12, γ-Glu-Homocysteine; 13, γ-Glu-Lys; 14, γ-Glu-Gln; 15, γ-Glu-Ala-Gly; 16, γ-Glu-Glu; 17, γ-Glu-Met; 18, γ-Glu-His; 19, Ophthalmate; 20, γ-Glu-Ser-Gly; 21, γ-Glu-Phe; 22, γ-Glu-Val-Gly; 23, γ-Glu-Norvaline-Gly; 24, γ-Glu-Arg; 25, γ-Glu-Citrulline; 26, γ-Glu-Homoserine-Gly; 27, γ-Glu-Thr-Gly; 28, GSSG; 29, GSH; 30, γ-Glu-Tyr; 31, γ-Glu-Ile-Gly; 32, γ-Glu-Leu-Gly; 33, γ-Glu-Asn-Gly; 34, γ-Glu-Asp-Gly; 35, γ-Glu-Homocysteine-Gly; 36, γ-Glu-Gln-Gly; 37, γ-Glu-Glu-Gly; 38, γ-Glu-Trp; 39, γ-Glu-Tyr-Gly. Experimental conditions: concentration of each peptide, 20 μmol $l^{-1}$. The numbers on the right are *m/z* for analyte ions of Q1 (protonated precursor ion) and Q3 (product ion) in multiple reaction monitoring mode. (Reproduced from [22] with permission from Elsevier.)

## 10.3 Application

As described in Section 10.2, there are two approaches that can be adopted in metabolomics, namely the targeted approach (metabolic profiling) and the nontargeted approach (metabolic fingerprinting) [24, 25]. The targeted approach focuses on quantitative analysis of a selected metabolite involved in a specific pathway or class of compounds. By contrast, the nontargeted approach is a global

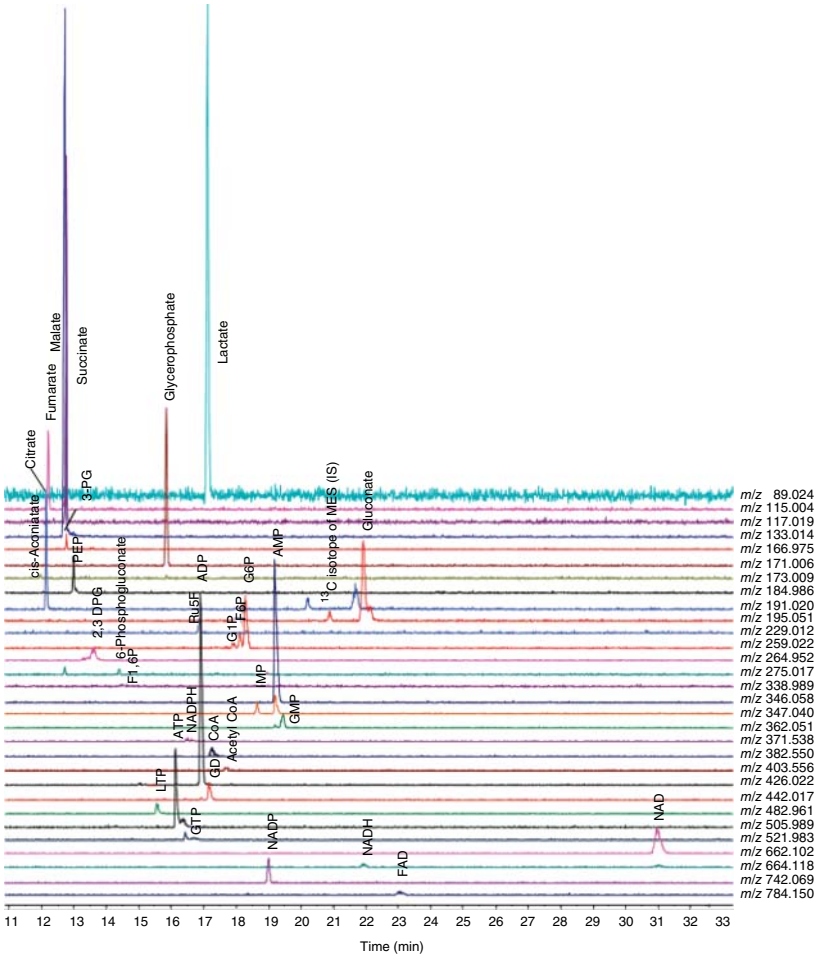


**Figure 10.3** Selected CE-TOFMS electropherograms for intermediate metabolites of glycolysis, pentose phosphate, and the TCA pathways extracted from mouse liver. (Reproduced from [23] with permission from American Chemical Society.)

screening approach that can classify samples based on their metabolite patterns (fingerprints), and not all metabolites must be identified and quantified. In the following sections, the proper definitions of targeted and nontargeted analyses are not necessarily adhered to in each application.

### 10.3.1 Blood

Serum and plasma are the most frequently used biofluid for metabolomics. In one study by Koike *et al.*, plasma samples were analyzed by CE-MS to explore

global metabolomic changes and identify candidate biomarkers of schizophrenia [26]. The plasma samples were collected from 30 patients with first-episode schizophrenia, 38 healthy controls, and 15 individuals with autism spectrum disorders and analyzed by CE-TOFMS. Increased levels of creatine and decreased levels of betaine, nonanoic acid, benzoic acid, and perillic acid were observed in the first-episode schizophrenia patient plasma. These metabolites could be promising biomarkers for several situations, such as differential diagnosis, determination of clinical stages, prediction of outcomes, and treatment responses.

Kume *et al.* validated the use of plasma metabolome analysis in a rat model of fatigue using CE-TOFMS [27]. Several fatigue-induced changes were observed in metabolic pathways, including in the urea cycle, proline metabolism, and branched-chain amino acid metabolism (Figure 10.4). Citrulline and hydroxyproline were identified for the first time as metabolic candidates that reflect complex fatigue in the rat model. These metabolites are promising diagnostic biomarkers for human chronic fatigue and/or chronic fatigue syndrome.

Alzheimer's disease (AD) is the most prevalent disorder among the various causes of dementia. According to the 2014 World Alzheimer report, there were an estimated 44 million people worldwide living with dementia, and the incidence of AD throughout the world is expected to triple by 2050 [28]. Therefore, it is important to discover early diagnostic biomarkers of AD. To address this, CE-MS-based metabolomics was applied to serum samples to obtain representative metabolites that could be used to distinguish between patients with AD, mild cognitive impairment, and healthy controls [29]. With progression of the disease, large increases were observed in the levels of choline, creatinine, asymmetric dimethylarginine, homocysteine–cysteine disulfide, phenylalanyl-phenylalanine, and different medium-chain acylcarnitines. At the same time, asparagine, methionine, histidine, carnitine, acetyl-spermidine, and C5-carnitine levels decreased. The elevation of serum phenylalanyl-phenylalanine is a potential biomarker of vascular risk in AD.

Metabolome analysis provides the ability to analyze many metabolites simultaneously and can be used to develop biomarker panel of multiple metabolites for clinical diagnoses. Soga and coworkers applied both CE-TOFMS and LC-MS/MS approaches to discover noninvasive and reliable biomarkers for rapid diagnoses of liver diseases [30]. They analyzed a total of 248 serum samples from nine different types of liver disease and healthy controls and found that the levels of γ-glutamyl dipeptides could be used to distinguish among different liver diseases. Furthermore, they developed multiple logistic regression models that enabled discrimination between a specific liver disease and other liver diseases (Figure 10.5). This research shows that γ-glutamyl dipeptides have the power to discriminate among different forms of hepatic disease.

Zeng *et al.* employed CE-TOFMS to discover novel biomarkers for hepatocellular carcinoma (HCC) [31]. They analyzed a total of 183 human serum specimens (77 for the discovery set and 106 for the external validation set), including from

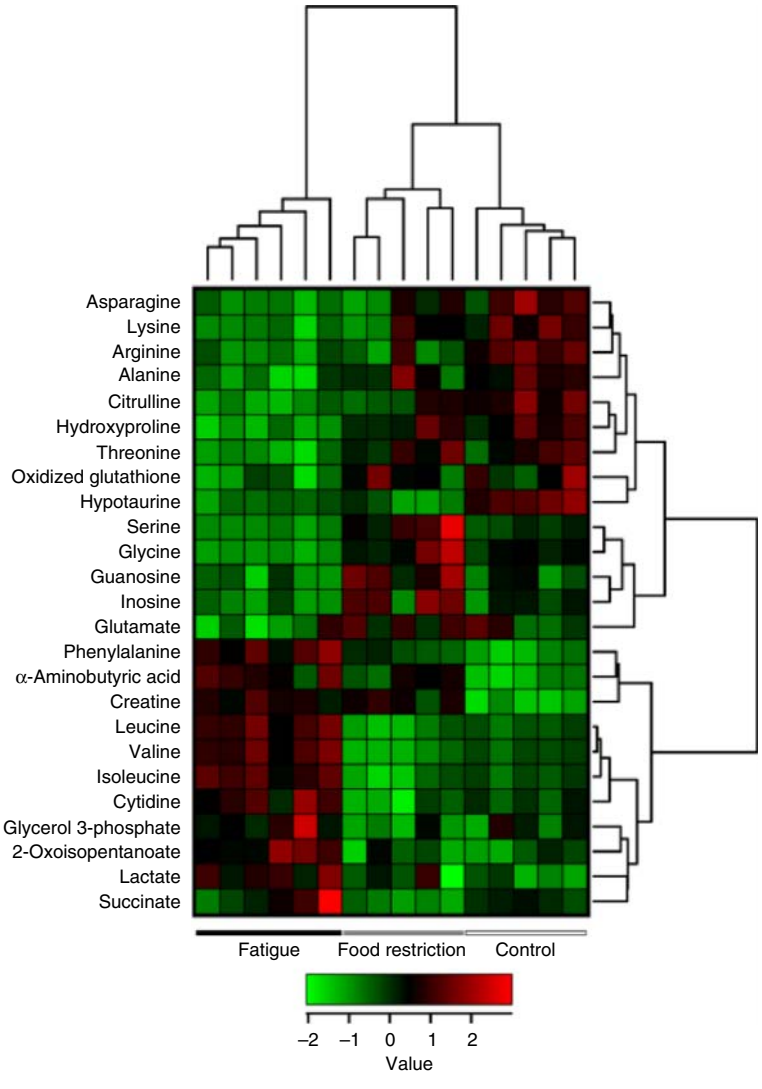


**Figure 10.4** Supervised hierarchical clustered heat map of 25 metabolites identified by one-way ANOVA. Each column shows the metabolic pattern of individual animals in the control, food-restricted, and fatigued groups. The quantity of each metabolite in the individual samples is expressed as a relative value obtained by the autoscaling method and is represented by a color scheme with red and green for high and low concentrations of metabolites, respectively. (From [27]. http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0120106. Used under CC BY 4.0 http://creativecommons.org/licenses/by/4.0/.)

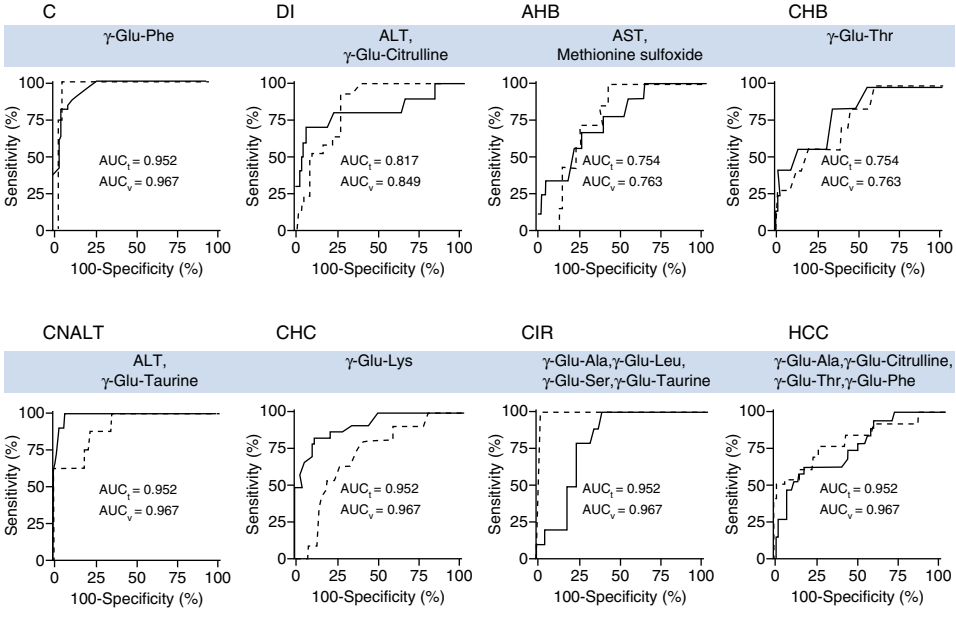


**Figure 10.5** Receiver-operating characteristic curve analysis of the ability of γ-glutamyl peptides alone or in combination with aspartate transaminase (AST), alanine transaminase (ALT), and methionine sulfoxide to discriminate each group from all other liver diseases and healthy controls. The solid and dashed curves represent the ROC curves for the training and validation cohorts, respectively. $AUC_t$ and $AUC_v$ in each panel indicate the area under the curve values in the training and validation cohorts, respectively. The group label indicates the group discriminated from all the other groups by a multiple logistic regression model. (Reproduced from [30] with permission from Elsevier.)

healthy controls and patients with cirrhosis and HCC. The concentrations of tryptophan, glutamine, and 2-hydroxybutyric acid were different in the HCC patients, compared with the cirrhosis patients and the healthy controls. A serum biomarker model consisting of these three metabolites showed excellent discrimination ability. The area under the curve (AUC) from receiver-operating characteristic (ROC) analysis was 0.969 in the discovery set and 0.99 in the validation set for diagnosing HCC from non-HCC. These results indicate that the development of a biomarker panel consisting of multiple metabolites has the potential for clinical diagnosis in future.

