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Review

Capillary zone electrophoresis: its applicability and potential in biochemical analysis

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ABSTRACT

Recent developments in capillary zone electrophoresis (CZE) are reviewed, starting with available instrumentation, a description of different operational modes and the most commonly used detection systems. Appropriate attention is paid to CZE–mass spectrometry coupling and coupling of electrophoretic and chromatographic procedures. The possibility of separating chiral molecules is also discussed. Examples of applications concern mainly amino acids, peptides, proteins, nucleic acids and their constituents.

CONTENTS

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LIST OF ABBREVIATIONS

CBQCA	3-(4-Carboxybenzoyl)-2-quinoline carboxaldehyde
CE	Capillary electrophoresis
CHES	2-(N-Cyclohexylamino)ethanesulphonic acid
CMEC	Capillary electrokinetic micellar chromatography
CZE	Capillary zone electrophoresis
DABSYL	4-(Dimethylamino)azobenzene-4'-sulphonyl
DANSYL	1-Dimethylamino-4-sulphonyl
ESI	Electrospray ionization
FAB	Fast atom bombardment
GC	Gas chromatography
hGH	Human growth hormone
HPCE	High-performance capillary electrophoresis
HPLC	High-performance liquid chromatography
MS	Mass spectrometry
PFEP	Perfluorinated ethylene–propylene
R.S.D.	Relative standard deviation
SDS	Sodium dodecyl sulphate

1. INTRODUCTION

Recent development in understanding of etiopathology of a number of diseases and advances in biomedical diagnostics and therapy constitute the basis for many demanding challenges in separation science. High sensitivity, easy quantification and high resolving power in the analysis of frequently very complex mixtures are the most common requirements. Even advanced high-performance chromatographic techniques fall frequently short in fulfilling these tasks. Moreover, facile independent separation procedures exploiting different separation principles are needed in order to obtain reliable results.

The traditional combination of chromatographic and electromigration techniques suffers from considerable drawbacks, particularly on the electromigration side: long running times, unsatisfactory reproducibility of gel properties (as revealed particularly in twodimensional separations), problems with reproducible

staining and zone quantification and the need for relatively large amounts of samples for analysis preclude gel electrophoretic separations from more effective applications in the biomedical and biotechnology fields. Other problems emerge from the fact that particularly in the biotechnology area enormous numbers of samples need to be analysed. From the very nature of gel electromigration techniques, it is obvious that they are not easily automated. The above-mentioned facts prompted attempts to develop more effective and more sensitive electromigration techniques with high resolving power, the result of which is reflected in the current research on capillary zone electrophoresis (CZE).

2. PRINCIPLE AND OPERATION MODES OF CAPILLARY ZONE ELECTROPHORESIS

Jorgenson and Lukacs [1–5] were the first to perform capillary zone separations. The principle of the method is very simple but its realization encounters a number of difficulties which, however, are likely to be overcome in the near future. The separation is carried out in a fused-silica capillary (less than 100 μm I.D.) with a voltage of 10–30 kV applied over a 50–100 cm capillary. Application of microcapillary columns results in a very efficient dissipation of heat generated by the electrophoretic process and in high plate counts. Most commonly 20–50 m*M* buffers of both basic and acidic pH can be used, although high-pH buffers are preferred in order to eliminate capillary wall charge and prevent, *e.g.*, separated protein species form adhering to the capillary wall. Early achievements including the theory of CZE were reviewed in the past and readers interested more in this area, including instrumentation rather than applications, are referred to those surveys [6–12].

In principle, we shall discuss the following three different operational modes (Fig. 1).

(1) Plain electrophoretic separations in which the solutes are separated on the basis of their effective charge. Such separations can only be, effected however, in modified capillaries in which the endoosmotic flow has been eliminated by inner capillary surface modification.

(2) Electrophoretic separations with endoosmotic flow. Here the velocity of the endoosmotic flow is as a rule much faster than electromigration of the separated solutes and both these migrations occur in opposite directions. Thus, *e.g.*, proteins are separated in a system in which a high voltage is applied to the positive pole of the capillary. Assuming that the pH of the buffer used is above the isoelectric point of any constituent of the sample, migration proceeds from the negative to the positive pole. If there were no endoosmotic flow, the sample would be flushed to the anodic electrode jar and would never pass the capillary. However, as this is not the case, and owing to the endoosmotic flow, the whole content of the capillary is moved to the cathodic end at a rate faster than electrophoretic separation of solutes. Individual separated species pass the detector's cuvette and those with low electrophoretic mobility are the last on the electropherogram.

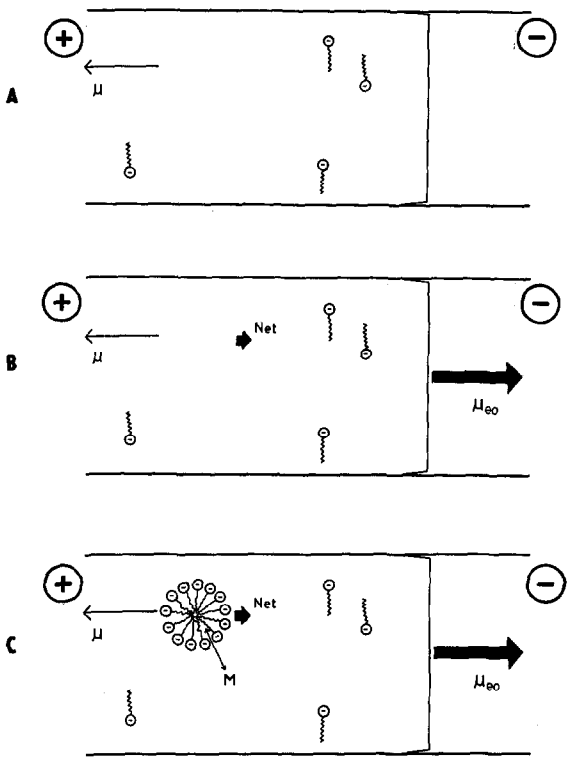

Fig. 1. Schematic representation of the three operational modes in capillary electrophoretic separations. (A) Simple electrophoresis without endoosmotic flow; (B) electrophoretic separation in the presence of endoosmotic flow; (C) micellar electrokinetic capillary chromatography in the presence of a detergent. μ_{eo} = Endoosmotic flow factor; μ = electrophoretic migration factor; Net = the difference between endoosmotic and electromigration flow; M = micelle. Negatively charged amphiphilic solute. Modified according to ref. 6.

(3) Separations in the presence of surfactants (capillary elektrokinetic micellar chromatography, CMEC). This mode can be applied to uncharged and hydrophobic species. The surfactant added forms charged micelles and separation occurs on the basis of differential partitioning between the electroosmotically driven mobile phase and the micellar phase. Both phases are moving at different velocities, the velocity of the micellar phase being slower [2,6].

Over the years, three closely associated techniques have appeared, namely capillary gel electrophoresis [13–15], CMEC [16–18] and CZE [19–21]. This review is devoted mainly to the last two techniques, with some emphasis on their application in the biomedical field. Let us mention for completeness that in electrochromatography a high voltage is applied along a liquid chromatographic column under pressurized flow. The method was independently described by

Knox and Grant [19] and Tsuda [22,23]. Readers interested in this technique are referred to those papers.

