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Review

Use of on-line mass spectrometric detection in capillary electrochromatography

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Abstract

Capillary electrochromatography (CEC) is a liquid phase analytical separation technique that is generally carried out with packed capillary columns by electroosmotically driven mobile phase at high electric field strength. The analytes are separated by virtue of the differences in their distribution between the mobile and stationary phases and, if charged in their electrophoretic mobilities as well. It is thus considered a hybrid of liquid chromatography and capillary electrophoresis and is expected to combine the high peak efficiency of capillary zone electrophoresis (CZE) with the versatility and loading capacity of HPLC. This review explores the potential use of on-line mass spectrometric detection for CEC. It discusses key design issues that focus on the physical and electrical arrangement of the CEC column with respect to the electrospray orifice inlet. The salient features of the sheathless, sheath flow and liquid junction interfaces that are frequently employed while coupling a CEC column to an electrospray ionization mass spectrometry system are also highlighted. Possible configurations of the CEC column outlet that would obviate the need for pressurizing the capillary column are also presented. While coupling CEC with MS both the nature of the interface and the configuration of the column outlet will determine the optimal arrangement. The review also discusses bandspreading that occurs when a connecting tube is employed to transfer mobile phase from the column outlet to the atmospheric region of the electrospray source with a concomitant loss in sensitivity. Selected examples that highlight the potential of this technique for a wide range of applications are also presented. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Mass spectrometry; Electrochromatography; Detection, CEC; Interfaces, CEC–MS

Contents

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1. Introduction

At present there is a great deal of interest in exploring the potential of capillary electrochromatography (CEC) for the analysis of biomolecules in the pharmaceutical industry [1–16]. The high separation efficiency and loading capacity of CEC makes it an attractive separation mode for coupling with mass spectrometry (MS), which has the ability to unambiguously identify analytes with high selectivity and sensitivity. The combination of CEC and MS overcomes many of the limitations of competing technologies. Capillary zone electrophoresis (CZE) offers high efficiency separations, but when combined with electrospray ionization mass spectrometry (ESI-MS) is limited to a small number of buffer systems and suffers from low sample loading capacity. Since CZE separates only charged analytes, micellar electrokinetic chromatography (MEKC), has been developed for the separation of neutral analytes. However, being based on the use of high concentration of surfactants, MEKC has relatively poor selectivity and lacks MS-compatibility. In contrast, CEC has good MS-compatibility, selectivity, sample loading capacity and general applicability. Thus, CEC coupled to MS could fill the gap in modern liquid phase analytical separation techniques employing fused-silica capillaries.

The principal detection method used in CEC so far has been ultraviolet detection (UV) with transmission through the unpacked part of the capillary immediately following the outlet retaining frit where the polyimide coating has been removed. This poses limitations on the sensitivity of detection since the path length is restricted to only the inner diameter (50–150 μm) of the capillary column. Furthermore, the capillary columns have reduced durability due to removal of the polyimide coating and are prone to breaking at the detection window. The above two problems can be overcome by using the recently developed high-sensitivity detection cell [17,18]. The high-sensitivity detection cell has a path length of 1.2 mm and has been shown to increase signal-to-noise ratio by a factor of 8 when compared to a 100 μm I.D. capillary column [18]. In addition, the fragile portion of the capillary can now be totally eliminated since the column is terminated after the outlet retaining frit and connected to the high sensitivity cell with the help of fittings. Since, there is no fragile polyimide-free zone, the robustness of the column is greatly improved and its handling is much easier. The use of laser-induced fluorescence (LIF) detection has also been reported for the analysis of 16 polynuclear aromatic hydrocarbons by CEC with on column detection limits of 10^{-9} and 10^{-10} *M* [19]. Mass spectrometric detection has an advantage over both UV–Vis and LIF detection techniques in that it can provide both molecular mass and structural information about the analytes. Such information is of particular importance in the pharmaceutical industry where it is imperative to positively identify all eluting variants and in qualitative characterization of complex biomolecules.

The coupling of CEC and MS has largely followed the groundwork developed in coupling capillary electrophoresis and mass spectrometry [20–22]. A capillary electrochromatographic system with both voltage and pressure gradients applied across a

packed 220 μm I.D. capillary was first coupled to a mass spectrometer with a fast ion bombardment (FAB) source [23]. The coupling of such a pressure-assisted electrochromatographic system to the mass spectrometer via electrospray ionization (ESI-MS) employing a sheath flow interface was later demonstrated by the same group [24]. Similarly, pressure-assisted CEC with ESI-MS detection has been employed for the separation and detection of peptide mixtures [3,25–28].

An electrochromatographic system without pressure-driven flow was first coupled to a mass spectrometer with FAB ionization source [9]. A packed capillary column followed by a long open capillary segment was employed for the separation of steroid hormones with modest efficiency. Lane and co-workers [7,8,29,30] have also demonstrated the feasibility of coupling CEC column without pressure assistance to an ESI-MS system. The geometry of the electrospray (ES) source dictated the use of a packed capillary columns at least 90 cm long, resulting in long elution times and relatively low separation efficiencies [8]. This system was later modified to accommodate shorter column lengths [7] and subsequently also coupled to an automated sample introduction system [30]. Very recently, CEC has been coupled to ESI-MS using columns with tapered outlets [10,31] that act as restrictors to retain the packing material and also serve as the electrospray needle.

2. Design considerations

The on-line combination of CEC and ES is nontrivial and more complicated than LC–ESI-MS. In general, the key design issue in the coupling focuses on the physical and electrical arrangement of the CEC capillary outlet with respect to the electrospray inlet orifice. There are three fundamental considerations in coupling CEC with ESI-MS. An electric field is applied across the packed capillary column to generate electroosmotic flow (EOF) that is responsible for bulk flow of eluent from the column inlet to outlet. Thus, the foremost consideration in interfacing CEC with ESI-MS is that an appropriate mechanism for maintaining electrical contact at the column outlet should be provided. This electrical contact will provide the return path for both CEC column and the electrospray current flow. The second consideration is that interface should also serve as an outlet buffer reservoir for CEC in order to prevent buffer depletion. These considerations are similar to those encountered with CZE–ESI-MS. In addition to the above two requirements, however, there is an additional requirement in CEC for the need to pressurize the outlet and/or the inlet buffer reservoir to facilitate continuous operation without any electric current breakdown.

Besides the above three considerations, one problem that is faced universally by researchers coupling CEC with ESI-MS is the limitation imposed on the column length by the commercially available instruments. Most of the instruments require columns in the range of 0.5–1 m in length, which is less than ideal in CEC since the electric field strength across the capillary column is significantly reduced even at the maximum applied voltage of 30 kV. This results in linear flow velocities in the range of 0.3–0.7 mm/s and long elution times with concomitant band spreading. Several researchers have circumvented this problem by using homemade experimental apparatus. One of the major problems with the homemade modules is that the column is generally present at ambient temperature so high temperature cannot be used as an operational variable and Joule heating can become an issue.

2.1. Interfaces

Several approaches have been designed to meet the requirement of providing a return path for CEC as well as electrospray current while interfacing capillary electrochromatography with mass spectrometry. As mentioned above, these are based on legacy of developments from interfacing high-performance liquid chromatography (HPLC) or CZE with ESI-MS [20–22]. The approaches include the use of (a) sheathless interface [32–34], (b) sheath flow interface [35] and (c) liquid junction interface [36,37]. In this section the salient features of these interfaces along with their advantages and disadvantages are outlined.