After blood sampling, parameters such as clotting time, incubation temperature, and the number of freeze–thaw cycles are important in metabolome analysis. Hirayama *et al.* assessed the effects of sampling procedures and storage conditions on the stability of charged metabolome profiles in serum and plasma using CE-TOFMS [32]. Overall, interindividual variations were larger than intraindividual ones, and profiles in plasma showed better stability than those in serum. The authors described that better metabolome profiles could be obtained from plasma stored for a shorter period of time, subjected to fewer freeze–thaw cycles.

### 10.3.2 Urine

Urine can be readily collected in large quantities noninvasively, which is advantageous compared with other biofluids. In addition, it poses low risk of infection to personnel, and the concentrations of metabolites are often higher than those in plasma [33]. For these reasons, urine has been used in metabolomics for biomarker discovery of various diseases.

Chen *et al.* optimized the parameters of moving reaction boundary, a technique for on-line sample enrichment in CE-MS, and applied it to urinary biomarker discovery for gastric cancer [34]. Urine samples from 28 gastric cancer patients and 14 controls were subjected to moving reaction boundary-based CE-MS analysis. They found that the levels of lactic acid, arginine, leucine, isoleucine, and valine were much higher in gastric cancer patients compared with the controls, while the levels of citric acid, histidine, methionine, serine, aspartic acid, malic acid, and succinic acid were much lower in gastric cancer patients compared with controls. In addition, they performed ROC analysis and obtained the AUC between gastric cancer patients and controls (AUC = 1.0), as well as between early and advanced stage patients (AUC = 0.847).

Alberice and coworkers performed urinary biomarker discovery for bladder cancer recurrence using both LC-MS and CE-MS [35]. A combined total of 27 metabolites from the two techniques, with 14 of these from CE-MS analysis, were identified as showing large differences between stable and recurrent patients with different tumor stages (low or high risk of recurrence). Among these metabolites, betaine, cysteine, histidine, tyrosine, carnosine, decanoylcarnitine, and uric acid showed the most potential as markers of disease stability. The metabolites *N*ε,*N*ε,*N*ε-trimethyllysine, *N*-acetyltryptophan, dopaquinone, leucine, and hypoxanthine were potential markers of tumor recurrence. These results indicate that urine can be used to identify prognosis biomarkers of various diseases.

Urine volumes can vary widely depending on water uptake and other pathophysiological factors such as kidney disease and diabetes. Consequently, several normalization procedures have been reported. However, the main problem with the use of urine in metabolomics is the lack of confirmed normalization methods. Twenty-four-hour urine collection is considered the "gold standard" and has been used in several studies [36–38], but the urine collection is inconvenient and patient noncompliance can lead to errors. As an alternative to 24-h collection, spot urine samples can be normalized using creatinine. However, it has been reported that the creatinine-based normalization method is affected by a multitude of patient factors, such as sex, age, physical activity, and muscle mass [39, 40].

Warrack *et al.* evaluated four normalization approaches using urine volume, osmolality, creatinine concentration, and mass spectra total useful signal (MSTUS, the total intensity of components that are common to all samples) [41]. They found that normalization to both osmolality and MSTUS improved the differentiation between groups of rats receiving either a low or a high dose

of a toxic compound. They recommended the use of osmolality, if available, and MSTUS or a related approach when osmolality was not suitable.

More recently, normalization to specific gravity (SG) has been evaluated for cattle urinary metabolome analysis [42]. Normalization based on SG was compared to freeze-drying, which is commonly used for normalization in cattle, and MSTUS. Among the metabolites detected by LC-MS, 77% were found in both freeze-dried and SG samples, and the same candidate biomarkers were obtained for screening for administration of anabolic steroids to cattle by orthogonal partial least squares analysis. SG shows potential as an alternative normalization strategy for human urine.

Urine samples usually contain bacteria, and their additional metabolisms during sample storage sometimes influence the metabolite concentrations [43, 44]. Saude and Sykes evaluated the effect of sample handling after collection on the metabolome of urine [44]. Addition of the preservative sodium azide ($10\,mmol\,l^{-1}$) or filtration of the urine could eliminate the influence of bacterial contamination, and the original metabolite concentrations of the urine were retained. In addition, the study showed that samples should be stored in a deep-freeze (−80 °C), and freeze–thaw cycles of sample should be minimized.

### 10.3.3 Other Biofluids

In addition to blood and urine, other biofluids are also used in metabolomics. Ramautar *et al.* applied sheathless CE-MS to nontargeted metabolic profiling of mice cerebrospinal fluid (CSF) [45]. Compared with the conventional sheath-flow CE-MS method, sheathless CE-MS does not use a sheath liquid, which means that the analyte is not diluted at the end of the capillary, and this increases the sensitivity. Using this system, approximately 340 molecular features with signal-to-noise ratios of greater than 10 were detected in mouse CSF. This system can be used for sensitive metabolomic profiling of volume-restricted biological samples.

CSF is considered a desirable biofluid for the detection of brain-related disorders, such as mild cognitive impairment and AD, because its composition is a direct reflection of metabolite production in the brain [46]. In one study, 85 CSF samples from patients with subjective cognitive impairment (control), mild cognitive impairment, and AD were subjected to CE-MS-based metabolomics [47]. A prediction model of AD progression was developed, and it could correctly classify 97–100% of the tested samples into their diagnosis group. In addition, choline, dimethylarginine, arginine, valine, proline, serine, histidine, creatine, and suberylglycine were identified as possible disease progression biomarker candidates. These results suggest that CSF is a promising biofluid for predicting AD progression.

Similar to urine, saliva is readily accessible biofluid. Sugimoto *et al.* analyzed saliva samples obtained from 215 individuals (69 oral, 18 pancreatic and 30 breast cancer patients, 11 periodontal disease patients, and 87 healthy volunteers) using CE-TOFMS. They identified 57 primary metabolites that could discriminate

among these diseases. In addition, they developed multiple logistic regression models and obtained high AUC values (0.865–0.993) to discriminate healthy controls from each disease. This study demonstrated that CE-MS can be readily and effectively applied to salivary metabolomics.

Although other biofluids, including sweat, tear fluid, and bile, could be used for CE-MS-based metabolomics, there are no recent publications on their application in the scientific literature. Sweat is an analytically friendly biofluid with very low protein concentrations [48], but can only be collected in limited volumes, which makes CE-MS an ideal analytical method for this biofluid.

### 10.3.4 Cell Cultures

From a biochemical point of view, CE-MS is especially suitable for global profiling of the central carbon metabolism. Metabolic pathways such as glycolysis, the pentose phosphate pathway, tricarboxylic acid (TCA) cycle, and amino acid metabolism play important roles in energy metabolism, and the intermediates involved in these pathways are highly polar and charged compounds [49]. Therefore, CE-MS is often applied in the studies of cellular metabolism.

In the field of dentistry, sodium fluoride (NaF) has the potential to act as an antibacterial material to inhibit the function of enolase in bacteria, but its site of action in human cells has not been identified. CE-TOFMS has been applied to detect target metabolites or metabolic pathways that are affected by NaF administration into cultured human oral squamous cell carcinoma cell lines [50]. In the early stages after administration, inhibition of both enolase activity and TCA cycle was observed. Meanwhile, in the later stages, gradual increases in the levels of oxidized products, such as oxidized glutathione and methionine sulfoxide, were observed. In addition, the AMP/ATP ratio, which is a putative marker of apoptosis, increased.

Kim *et al.* performed a comparative study of metabolic content between fetal and adult hepatocytes using CE-TOFMS [51]. They identified 211 metabolites (116 anions and 95 cations) from hepatocytes that were cultured *in vitro*. The quantities of most metabolites related to the glycolysis/gluconeogenesis, TCA, and urea cycles were lower in fetal hepatocytes than in adult ones. These results provide fundamental information for studies on the metabolic functions of human fetal and adult hepatocytes.

Generally, culture media contain high concentration of salts, which could result in instability in the electrospray ionization and shifts in the migration times. Therefore, the methods used to prepare cell cultures for metabolomics are important. Simó *et al.* investigated four metabolite purification approaches, including methanol deproteinization, ultrafiltration, and two SPE methods, for the metabolomic profiling of human HT-29 colon cancer cells using CE-TOFMS [52]. Narrow peaks were obtained for a desalted sample prepared by SPE with a polymer-based sorbent. By comparison, a slight increase in the peak width,

as indicated by the theoretical plate number, was observed in a nondesalted sample prepared by methanol deproteinization. However, despite this increase in peak width, methanol deproteinization showed good potential for metabolomics profiling. In addition, SPE showed good extraction efficiency with different selectivity compared to the other methods.

The same group further improved the sample preparation protocol for cell metabolomics and applied it to the examination of metabolic changes in the polyamines pathway caused by the potent ornithine decarboxylase inhibitor difluoromethylornithine in HT-29 cells [53]. Using the optimized protocol, 10 metabolites, including putrescine, ornithine, γ-aminobutyric acid, oxidized and reduced glutathione, 5′-deoxy-5′-(methylthio)adenosine, *N*-acetylputrescine, cysteinyl-glycine, spermidine, and one unknown compound, were found to be significantly ($p < 0.05$) altered with difluoromethylornithine treatment. In addition, minor modifications of metabolic pathways related to the intracellular thiol redox state were also observed.

Combined with a recent large improvement in the sensitivity of MS, much progress has been made in the development and application of CE-MS to single-cell metabolomics by Sweedler's group [54–56]. More recently, Aerts and colleagues developed a combined system using visualized whole-cell patch clamp recording and CE-MS [57]. This system enabled selection of specific cells, characterization of their physiological properties, collection of a small volume of an individual cell's cytoplasm, and subsequent CE-MS-based metabolome analysis. Using this system, they analyzed three neurons and an astrocyte obtained from rat thalamus (Figure 10.6). This technology provides the ability to link the physiological activity of neurons and astrocytes with their neurochemical state.

### 10.3.5 Tissue

Tissue-based metabolome analysis is one of the most important research strategies for understanding disease mechanisms because primary changes before disease development can be detected in the relevant organs. For this type of research, tissue samples are usually obtained from animal models.

Maekawa *et al.* performed global metabolome analysis of myocardial tissue from the left ventricles of J2N-k cardiomyopathic hamsters, which exhibit similar symptoms to those of human dilated cardiomyopathy (DCM) [58]. J2N-k hamsters were compared with J2N-n healthy controls at 4 (presymptomatic phase) and 16 (symptomatic phase) weeks of age. Charged metabolites and lipids from the myocardial tissue were analyzed by CE-MS and LC-MS/MS, respectively. Reduced levels of metabolite intermediates from glycolysis, the pentose phosphate pathway, and TCA cycle, combined with huge decreases in triacylglycerol levels, suggested that decreased energy production leads to cardiac contractile dysfunction in the symptomatic phase. In addition, a mild reduction in glutathione and a compensatory increase in ophthalmic acid suggested increased

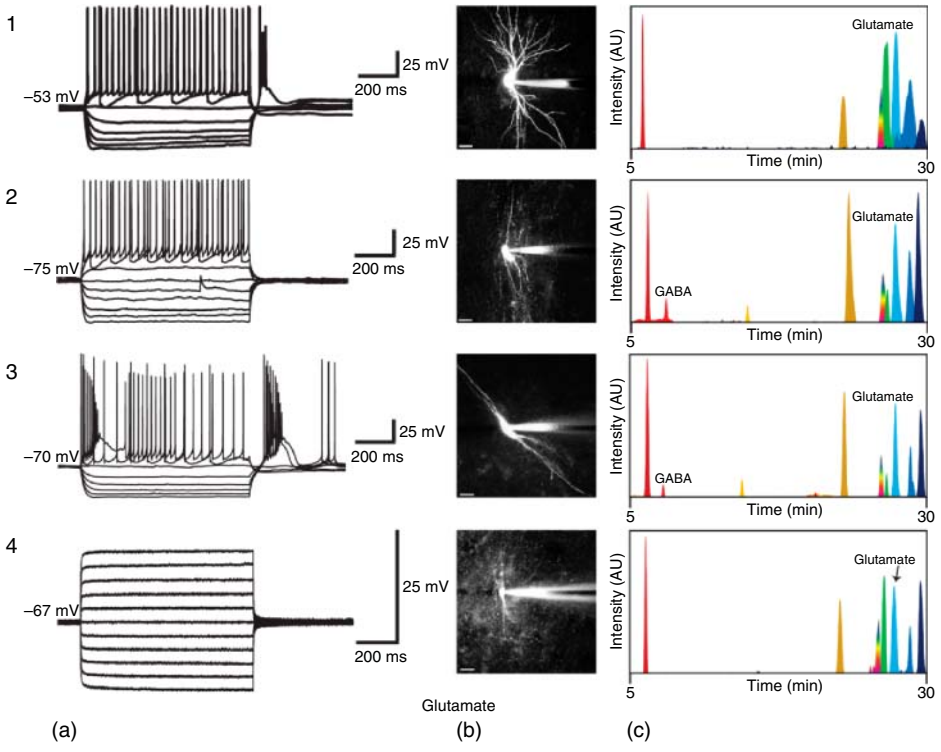


**Figure 10.6** Data obtained from the following four distinct cell types: (1) ventral basal thalamocortical neuron, (2) nonburst-firing thalamic reticular nucleus neuron, (3) bursting thalamic reticular nucleus neuron, and (4) astrocyte. (a) Electrophysiological recordings of the individual cells (1–4) shown in the (b) photomicrographs (scale bar = 20 μm). (c) Extracted ion chromatograms corresponding to the cytoplasm sampled from the neurons and glial cells are shown. Peaks correspond to ornithine (dark red), GABA (light red), glycine (yellow), serine (gold), tryptophan (rainbow), glutamine (light green), glutamic acid (light blue), tyrosine (dark blue), and proline (indigo). (Reproduced from [57] with permission from American Chemical Society.)

oxidative stress in the diseased tissue (Figure 10.7). These finding provide new mechanistic insight into DCM pathogenesis.