After considering the above operational modes, one is tempted to ask why isoelectric focusing is not much exploited in capillary systems. This is naturally possible by dividing the whole separation process into two discrete steps: focusing and elution [24–27]. Focused zones (first step) are eluted in the second step either by hydrodynamic flow (by applying an adequate constant pressure to the sample side of the cappillary) or by introducing endoosmotic flow (by changing the ionic concentration in none of the electrode jars). This approach, however, has not yet achieved wide application. On the other hand, it is clear that the selectivity of the separation of substances is given by the differences in the effective mobilities of the separated solutes, which may be optimized by acid–base equilibria, *i.e.*, by pH changes. When the sample contains solutes that exhibit close mobilities and covering a wide range of pK values (a frequent situation in physiological samples), it is difficult to find a fixed pH value at which the individual constituents of the sample can be separated with good quality within a reasonable period of time. For this purpose, dynamic pH gradients [28,29] were introduced into the CZE systems. This can be realized by using a three-pole separation column [30] or more conveniently by applying a programmed dynamic change of pH in one electrode chamber during the analysis [25]. The method of operation of such a system is evident from Fig. 2.

3. AVAILABLE INSTRUMENTATION

The early attempts to obtain CZE separations were invariably done with laboratory-made equipment [e.g., 31]; currently, however, at least six types of apparatus are commercially available or will be soon (Microphoretic Systems, Sunnyvale, CA, USA; Applied Biosystems, Foster City, CA, USA; Bio-Rad Labs., Richmond, CA, USA; Beckman Instruments, Palo Alto, CA, USA; Dionex, Sunnyvale, CA, USA; ISCO, Lincoln, NE, USA).

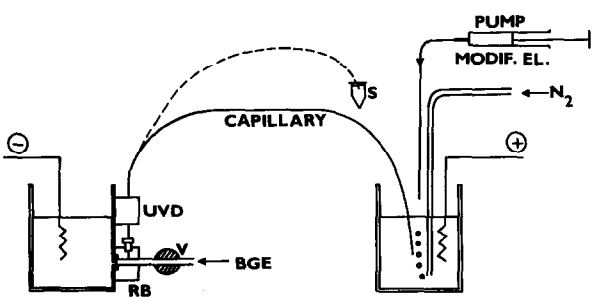

Fig. 2. Schematic representation of the set-up for electrophoretic analysis in a dynamic pH gradient (from ref. 25, with permission). S = Sample; UVD = UV detector; RB = refilling block; V = valve; BGE = background electrolyte.

The set-ups of the various types of apparatus are similar and may be exemplified by the Dionex capillary electrophoresis system (Fig. 3). The apparatus features menu-driven programming which offers full control of all the instrument's parameters. Methods can be stored in the non-volatile RAM and schedules can be set up so that the system will run experiments automatically. An automated rinse and refill system increases reproducibility and allows buffer changes even if the apparatus is working in the automated mode. Three methods of sample introduction (see also Section 3.2) are offered, namely pneumatic, by electromigration and by gravity. The device is supplied with a 30-kV power supply offering either a positive or negative pole at the high-voltage end. Also the choice of constant-voltage, -current or -power mode can be selected according to operator's requirements and, moreover, the controlled parameter can be varied with time to generate electrical gradients. The machine is supplied with a fibre-optic UV absorbance or fluorescence detector. The seaparation capillary is located loosely in a Plexiglas box and is captured in V-shaped grooves so that it is automatically aligned on installation and is easily exchangeable. The advantage of the fibre-optic detector is that capillaries of different diameters can be accommodated without a decrease in performance. Samples are supplied from a carousel that has random access to 40 positions occupied by 500-μl microcentrifuge vials.

The machine supplied by Applied Biosystems is also fully automated, with a freely located capillary. The capillary is thermostated, however: it has fewer sample positions on the carousel and it does not use fibre-optics in the detector. So far it is supplied with a UV detector only (Fig. 4).

Temperature control within the capillary is an important factor that should not be neglected. The flat electrophoretic profile of migration velocity assumed in CZE separations may be distorted or even eliminated owing to Joule heat gener-

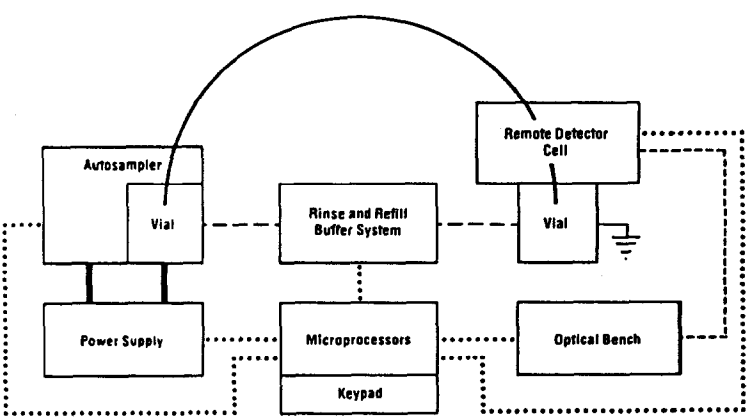

Fig. 3. Schematic representation of the set-up of the Dionex capillary electrophoresis system. Short dashed lines, fibre optics; long dashed lines, liquid lines; solid lines, high-voltage lines; dotted lines; communications. The separation capillary is represented by the arch at the top.

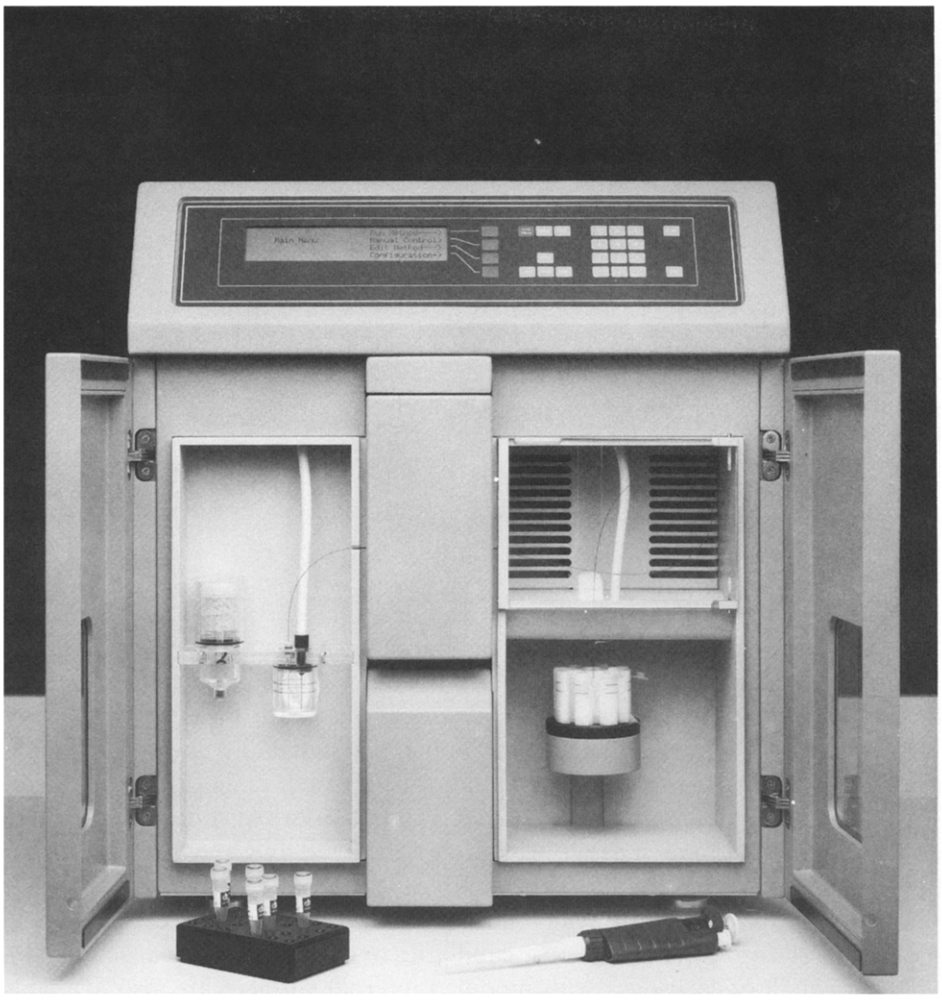

Fig. 4. Apparatus for capillary electrophoresis supplied by Applied Biosystems. The capillary is freely located between the left and right open compartments. Sample is introduced from the set of vials located in the right-hand compartment.

ated within the capillary. Grushka *et al.* [32] assumed that in real separations the migration velocity across the capillary profile has a parabolic shape. The plate height in CZE is a function of the capillary radius, the strength of the applied field and buffer concentration. Large applied voltages broaden solute peaks if the capillary is not cooled and decreased buffer concentrations allow the use of capillaries of larger diameter. The theoretical basis of the above conclusions was presented in detail [32].