2.1.1. Sheathless interface

In sheathless design [32,33] the CEC column exit

has an external taper and the outside surface is coated with gold to complete simultaneously the CEC and electrospray electrical paths (see Fig. 1a). The packed CEC column with the external taper is used directly to spray the mobile phase into the electrospray region. The external taper acts as a restrictor there by avoiding the need for a pressurized reservoir at the outlet [10]. No additional sheath liquid is employed, so there is no dilution of the analyte zone and hence sensitivity obtained with this method is higher than that with the sheath flow or liquid junction approach [22,38]. A sheath gas, such as SF_6 is often used with these interfaces to prevent corona discharge due to higher electrical field strengths present at the sharpened tips [21,22]. The lack of sheath liquid restricts the choice of buffers to those that are suitable for electrospray, i.e., volatile buffers with low surface tension and low conductivity [20,22]. The metal coating present at the sharpened tips, normally gold or silver however has a limited lifetime [20]. In addition appearance of doubly-charged adduct ions such as $[M+H+Ag]^+$, makes molecular mass determination more problematic [21]. An additional disadvantage is that since the column is used to spray the mobile phase and the analytes directly into the electrospray region, so no effective method of thermostating the CEC column has been demonstrated. However, this interface has

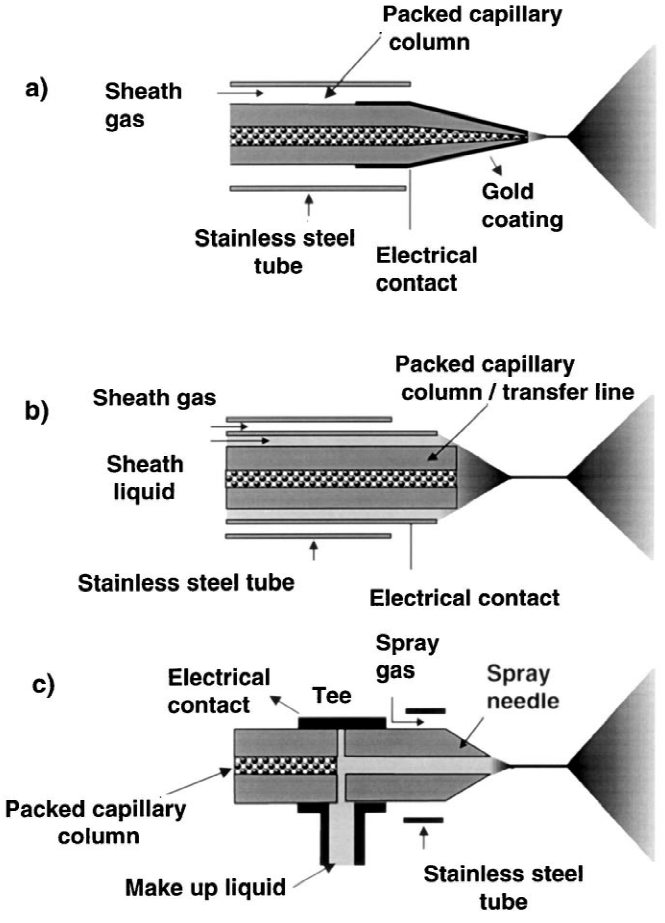

Fig. 1. Schematic illustration of different interfaces for coupling capillary electrochromatography with mass spectrometry. (a) Sheathless interface, (b) sheath flow interface, (c) liquid junction interface (adapted from Ref. [22]).

the best sensitivity since there is no extra column band broadening effect and no dilution due to the sheath liquid.

2.1.2. Sheath flow interface

The most widely used method for interfacing CEC with electrospray is the sheath flow interface [10,31,35]. In this arrangement, (shown in Fig. 1b) the packed capillary column is introduced into the atmospheric region of the electrospray ionization source through a narrow metal tube, which delivers a sheath liquid to the column exit. As the sheath liquid flows through the tube it mixes with the column effluent and forms a stable electrospray Taylor cone. The sheath liquid while flowing through the metal tube, which is at ground potential (or a fixed electrical voltage), simultaneously completes the electrical path for CEC column and the electrospray ionization. A third concentric tube may deliver a gas flow that can assist in the spray formation via nebulization and/or scavenge free electrons to prevent corona discharge especially in the negative ion mode. The use of sheath or nebulizing gas relaxes the requirements of low surface tension and ionic conductivity for the buffer. The sheath liquid is introduced at a flow-rate of a few μl/min, whereas the EOF contributes a few nl/min. In this way the electrospray process is dominated by the sheath liquid and the limitations on the CEC buffer composition may be relaxed. Sheath liquid with excellent electrospray characteristics can be chosen to optimize post-column solution chemistry and improve ionization efficiency. The physical area where the CEC and sheath liquid combine and mix has a small but definite volume so that band broadening due to diffusion must be considered. Nonetheless, the effect of mixing dead volume on the separation integrity can be observed [39]. It has been noted that the relative distance the CEC column protrudes from the sheath liquid may have serious consequences on the performance and hence designs have been developed that allow for easy manipulation and optimization of this parameter [39,40]. The sheath liquid technique has the potential of substantially increasing the background noise [38]. Hence, alternative arrangements of the spray tip with respect to the electrospray sampling orifice such as the orthogonal spray geometry and Z flow have been developed. The sheath liquid also acts as the outlet reservoir for CEC and is frequently different in composition from the inlet buffer reservoir. This results in a discontinuous buffer system and has been found to cause significant changes in the migration order of the analytes [31,41]. In the sheath liquid approach the mobile phase from the column is diluted and consequently results in lower sensitivity compared to the sheathless interface. Also the ions of the sheath liquid compete for charge with the analytes during the electrospray process again resulting in loss of sensitivity. The CEC column outlet configuration in the sheath flow interface can either be an internal taper or no taper or a connecting tube as described in the next section.

2.1.3. Liquid junction interface

A liquid junction interface [36,37] employs a stainless steel tee (as shown in Fig. 1c) to establish electrical contact and provide additional make-up flow to the buffer. The exit of the CEC capillary column and the end of the electrospray needle are positioned in the center of a low dead volume tee opposite each other with a gap of 10–25 μm. The make-up liquid is delivered through the third branch of the tee piece and also acts as an outlet buffer reservoir. The interface may however prove cumbersome due to difficulty in aligning the column exit and the transfer capillary in the junction [42]. The make-up liquid dilutes the analyte zone and additional band broadening takes place in the connecting tube as discussed below. A packed capillary column terminated after the outlet frit is the most appropriate CEC column outlet configuration for the liquid junction interface.

2.2. Configuration of column outlet for CEC–ESI-MS

In CEC the packed segment of the capillary column is followed by an open segment to facilitate UV detection [13] and the retaining frit between the two segments is believed to act as nucleation site for bubble formation. Knox and Grant [43] recommended the use of pressurized inlet and outlet reservoir to suppress bubble formation and most CEC units embody this feature. However, such a pressurized system is not compatible with the ESI-

MS system since the ESI source is at atmospheric pressure. Three different configurations of column outlet (illustrated in Fig. 2) are seen in literature for coupling CEC with ESI-MS.

2.2.1. Columns with fritted termination

Several researchers [11,29,44–46] have reported using just the packed portion of the capillary column, i.e., without the open connecting tube for CEC–ESI-MS. Thus, as illustrated in Fig. 2a the column is terminated after the outlet retaining frit and the effluent may be electrosprayed directly into the atmospheric region of the ion source. Fig. 3 illustrates the separation of a mixture of thiazide diuretics [11] by CEC with ESI-MS detection in the negative mode. The CEC column terminated after the outlet retaining frit was interfaced to the MS system in the sheath flow arrangement. Such columns were also used for separation and identification of positively charged and neutral nucleoside and DNA adducts [44,45] by CEC–ESI-MS employing a liquid junction interface. The make-up flow comprising methanol (MeOH)–water containing 1% acetic acid (70:30, v/v) was infused at a flow-rate of 0.3–0.8 μl/min. A field strength of 500–700 V/cm was typically applied across the packed capillary columns.