Nonsteroid anti-inflammatory drugs (NSAIDs) are among the most frequently prescribed drugs. They have serious side effects of gastric mucosal ulceration and gastric hemorrhage. Takeuchi *et al.* applied a shotgun approach based on CE-TOFMS profiles of endogenous metabolites to identify new biomarkers of NSAID-induced gastric injury in rat stomach tissue [59]. The levels of citric acid, *cis*-aconitic acid, succinic acid, 3-hydroxybutanoic acid, *o*-acetyl carnitine, proline, and hydroxyproline were decreased in the NSAID stomach tissue compared with the healthy tissue. Four of these metabolites were correlated with changes in serum. The authors concluded that these changes were caused

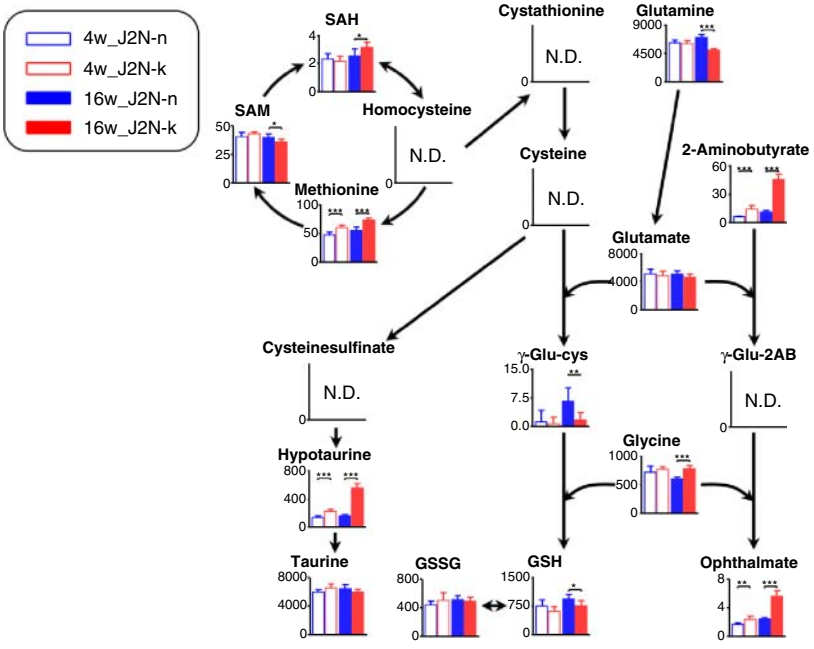


**Figure 10.7** Quantification of metabolites involved in glutathione biosynthesis and related pathways. The columns represent average concentrations (nmol $g^{-1}$ tissue), and the error bars indicate the standard deviation. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; and N.D., not detected. (Reproduced from [58] with permission from Elsevier.)

by NSAID-induced depression of mitochondrial function and activation of collagenase by lesions in the stomach.

Typically, it is difficult to collect control (or normal) tissue in cases when metabolome analysis is performed on human tissue samples. To overcome this problem, resected tissue can be divided into affected and nonaffected regions, and both tissues can be analyzed simultaneously. This method can clarify what metabolites are specific to the affected region and reduce interindividual bias.

Kami *et al.* compared metabolomic profiles (CE-TOFMS) and levels of enzymes involved in phosphorylation (nanoLC-MS/MS) between normal and tumor tissues, which were surgically resected pairwise from nine lung and seven prostate cancer patients. Concentrations of most amino acids, especially branched-chain amino acids, were significantly higher in both tumor tissues. Essential amino acid concentrations were particularly high in tissues from poorly differentiated prostate cancers compared with those from moderately differentiated prostate cancers. High lactic acid concentrations and elevated activating phosphorylation levels of phosphofructokinase and pyruvate kinase in lung tumors were indicators of hyperactive glycolysis. Integrating metabolomics with analysis of enzymes involved in phosphorylation could increase understanding of tissue-specific tumor microenvironments.

### 10.3.6 Plants

CE-MS is often used in plant metabolomics. For example, aphid infestations can result in serious yield losses for crops, and cultivation of aphid-resistant varieties of plants is a promising approach for pest control. Sato *et al.* applied CE-TOFMS to elucidate the aphid-resistance mechanism for two soybean varieties, Tohoku149 (strong resistance to aphids) and Suzuyutaka (susceptible to aphids) [60]. Comparative metabolomics analysis showed that the concentrations of citrate, amino acids, and their intermediates were higher in Tohoku 149 than in Suzuyutaka (Figure 10.8). In addition, concentrations of several metabolites, including *trans*-cinnamate, shikimate, and 5-aminovalerate, which are involved in the production of secondary metabolites such as flavonoids and alkaloids, were drastically reduced 6 h after aphid introduction. Furthermore, several TCA cycle intermediates increased in Tohoku149 48 h after aphid introduction, possibly indicating that the ability for recovery from damage is higher in the aphid-resistant variety than in the susceptible variety. CE-MS-based metabolome analysis may contribute to the understanding of the effects of pest infestations in crops.

Indole 3-acetic acid (IAA), a naturally occurring auxin, regulates many morphological and physiological phenomena in plants. To gain insights into the role of IAA in the regulation of primary metabolism, a comprehensive analysis of primary metabolites in wild type (WT) and several auxin-signaling mutants of *Arabidopsis thaliana* has been performed using CE-MS [61]. The responses of primary metabolites after a 60 min treatment with exogenous IAA in both WT and three auxin-signaling mutants were investigated. Levels of several amino acids, including γ-aminobutyric acid (GABA) in WT roots and glycine and alanine in WT shoots, changed after IAA treatment, but these changes were not observed in the mutants. Levels of glucose 6-phosphate in roots and succinic acid in shoots also changed in WT *A. thaliana* with IAA treatment. These results indicate that some metabolic processes are regulated by IAA.

## 10.4 Conclusions

Currently, there is no single analytical platform that can be used for profiling all metabolites in a given sample in a single run. Therefore, studies have employed several analytical techniques, such as GC-MS, LC-MS, CE-MS and NMR, according to the purpose of the study. Among these techniques, CE-MS is particularly suited to the separation of polar and charged metabolites and can provide information that is complementary to that from the other analytical techniques mentioned earlier.

However, relatively poor concentration sensitivity is an often-described disadvantage of CE-MS. One possible way to overcome this drawback is to apply sheathless CE-MS. Recently, a porous-tip-type sheathless CE-MS interface, called the

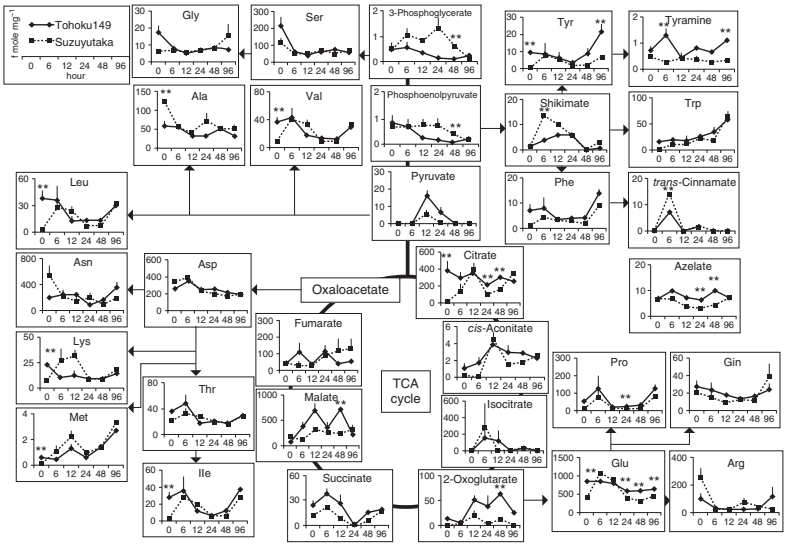


**Figure 10.8** Changes in the level of representative primary metabolites after aphid introduction. The data points are mean values and the error bars indicate the standard error. Asterisk denotes statistical significance with $p < 0.01$. (Reproduced from [60] with permission from Elsevier.)

*CESI 8000 system*, was introduced on the market from SCIEX. Several groups have reported its application in the field of metabolomics [45, 62, 63].

On-line sample preconcentration techniques are another way to improve the sensitivity of CE-MS. A recent review highlighted the applications of on-line sample preconcentration techniques to CE, including field-amplified stacking, transient isotachophoresis, pH-mediated stacking, and sweeping [64]. However, some of these methods require high concentrations of nonvolatile buffers, which limit their use in CE-MS. Future research is expected to target development of novel stacking methods that can be applied for the analysis of trace levels of metabolites in biological samples.

Capillary electrochromatography, a hybrid technique of LC and CE, has emerged as a powerful separation tool for simultaneous analysis of charged and neutral compounds. Capillary electrochromatography offers high efficiency, high selectivity, and low sample consumption. Although few studies have reported its application in metabolomics [65, 66], it has the potential to become a next-generation analytical platform.

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# 11
# CE-MS for Clinical Proteomics and Metabolomics: Strategies and Applications

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## 11.1
## Introduction

Recent advances in analytical technologies and bioinformatics tools over the past decade have created most of the prerequisites required for successful proteomics and metabolomics initiatives. In the field of proteomics, reversed-phase liquid chromatography–mass spectrometry (RPLC-MS) remains the predominant instrumental platform used for peptide mapping and characterization of intact proteins [1, 2]. In contrast, various analytical techniques with complementary separation and detection mechanisms are often used in metabolomics in order to analyze a diverse range of compounds due to their wide structural diversity and physicochemical properties [3, 4].

In this chapter, we highlight the potential of capillary electrophoresis–mass spectrometry (CE-MS) for clinical proteomics and metabolomics studies. CE-MS has been used for the characterization of a wide range of analytes, including (endogenous) metabolites, drug-related compounds and proteins, as well as intact cells and subcellular components (i.e., organelles). The high separation efficiency of CE for the analysis of charged compounds makes this technique well-suited for quantitative analysis of polar/ionic analytes in complex (biological) samples with minimal sample pretreatment. As compounds are separated on the basis of differences in their intrinsic electrophoretic mobility (i.e., charge-to-size ratio), the separation mechanism of CE is fundamentally different from most equilibria-based chromatographic techniques. Also, various separation modes can be explored in CE by the use of additives in free solution, such as micelles or chiral selectors, in the background electrolyte (BGE). For example, micellar electrokinetic chromatography (MEKC), which employs micelles as pseudostationary phases in the BGE, can be used for the separation of both neutral and charged analytes. Still, the coupling of MEKC to MS is challenging when using electrospray ionization (ESI-MS) that often requires partial filling techniques to avoid ion suppression/enhancement effects. Among the various CE separation modes, capillary zone electrophoresis (CZE; normally referred to as *CE* and also in this chapter) is most often used due to its compatibility with ESI-MS when

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using aqueous buffer solutions. Furthermore, CE separations can often be performed directly on volume-restricted and highly saline biological samples without complicated or time-consuming sample pretreatment. Other attractive features of CE include the very low sample and reagents consumption and the use of open tubular fused-silica capillaries instead of more expensive LC columns, as well as the prediction of solute electromigration behavior based on their intrinsic physicochemical properties. From a viewpoint of concentration sensitivity, the poor sample loadability (i.e., nanoliter injection volumes) can be considered a limitation of CE-MS. Alternatively, chromatographic and/or on-line electrophoretic-based preconcentration can be used in order to circumvent this issue [5, 6].

Until recently, the coaxial sheath-liquid interface has been primarily used for the coupling of CE to MS [7]. In this setup, the sheath liquid is provided coaxially to the end of the CE capillary as a terminal electrolyte reservoir, thereby providing a closed electrical contact and a makeup liquid flow and nebulizer gas to stabilize spray formation. This configuration enables independent optimization of BGE conditions for separation from sheath-liquid composition for solute ionization. For many applications, this interface provides good overall robustness and analytical figures of merit. However, post-capillary dilution of the effluent and suctioning effects can further reduce concentration sensitivity, as well as separation efficiency. The field of CE-MS has benefitted from recent developments in more sensitive interface designs that operate ideally under lower flow regimes. In this context, the porous tip sheathless interface, the flow-through microvial interface, and the electro-osmotic flow (EOF)-driven (or electrokinetic) sheath-liquid interface have emerged as important recent developments in CE-MS [8–10]. Several recent reviews provide an in-depth overview of these nanoflow-based interface designs for CE-MS and on their benefits for cutting-edge proteomics and metabolomics studies [11–15]. For an update of the *state of the art*, we refer to Chapter 2 of this book.

Although CE-MS has emerged as an attractive complementary technique for both proteomics and metabolomics studies, the use of this technology in these fields is still underrepresented as compared to LC-MS. Constraints such as poor concentration sensitivity, migration time variability, method robustness, and lack of standardized operating conditions has hindered widespread use of CE-MS technology. Moreover, most vendors do not provide adequate training support for new users or robust CE-MS workflows that are fit for purpose, unlike more established LC-MS-based proteomics and metabolomics methods. However, recent advances in CE-MS interface designs and method development now place this technology in a new perspective. In this chapter, attention is devoted to the use of recently developed CE-MS methodologies relevant to clinical applications as related to proteomics and metabolomics studies. Concerning proteomics, the focus will be on bottom-up proteomics studies (i.e., the analysis of (complex) peptide mixtures obtained after enzymatic digestion of the protein sample) and the profiling of native peptides in biological samples. The potential of CE-MS for intact protein analysis is covered by Haselberg *et al.* in this book. Contemporary studies reporting various methodological developments with

practical strategies to obtain reproducible data with CE-MS are also discussed. Special attention is paid to studies in which CE-MS has been compared to other MS-based techniques for proteomics and metabolomics studies. Finally, some general conclusions and perspectives are provided.