The Bio-Rad HPE 100 system is unique in that it uses a capillary inserted in a cartridge, which avoids occasional problems with the fragility of the loosely

mounted capillary. Only a UV detector is featured. Another important feature is the use of capillaries with a coated internal surface which alters (or even eliminates) endoosmotic flow and is claimed to minimize problems with capillary adsorption of high-molecular-mass solutes (proteins). However, the price of such capillary-containing cartridges is high.

ISCO recently introduced two capillary electrophoresis systems at the Second International Symposium on High Performance Capillary Electrophoresis, San Francisco, CA, January 29–31, 1990. One system is fully automated and equipped with a 40-position autosampler, temperature control, buffer switching and data acquisition, and can be joined to a sample collector which, however, is not yet marketed. The second capillary electrophoresis system is a compact, inexpensive device equipped with a high-sensitivity absorbance detector and dual-mode injection capability.

The extremely high costs of these equipments (up to US $ 50 000) obviously prevent them from more rapid introduction into many laboratories.

3.1. Capillary columns

Fused-silica capillaries almost invariably obtained from Polymicro Technologies (Phoenix, AZ, USA) are used, which of course does not preclude the application of other products. These capillaries are supplied with a wide range of inner diameters (5–530 μm) with an outside polyimide protecting sheet that makes them mechanically stronger. The advantage of polyimide is its high thermal stability which, however, is not needed for CZE and therefore silicone rubber-coated capillaries can also be used as well (Institute for Phycis and Mechanics, Czechoslovak Academy of Science, Bratislava, Czechoslovakia).

As far as the commercially available instruments are concerned, capillaries can be obtained separately either as loose capillaries or cartridge adjusted (see above), and even capillary cartridges with modified internal surfaces are also available (Bio-Rad Labs.).

3.2. Sampling and sampling automation

Sample introduction is a crucial point in operating a CZE apparatus. Two methods of introducing the sample are most frequently used, in addition to other existing possibilities: in electromigration injection [3,4,33] the anodic end (provided that the endoosmotic flow is directed to the cathode) is disconnected and the buffer is replaced with a sample vial connected to the anode. Then a voltage is applied for a period ranging from 0.05 to 0.5 min, which causes the sample to migrate into the capillary. After the injection voltage pulse, the sample vial is replaced with the anodic electrode jar and the electrophoresis may proceed. Because of the electrophoretic migration during sample application, discrimination occurs within the solutes applied: those with low electrophoretic mobility move

with the sample front whereas those with high electromigration are introduced last. In practice, problems arising from this effect are rare and enrichment of the sample in the electrophoretically slowly moving solutes represents mostly a theoretical possibility. The amount of sample applied can be regulated by changing not only the time of the sample-introducing pulse but also the sampling voltage.

Under optimum circumstances, the sample is applied in the running buffer; otherwise one must bear in mind that electromigration is also affected by the conductivity of the sample solution. The peak area increases linearly with increasing resistance of the sample solution. Automated sample introduction offers the best results regarding reproducibility [4.1% relative standard deviation (R.S.D.)] [33]. Contrary to the different assumptions about the disadvantages of electrophoretic sample application, Rose and Jorgenson [33] reported that electromigration sample application has virtually no effect on the separation efficiency. Compared with hydrodynamic flow injection (see below), electromigration injection has the advantage of a rectangular flow front.

Hydrodynamic flow injection [33,34] (siphoning, gravity injection) is accomplished either by placing the sample end of the capillary in the sample vial, which is elevated to a certain height difference, Δh, higher than the grounded (detection) end of the capillary, and the sample is allowed to flow inside for a definite period of time. Alternatively, the sample vial is left at the same height as the cathodic (opposite) end of the capillary and the sample is applied by introducing suction to the air-tight cathodic electrode jar. These methods of sample introduction are not affected by the sample composition: the applied height differences are *ca.* 15 cm and typically the sample introduction time is *ca.* 5–10 s. According to Rose and Jorgenson [33], with automated hydrodynamic sample introduction a reproducibility of 2.9% R.S.D. can be easily achieved.

There are three other methods of sample introduction into the capillary, which are much less common than those described above. In the electric sample splitter described by Deml *et al.* [35], the sample migrates into two circuits in which the splitting ratio is given by the ratio of corresponding split currents (Fig. 5). The dosing capillary (amount injected $\approx$ 1 μl) is of I.D. 500 μm at a distance of 10 cm from the splitting point. The separation capillary in this instance, however, is far beyond the optimum I.D. for good CZE separations (200 μm). A major difficulty with this system is the problem of adapting the device to separation capillaries of smaller diameter. The accuracy of sample introduction through the sample splitter is less than 3% R.S.D. This system, however, has the advantage of preventing the overloading effects that occur if a sampling valve is used and it can be expected that modules of this type will be used in coupling CZE with high-performance liquid chromatography (HPLC).

A rotary-type sample injector was introduced by Tsuda *et al.* [36]. An injector of this type must be constructed from non-metallic materials (in this instance ceramics and PTFE), as otherwise bubbles are generated by electrochemical reactions at the metal surfaces. The system described had a volume of 350 nl and was

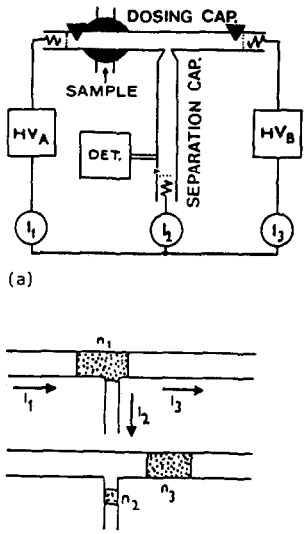

Fig. 5. (a) Scheme of the arrangement and operation of the electric sample splitter (adapted from ref. 35, with permission). ▼, Operating valves. (b) Principle of operation. I_1, I_2 and I_3 = currents; n_1, n_2 and n_3 = samples; HV_A and HV_B = high-voltage sources.

connected to a 200 μm I.D. capillary. The method of operation is evident from Fig. 6. This system is unlikely to find wide acceptability for several reasons. First, there are construction difficulties encountered in scaling down to permit small-volume injections; further, there is a need to operate the sampling device in an

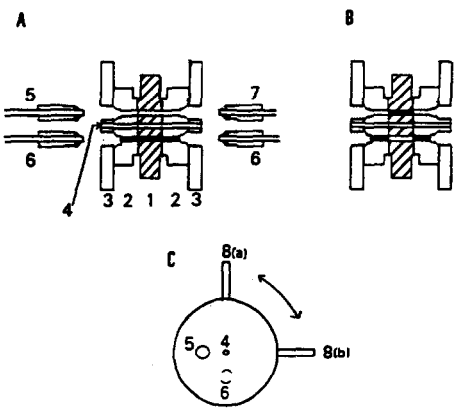

Fig. 6. Schematic representation of the rotary injector. 1 = Rotor; 2 = stator; 3 = plate for setting rotor and stator; 4 = central pin; 5 = tubing between injector and reservoir; 6 = sample introduction tubing; 7 = capillary column; 8 = knob. (A) Loading position; (B) injection position (adapted from ref. 36, with permission).

automated mode for safety reasons; and finally there are problems involved in attaching the injector to small-bore capillaries. Therefore, in spite of the theoretical advantages of the convenient determination of amounts of solutes injected and high reproducibility, the system is unlikely to find wider applicability in CZE.