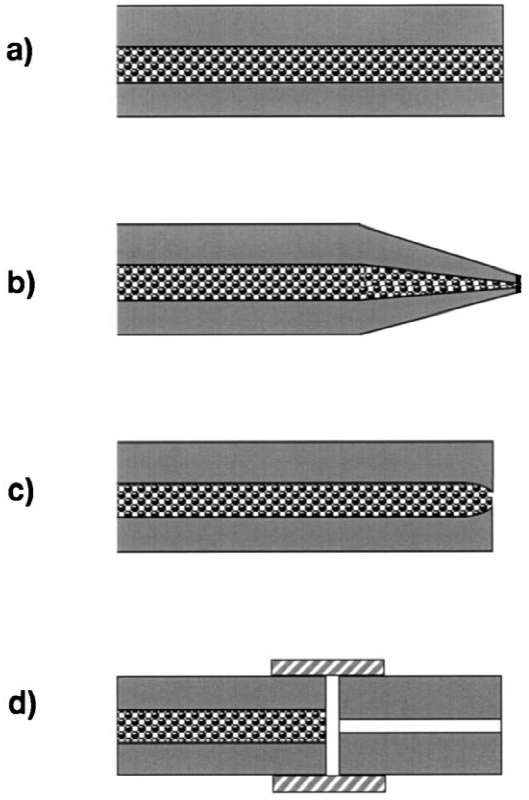

Fig. 2. Configuration of column outlet for CEC–ESI-MS. Columns with (a) fritted termination, (b) external taper, (c) internal taper, and (d) connecting tube acting as transfer line.

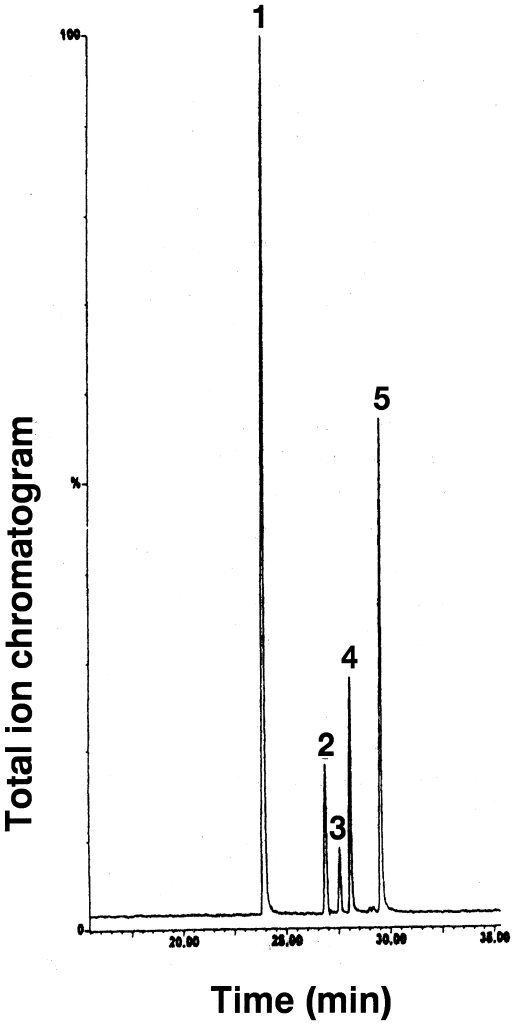

Fig. 3. Total ion chromatogram illustrating separation of a mixture of thiazide diuretics by CEC with ESI-MS detection (reproduced with permission from Ref. [11]). Peaks in order of elution, (1) hydroflumethiazide, (2) methylclothiazide, (3) metolazone, (4) epitizide and (5) bendrofluazide.

2.2.2. Columns with tapered ends

Tapered capillaries have been employed as flow restrictors in open tubular supercritical fluid chromatography [47–51] and with metallized tips also for CE–ESI-MS in a sheathless configuration [33,52–54]. There are two different types of tapering configurations possible for CEC columns [10,31].

A packed capillary column with externally tapered outlet [10] is illustrated in Fig. 2b. Whereas drawing of fused-silica capillaries through a high-temperature flame readily yields tapered capillaries with reduced outer and inner diameters, such externally tapered capillary ends are however very fragile. Capillary columns with internal tapers [31] where only the inner diameter is reduced offer an alternative (Fig. 2c). Internal tapers are prepared by melting the tip of the open capillary in a high-temperature flame thereby sealing its end. Then by carefully grinding the sealed end with a ceramic tile an opening of approximately 10 μm is produced. Capillaries with such internal tapers can be prepared reproducibly and they are more robust than capillaries with external tapers. A taper (external or internal) to approximately 10 μm I.D. at the exit end of the capillary column is sufficient to retain 3 μm stationary phase particles. Upon packing the stationary phase into the capillary tube, the particles arrange themselves in such a way that a porous plug is formed at the capillary outlet. This plug is sufficiently permeable and can be used in CEC as a replacement for the retaining frit.

Columns with both externally and internally tapered ends have been used in either sheathless or sheath flow arrangement.

2.2.3. Columns with connecting tubing acting as a transfer line

Another approach to coupling CEC to MS is the use of a piece of fused-silica capillary as connecting tubing [55]. The advantage of this approach is that the CEC column can be placed in a temperature controlled environment. Temperature control in CEC is important since, as in HPLC, temperature affects the selectivity and efficiency of the separation as well as the reproducibility of the elution times. In addition, as in CZE, temperature control is important in CEC to avoid untoward effects of Joule heating. In this arrangement, illustrated in Fig. 2d, it is best to electrically ground the CEC column right after the terminating frit [55]. The connecting tube is coupled to the packed CEC column via a zero dead volume union. The grounding of the CEC column at the stainless steel union ensures that there is no electrical discontinuity in the CEC system. Consequently, the flow of eluent through the connecting tubing is strictly parabolic and gives rise to significant band broadening [55,56]. The fused-silica tubing is chosen such that its inner diameter is smaller than the diameter of the packed CEC column, typically 25 μm [55]. The backpressure generated due to flow through the open tube is enough to prevent degassing of the buffer at the CEC column outlet. The smaller the diameter of the tube and greater the length, the greater the backpressure and hence the more robust the system will be in terms of continuous CEC operation. CEC columns coupled to a transfer line are generally used in a sheath flow and liquid junction configuration but will suffer from buffer depletion effects.

3. Band spreading

Invariably, dispersion occurs between the packed column and MS source decreasing chromatographic performance [55]. In CEC with UV detection, the column is one-piece with the electric field applied across the whole column, i.e., from the inlet of the packed column to the outlet of the open tube section. The exact nature of the flow profile as the solute band exits from the packed to open tube section is not well understood [55,56]. While coupling CEC with MS, both the nature of the interface and the configuration of the CEC column outlet, determine the optimal arrangement. Besides, bandspreading dilution factor and buffer depletion effects are also important considerations. In this section we examine the effect on dispersion of various arrangements and offer suggestions to the most practical way of connecting CEC columns to a mass spectrometer.

3.1. Sheathless interface

In the sheathless interface arrangement where the tip of the capillary is drawn into a narrow point and then coated with gold, the eluent from the CEC column is sprayed directly into the electrospray

source so there minimal extra column band broadening of the analyte zone [10]. The sheathless interface offers maximum sensitivity since there is no dilution of the analyte zone by the sheath liquid. There is, however, a significant amount of buffer depletion because of the absence of outlet buffer reservoir that can cause substantial differences in retention times and reproducibility of the system.