## 11.2 Clinical Proteomics

Although CE-MS has a relatively long history for the analysis of relatively simple proteins and protein hydrolysates (e.g., tryptic digest of BSA or cytochrome *c*), the technology is still in its infancy for clinical-based proteomics studies. Recent CE-MS applications in the proteomics field have been primarily focused on demonstrating the utility of new interface designs to increase concentration sensitivity in order to provide deeper proteome coverage [13]. However, further studies are needed to better evaluate the performance of these recently developed CE-MS methods for peptide mapping relative to decades of effort contributed by hundreds of research groups for LC-MS-based proteomics. On the other hand, CE-MS using a classical coaxial sheath-liquid interface has been used extensively for the characterization of native peptides in human urine samples in a clinical context over the past few years [16–19]. In this section, technological issues associated with the application of CE-MS in the field of clinical proteomics are discussed. Attention will be paid to sample pretreatment, CE-MS separation conditions, data analysis, and validation as required for achieving reliable results.

### 11.2.1 Sample Pretreatment

Direct analysis of (native) peptides in complex biological samples by CE-MS requires a sample pretreatment step in order to remove compounds such as salts, lipids, and high-molecular-weight proteins as they may affect analytical performance, including migration time reproducibility and separation efficiency. Concerning the analysis of native peptides in human urine samples by CE-MS, Theodorescu *et al.* developed a sample pretreatment protocol, which has been used for clinical studies for almost a decade now [20]. Briefly, human urine samples were prepared in an aqueous solution of 2 M urea, 10 mM ammonium hydroxide, and 0.02% sodium dodecyl sulfate. High-molecular-weight proteins were removed by ultracentrifugation (employing 20 kDa MWCO cut-off filters), and the filtrate was then applied onto an RP C2-column to remove urea and salts. Polypeptides were eluted with 50% acetonitrile containing 0.5% formic acid, and the processed samples were then resuspended in 50 µl of HPLC-grade water before injection. The final sample does not contain large proteins, which may adsorb onto the surface of the fused-silica capillary wall while nonvolatile and abundant salts/solutes (e.g., sodium and urea) are removed as they may contribute

to ion suppression in ESI-MS. CE-MS analysis was generally performed using a bare fused-silica capillary (50 μm i.d. and 90 cm length) at low-pH separation conditions employing 1% acetic acid (pH ~ 2) as the BGE. The optimized analytical workflow provided an average recovery of about 85% for selected peptides with a detection limit of 1 fmol. Sample pretreatment protocols for native peptide profiling in human plasma and bile samples by CE-MS have also been reported [16, 21, 22]. Overall, the developed sample pretreatment procedure has been used in a consistent way for the analysis of urinary native peptides in more than 20 000 samples with CE migration time deviation between 2% and 5% [17].

In MS-based bottom-up proteomics studies, complex mixtures of proteins are isolated from the biological sample of interest and enzymatically cleaved into peptides via digestion with trypsin. The resulting protein digest may be desalted using a C18 Sep-Pak column or directly lyophilized and subsequently dissolved in a CE-MS-compatible solvent [23, 24]. In most cases, the digestion step is performed in an offline manner; however, various groups have developed systems integrating on-line proteolytic digestion with protein separation. In such approaches, a mixture of proteins is separated, passed through a reactor containing an immobilized proteolytic enzyme, and the resulting peptide mixture analyzed by CE-MS [25, 26]. Recently, Li *et al*. developed a two-dimensional CE system, which contains a replaceable enzymatic microreactor for on-line protein digestion [27]. In this system, proteins were separated in the first CE capillary and subsequently digested by trypsin that was immobilized on magnetic beads. The resulting peptides were transferred into the second CE capillary and separated prior to ESI-MS. Old beads were flushed to waste and replaced with a fresh plug of beads, which was captured by a pair of magnets at the end tip of the first CE capillary (Figure 11.1). During peptide separation in the second capillary, a new plug of protein was introduced into the microreactor and underwent digestion. Whereas the system could be

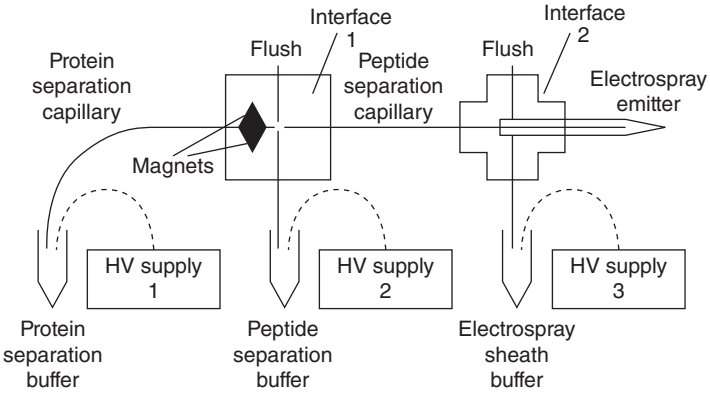


**Figure 11.1** Scheme of the two-dimensional CE system containing a replaceable enzymatic microreactor for on-line protein digestion. The magnets hold the trypsin-modified magnetic beads, forming the replaceable microreactor. High-voltage connections are shown as dashed curves. (Reproduced from [27] with permission from Elsevier.)

used for more than 2 months without replacing the capillary for a few model proteins, the potential for the analysis of biological samples has not yet been demonstrated.

In some cases, prior to CE-MS analysis, protein digests are first fractionated by RPLC in order to reduce sample complexity as a way to improve proteome coverage. A fractionation step may further reduce the number of comigrating peptides during CE analysis by decreasing the number of analytes present in each peak, thereby increasing the electrophoretic peak capacity. Pioneering work in this context was recently demonstrated by the Lindner group, where RPLC fractions were combined with sheathless CE-MS as an alternative workflow for quantitative proteomics [28]. Proteins extracted from a SILAC (stable isotope labeling by amino acids in cell culture) labeled and an unlabeled yeast strain were mixed and digested enzymatically in solution. The resulting peptides were fractionated using RPLC and analyzed by sheathless CE-MS, providing a total of 28 538 quantified peptides, which corresponded to 3272 quantified proteins. In this case, CE-MS analysis was performed using a neutral capillary coating, providing a high peak capacity at ultralow-flow separation conditions ($<10\ \mathrm{nl\ min^{-1}}$) as required for improved sensitivity and protein coverage. Moreover, without using enrichment strategies, 1371 phosphopeptides present in the CE-MS data set could be quantified, of which 49 were differentially regulated in the two yeast strains. In the proposed methodology, RPLC and CE-MS analyses are fully automated. Overall, this new approach shows strong potential for clinical proteomics studies.

### 11.2.2 Separation Conditions

The main separation mode used in CE-MS for the analysis of peptides is zonal electrophoresis in free solution. The separation mechanism of this CE mode is relatively straightforward since peptides are resolved on the basis of their effective charge-to-size ratio. In general, bare fused-silica capillaries employing volatile buffers, such as acetic acid or formic acid, at low pH (<3) conditions are generally employed for peptide analysis using CE-MS. Under these conditions, most peptides migrate toward the ion source together with the EOF and detected using ESI-MS in positive ionization mode.

Method stability is of utmost importance for a clinical proteomics study. Although the electrophoretic mobility of an ion is more reliably measured in CE when using a neutral reference marker to correct for changes in the EOF, in most cases, separations of peptide migration times are reported similarly to chromatographic separations. In CE, adsorption of analytes and/or matrix components to the capillary inner wall may affect the EOF, which, in turn, may result in poor apparent migration time reproducibility. Especially, when bare fused-silica capillaries are used, separation efficiencies may also be compromised due to adverse analyte–capillary wall interactions. In this context, the Mischak group developed a procedure to effectively recondition the CE capillary between

consecutive runs in order to remove potentially adsorbed components from the capillary wall in conjunction with precolumn sample workup of urine samples [29].

Adsorption effects in fused silica-capillaries may also be alleviated by modifying the inner wall of fused-silica capillaries with polymer coatings [30]. This approach has been successfully applied in various CE-MS-based proteomics studies [31–33]. When employing such systems, special attention has to be given to the stability of the coating under the used experimental conditions in order to prevent any leakage of the used polymer toward the MS. Therefore, further work is required in order to demonstrate the long-term performance of such systems for the analysis of complex biological samples that vary widely in their chemical composition, such as ionic strength, pH, and osmolality. Apart from minimizing the analyte–capillary wall interactions, coated capillaries can also be used to improve the resolution of the electrophoretic separation. For example, the Lindner group used a polyethyleneimine (PEI)-coated capillary and an M7C4I-coated (full chemical name is 1-(4-iodobutyl) 4-aza-1-azoniabicyclo[2, 2, 2] octane iodide) capillary, which generate an EOF toward the MS under reverse polarity conditions (−25 kV) using 0.1% formic acid (pH 2.7) as BGE for peptide analysis [34, 35]. Under these conditions, the positively charged peptides migrate in the opposite direction, that is, toward the cathode; however, due to the high positive charge of the capillary coating, the magnitude of the generated EOF exceeds the electrophoretic mobility of the peptides, and in this way, peptides are dragged by the EOF toward the MS. As shown in Figure 11.2, the magnitude of the EOF and thus the (effective) separation window for the analysis of peptides can be tuned by the type of capillary coating. For example, the PEI coating generates a stronger EOF as compared to the M7C4I-coating (which is less positively charged), resulting in a relatively fast separation time for the peptides. In principle, such systems provide a wider separation window as compounds migrate after the EOF. However, the use of reversed-polarity separation conditions in CE-MS can contribute to inadvertent corrosion and capillary outlet blockages when using conventional stainless-steel emitters [36]. Busnel *et al.* evaluated a neutral-coated capillary based on polyacrylamide when using sheathless CE-MS analysis of an *E. coli* tryptic digest using 10% acetic acid (pH 2.2) as the BGE [37]. A positive pressure of 0.5 psi was applied at the inlet of the CE capillary in order to stabilize spray formation that corresponds to about 5 nl $min^{-1}$. The peak capacity obtained by this system was circa 300, taking into account a separation window of 60 min and an average value for the peak width at half height of 11 s. Such a high peak capacity clearly demonstrates the high separation power of this system for the profiling of peptides in complex samples.

The potential of multidimensional separation systems employing capillaries and microfabricated channels, such as two-dimensional (2D) CE, CE coupled to capillary LC, and microfluidic devices, has also been explored for proteomics studies [38]. In comparison to conventional methods, these systems provide faster analysis times and higher separation efficiencies. For example, Geiger *et al.* developed a novel 2D separation platform that combines nano-RPLC directly, that is, without

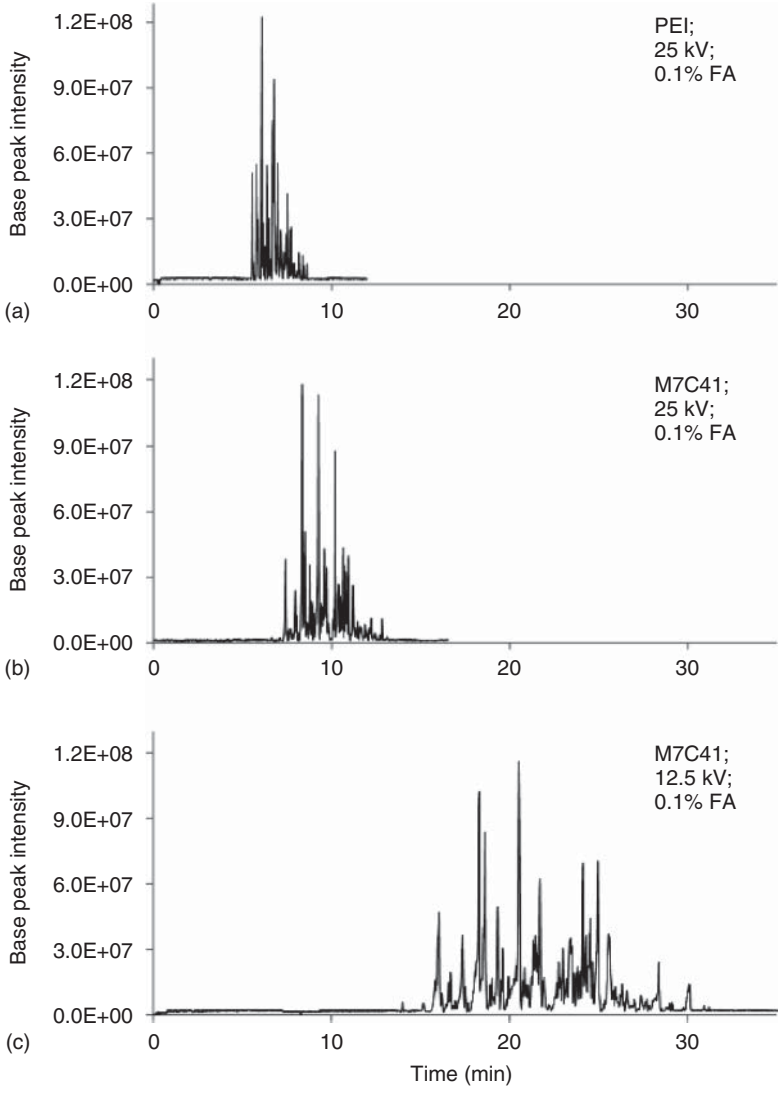


**Figure 11.2** Base peak electropherograms of rat testis H1 histones digested with endoproteinase Arg-C obtained with sheathless CE-MS using positively charged capillaries. (a) PEI-coated capillary, separation voltage of −25 kV. (b) M7C4I-coated capillary, separation voltage of −25 kV. (c) M7C4I-coated capillary, separation voltage of −12.5 kV. BGE: (pH 2.7)0.1% (v/v) formic acid. Sample amount: 6.15 ng (300 fmol). Capillary length: 100 cm with porous tip. Inner diameter 30 μm, outer diameter 150 μm. (Reproduced from [35] with permission from American Society for Biochemistry and Molecular Biology.)

using a complicated injection or sample modulation, with micro free-flow electrophoresis for high-resolution peptide analysis. The 2D system provided a peak capacity of 2352 for the analysis of a tryptic digest of BSA in a 10 min separation window [39]. So far, a variety of CE and LC modes have been used for the development of 2D separation systems employing new interfacing designs. These systems have been used for the proteomic analysis of various biological samples, ranging from a single cell to tumor tissues. For a comprehensive overview, we refer to the work of Xu *et al*. [38].