One of the main advantages of CZE is its ability to handle extremely small amounts of solutes and samples, which makes it amenable to such operations as the analysis of single cells or discrete tissue regions. In order to perform such analyses, three types of microinjector systems have been developed. All of them employ heated glass capillaries that are pulled to form very small tip diameters. The microinjectors are connected to the sample (anodic) end of the capillary, extending it into a very small tip area [37,38].

Next we shall briefly describe the different construction features: basically microinjectors can be constructed with either one or two barrels. As shown in Fig. 7a, the carbon fibre electrode (10 μm O.D.) is inserted into the barrel which connects the microinjector to the high-voltage source. This construction, although simple and versatile at first glance, proved unsuitable owing to poor reliability and the fact that electrolysis occured around the carbon fibre if the glass was not tightly sealed to the fibre. Replacing the carbon fibre with a platinum wire, as shown in Fig. 7b, improves the system considerably. This system can be used with ultra-small tip diameters as their size is no longer limited by the diameter of the carbon electrode. Further, filling of the device with the sampling buffer will cause endoosmotic flow in the microinjector, thus making it suitable for continuous sampling. The third arrangement, shown in Fig. 7c, demonstrates a microinjector with an electrode placed outside the sampling tip. Because of the arrangement there is no risk of electrolytic phenomena and the system proved useful for taking samples from single nerve cells.

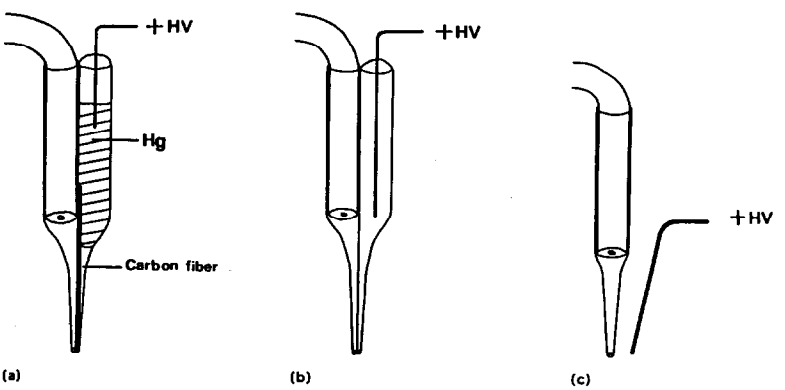

Fig. 7. Schematic representation of different constructions of microinjectors (from refs. 37 and 38, with permission). HV = high voltage.

4. DETECTION SYSTEMS

Detection of the separated species is a problem of its own. In the original version of CZE, solutes were detected on the basis of their UV absorbance; however, more recently other detection principles have been applied in this area [fluorescence, diode array, CZE–mass spectrometry (MS) coupling]. For absorbance detection even advanced HPLC detectors can only be used with limitations as their volume is still far too large and their coupling to the electrophoretic capillary represents a considerable technical problem. In most papers published so far the detection cuvette is represented by a part of the capillary column (about 1 cm) from which the protecting polyimide or silicone rubber sheet has been removed.

4.1. Absorbance detection

The most widespread method of detection in CZE is UV absorption, not only because of its nearly universal application to different kinds of solutes but also because the perspectives of this technique are seen in the analysis of proteins, nucleic acids and their constituents. Reports on UV detection systems and procedures are numerous [31,39–70].

Although principally unsuitable for CZE, many researchers have attempted to modify commercially available HPLC UV detectors for this separation technique. The following problems have to be overcome in such a situation: the detector must be modified in such a way as to allow insertion of the capillary and to hold it firmly in a definite position, and the slits must be sufficiently small to illuminate the capillary only, this consequently leading to considerable losses in the light beam intensity and putting extreme requirements upon the detector's sensitivity. Even with both these restrictions, several workers have succeeded in constructing useful modifications [34,39–45,50,51,57,58,61,64–67,71].

A typical example of such an adaptation was published by Walbroehl and Jorgenson [60]. A pen-ray cadmium lamp that emits radiation from two sides was used to obtain the sample and reference beam. The original slits were replaced by 100-μm pinholes to minimize stray light while illuminating the capillary cuvette sufficiently. Detection limits were 18 fmol for lysozyme at 229 nm with a 75 μm I.D. capillary. In experiments with a custom-made UV detector using a normal deuterium lamp (Hamamatsu), we were able to achieve detection limits of 13–15 fmol for 75 and 100 μm I.D. capillaries at a detection wavelength of 220 nm in the separation of collagen polypeptide chains and their polymers [69].

Two original constructions of CZE UV absorbance detectors have been published so far, namely those of Prusik *et al.* [46] and Foret *et al.* [68]. The first system is based on a fixed-wavelength detector (254 nm) using a high-frequency excited electrodeless low-pressure iodine discharge lamp. A silicon photovoltaic detector with high-gain amplifier was used to detect light power of the order of

10^{-12} W. It operated with a 50 μm I.D. capillary (detector volume 1.6 nl). The detection limit was found to be 67 fmol for phenol (in an electrokinetic micellar separation). The device constructed by Foret *et al.* [68] worked with two optical fibres (200 μm O.D., fused-silica core), which were butted against the capillary column 180° apart. One of the fibres was connected to a mercury lamp and served as the light source, while the other directed the transmitted light to a photomultiplier tube for detection.

4.2. Fluorescence detection

Commercially available fluorescence detectors are generally unsuitable for modifications compatible with the very small capillaries used in CZE. In the original work of Jorgenson and Lukacs [2–5], a simple detector as constructed by Guthrie and Jorgenson [72] was used. This detector was originally designed for use in open-tubular liquid chromatography. It has a high-pressure mercury lamp with glass filters for the selection of the excitation wavelength. Fluorescent light was measured at right-angles to the emission source and the detection limit was reported to be 19 fmol of riboblavin with a 63 μm I.D. capillary (however, the detection unit was operated as a part of an open-column chromatographic system). Later this detector was improved [73] by inserting a double monochromator to achieve more precise wavelength discrimination and to decrease the stray light. The detection limits with this improved device were as low as 2 fmol for α-chymotrypsinogen with a 75 μm I.D. capillary.

Further attempts to decrease the detection limits and increase the light gain on the detection side of the capillary led a number of workers to use laser excitation sources for fluorescence measurements [74–82]. Helium–cadmium (He–Cd) lasers at 325 nm wavelength and 5–10 mW power are most widely used. The advantage of using lasers is based on the better focusing possibility of the emitted light, which allows a more effective transfer of the excitation energy to the capillary. Moreover, lower levels of stray light can be achieved together with better monochromaticity. Laser-based detectors are essential for capillaries smaller than 50 μm.

Another laser fluorescence detection system was described by Zare and co-workers [74,78] and Sepaniak and co-worker [76,77]. They used the usual 325-nm He–Cd laser with a 75 μm I.D. capillary (cuvette volume 0.5 nl), the emitted fluorescence light being collected by means of an optical fibre and passed through a fast monochromator and photomultiplier. The detection limit with this device was reported to be 2 fmol for dansylamino acids. In another version of the laser-excited fluorescence detector desorbed by Kuhr and Yeung [83], the He–Cd laser beam was focused on a 50-μm spot in the fused-silica capillary mounted at the Brewster's angle to minimize light scattering and the emitted light was collected by means of a microscope objective and finally detected through a photomultiplier. In this way a detection limit of 10 amol (for dansylated amino acids) was achieved.