3.2. Sheath flow interface

3.2.1. Arrangement 1

The configuration of the CEC column is such that the outlet is either terminated right after the retaining frit without further modification [11,29,46] or has an internal [10,31]. The eluent from the packed CEC column is electrosprayed directly into the mass spectrometer. Thus, there is no extra column band spreading. The sheath liquid acts as the outlet buffer reservoir thereby avoiding buffer depletion. The analyte zone is however diluted by the sheath flow. The dilution factor defined as ratio of the total volumetric to CEC flow-rates is represented as:

$$D = \frac{F_{\text{total}}}{F_{\text{CEC}}} = \frac{F_{\text{CEC}} + F_{\text{Sheath}}}{F_{\text{CEC}}} \quad (1)$$

Thus, as the sheath flow-rate increases the analyte zone is further diluted. For typical flow-rates of 0.283 μl/min and 1 μl/min for CEC and sheath liquid, respectively, the analyte zone is diluted by a factor of 4.53.

3.2.2. Arrangement 2

In this arrangement the CEC column is electrically ground right after the terminating frit [55]. A piece of fused-silica capillary acts as a connecting tube between the packed column and the mass spectrometer. When the electrical ground connection is made at the junction between the packed column and the open tube, the flow in the open tube is parabolic in nature [55,56]. The magnitude of dispersion then depends on the dimensions of the connecting tube. The dispersion factor, *f*, which is defined as the ratio of the total temporal peak width ($w_{\text{temporal,total}}$) to the peak width in the packed CEC column ($w_{\text{temporal,CEC}}$), can be represented as [55]:

$$f = \left(\frac{w_{\text{temporal,total}}}{w_{\text{temporal,CEC}}}\right) = \left(1 + \frac{u_{\text{CEC}}^2}{u_{\text{t}}^2} \cdot \frac{\sigma_{\text{s,t}}^2}{\sigma_{\text{s,CEC}}^2}\right)^{1/2} \quad (2)$$

where, $\sigma_{\text{s,t}}$ and $\sigma_{\text{s,CEC}}$ are the spatial standard deviations in the transfer line and the packed capillary column, respectively. Similarly, u_{t} and u_{CEC} are the linear flow velocities through the transfer line and the packed CEC column, respectively.

For a sheath flow interface where a fused-silica capillary is used as a connecting tube this factor can be derived following the procedure outlined by Boughtflower et al. [55] as:

$$f = \left(1 + \frac{u_{\text{CEC}}}{u_{\text{t}}} \cdot \frac{L_{\text{t}}}{L_{\text{CEC}}} \cdot \frac{N_{\text{CEC}}}{96\epsilon t_0} \cdot \frac{d_{\text{t}}^2}{D_{\text{diff}}}\right)^{1/2} \quad (3)$$

and can further be represented as:

$$f = \left(1 + \frac{d_{\text{t}}^2}{d_{\text{CEC}}^2} \cdot \frac{L_{\text{t}}}{L_{\text{CEC}}} \cdot \frac{N_{\text{CEC}}}{96\epsilon t_0} \cdot \frac{d_{\text{t}}^2}{D_{\text{diff}}}\right)^{1/2} \quad (4)$$

where, ϵ, is the porosity of the packed CEC column; d_{t} and d_{CEC}, are the diameters of the connecting tubing and the packed CEC column, respectively; L_{t} and L_{CEC}, are the lengths of the connecting tubing and the packed CEC column, respectively; D_{diff}, is the diffusion coefficient; N_{CEC}, is the efficiency of the CEC column and t_0, the time taken by an uncharged, unretained tracer to reach the end of the CEC column.

Eq. (2) suggests that the dispersion factor is a square function of the diameter of the connecting tube, d_{t}, as well as the ratio of the diameters of the two tubes, $(d_{\text{t}}/d_{\text{CEC}})^2$. It is however only a linear function of the ratio of length of connecting tube to the packed CEC column, $L_{\text{t}}/L_{\text{CEC}}$. This dependence is illustrated in Fig. 4. Thus, for a 20 cm×100 μm CEC column, where an EOF velocity of 1 mm/s generates 100 000 plates, the dispersion factor due to a 10 cm open fused-silica connecting tube that has an inner diameter of 100 μm is 6.83. The dispersion factor for a 25 μm I.D. capillary however decreases dramatically to 1.08 (only 8%). Thus, a 25 μm I.D. capillary column may be most appropriate as a transfer line. This has been illustrated by Boughtflower et al. [55] who have experimentally determined the dispersion factor at different positions downstream of the packed capillary column.

The magnitude of dilution factor in this arrangement is the same as that for arrangement 1. The

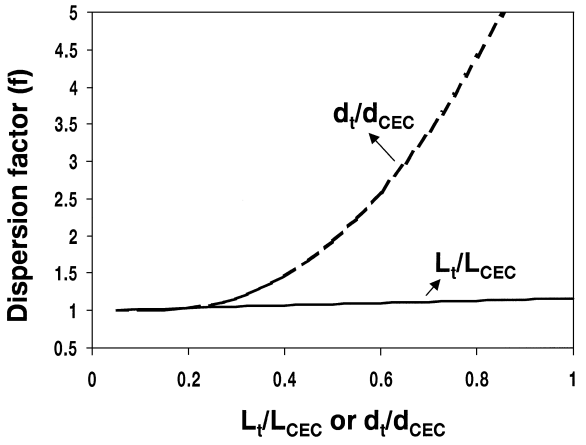

Fig. 4. Dependence of dispersion factor (f) in connecting tube on the ratio of diameters, (d_t/d_{CEC}) and lengths, (L_t/L_{CEC}). Calculations based on Eq. (2). 20 cm (L_{CEC})×100 μm (d_{CEC}) packed CEC column, with an electroosmotic flow velocity ($u_{CEC}=L_{CEC}/\epsilon t_0$) of 1 mm/s generating 100 000 (N_{CEC}) plates for an analyte diffusing at 10^{-5} cm^2/s (D_{diff}).

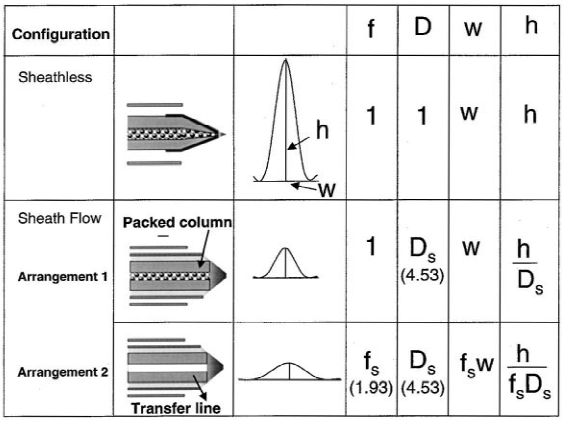

Fig. 5. Schematic illustration of the effect of dispersion (f) and dilution (D) factor on peak height and width in (a) sheathless and (b) sheath flow configuration. In arrangement 1 of the sheath flow configuration the column has a retaining fit or a taper at the outlet whereas in arrangement 2 a connecting tube acts as a transfer line between the packed CEC column and the mass spectrometer. The dilution factor ($D=4.53$) is calculated for CEC and sheath flow-rates of 0.28 and 1 μl/min, respectively. The dispersion factor for arrangement 2 is calculated from Eq. (2) for a 10 cm×50 μm transfer line. Other parameters as in Fig. 4.

sensitivity of this system is however much worse since the peak became broad as well as dilute. The resulting effect, which can be physically interpreted as a loss in peak height, is illustrated in Fig. 5. In the sheathless interface, the dispersion (f) and dilution (D) factor, each equals unity. In arrangement 1 there is no dispersion since the analyte is electrosprayed directly ($f=1$) but the peak is diluted by the sheath flow ($D=D_s$). Thus, a peak that elutes from a sheathless interface with a peak height of h and width of w (s) will in arrangement 1, exhibit the same peak width (width$=w$), but the peak height (height$=h/D_s$) is reduced by the dilution factor. Again, in arrangement 2 where the analyte peak disperses in the connecting tube, the peak width increases by the dispersion factor (width$=f_s w$) with a concomitant decrease in the plate height (height$=h/f_s$). The peak height however further decreases due to the dilution by the sheath liquid (height$=h/f_s D_s$). Thus, the sensitivity in arrangement 2 is much worse than that in arrangement 1 as well as the sheathless design.