Over the past few years, various novel ion source interfaces have been developed for coupling CE to MS, including ESI and MALDI [12, 15]. For an overview of the new ion source designs for CE-MS, we refer to the work of the David Chen group included in this book. In short, the use of these approaches often significantly improves concentration sensitivity with lower detection limits for peptides as compared to conventional coaxial sheath-liquid interfaces, resulting in deeper proteome coverage. The next step is to demonstrate the potential of CE-MS when using recent flow/sheathless interfaces that are suitable for large-scale analyses of clinical samples.

### 11.2.3 Data Analysis and Validation

Most "-omics" studies published to date have been hampered by poor experimental design (i.e., cohort selection, sample collection/storage, sample pretreatment, etc.), lack of adequate method validation, quality assurance practices, do not include independent replica cohorts, and, in some cases, are not adequately anchored to relevant clinical outcomes [40]. Therefore, to obtain reliable results in the field of clinical proteomics, a proper study design is crucial. In this context, Mischak *et al*. developed an analytical workflow for the profiling of native peptides in human urine in which every aspect, that is, experimental design, preanalytics, CE-MS analysis, data processing, and evaluation, was carefully considered in order to obtain reliable data and reduce false discoveries [16, 17, 41–43]. In contrast to other separation techniques, migration time variability in CE-MS is relatively large, and therefore, the development of effective time alignment strategies is necessary for comparative profiling studies. The Mischak group employs a local regression tool for the alignment of migration times, and a linear algorithm is applied for *m/z* recalibration [29]. Alternatively, a time-warping algorithm can be used to better correct for nonlinear changes in ion migration times in CE-MS [44]. Determination of relative peptide abundances in human urine can be performed by comparison with endogenous collagen fragments as internal standards, which also corrects for changes in hydration status when relying on randomized single-spot urine specimens, since they are excreted consistently in human urine. This approach has been used for native peptide profiling studies in urine samples from patients with chronic kidney disease (CKD) [29]. MosaiqueVisu software was developed for peak identification, data deconvolution, and the display of normalized peptide abundance in a three-dimensional format [45].

The final outcome of the developed analytical workflow is a peak list showing each peptide defined by its molecular mass, corrected migration time, and normalized signal intensity. Overall, standardized operating protocols for urine specimen collection, storage, and sample processing are needed when conducting large-scale clinical studies in order to reduce biological variability, such as azide-based preservatives to prevent bacterial growth and preanalytical proteolysis artifacts.

For clinical studies, it is essential not only to use a validated analytical workflow for the analysis of biological samples but also to repeat the same study using independent sample cohorts with adequate statistical power that can be conducted consistently across different laboratories. Until now, few studies have demonstrated the use of CE-MS for large-scale analyses of biological samples. For example, CE-MS has been used for the discovery of the urinary biomarkers for the diagnosis of kidney disease. Good *et al.* developed a CE-MS-based proteomics analytical workflow for the profiling of urinary peptides in 230 patients with CKD and in 379 healthy subjects [18]. Figure 11.3 shows typical urinary peptide patterns measured by CE-MS in human urine samples from patients with CKD and healthy controls. CE-MS analysis was performed at low pH (1% acetic acid as BGE, pH ~2) conditions using a 90 cm (50 µm i.d.) bare fused-silica capillary, which was coupled to a TOF-MS instrument via a sheath-liquid interface (using a flow rate of 200 nl $min^{-1}$, without nebulizer gas). Experimental details concerning

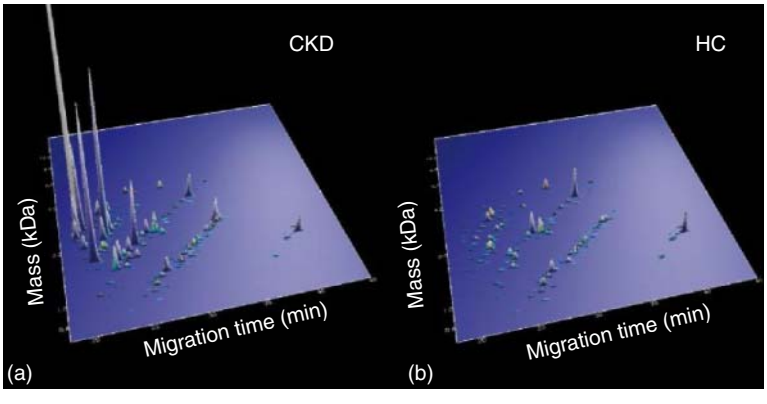


**Figure 11.3** Urinary peptide profiles distinguishing patients with chronic kidney disease (CKD) from healthy subjects. Compiled data sets of urine samples from 230 patients with CKD (a) and 379 healthy control subjects (b) are shown. Normalized molecular mass (*y*-axis) is plotted against normalized CE migration time (*x*-axis). The mean signal intensity is represented in three-dimensional depiction. CE-MS analysis was performed at low pH (1% acetic acid, pH ~2) using a 90 cm (50 µm ID) capillary, coupled to TOF MS via a sheath-liquid interface (flow rates in the range of 200 nl $min^{-1}$, without nebulizer gas) in the mass region 350–3000 *m/z*. The sample was injected for 99 s using 1 psi for injection (circa 60 nl). (Reproduced from [18] with permission from American Society for Biochemistry and Molecular Biology.)

the sample pretreatment used before CE-MS analysis are provided in Section 11.2.1. In total, 273 peptides were found as potential biomarkers for the detection of CKD using a Support Vector Machine model, and for the correlation analysis of each peptide biomarker, a Spearman's rank correlation coefficient was used. To evaluate whether this set of 273 peptides could be used as a classifier for CKD, a validation study was performed using an independent cohort of 144 human urine samples, revealing a sensitivity of 85% (95% CI, 77.5–91.4) and a specificity of 100% (95% CI, 89.6–100.0). Zurbig *et al.* evaluated the applicability of this panel of 273 peptides as classifier for CKD (CKD273) for the early prognosis of diabetic nephropathy (DN) in a longitudinal cohort of 16 patients with type 1 diabetes and 19 patients with type 2 diabetes [46]. In a total of 316 baseline and follow-up urine samples, CKD273 predicted macroalbuminuria 3 to 5 years before the clinical onset during follow-up with an AUC of 0.93 (statistical significance was assumed at $p < 0.05$), as compared to using the urinary albumin secretion rate procedure, which provided an AUC of only 0.67. Overall, the findings from these studies resulted in the initiation of the PRIORITY trail in which urine samples involving more than 3000 diabetic patients at risk of developing DN will be evaluated by using the CE-MS-based urinary proteomics approach. This work represents the first study demonstrating the potential of CE-MS for clinical proteomics in a large multicentric interventional trial. For a comprehensive overview of CE-MS-based clinical proteomics studies with emphasis on the characterization of urinary peptides, we refer to the work of Pejchinovski *et al*. [17].

Until now, the clinical utility of CE-MS in the field of proteomics has only been shown by a selected number of groups. However, the long-term robustness of CE-MS platforms employing new sheathless/low flow interface designs still needs to be further demonstrated for clinical proteomics studies. At present, no single vendor provides a complete work package and full service support for CE-MS instrumental methods unlike chromatographic-based MS techniques relying on reversed-phase, hydrophilic interaction, and aqueous normal-phase column technologies. Moreover, research groups employing CE-MS often use *in-house* developed procedures for migration time alignment, peak picking, and normalization of the data. In this context, the availability of a general, freely accessible software tools for the preprocessing of CE-MS data is also highly needed from a viewpoint of interlaboratory validation studies and round-robin trials for proficiency testing using standardized operating protocols, which has been lacking to date. Support from vendors in the development of such tools would strongly facilitate this process. Recently, an interlaboratory validation study comprising an international team of 13 laboratories from academia and biopharmaceutical companies was formed to evaluate the reproducibility and robustness of a standardized CE-MS protocol for peptide mapping of a BSA tryptic digest [47]. Migration time, peak height, and peak area of 10 representative target peptides of trypsin-digested bovine serum albumin were determined by every laboratory on two consecutive days. For relative migration time, the repeatability was between 0.05% and 0.18% RSD and the reproducibility between

0.14% and 1.3% RSD. For relative peak area, the repeatability and reproducibility values obtained were 3–12% and 9–29% RSD, respectively, demonstrating that CE-MS is adequately robust to enable a method transfer across multiple laboratories for peptide mapping studies despite using different instrumental configurations, including mass analyzers and ion sources.

### 11.2.4 Comparison of CE-MS with Other Techniques

In this section, several recent studies are highlighted in which CE-MS has been compared to other MS-based techniques for proteomics studies. The group of Lindner compared the performance of a sheathless CE-MS method employing a porous tip interface with nano-RPLC-MS by analyzing Arg-C-digested rat testis linker histones [34]. When the same sample amount was applied to both analytical methods, more peptides were detected by sheathless CE-MS as compared to nano-RPLC-MS. The number of identified peptides obtained by sheathless CE-MS and nano-RPLC-MS was evaluated as a function of their molecular mass (Figure 11.4), which clearly indicated that lower molecular mass/hydrophilic peptides were identified by sheathless CE-MS than by

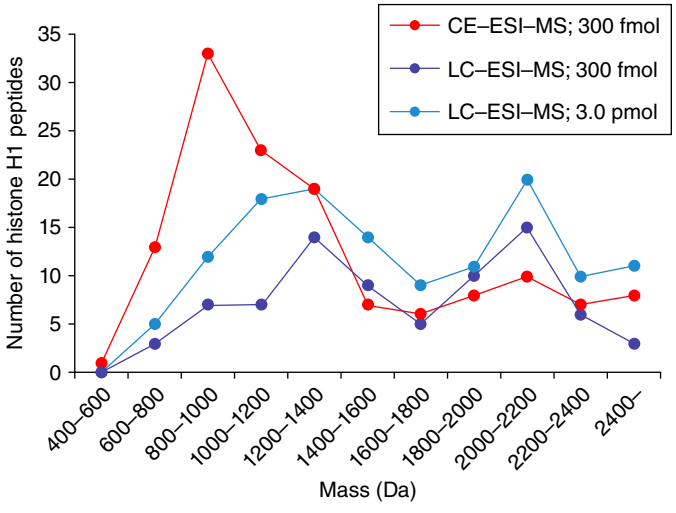


**Figure 11.4** Mass distribution of histone H1 peptides identified from perchloric acid extracted from rat testis digest using CE-MS and nano-RPLC-MS. CE conditions: M7C4I-coated capillary (length, 100 cm with porous tip; i.d., 30 µm); BGE, 0.1% (v/v) formic acid; separation voltage, −25 kV; nano-RPLC-MS was performed with a homemade fritless column: packed 10 cm with 3 µm reversed-phase C18 (Reprosil). The gradient (solvent A, 0.1% formic acid; solvent B, 0.1% formic acid in 85% acetonitrile) started at 4% B. The concentration of solvent B was increased linearly from 4% to 50% during 50 min and from 50% to 100% during 5 min. A flow rate of 250 nl $min^{-1}$ was applied. (Reproduced from [34] with permission from American Chemical Society.)

nano-RPLC-MS due to their poorer retention. For example, more than 60% of the peptides identified by sheathless CE-MS had a mass below 1400 Da, thereby showing the complementary nature of CE-MS for peptide profiling. Mullen *et al.* also evaluated the performance of CE-MS using a coaxial sheath-liquid interface and of nano-RPLC-MS for the profiling of native peptides in human urine [48]. Both techniques provided comparable results in terms of the number of peptides detected. However, CE-MS offered better resolution in the mass range between 1000 and 2000 Da, whereas nano-RPLC-MS was more favorable for peptides between 2000 and 3000 Da. Moreover, CE-MS could detect more peptides with a mass below 1000 Da since they are prone to ionization suppression when coeluting in the void volume in nano-RPLC-MS. A similar finding was reported by the Lindner group [34]. An additional advantage reported for CE-MS was the absence of sample carryover effects.