Generally, laser-excited fluorescence detection is classified in the category of ultra-sensitive detection methods [84]. This detection method is frequently applied to amino acid analysis; however, there are two basic problems: protein hydrolysis at subnanogram levels is difficult to perform and there is a general lack of suitable derivatization reagents that would match the wavelength offered by commonly used lasers (He–Cd at 325 nm, argon ion at 488 nm). The latter limitation is the reason why, *e.g.* fluorescein isothiocyanate derivatization is used for primary amines. Its fluorescence parameters match the argon ion laser well. Dovichi and co-workers [81,85] were able to reach detection limits of 10^{-21} mol (10^{-12} *M* solutions were analysed).

As frequently occurs when extreme detection limits are claimed, the above results were obtained with artificial samples, and it is expected that actual biological samples will require additional problems to be solved before comparable sentitivities can be achieved. Moreover, fluorescein isothiocyanate is far from being an ideal label, and Hsieh and Novotny [86] were reportedly unable to separate all common amino acids derivatized with this reagent. Another drawback is that fluorescein isothiocyanate is fluorescent itself. Therefore, Novotny *et al.* [84] attempted to synthesize and introduce new fluorescent labels that would be devoid of all the above drawbacks. They introduced 3-(4-carboxybenzoyl)-2-quinoline carboxaldehyde (CBQCA), which forms fluorescent isoindole derivatives with both amino acids and peptides. Problems were encountered, however, with lysine, because it has two primary amino groups. Oates and Jorgenson [87] tried to overcome this drawback by blocking the ε-amino group prior to hydrolysis. This is fine so far as lysine is concerned, but concomitantly the N-terminal amino acid is lost for analysis as its free amino group is also methylated. The minimum detectable amount after 3-(4-carboxybenzoyl)-2-quinoline carboxaldehyde derivatization was 1.4 amol of glycine ($7 \cdot 10^{-9}$ *M* solution analysed). As isoindoles belong to the most intensively fluorescent compounds, further progress in achieving even lower detection limits can be expected (Fig. 8).

Fluorescence detection is considerably less versatile than detection by absorbance. Although established methods have been developed for fluorescence labeling, the derivatization step still persists in many situations and may be a source of problems (dansylation, fluorescamine, etc.). In order to overcome this type of problem some workers attempted to use indirect fluorescence detection [83], the fluorescent compound (ion) being added to the electrophoresis buffer. The result is either displacement or ion pairing with the fluorophore. Consequently, the signal is independent of the spectral properties of the separated solute. Fluorescence detectors used for this purpose are identical with those described above. Thus, *e.g.*, Kuhr and Yeung [83] used a system in which 1.0 m*M* salicylate was added to the running buffer to provide background fluorescence. Detection limits of 20 fmol were achieved with a set of physiological amino acids. The limitations to the sensitivity of the system are two-fold: the background fluorescence should be as low as possible and the flicker noise of the laser must be eliminated. If the

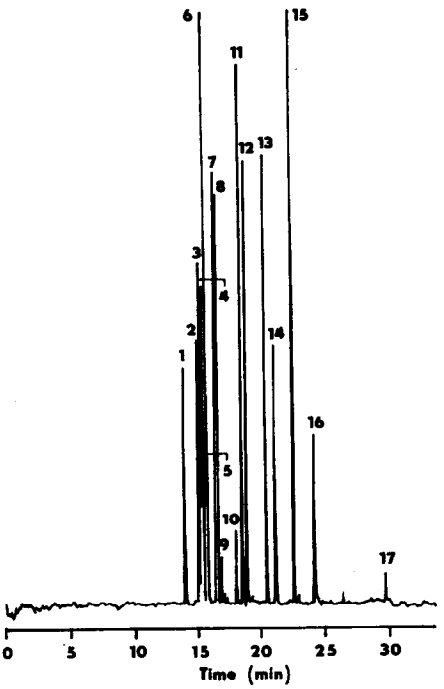

Fig. 8. Electropherogram of a mixture of standard amino acids derivatized with CBQCA. Peaks: 1 = Arg; 2 = Trp; 3 = Tyr; 4 = His; 5 = Met; 6 = Ile; 7 = Gln; 8 = Asn; 9 = Thr; 10 = Phe; 11 = Leu; 12 = Val; 13 = Ser; 14 = Ala; 15 = Gly; 16 = Glu; 17 = Asp. The concentration of each amino acid in the injected sample was $8.71 \cdot 10^{-6}$ *M*; 100 cm × 50 μm I.D. × 184 μm O.D. capillary (70 cm to the detector) (from ref. 84, with permission).

system is optimized along these lines, then detection limits comparable to those obtained with fluorescence labelling can be achieved. Although not widely used at present, the method has wide perspectives for most diverse categories of solutes separated (see also ref. 88).

Other types of arrangements are also possible. Nardi *et al.* [89] recently reported the resolution of α-, β- and γ-cyclodextrins using indirect photometric detection; as cyclodextrins are neutral, their mobility in the electric field was ensured by the formation of inclusion complexes with benzoic acid, which also served as the UV-absorbing constituent of the background electrolyte. Indirect UV detection was possible owing to the special shift of the complexed species.

As CZE is intended for application also in macromolecular separations (*e.g.*, proteins) there is one serious additional problem to be solved. Labelling agents which react with amino groups are widely used for peptide and protein detection; however, the labelled compounds often have more than one amino group. Therefore, these analytes can be labelled at several sites. As the introduction of a fluorescent label mostly leads to the disappearance of one positive charge, it may easily be predicted that unequal precolumn labelling will result in multiple peaks

of the same parent species on the electropherogram. This led some researchers to attempt to construct a postcolumn derivatization system [82]. The reactor consists of three fused-silica capillaries and a stainless-steel tee-fitting. The capillaries represent the electrophoretic, reaction and reagent sections, respectively. Helium pressure is used to pump the fluorescent label into the reactor through the tee-fitting, where it reacts with the electrophoretic capillary effluent and the resulting fluorescence is measured at the end of the reaction section where part of the coating is removed from the surface of the capillary. The reaction capillary was 40 cm × 100 μm I.D. × 375 μm O.D. The reagent capillary was 256 μm O.D. × 150 μm I.D. It is evident that fluorescence labelling in this system must be done with a non-fluorescent reagent and only the reaction products should fluoresce (*o*-phthalaldehyde).

4.3. Raman spectroscopic detection

Raman spectroscopy represents a promising detection technique in CZE. The first equipment for this purpose was described by Chen and Marris [90], based on a conventional Raman spectrometer equipped with a Weite cylinder around a section of the capillary (Fig. 9). A laser beam is focused onto the capillary and the resulting scattered radiation is collected by a series of ten optical fibres (200 μm O.D.) which are assembled around the flow cell. The scattered radiation is directed by the optical fibres to a monochromator and photomultiplier tube. A He–Cd