3.3. Liquid junction interface

The dispersion factor for the liquid junction interface is different from that for the sheath flow since now the flow through the open tube is a combination of the CEC flow-rate and the make-up flow-rate. Thus, the linear flow velocity through the open tube can be given as:

$$u_t = (F_{CEC} + F_{lj}) \cdot \left(\frac{4}{\pi d_t^2}\right) = \left(\frac{d_{CEC}^2}{d_t^2}\right) \cdot \epsilon u_{CEC} + F_{lj} \cdot \left(\frac{4}{\pi d_t^2}\right) \quad (5)$$

where, F_{lj} is the liquid junction volumetric flow-rate.

The dispersion factor for this case can thus be calculated by substituting Eq. (5) in Eq. (3):

$$f = \left[1 + \frac{L_t}{L_{CEC}} \cdot \frac{u_{CEC}}{\left(\frac{d_{CEC}^2}{d_t^2}\right) \cdot \epsilon u_{CEC} + F_{lj} \cdot \left(\frac{4}{\pi d_t^2}\right)} \cdot \frac{N}{96 \epsilon t_0} \cdot \frac{d_t^2}{D_{diff}} \right]^{1/2} \quad (6)$$

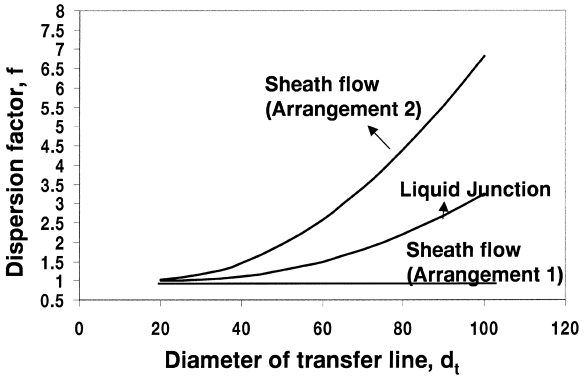

Fig. 6. Dependence of dispersion factor on the inner diameter of the connecting tube in sheath flow and liquid junction interface. The length of the connecting tube (sheath flow)/transfer line (liquid junction) is assumed to be 10 cm. Other parameters as in Fig. 4.

A comparison of the dispersion factor in the liquid junction interface and sheath flow interface is provided in Fig. 6. It is assumed that a 20 cm×100 μm packed CEC column with an EOF velocity of 1 mm/s generates a 100 000 plates for an analyte diffusing at $1 \cdot 10^{-5}$ cm^2/s. The length of the connecting tube for sheath flow and transfer line for liquid junction interface is assumed to be 10 cm. The sheath flow and make-up flow are delivered at a rate of 1 μl/min (i.e., $D=4.53$). It is seen in Fig. 6 that the dispersion factor is higher for sheath flow (with connecting tube, i.e., arrangement 2) than liquid junction interface assuming there are no other sources of extra column bandspreading. For example, for a 25 μm inner diameter connecting tube the dispersion factor in the sheath flow arrangement in 1.08 while it is only 1.02 in liquid junction interface for the same inner diameter transfer line. Also, the increase in dispersion upon increasing the inner diameter of the connecting tube is higher in sheath flow than in liquid junction interface. For example, upon increasing the inner diameter of the connecting tube from 25 to 50 μm the dispersion increases from 1.08 to 1.93 for sheath flow but only from 1.02 to 1.26 for liquid junction interface. Thus, for a given magnitude of dispersion factor (1.5), a larger diameter transfer line can be used in liquid junction (60 μm) than in sheath flow interface (40 μm). Typically, the difficulty in aligning the CEC column and the transfer line in a liquid junction interface may lead to a higher dispersion factor than that theoretically predicted.

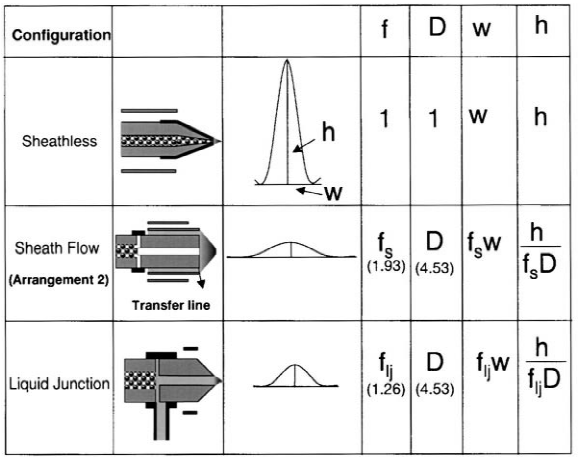

Fig. 7. Schematic illustration of the effect of dispersion factor (f) and dilution factor (D) on peak height and width in (a) sheathless, (b) sheath flow with connecting tubing acting as a transfer line and (c) liquid junction interface. The dilution factor ($D=4.53$) is calculated for CEC and sheath flow-rates of 0.28 and 1 μl/min, respectively. The dispersion factor is calculated from Eqs. (2) and (3) for sheath flow and liquid junction interface, respectively, for a 10 cm×50 μm transfer line. Other parameters as in Fig. 4.

The dilution factor in the liquid junction configuration is the same as the sheath liquid configuration provided the make-up and sheath liquid flow-rates are identical. Fig. 7 schematically, compares the width and peak height in liquid junction and sheath flow (with connecting tube) configuration. Again, the liquid junction interface may provide a better sensitivity than the sheath flow interface with connecting tube (arrangement 2).

4. Examples and modes of operation

4.1. Open tubular capillary electrochromatography

In open tubular capillary electrochromatography (OTCEC) the retentive stationary phase is present only on the walls of capillary columns that have dimensions typically less than 20 μm. Separation of

analytes occurs by interaction with the stationary phase and if charged by their electrophoretic mobility as well. OTCEC has certain advantages compared to packed columns. OTCEC columns with inner diameters around 10 μm have been found to have smaller plate height compared to packed columns due to the lack of band broadening effects associated with the presence of stationary phase particles and column end frits [57–60]. High concentration sensitivity is also an advantage of OTCEC since columns of extremely small dimensions can be used. In spite of these advantages, OTCEC columns have a major limitation of relatively low sample loading capacity. These columns are not widely used in HPLC separations mainly because of the difficulties associated with the sample injection and detection. The small injection volume (nl–pl range), requires split devices in HPLC, which results in sample wasting and complexity of instrumentation. In the CEC mode, however samples can be injected electrokinetically, thus dramatically decreasing sample consumption while concomitantly avoiding complexity of split injection. Although, the extremely small diameter of OTCEC columns makes optical detection difficult, they are compatible with the concentration sensitive detection methods such as ESI-MS. Thus, the major disadvantage of using open tubular columns in liquid chromatography may be overcome in a CEC system with ESI-MS as the detection method. Fig. 8 shows UV trace and TIC of a gradient OTCEC separation of a tryptic digest of a horse heart myoglobin. Among the peaks shown in TIC, obtained with an ion trap/reflectron time-of-flight mass spectrometer, at least 15 (including co-eluting components) could be assigned to the expected fragments covering about 90% of the protein sequence [57]. A