Recently, Wang *et al*. developed an on-line micro solid-phase extraction (SPE) CE-MS system using a sheathless porous tip interface for the analysis of digests of complex protein mixtures [49]. The micro-SPE column was used to both concentrate samples and perform a multistep elution to improve separation performance prior to on-line preconcentration via transient ITP (tITP)-CE-MS/MS analysis. This integrated and highly sensitive hybrid analytical platform was optimized and evaluated for the analysis of complex proteomic mixtures in comparison to both conventional nano-RPLC-MS/MS results over the same analysis time and CE-MS/MS using conventional small-volume sample injections without on-line SPE workup and tITP preconcentration. Complementary peptide and protein identifications were found with larger quantities (100 ng) of a *Pyrococcus furiosus* (Pfu) tryptic digest; however, with mass-limited amounts (5 ng), CE was three times more effective at identifying proteins (Figure 11.5). Sun *et al.* developed a CE-MS method that provided a wide separation window (up to 90 min) and a high peak capacity (~300) for the analysis of complex proteome digests when coupled to an Orbitrap Fusion MS via an electrokinetically pumped sheath-flow interface [50]. CE-MS/MS analysis of a HeLa cell proteome digest in approximately 100 min resulted in the identification of more than 10 000 peptides and 2100 proteins, which was nearly an order of magnitude better as compared to data obtained with previous CE-MS methods developed by the same group. The same sample was also analyzed by nano-RPLC-MS, which generated more than 40 000 peptides and 5000 proteins. Actually, 70% of the peptides observed by CE-MS could also be detected by nano-RPLC-MS. Molin *et al.* evaluated the performance of CE-MS and MALDI-MS for the detection of CKD in a cohort of 137 urine samples (62 cases and 75 controls) [51]. On the basis of a mass deviation of <50 ppm (a linear regression algorithm was applied for *m/z* recalibration using reference standards), 12 identical peptides were found by both techniques, which were significantly different between cases and controls. In this case, CE-MS provided better separation performance in terms of peptide resolution with better disease prediction accuracy rates. The authors recommended to use

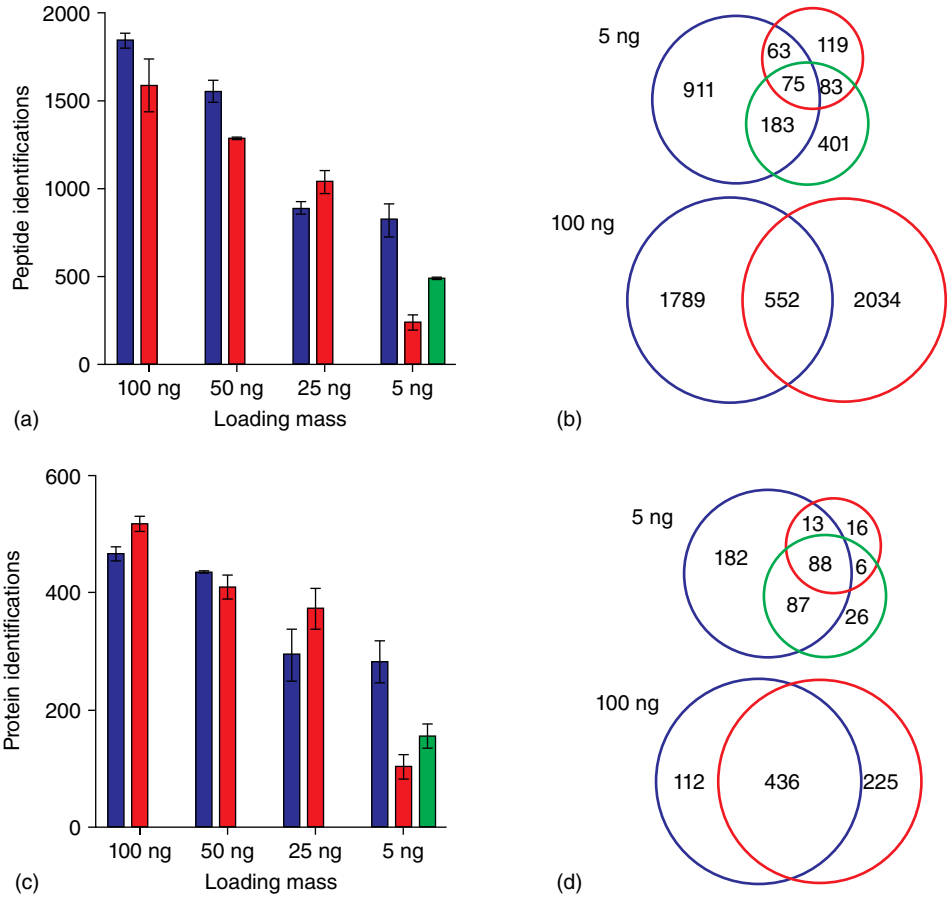


**Figure 11.5** Peptide (a) and protein (c) identification sensitivity comparison of micro-SPE-CE-MS/MS (blue), direct injection CE-MS/MS (green), and nano-LC-MS/MS (red) of *Pyrococcus furiosus* (Pfu) tryptic digests run in duplicate. Venn comparisons of identified peptides (b) and proteins (d) from combined duplicate runs of 5 and 100 ng of *Pfu* digests using micro-SPE-tITP-CE and nano-LC. (Reproduced from [49] with permission from American Chemical Society.)

MALDI-MS as a primary screening approach for CKD, whereas CE-MS for those patients that scored negative for CKD in the MALDI-MS analysis, thereby reducing the overall costs and time of such a program.

On the basis of the results of the comparative studies, it can be concluded that CE-MS is highly complementary to nano-RPLC-MS for proteomics studies. Therefore, for proteomic-based studies, CE-MS offers an orthogonal separation platform to nano-RPLC-MS, which is needed for expanding the overall peptide coverage as required for biomarker discovery.

## 11.3 Clinical Metabolomics

Until now, CE-MS has only been used by a limited number of groups for metabolomics with the majority of the studies reported by Soga and coworkers [4, 52–59]. Over the past few years, significant advancements have been made in CE-MS approaches for metabolic profiling studies that address major technical limitations, including sensitivity/metabolome coverage, sample throughput, data preprocessing, and unknown metabolite identification [60–63]. In this section, attention is paid to recent CE-MS strategies developed for clinical metabolomics studies.

### 11.3.1 CE-MS Strategies for Clinical Metabolomics

The Human Metabolome Database currently contains more than 40 000 metabolite entries, of which a major part is comprised of lipids and extrinsic solutes derived from food/diet. As a result, multiple separation modes are needed in MS-based metabolomic studies in order to achieve adequate selectivity for resolution of different classes of metabolites in complex biological samples [4, 64]. Thus, efficient sample pretreatment techniques in conjunction with complementary instrumental platforms are required to characterize the human metabolome [65]. In most cases, sample pretreatment strategies used in CE-MS-based metabolomics encompass sample dilution, solvent extraction, ultrafiltration, and/or SPE [53, 61, 66]. Optimization of sample pretreatment for CE-MS is key for obtaining reliable metabolic profiles in biological samples [66]. Although analysis of charged lipids is feasible by CE-MS when using a nonaqueous BGE , the vast majority of reports to date have focused on analysis of polar/ionic metabolites using an aqueous buffer system that may include small amount of organic solvent modifier (5–10% v/v). Overall, CE-MS is ideal for diverse classes of highly polar metabolites (e.g., organic acids, nucleotides, peptides) and their intact conjugates (e.g., glycine, sulfate, or glucuronide) that are poorly retained by conventional reversed-phase LC-MS or undergo excessive band broadening in HILIC separations.

In general, the major aim in metabolomics is to analyze as many (endogenous) molecular features as possible in a given biological sample with adequate metabolic coverage. However, this is not necessarily required in order to obtain insights into biomedical or clinical problems. For example, Christians *et al*. [67] suggested that screening for changes in selected metabolic pathways using a set of validated and quantitative analytical platforms represents a more effective approach for biomarker discovery than a global metabolomics strategy, which is subject to bias due to complicated and time-consuming time alignment and peak-picking algorithms, as well as data overfitting when using multivariate statistical methods. Indeed, a large majority of detected molecular features generated during spray formation in ESI-MS represent artifact signals, background adducts, and in-source fragments that can contribute to false discoveries if original data is not

rigorously filtered [68]. In biology-driven/targeted CE-MS-based metabolomics, sample pretreatment can be directed to the metabolites of interest and internal standards can be used for the reliable quantification of metabolites [69, 70]. Until now, most CE-MS-based metabolomics studies have been carried out with a coaxial sheath-liquid interface [60, 61]. In general, low-pH (~2) and high-pH (~9) BGE conditions in conjunction with ESI (+) and ESI (−) are used for the analysis of cationic/zwitterionic and anionic metabolites, respectively [52, 63]. For anionic profiling, Soga *et al.* employed a capillary coated with a triple layer of polybrene–dextran sulfate–polybrene (PB-DS-PB) using 50 mM ammonium acetate (pH 8.5) as BGE under reverse-polarity conditions [71]. However, a recent cross-platform comparison for primary polar metabolites involving GC-MS, LC-MS, and CE-MS highlighted that the triple-coating procedure under anionic conditions was unreliable and complicated [72]. Shortly after the publication of this paper, Soga *et al.* showed that replacing stainless steel with platinum as ESI spray needle greatly improved the method performance and long-term stability when employing CE with cationic-coated coatings under reversed polarity for anionic metabolic profiling [36]. However, a platinum needle is not required for anionic metabolic profiling under normal-polarity CE conditions since electrolytic corrosion at the anode is avoided. Various studies also reported that analyte ion responses in CE-MS using negative ionization are often relatively low [73, 74]. To improve the ionization efficiency of anionic metabolites, Kok *et al.* evaluated various BGE and sheath-liquid compositions using normal polarity CE [75]. The inclusion of triethylamine (TEA) in the BGE and sheath liquid, and the exclusion of ammonium acetate, appeared to be an effective way to enhance metabolite responses in CE with MS detection in negative ionization mode. Comparisons with common negative ionization CE-MS methods for metabolic profiling, which employ ammonium acetate in the BGE and sheath liquid, demonstrated that the metabolic coverage of human urine was increased using TEA as a BGE, revealing more than twice the number of molecular features. However, use of TEA as a buffer can be problematic as the ion-pair reagent can contribute to major ion suppression or enhancement effects when operating the same instrument under positive ion conditions without extensive cleaning of ion source and capillary inlet to mass spectrometry. Recently, alkaline ammonia-based buffers (pH >9) have been shown to react with polyimide outer coatings used to impart flexibility to fused-silica capillaries, which contribute to frequent capillary fractures and poor long-term performance often reported in CE-MS. In this case, aminolysis of polyimide is prevented by the use of weakly alkaline ammonia buffers (pH <9), frequent rinsing of capillary in stable buffer systems between runs (e.g., 1 M formic acid), and use of alternative buffers or chemically more resistant capillary coatings in CE-MS. As a result, careful optimization and rigorous validation of alkaline buffer conditions are needed in order to ensure reproducible yet robust performance for anionic metabolite profiling in diverse biological specimens across different laboratories.

Over the past few years, the benefits of using new ion source interface designs for CE-MS in metabolomics research have been examined, such as the

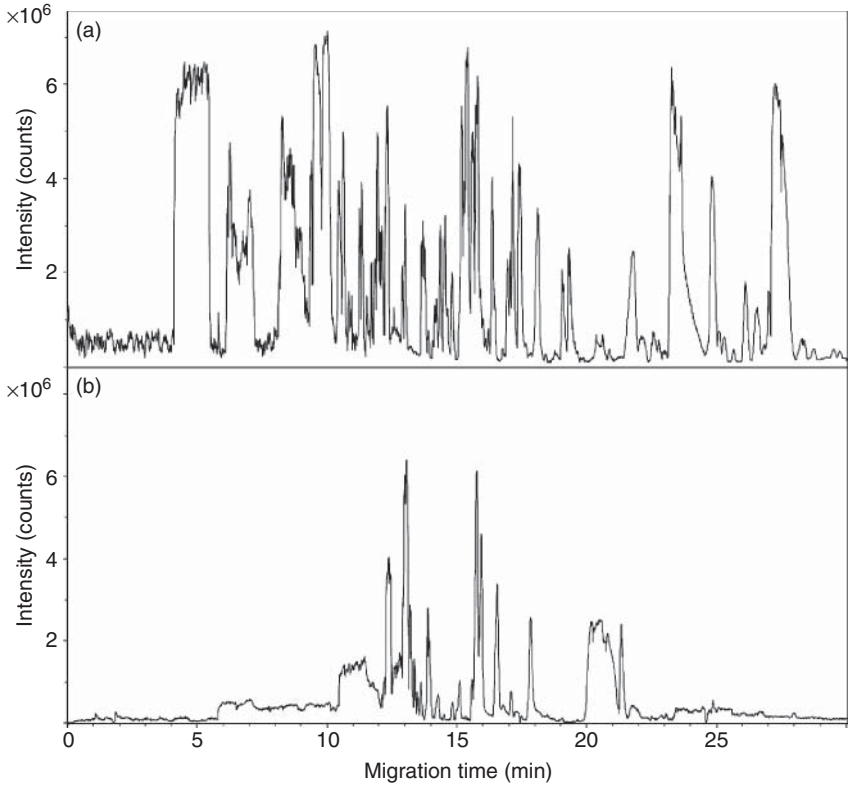


**Figure 11.6** (a) Base peak electropherogram (*m/z* 50–450) of human urine obtained with sheathless CE-MS using a porous tip sprayer. Conditions: BGE, 10% acetic acid (pH 2.2); sample injection, 2.0 psi for 30 s (1% of capillary volume). (b) Base peak electropherogram (*m/z* 50–450) of human urine obtained with CE-MS using a sheath-liquid interface. Conditions: BGE, 10% acetic acid (pH 2.2); sample injection, 0.5 psi for 30 s (1% of capillary volume). (Reproduced from [76] with permission from American Chemical Society.)

sheathless porous tip interface and the flow-through microvial interface [76, 77]. For example, more compounds were detected in human urine when employing CE-MS with a low flow, sheathless porous tip interface as compared to CE-MS based on a coaxial sheath-liquid interface (Figure 11.6), resulting in nanomolar detection limits for urinary metabolites and a threefold increase in detected molecular features [76]. However, the long-term performance of these new CE-MS interfaces needs to be evaluated in more extended studies analyzing large numbers of diverse clinical samples. For instance, control charts derived from intermittent analyses of pooled urine samples as quality controls (RSD = 11%, $n = 87$) provide a good indicator for reliable population-based nutritional iodine status determination without system bias (Figure 11.7) when using CE with UV detection for 800 × 24 h urine specimens over 5 weeks of continuous analysis [78].