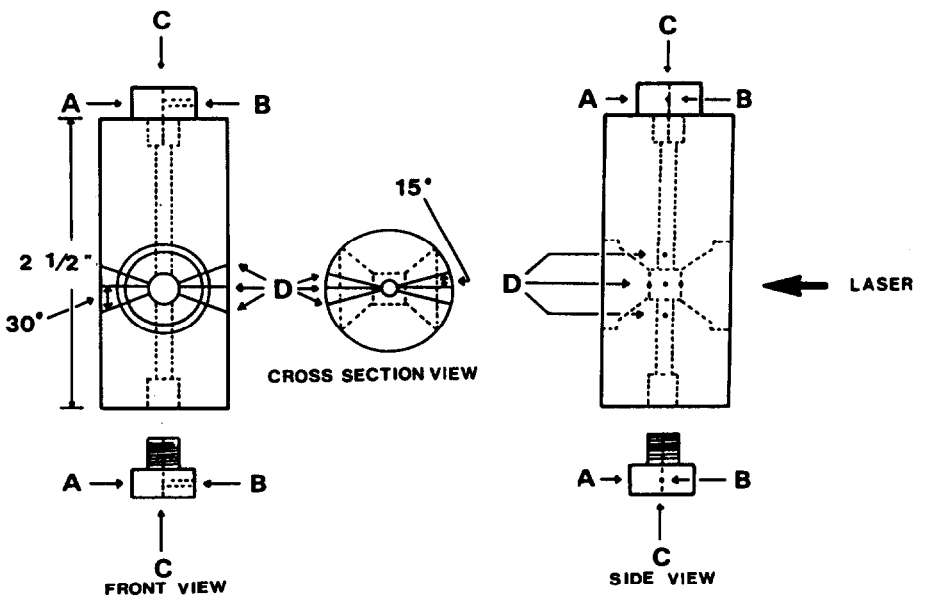

Fig. 9. Schematic representation of the capillary Raman spectroscopic detector. A = Cap; B = set screw; C = bore for inserting the capillary; D = bore for inserting optical fibre (from ref. 90, with permission).

laser (40 mW) was used to generate the 442-nm line which served for excitation. Scattered light at 471.2 nm corresponding to the N=N stretch was monitored. The detection limits were $2.5 \cdot 10^{-6}$ mol for methyl orange and methyl red. Obviously the detection limit is of the same order as with UV spectrometry, but further improvements can be expected, particularly in decreasing the minimum amount needed for detection and in acquiring Raman spectra on-line.

4.4. Electrochemical detection

4.4.1. Potentiometric detection

The first attempts to use potentiometric detection in capillary electrophoresis were reported by Virtanen over fifteen years ago [91]. Nowadays both the basic modes, *i.e.*, the two-electrode and one-electrode systems, are used in connection with CZE. In the one-electrode system potential changes are measured between the indicator and the reference electrodes under the conditions of an open circuit or in the presence of a small current applied to the indicator electrode. The two-electrode systems are generally referred to as conductivity detectors. Here the potential changes between two electrodes are measured under the condition of a small current applied to them.

The first systematic attempts to use conductivity detection were reported by Mikkers *et al.* [92], Deml *et al.* [35] and Foret *et al.* [68]. In the early work of Mikkers *et al.* [92], the peak heights obtained by means of conductivity detection in capillary electrophoresis were very imprecise and therefore conductance peak areas were used as the basis for quantification. The detection limits were in the picomole range. Foret *et al.* [68] used an off-column conductivity detector consisting of three wires located in a polyester resin block and attached to the grounded (cathodic) end of the capillary. An advanced device for conductivity detection was reported by Huang *et al.* [93], consisting of two 25 μm O.D. platinum wires sealed into 40-μm holes penetrating the walls of a fused-silica capillary (Fig. 10). The holes in the capillary wall are located on opposite sides of the capillary and are drilled with a computerized CO_2 laser. Exactly opposite location of the electrodes is an essential condition for avoiding disturbances when the electric potential is applied. The voltage change between the two platinum electrodes is measured and amplified by means of an a.c. conductivity circuit with an oscillation frequency of 3.5 Hz. This detector can work with 75 and 50 μm I.D. capillaries and its limit of detection was 10^{-7} *M* for Li^+. Application to amino acid detection showed negative peaks, which reflects the fact that the mobility of common amino acids is slower than that of common electrolyte ions. The disadvantages of this type of detector are mainly the baseline drift, which is difficult to eliminate, and high detection limits. However, the system is universally applicable to the analysis of ionic species.

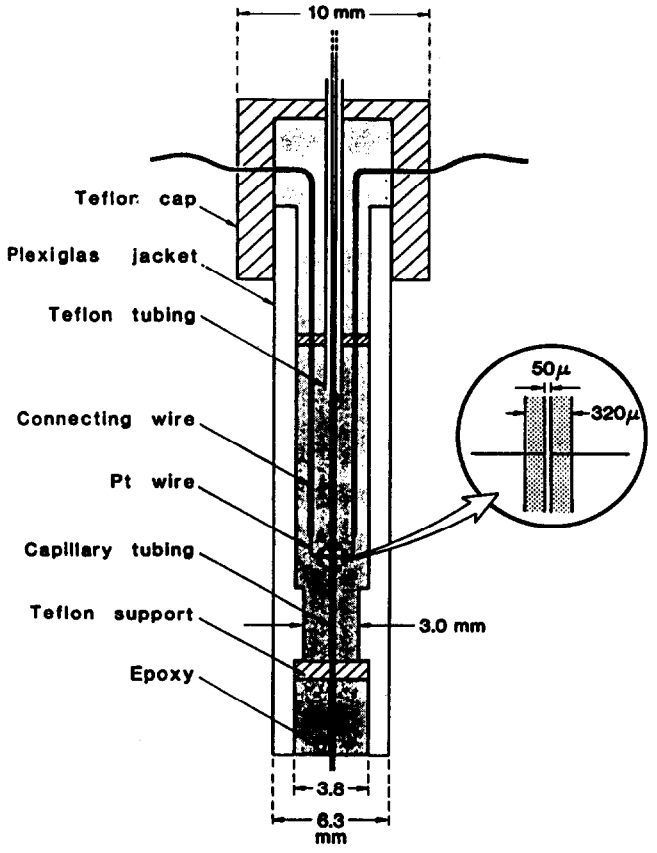

Fig. 10. Schematic representation of the conductivity detection cell (from ref. 93, with permission).

4.4.2. Amperometric detection

From the nature of CZE, it emerges that amperometric detection is basically incompatible with the electric current produced in the capillary on the application of the 10–30 kV separation potential. The electric current produced by this voltage is about six orders of magnitude greater than those obtained in an amperometric detector. Consequently, if amperometric detection is to be used, the detector must be electrically isolated from the applied separation potential.

In a series of papers, Wallingford and Ewing [38,94–96] developed an amperometric detector interface shown schematically in Fig. 11. The interface consists of an electrically conductive joint placed at the cathodic (detection) end of the capillary. This construction allows independent application of the running and detection potential across the capillary and the detection segment. The solutes are driven through the detector by the endoosmotic flow created in the separation capillary. Detection is effected by inserting a single carbon fibre into the end of the capillary and the current between this electrode and the reference electrode

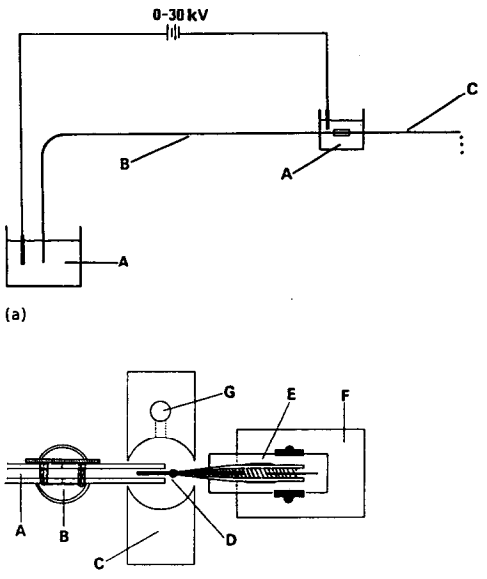

Fig. 11. Schematic representation of electrophoresis arrangement for amperometric detection. (a) Overall assembly. A = Buffer reservoirs; B = separation capillary; C = detection capillary. (b) Top view of the detection system. A = Capillary; B = porous glass joint assembly; C = Plexiglas block; D = carton filter electrode; E = microscope slide; F = micromanipulator; G = reference electrode (from refs. 38 and 94–96, with permission).

located in the body of the detector is recorded. This detection system was applied to a 26 μm I.D. capillary and detection limits were 200–5000 amol for catechols, 6 amol for 5-hydroxytryptamine and 22 amol for isopropylnoradrenaline. As far as the diameter is concerned, the separation capillary used in these experiments is one of the smallest reported so far. Such extremely narrow-bore capillaries can be used only with this type of detector and are possible owing to the small-diameter electrode. Smaller I.D. capillaries also exhibit smaller annular flow regions around the electrode, resulting in greater coulombic efficiency and, consequently, higher sensitivity.