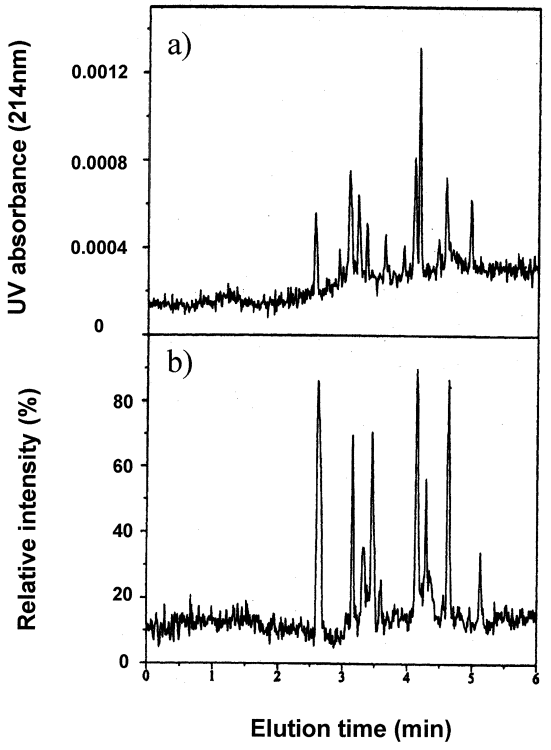

Fig. 8. Open tubular capillary electrochromatographic (OTCEC) separation of tryptic horse heart myoglobin digest. Detection; (a) UV, (b) total ion chromatogram (TIC) obtained by an ion trap reflectron time-of-flight mass spectrometer. (Reproduced with permission from Ref. [57]).

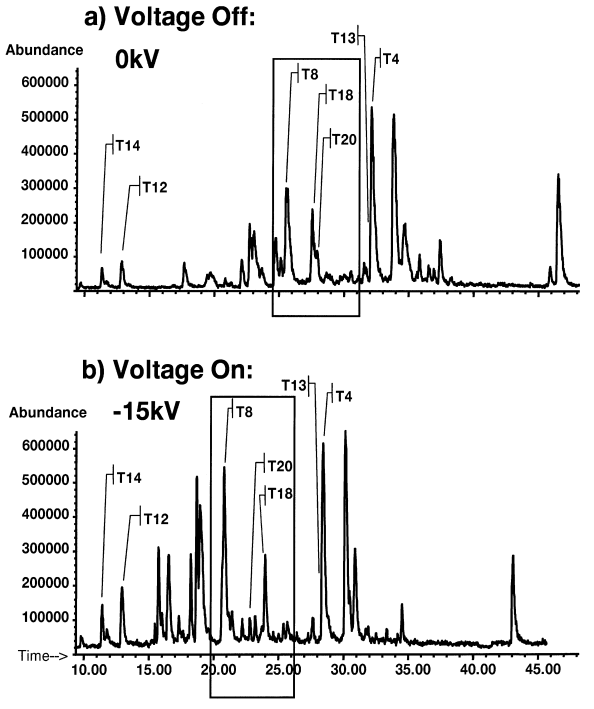

Fig. 9. Effect of application of voltage on tryptic digest of recombinant human growth hormone. Column, 25 cm×100 μm fused-silica capillary column packed with 5 μm Vydac octadecyl stationary phase; mobile phase, buffer A: 0.1% TFA–water, buffer B: 0.09% TFA–acetonitrile; gradient, 0% B for 5 min; 0–60% B in 60 min. (Reproduced with permission from Ref. [25]).

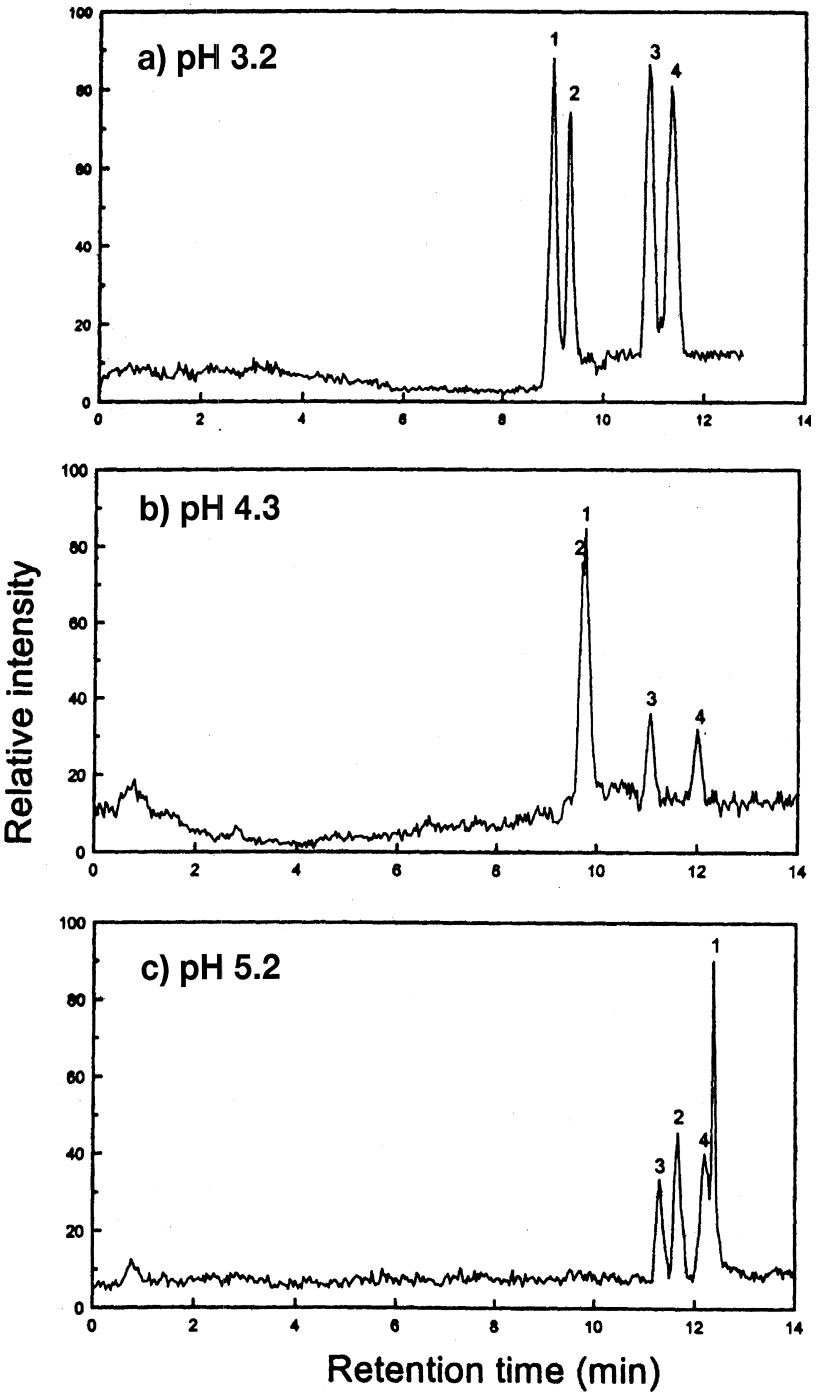

Fig. 10. Effect of pH on the separation of peptides on a mixed mode stationary phase by electrically assisted capillary liquid chromatography (reproduced with permission from Ref. [28]). Peaks: 1, angiotension; 2, methionine enkephalin-Arg-Phe; 3, methionine enkephalin; 4, leucine enkaphlin.

sheathless interface with an external taper at the column outlet was used for the above separation.