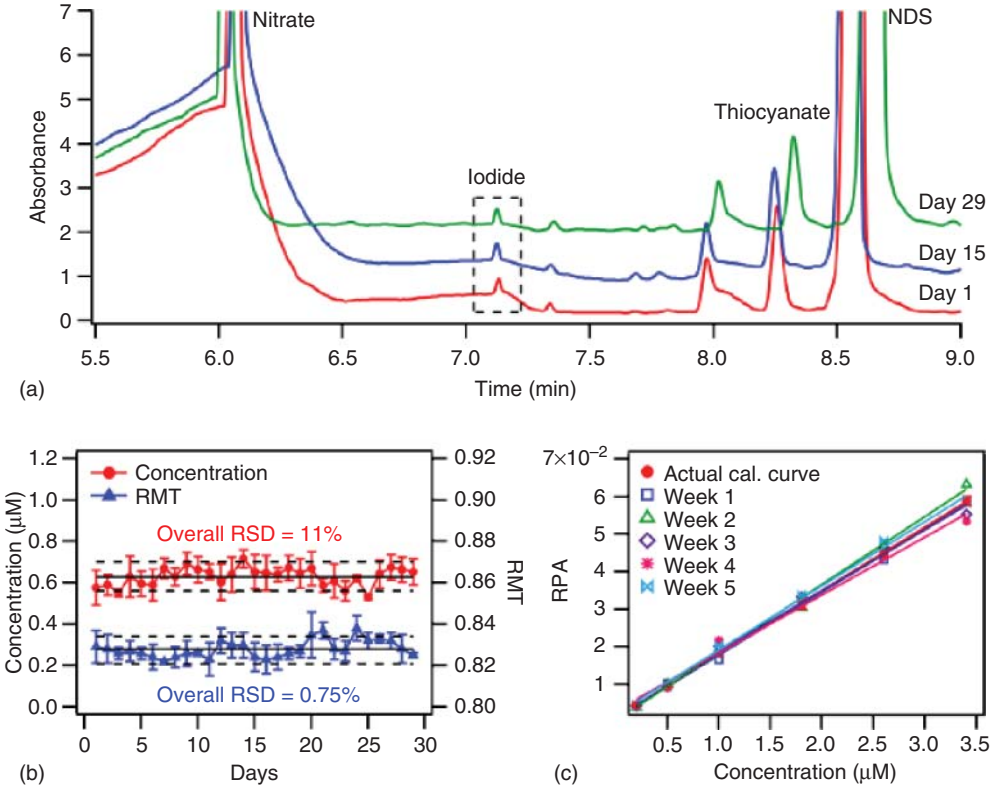


**Figure 11.7** (a) Representative electropherograms of pooled 24 h urine samples as quality controls analyzed intermittently over 29 days to demonstrate method robustness for population-based iodine nutrition assessment for large-scale epidemiological studies. (b) Control chart summarizing intermediate precision performance for CE method based on measurement of urinary iodide concentration (μM) and relative migration time (RMT) with an overall variance of 11% and 0.75% ($n = 87$), respectively. (c) Reliable quantitative performance of CE also demonstrated by external calibration curves derived from analyzing iodide calibrants with an internal standard over 5 weeks with consistent sensitivity relative to original calibration curve performed at the start of the study. (Reproduced from [78] with permission from American Chemical Society.)

In this case, on-line preconcentration with sample self-stacking enabled direct analysis of dilute urine samples for sub-micromolar detection of urinary iodide in large-scale epidemiological studies to assess population-based risk for iodine deficiency based on criteria from the World Health Organization. Similarly, daily and weekly maintenance protocols (e.g., daily electrode assembly cleaning to minimize sample carryover) while operating under standardized operating conditions are critical for ensuring robust performance when using CE-MS for global metabolic profiling of complex biological samples. A similar approach has been demonstrated for quantitative yet robust analysis of chloride, sulfate, and/or

sulfite content in volume-restricted biological fluids as required for clinical diagnostic applications, such as pilocarpine-stimulated sweat specimens for confirmatory testing of screen-positive infants with cystic fibrosis [79]. However, until now, this approach to method validation and robustness testing has been rarely performed in CE-MS-based metabolomics studies to date. This is particularly relevant when assessing the utility of the new CE-MS interface designs for increasing concentration sensitivity and expanding metabolic coverage, which must also ensure adequate robustness as needed in large-scale epidemiological or clinical applications.

The development of robust, sensitive, high-throughput, and low-cost analytical technologies is of utmost importance for metabolic profiling in longitudinal studies with clinically relevant biochemical coverage. In most cases, separations represent a major bottleneck to high-throughput MS-based metabolomics studies due to the time requirements for sample elution and column reconditioning, as well as considerable efforts at time alignment and peak picking during data preprocessing of large numbers of samples generated from the same instrument as well as across different platforms operated in parallel. In this context, Kuehnbaum *et al.* recently developed a multiplexed separation platform based on multisegment injection (MSI) CE-MS method for untargeted

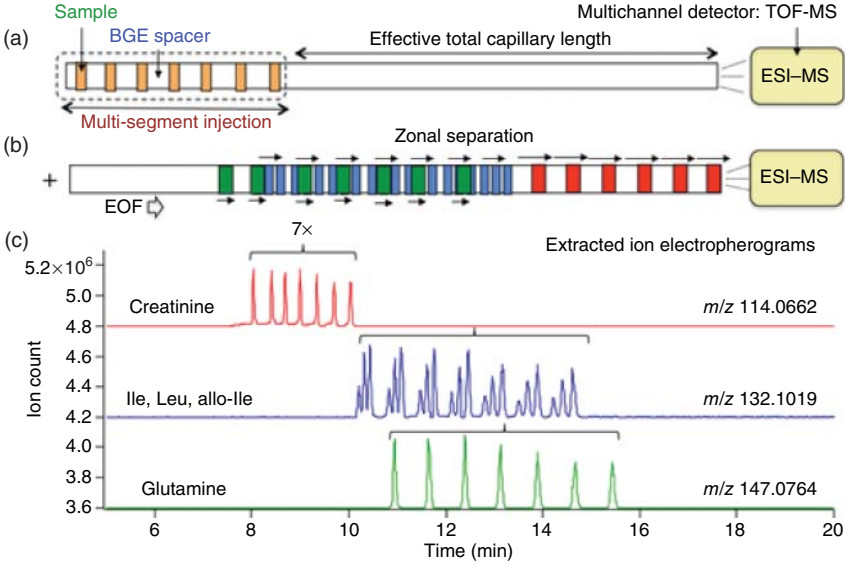


**Figure 11.8** (a) Multiplexed separation based on serial injection of seven discrete sample segments within a single capillary by MSI-CE-MS; (b) ions migrate as a series of zones in free solution prior to ionization; (c) the procedure enables reliable quantification of polar metabolites and their isomers in different samples as ionization occurs within a short-time interval (≈2–6 min) under steady-state conditions with fast data acquisition by TOF-MS. (Reproduced from [80] with permission from American Chemical Society.)

metabolite profiling, which increased sample throughput up to one order of magnitude without loss in separation performance [80]. A serial hydrodynamic injection of seven or more discrete sample segments within a single capillary enabled isomeric resolution without ion suppression when using a single TOF-MS mass analyzer with fast data acquisition (Figure 11.8). MSI-CE-MS thus allows for a higher effective sample throughput (≈3–4 min/sample) without complicated hardware modifications or expensive infrastructure investment. However, rigorous optimization of the serial injection procedure was critical as shorter buffer spacer plugs introduced in between sample segments resulted in loss of resolution of metabolites and their isomers between adjacent sample segments. Overall, an acceptable precision was obtained for the quantification of selected polar metabolites in human plasma filtrates (RSD ≈ 10%, $n = 70$). Moreover, MSI-CE-MS facilitates data preprocessing in metabolomics based on signal pattern recognition of temporally resolved ion responses. The authors also introduced an accelerated data workflow for biomarker discovery when using multiplexed separations by using a dilution trend filter on a pooled quality control sample to exclude redundant ions and background signals prior to univariate or multivariate data analysis (Figure 11.9) [81]. In this way, reliable molecular features common to most biological samples are selected for subsequent data analysis, provided that they satisfy three major criteria, namely ion responses have adequate precision (RSD < 40%, $n = 3$), signals do not originate from a blank sample, and ion signals exhibit a linear response change ($R^2 > 0.900$) upon serial dilution that is dependent on their natural abundance. As a result, a list of authentic yet reproducible ions (i.e., known or unknown metabolites) defined by their characteristic accurate mass and relative migration time (*m/z*:RMT) is generated after untargeted metabolite profiling of a representative pooled sample that is subsequently used for targeted metabolite profiling of individual samples from a cohort in a clinical study. Unlike conventional MS-based data workflows in metabolomics, this approach avoids time-consuming data preprocessing operations involving large numbers of individual samples that are subject to systematic bias when comparing different time alignment and peak-picking algorithms used in various commercial and open-access software [82].

In CE-MS-based metabolomics studies, basically all types of MS analyzers have been hyphenated to CE, that is, single quadrupole, ion trap, triple quadrupole, time-of-flight (TOF), quadrupole time-of-flight (Q-TOF), and Fourier transform–ion cyclotron resonance (FT-ICR) [61]. For biology-driven/targeted metabolomics studies, single-quadrupole and triple-quadrupole instruments are often used, especially the latter can provide a very high sensitivity and selectivity when operated in selective reaction monitoring mode. TOF instruments provide a high resolution and mass accuracy with errors below 5 ppm (or even lower when using internal calibration), and due to their intrinsic high spectral acquisition rate, these instruments are fully compatible with relatively fast and highly efficient CE separations. Moreover, TOF instruments allow for the determination of the elemental compositions of compounds on the basis

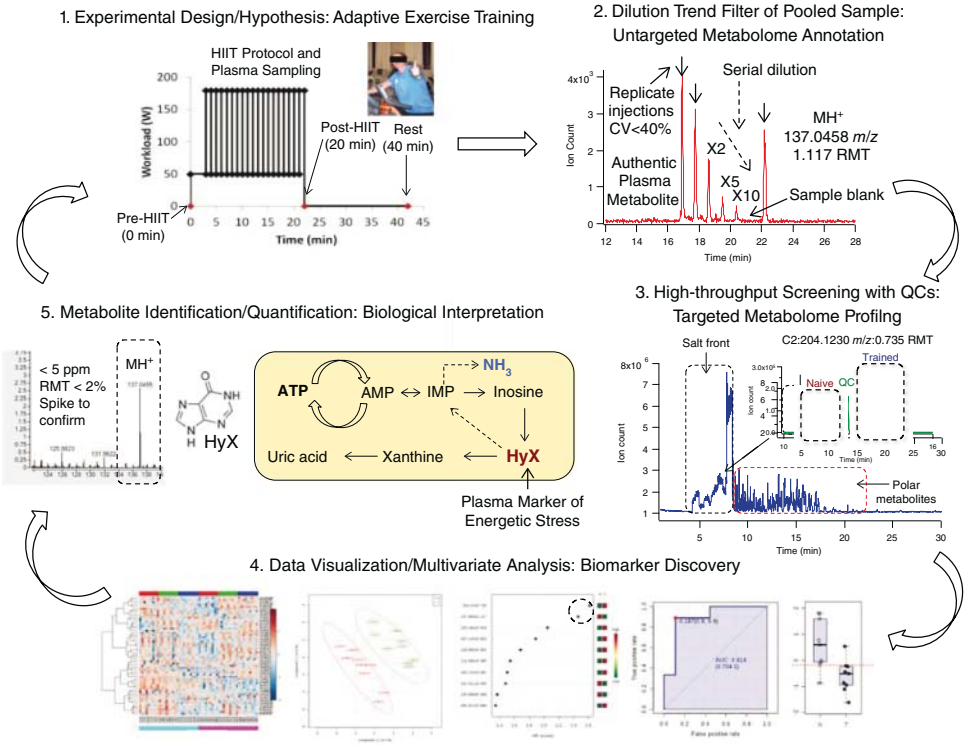


**Figure 11.9** An accelerated workflow in metabolomics for biomarker discovery that takes advantage of multiplexed separations by MSI-CE-MS, such as a seven-segment format captures dynamic metabolomic responses to strenuous exercise for individual subjects, as well as their adaptive responses to exercise training. Unlike conventional MS-based data workflows, a single dilution trend filter of a pooled QC sample is used as a primary screen to certify reproducible plasma-derived molecular features while rejecting signal artifacts based on signal pattern recognition. This allows for targeted analysis of authentic metabolites from individual samples with high data fidelity that avoids data overfitting and false discoveries when using multivariate statistical methods. Unambiguous identification and quantification of lead plasma markers (HyX) and their associated metabolic pathway (purine degradation) provide deeper insight into exercise responsiveness that differ between subjects. (Reproduced from [81] with permission from Wiley.)

of their isotopic patterns, thereby providing an additional analytical dimension for compound identification. For these reasons, TOF instruments have often been used in CE-MS-based metabolomics studies. Q-TOF instruments can provide accurate mass information for product ions, which is very useful for the identification of (unknown) compounds. FT-ICR instruments provide a very high mass resolution (>1 000 000) and the most accurate mass measurements (<1 ppm); however, the inherently slow scan speed and high costs of this analyzer have limited its use in CE-MS-based metabolomics studies.