4.5. Radioactivity detection

Labelled radioactive compounds are widely applied in biomedically oriented research. Radioisotope detection appears appealing also for other reasons. State-of-the-art radiation detection technology offers extremely high sensitivity; also, radioactive metabolites elicit the detector's response. In addition, the radioactively labelled molecule possesses the same properties as the parent species, permitting tracer studies and the measurement of absolute concentrations of the labelled compound. Altria *et al.* [97] and Berry [98] designed a radioactivity detector based on passing a capillary (2 cm long) through a solid block of scintillator material.

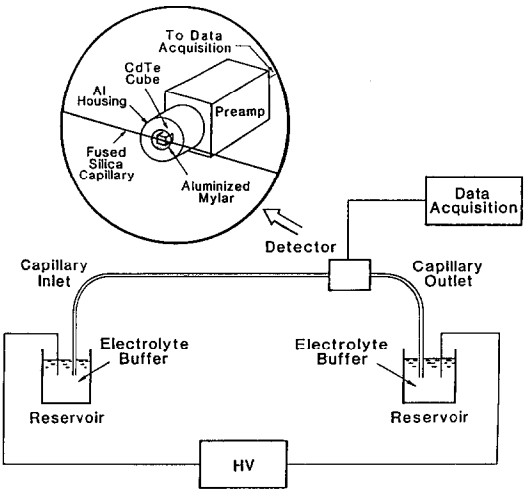

Fig. 12. Arrangement of the on-line radioactivity detector. The arrangement of the detection cell is shown in the inset (from ref. 99, with permission).

More recently, Pentoney *et al.* [99] reported another version of the radioactivity detector (Fig. 12). The detector probe is made of a 2-mm cadmium telluride probe which is mounted in plastic and located behind a thin film of aluminized Mylar (1.5 mm from the face of an aluminium housing). The cadmium telluride detector is shielded with a lead aperture (2 mm × 0.2 mm thick). The front of the aluminium housing is joined to a sensitive miniature preamplifier and is in direct contact with the polyimide-coated fused-silica capillary–lead aperture assembly. The detection volume with a 100-μm capillary was 15 nl (using a 2-mm section of the capillary for detection). It must be emphasized that with a radioactivity detector in CZE, the residence of individual solutes within the detector must be determined separately for each component because the separated sample zones travel with different velocities according to their speed of electrophoretic migration. In this respect the CZE radioactivity detector differs from the detectors used in chromatography, where the residence time for every separated solute is the same.

Recently a new construction of γ-ray detector for CZE was described by Altria *et al.* [100] (Fig. 13). The separation capillary is closely attached to the surface of the crystal for maximum response; however, the collection efficiency is less than 50%. The precise length of the capillary exposed to the scintillator crystal was adjustable by using two lead sheets between the crystal surface and the axis of the capillary to form a slit. The optimum slit width was determined by electrophoresing a pertechnate solution and determining the signal obtained *versus* zone broadening with varying slit width. It was demonstrated that a slit width of 20 nm, corresponding to a zone volume of 8.84×10^{-2} μl, was a reasonable compromise between peak efficiency and signal strength. The loss in efficiency was about 10%.

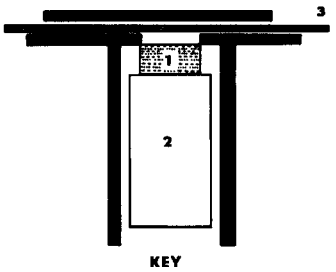

Fig. 13. Schematic representation of the γ-ray detector (according to Altria *et al.* [100], with permission). 1 = Scintillation crystal; 2 = photomultiplier tube; solid lines, lead shielding; 3 = capillary.

The detector was shown to have a linear response within the range 10–500 Bq cm^{-3}, which corresponds to $5.1 \cdot 10^{-17}$–$2.55 \cdot 10^{-15}$ g cm^{-1} of Tc^{99m}. The practical applicability of this detector was demonstrated with Tc^{99m}–dimercaptosuccinic acid chelates. These compounds with Tc in oxidation state III or V are used in radiopharmacy for imaging tumours and kidneys. A variety of separation conditions were investigated.

With a carrier electrolyte containing 0.02 *M* benzyltrimethylammonium bromide at pH 7.0 and -30 kV, a signal for Tc^{V} radioactivity was obtained at 7.2 min, indicating the ionic nature of the separated complex (this electrolyte was shown to have zero electroendoosmotic flow). With a phosphate buffer (0.02 *M*, pH 7.0) at -30 kV (anodic electroosmotic flow) the signal was obtained at 21.1 min, indicating again the anionic nature of the chelate under these conditions (obviously the ionic nature of the chelate depends more on the pH used than on the nature of the buffer). When a Tc^{III} complex was electrophoresed in 0.02 *M* benzyltrimethylammonium bromide buffer (see above), the signal was obtained at 13.6 min. This lower retention time is indicative of a lower anionic state of the complex and/or lower coordination number. Taking into account the fact that at pH 7.0 the respective chelates have charge -1 (III) and -3 (V), the ratio of the migration times should be 3; however, 1.89 was found, indicating that the coordination numbers of the two states must differ. This is not only a good example of how CZE with a radioactivity detector can be used in the analysis of radiopharmaceuticals, but also what conclusions regarding the structure of the separated solutes can be drawn from such results.

Naturally, the sensitivity of a radioactivity detector can be increased by increasing the residence time within the detection zone (equal to the increased time in scintillation counting). Such an increase in residence time can be realized by slowing the flow in the capillary during a period when the solute passes the detector by a temporal decrease in the applied voltage. Hence sophisticated voltage programming leads to a considerable increase of the system's sensitivity. It was claimed that limits of detection of 10^{-11} *M* can be achieved [100].

4.6. CZE–mass spectrometry (MS) coupling

MS is potentially an ideal method of detection for capillary electrophoretic techniques. At present basically two types of spectra are exploited, namely those arising from fast atom bombardment (FAB) or electrospray ionization (ESI). Both have advantages and disadvantages. The flowing FAB interface is attractive because of its compatibility with commercially available mass spectrometers, but problems arise from the necessity to use FAB matrix substances, the need to minimize the pressure gradient across he capillary and, the constraints of the FAB method as such.

Historically, the ESI interfaces were the first to be used [101–103]. A schematic representation of such a system is presented in Fig. 14. The main difference from all other CZE systems is that the detection end of the capillary is not placed into the cathode reservoir. Instead, either a metal needle or a thin film of metal deposited on the capillary end ensures electrical contact with the buffer solution and thus it is possible to create an appropriate voltage potential along the capillary. ESI is done at atmospheric pressure and hot nitrogen flowing across the electrospray interface removes the solvent before the ions enter the mass analyser.