4.2. Assisted capillary chromatography

Assisted capillary chromatography can be divided into two categories, i.e., pressure-assisted capillary electrochromatography (PCEC) or electrically-assisted capillary liquid chromatography (ECLC). Although, the above terms are used interchangeably, a distinction can be drawn between the two methods. PCEC is used to refer to all methods where electroosmosis is responsible for the flow of eluent from the inlet to the outlet of the capillary column [23,24]. The additional pressure either assists the bulk flow due to EOF or is an unavoidable feature of gradient elution module. In contradistinction, ECLC is used to describe all methods where the bulk flow in due to the pressure generated by the pump [25–28,61]. The voltage applied across the capillary column provides an orthogonal separation and selectivity tuning mechanism. A small amount of EOF may be generated depending upon the nature of stationary and mobile phases.

PCEC with negative ion electrospray MS has been demonstrated for a mixture of aromatic glucuronides as well as food colors [24]. A 250 μm I.D. fused-silica capillary tubing packed with Nucleosil C_{18} packed stationary phase was used for CEC. This capillary column was coupled to a 40 μm I.D. fused-silica transfer capillary via a zero dead volume union. The transfer capillary was interfaced to an ESI-MS system in a sheath flow arrangement.

ECLC coupled with ESI-MS [25–28] has been employed for separation of tryptic digest of proteins. Traditionally, such separations are performed by reversed-phase HPLC with mobile phase containing trifluoroacetic acid (TFA). TFA acts as an ion pairing reagent and prevents secondary interactions between the peptides and the silanol groups on octadecylated siliceous stationary phases. Under these low-pH conditions and with conventional HPLC stationary phases (that have moderate to heavy endcapping) the amount of EOF generated upon application of voltage is however small. The charged peptides migrate electrophoretically in addition to interacting hydrophobically with the stationary phase and thus exhibit altered selectivity. This is illustrated in Fig. 9, which shows the effect of application of voltage on tryptic digest of recombinant human growth hormone [25].

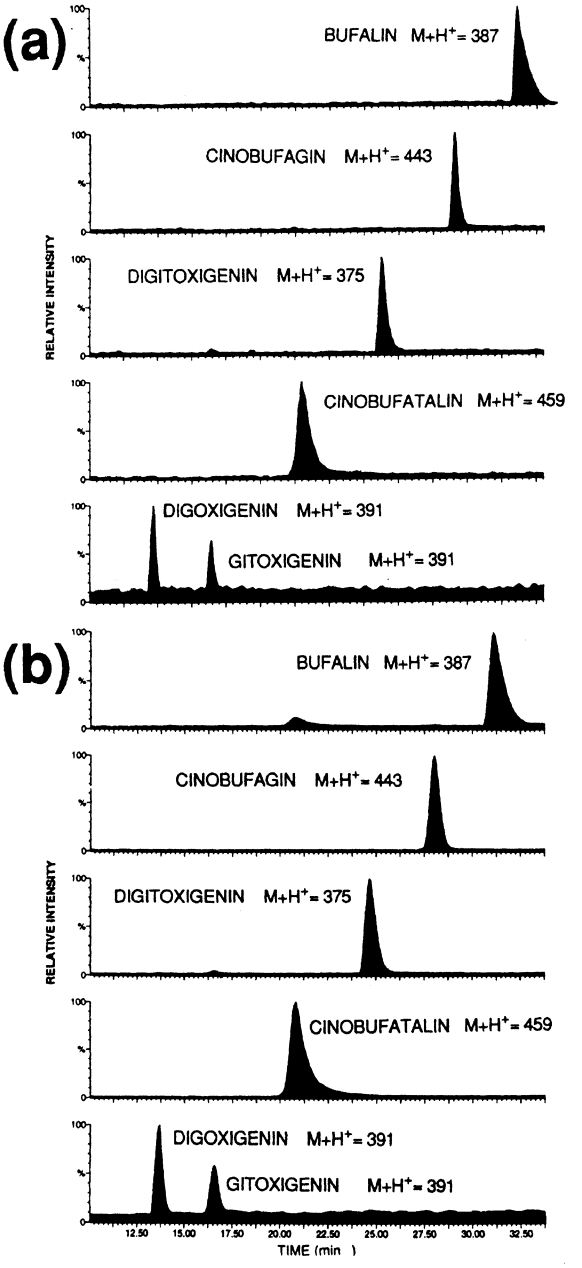

Fig. 11. Reconstructed ion chromatograms for the separation of a mixture of steroids by CEC with ESI-MS detection using a column with external taper at the outlet in (a) sheathless and (b) sheath flow arrangement (reproduced with permission from Ref. [10]).

A mixed-mode stationary phase (reversed-phase/anion-exchange) was used for investigating the separation of peptide mixtures in electrically assisted CEC coupled to an electrospray ionization ion trap/reflectron time-of-flight mass spectrometer [28]. The stationary phase comprised of both ODS and dialkyl amine groups. Fig. 10 shows the effect of changing pH on selectivity. ECLC thus, offers enormous potential for tweaking minor differences between analytes for altering selectivity

4.3. Packed column capillary electrochromatography

Numerous applications of separation of steroids by CEC–MS are available. A mixture of six steroids belonging to the cardenolide (five-membered lactone ring) and bufadienolide (six-membered lactone ring) family has been analyzed by CEC–ESI-MS [10]. Externally tapered CEC columns were interfaced with ESI-MS in both sheathless and sheath flow arrangement as illustrated in Fig. 11. The ion intensity was 20-fold higher in the sheathless arrangement than in sheath flow arrangement. In another application, a steroid mixture comprising of hydrocortisone, dexamethasone caffeine and betamethsone-17-valerate was separated by a 100 μm I.D. C_6/SCX CEC column using a sheath flow interface for MS detection [30]. The column was terminated after the outlet retaining frit and then used without further modification. The above arrangement was also used for the separation by CEC–ESI-MS of 13 structurally related compounds from parent drug candidate. This example highlights the potential use of CEC in high-throughput drug discovery schemes [46]. Fig. 12 shows the separation of fluticasone propionate from its four impurities and cefuroxime axetil diastereoisomeric pairs by CEC on a column packed with 3 μm Hypersil C_{18} stationary phase [8]. Separation of Salbutamol and Salmeterol (Glaxo compounds) was demonstrated on a C_6/SCX CEC column. The system comprised of an automated