### 11.3.2 Data Analysis and Clinical Validation

In general, CE-MS metabolic profiling studies provide relatively large and complex data sets. Baseline correction, data normalization, and migration time alignment, as well as detection of significant differences between metabolic profiles using multivariate data analysis, can be performed using similar procedures as developed for LC-MS. However, for CE-MS, tailor-made procedures are required due to the relatively large variation in migration times between runs due to differences in the EOF as compared to reversed-phase LC-MS. Also, appropriate software tools are needed to accommodate ion peak dispersion/symmetry characteristic to CE. For instance, electrophoretic mobility as opposed to migration time is an intrinsic physicochemical property of a solute relevant to CE separations that can be measured with better precision when using a neutral EOF marker. In fact, changes in viscosity-corrected electrophoretic mobility under isothermal conditions has long been used for reliable determination of apparent binding constants involving biomolecular interactions in free solution in affinity CE applications [83]. Alternatively, the use of dynamic coatings to the capillary wall has been shown to provide better long-term migration time stability notably when analyzing highly saline biological samples that vary considerable in ionic strength [54, 73]. Similarly, determination of relative migration times (RMTs) using one or more internal standards that are needed to correct for variations in sample injection volume for improved quantitative performance can also be used to reduce migration time variability with RSD below 1% [78]. Alternatively, a genetic algorithm designed for alignment of CE-MS data using accurate mass information was developed [84]. The usefulness of this algorithm was shown for urine samples of mice analyzed by CE-MS. RSDs for migration time were calculated for three peaks at the beginning, middle, and end of the electropherogram and ranged from 5.6% to 6.5% before alignment. After application of the alignment procedure, the RSDs significantly improved and ranged from 0.12% to 0.99%. Although the new algorithm significantly reduced migration time variation in the aligned datasets notably when using ultrahigh-resolution TOF-MS instrument, the applicability was only shown for a limited number of urine samples. Sugimoto *et al.* also developed a software tool for processing metabolomic data when using CE-MS, named Java application for Differential Analysis of Metabolite Profiles (JDAMP), which allows users to identify the metabolites that vary between two groups [44]. Main features include baseline subtraction, data normalization and alignment, visualization on 2D plots ($m/z$ and time axis) with matching metabolite standards, and detection of significant differences between metabolite profiles. The availability of an open-access software package for standardized data preprocessing across different vendor platforms is urgently needed to expand interest in CE-MS-based metabolomics studies.

A major obstacle in metabolomics for biomarker discovery is the identification of a large fraction of unknown yet clinically relevant metabolites in complex biological samples when purified standards and MS spectral database entries

are unavailable. In CE-MS, metabolites are generally identified by matching their (relative) migration times and *m/z* values with those of authentic metabolite standards. The lack of commercially available metabolite standards often prevents unambiguous identification of molecular features. In LC-MS-based metabolomics, fractionation steps may be employed to facilitate the compound identification process using NMR; however, such an approach is difficult to apply in CE-MS due to small injection volumes. However, a major advantage of CE is the ability to predict ion electromigration behavior in free solution based on their physicochemical properties derived from a chemical structure. To facilitate metabolite identification, Sugimoto *et al.* recently developed a mathematical model using support vector regression to identify unknown peaks based on the predicted migration time and accurate *m/z* values [85]. The model provided good correlations between the predicted and measured migration times for 375 cationic metabolite standards. Inclusion of the predicted migration time significantly reduced the number of putative isobaric/isomeric chemical structures for a defined molecular formula. The group of Britz-McKibbin modeled the migration behavior of polar/ionic metabolites in CE as a complementary qualitative tool to support MS characterization based on molecular volume, valence charge, and weak acid dissociation constant ($pK_a$) [56]. Computer simulations using Simul 5.0 were used to better understand the dynamics of analyte electromigration, as well as aiding *de novo* identification of unknown compounds. Overall, there was a good agreement between computer-simulated and experimental electropherograms for several classes of cationic metabolites as reflected by their RMT with an average error of <2.0%.

A unique advantage of CE-MS is that high-efficiency separations of polar/ionic metabolites are achieved with effective desalting of complex biological samples; thus, solute ionization occurs under steady-state conditions without matrix-induced ion suppression/enhancement effects. As a result, accurate quantification of metabolites can be realized without stable-isotope-labeled internal standards as required for LC-MS with gradient elution. In some cases, precolumn chemical derivatization can be used to boost concentration sensitivity in CE-MS for weakly ionizable and/or labile classes of metabolites, such as reduced thiols [86]. However, it is crucial to implement a quality assurance practice that includes pooled samples as quality controls in order to assess system stability when analyzing randomized samples during large-scale clinical studies [57, 80, 87]. However, to accelerate biomarker discovery and its successful translation into clinical practice, metabolomic studies need to integrate complementary "omics" data that is anchored to relevant physiological and clinical outcomes. Also, a single, ratiometric, or panel of biomarkers developed for population-based screening, diagnostic testing, or monitoring treatment responses to intervention must demonstrate acceptable positive predictive value and cost-effectiveness to improve patient care relative to current practices or methods. Accurate biomarker quantification along with its intrinsic chemical stability (i.e., impact of sample storage/collection) and natural distribution in unaffected/healthy control relative to an affected/disease population also requires rigorous validation with adequate

statistical power. As outlined earlier, metabolite identification is still a major challenge in metabolomics studies that is absolutely required for interpretation of the biological significance and clinical relevance of aberrant metabolism in human health [88].

### 11.3.3 Comparison of CE-MS with Other Techniques

In this section, a few recent studies are highlighted in which CE-MS has been compared to other MS-based techniques for metabolomics studies. For an overview of reported studies in which CE-MS was used in conjunction with direct MS, LC-MS, and/or GC-MS for the analysis of metabolites, we refer to the work of Kok *et al.* [89]. Ibanez *et al.* compared RPLC-MS, HILIC-MS, and CE-MS for metabolic profiling of colon-cancer cells using different MS instruments, yielding, after filtering out redundant responses from the same ion (i.e., isotopes, adducts, fragment ions, etc.), 2176, 1077, and 2890 molecular features, respectively [90]. For HILIC-MS analyses, samples were diluted, and as a result, a lower number of molecular features was observed. In total, 32, 12, and 22 compounds were identified with respectively RPLC-MS, HILIC-MS, and CE-MS, which were significantly different ($p < 0.05$) in colon-cancer cells treated with dietary polyphenols. Of these compounds, six were detected by both CE-MS and RPLC-MS and two with HILIC-MS and RPLC-MS. There was no overlap in differentially expressed compounds associated in colon-cancer cells detected by CE-MS and HILIC-MS. Ramautar *et al.* used CE-MS and RPLC-MS to study gender differences in metabolic profiles in human urine [91]. Using multivariate data analysis, males and females could be distinguished. Ten times more discriminatory compounds were observed with RPLC-MS as compared to CE–MS (i.e., 300 vs. 27). The metabolites identified with CE-MS coeluted in the void volume when analyzed with RPLC-MS. Moreover, with CE-MS, ions were detected with lower masses mainly observed in the range of 100–150 *m/z*, whereas with RPLC-MS, 95% of the compounds had masses above 150 *m/z*. Naz *et al*. used CE-MS, GC-MS, and RPLC-MS for global metabolic profiling of lung tissue extracts from mice [92]. Significantly more compounds could be detected and identified with LC–MS (1115 metabolites) as compared to CE–MS (85 metabolites) and GC–MS (69 metabolites) (Figure 11.10). However, RPLC–MS was employed both in positive and negative ionization modes, while CE–MS and GC–MS were only used in positive ESI mode. RPLC-MS and GC-MS yielded the greatest overlap in detected compounds (21 metabolites), followed by the combined use of CE-MS and RPLC-MS (20 metabolites). Common metabolites detected with GC-MS and RPLC-MS were mainly fatty acids, sterols and sugars, while most amino acids were found with both CE-MS and RPLC-MS. Only seven compounds were detected with all three analytical technologies. Kok *et al.* recently compared a developed CE-MS method for anionic metabolic profiling in urine with HILIC-MS under negative ion mode conditions [93, 94]. Overall, peak area RSDs were similar; however, HILIC-MS provided better retention

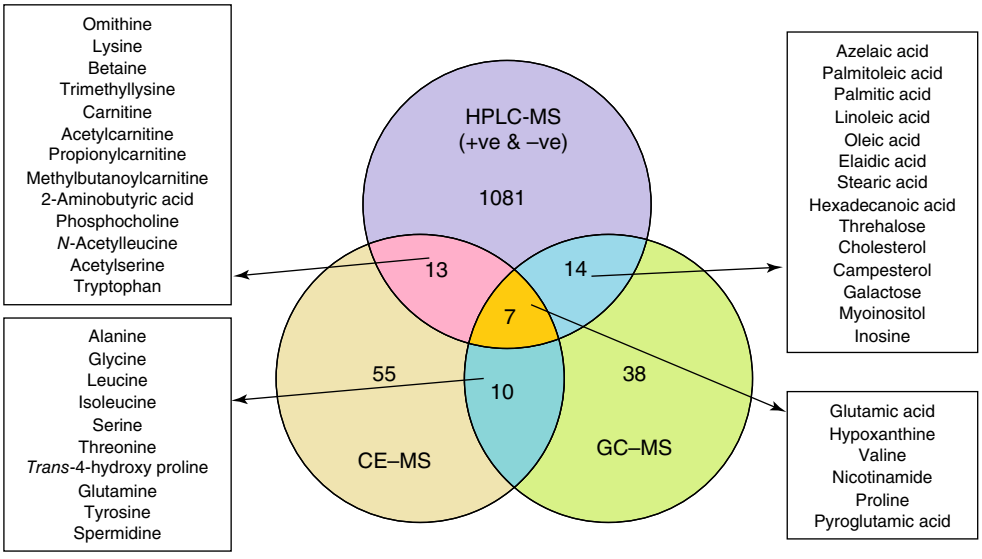


**Figure 11.10** Venn diagram showing the number of identified molecular features detected by RPLC-MS, GC-MS, and CE-MS in a pooled extract of a mouse lung. For experimental details see [86]. (Reproduced from [92] with permission from American Chemical Society.)

time repeatability and up to 80-fold lower detection limit for test metabolites as compared to CE-MS. Metabolic profiling of rat urine by HILIC-MS resulted in the detection of 1360 molecular features, whereas 347 molecular features could be detected by CE-MS. The HILIC retention and CE migration times of the 144 common molecular features were not correlated. Although significant more molecular features could be detected by HILIC-MS, the total number of known features is low in HILIC-MS as compared to CE-MS. The concentration sensitivity of CE-MS can be further improved and, as a result, the metabolic coverage, by employing a sheathless interface and on-line sample preconcentration techniques.

One of the major advantages of CE-MS as a microseparation platform is its low intrinsic mass sensitivity requiring only nanoliters of sample volume injected on-column that is ideal for single-cell metabolomics studies that include integrated protocols for neuron isolation, cell extraction, and metabolite analyses [95]. For example, Nemes *et al.* demonstrated metabolic differences between individually cultured neuron cell types derived from the central nervous system of Aplysia californica that were influenced by overnight culture conditions [96]. CE is also amenable to high-efficiency separations of intact cells, as well as subcellular organelles such as isolated mitochondria, which are needed to better understand cell heterogeneity relevant to aging and disease risk [97]. Further work is needed to expand single-cell metabolomics applications when using multidimensional

separations coupled to ESI-MS as a way to further enhance peak capacity and overall metabolome coverage [98]. Overall, these studies clearly demonstrate the complementarity of CE-MS in comparison to other MS-based instrumental techniques for global metabolic profiling of complex biological samples, including unique applications in single-cell and organelle-specific metabolomics studies.

## 11.4 Conclusions and Perspectives

Over the past few years, CE-MS has gained increased attention for proteomics and metabolomics studies, especially with the development of new interface designs to enhance concentration sensitivity. Moreover, there is growing interest in applying multiple/orthogonal separation platforms in order to increase the coverage in both proteomics and metabolomics studies notably for polar/ionic metabolites and hydrophilic peptides that are not well resolved when using conventional chromatographic columns. As outlined in this chapter, various groups have clearly demonstrated several distinct advantages of CE-MS for this purpose, including minimal sample workup of complex biological samples, higher sample throughput based on serial sample injections, and prediction of ion migration behavior to assist unknown identification. Several groups have also developed complete analytical workflows for the reliable profiling of peptides and metabolites in biological samples [16, 80, 92]. However, compared to other analytical techniques, the applicability of CE-MS for large-scale clinical studies has been limited with few stringent interlaboratory validation studies using standard operating protocols and quality assurance practices to demonstrate long-term robustness. In general, the CE-MS approaches reported in this chapter offer a promising approach for clinical proteomics and metabolomics notably when used in conjunction with accelerated data workflows for biomarker discovery based on multiplexed separations. The usefulness of recent low-flow/sheathless interfacing techniques has clearly been demonstrated for improving the concentration sensitivity of CE-MS. However, the figures of merit for large-scale clinical studies still need to be determined. We anticipate that CE-MS will play a key role in those clinical and biomedical studies for volume- or mass-limited biological samples as well as novel applications involving single cell or subcellular metabolome characterization.

### Abbreviations

| | |
|---|---|
| BGE | background electrolyte |
| CE-MS | capillary electrophoresis – mass spectrometry |
| CKD | chronic kidney disease |
| DN | diabetic nephropathy |
| EOF | electro-osmotic flow |

| | |
|---|---|
| MEKC | micellar electrokinetic chromatography |
| PB-DS-PB | polybrene–dextran sulfate–polybrene |
| PEI | polyethyleneimine |

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