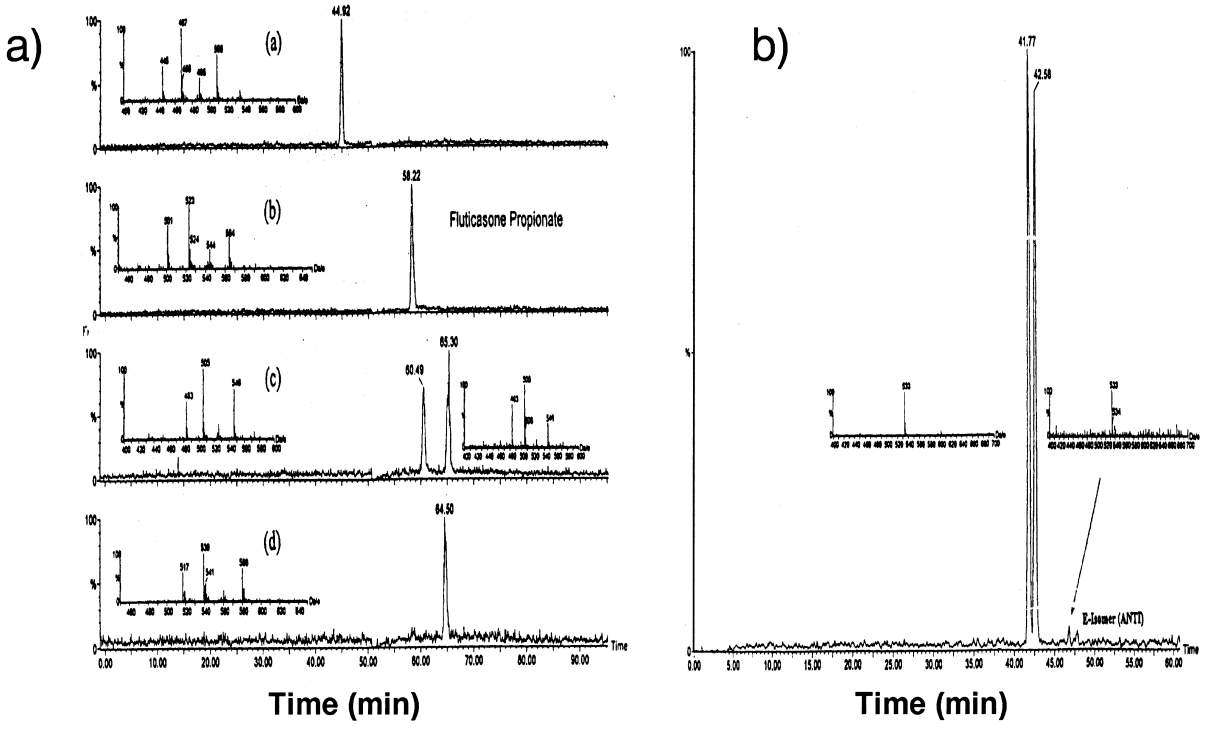

Fig. 12. Reconstructed ion chromatogram of separation by CEC–ESI-MS of (a) fluticasone propionate from its impurities and (b) cefuroxime axetil diastereoisomeric pairs (reproduced with permission from Ref. [8]).

injection module and a sheath flow electrospray interface [29].

CEC has been successfully used for the separation of complex mixtures of neutral isomeric compounds derived from in vitro reaction of carcinogenic hydrocarbon (benzo[*g*]-chrysene and 5,6-dimethylchrysene) dihydrodiol epoxides with calf thymus deoxyribonucleic acid (DNA) [45]. Fig. 13 illustrates CEC–ESI-MS detection of the above DNA adduct mixture.

Electroosmotically driven solvent gradients were employed for the separation of 12 PTH-amino acids by reversed-phase CEC with a time-of-flight mass spectrometer serving as the detector [31]. Capillary columns with an internal taper at the column outlet were used in the sheath flow configuration. Fig. 14 shows the effect of sheath liquid composition on the signal intensity, resolution and analysis time for the separation of six PTH-amino acids.

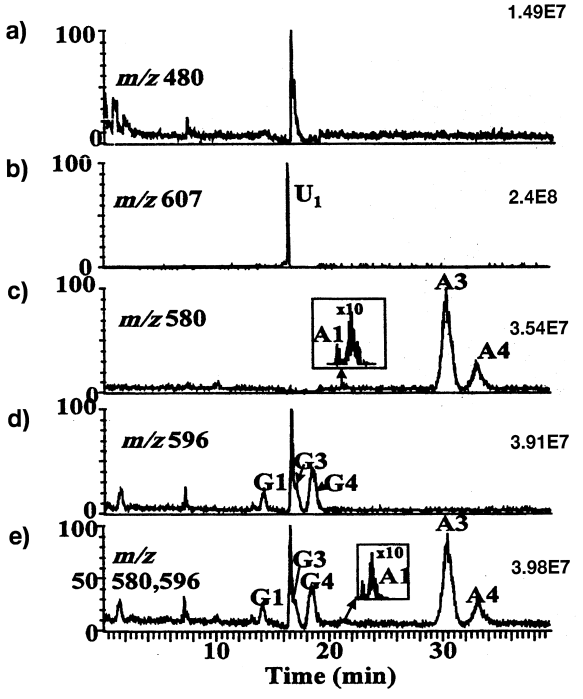

Fig. 13. CEC–ESI-MS analysis of a reaction mixture of anti-benzo[*g*]chrysene-11,12-dihydrodiol-13,14-epoxide with calf thymus DNA (reproduced with permission from Ref. [45]). (a–d) Reconstructed ion chromatograms for *m*/*z* 480, 607, 580 (dA adducts) and 596 (dG adducts), respectively, (e) a combined chromatogram of (c) and (d).

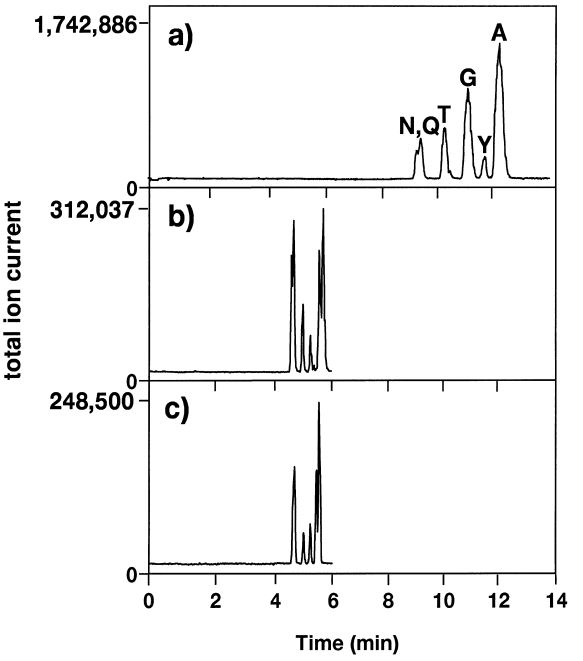

Fig. 14. Total ion chromatograms illustrating the effect of sheath liquid composition on the separation of PTH-amino acids by CEC with ESI-MS detection using a column with internal taper at the outlet. Sheath liquid: (a) neat methanol; (b) 0.2 m*M* ammonium acetate in water–methanol (1:9, v/v); (c) 2 m*M* ammonium acetate in water–methanol (1:9, v/v). (Reproduced with permission from Ref. [31]).

5. Conclusions

Electrochromatography is rapidly gaining acceptance in the bioanalytical community. Improvements in stationary phases, coupled with developments in commercially available instrumentation have helped to move CEC from a technique largely of theoretical interest to one of immediate applicability to ''real world'' analytical problems. Improvements in interfacing techniques between CEC and MS, will undoubtedly play a key role in bringing out the full potential of this technique. In a decade, we may well look back on the late 1990s as a pivotal point in the evolution of this modern analytical technology.

6. Nomenclature

D	Dilution factor, dimensionless

d_t Inner diameter of the connecting tube, m
d_{CEC} Inner diameter of the packed CEC column, m
D_{diff} Diffusion coefficient, m^2/s
ϵ Interstitial porosity, dimensionless
F_{CEC} Volumetric flow-rate through the CEC column, μl/min
F_{Sheath} Volumetric sheath flow-rate, μl/min
F_{Total} Sum of CEC and sheath flow-rate, μl/min
f Dispersion factor, dimensionless
L_t Length of the connecting tube, m
L_{CEC} Length of the packed CEC column, m
N_{CEC} Efficiency of the packed CEC column
t_0 Time taken by an uncharged and unretained tracer to reach the CEC column end, s
u_{CEC} Linear flow velocity of the uncharged and unretained tracer, m/s
F_{lj} Flow-rate of the make-up liquid in the liquid junction interface, μl/min

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