The system is combined with a quadrupole mass filter to permit on-line detection. An example of a separation is shown in Fig. 15, where the separation of five quaternary ammonium salts is presented (total-ion reconstructed electropherogram). The peaks correspond to 10^{-6} M of each component (14–17 fmol injected). The detection limits of this system are as low as 10 amol. The highest sensitivites are achieved under conditions that facilitate desolvation and minimize cluster formation. The latter problem occurs particularly with aqueous buffers. In most experiments of this type, buffers containing 50% of methanol are used to increase volatility. The flowing liquid sheath interface offers the possibility of controlling the electrosprayed liquid (its composition and flow-rate) independently of the CZE buffer. The importance of this construction is based on the fact that the relatively high-ionic-strength buffers used in CZE are generally not com-

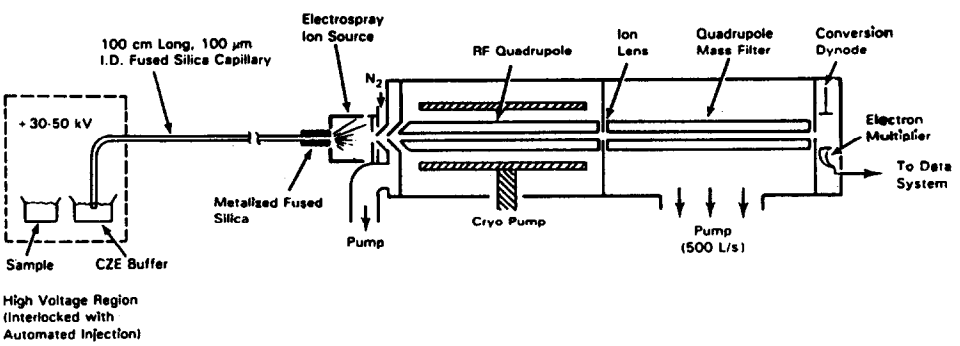

Fig. 14. Schematic representation of CZE–MS coupling (from refs. 101–103, with permission).

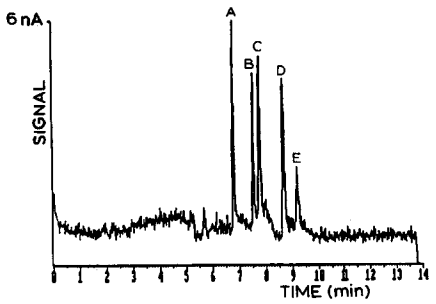

Fig. 15. Representation of a reconstructed ion electropherogram of quaternary ammonium salts: concentration 10^{-6} *M*; CZE–MS system. Peaks: A = tetramethylammonium bromide; B = trimethylphenylammonium iodide; C = tetraethylammonium perchlorate; D = tetrapropylammonium hydroxide; E = tetrabutylammonium hydroxide (from ref. 101, with permission).

patible with ESI. Electrical contact in this construction is established through the liquid sheath (methanol, acetonitrile, acetone, etc.) containing (for protein analysis) a small proportion of water.

Early attempts to use CZE–MS coupling related to the above quaternary ammonium salts, dipeptides and amines. Recently, however, a number of other applications have appeared, including a wide selection of proteins [104–108].

Hallen *et al.* [109] undertook a systematic investigation of ESI designs for the introduction of samples into the ion mobility spectrometer; they used metallized-

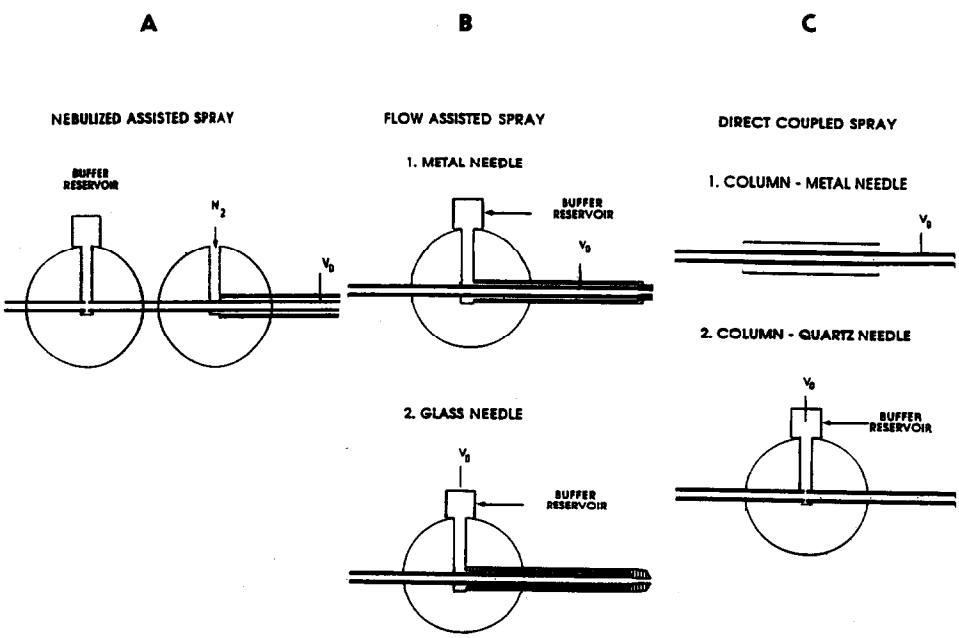

Fig. 16. Schematic representations of different CZE–MS interfaces (from ref. 109, with permission).

assisted spray (Fig. 16A), flow-assisted spray (Fig. 16B) and direct-coupled spray interfaces (Fig. 16C). Of these the last two were the most promising.

In the flow-assisted interface, the separation column is inserted through a tee-fitting to the end of the electrospray needle. A sheath flow of a secondary buffer is introduced by the tee-connector, which ensures contact with the electrophoretic buffer at the end of the column. Potential is established by placing an electrode in the sheath flow buffer or through a metal needle. Establishing the terminal potential through the buffer is necessary when using a glass electrospray needle. Obviously this system resembles that of Smith *et al.* [102] described briefly above.

The direct-coupled interface is analogous to that used in pumped flow systems. Hallen *et al.* [109] reported two modifications of this approach, one with a metal needle and the other with a fused-silica needle. In the first instance the terminal voltage is applied directly to the metal needle. With the fused-silica needle the terminal voltage is applied through the connector joint by an external buffer solution.

It was claimed that the CZE–MS system operated not only with a high separation efficiency but also with good ion mobility reproducibility. The number of theoretical plates reported was $3 \cdot 10^3$ (for tetrabutylammonium iodide) with ion mobilities reproducible with 1.43% R.S.D. However, the method has some inherent problems. Attempts to maintain the spectrometer as hot as possible occasionally result in vaporization of the solvent in the interface, creating an unstable spray and inhomogeneities in the electric field, with consequent disturbances in electrophoretic migration.

The development of FAB ion mobility MS interfaces with flowing liquid streams by Caprioli *et al.* [106], referred to as a flowing liquid stream FAB interface or frit FAB, has provided an MS interface particularly suited for the analysis of non-volatile and possibly thermally labile analytes that are so frequently present in biological samples. Moseley *et al.* [104] exploited these data for constructing a coaxial flow FAB interface, which was used for coupling CZE with tandem sector MS. However, successful attempts to combine FAB with CZE were already reported by Minard *et al.* [110], De Wit *et al.* [111] and Moseley *et al.* [112]. It should be emphasized here that the low flow-rates in CZE place extremely high requirements on the construction of the interface for CZE–FAB-MS coupling.

The coaxial continuous flow CZE–FAB-MS interface is shown schematically in Fig. 17. The CZE fused-silica capillary is inserted into a sheath fused-silica capillary column using a stainless-steel tee-fitting to accommodate the two columns. Both coaxial capillary columns terminate at the FAB probe tip, which is electrically insulated from the probe shaft. The 8-kV FAB probe tip is used as the electrical ground of the CZE system. The active electrophoretic transport moves the analytes through the CZE column towards the FAB probe tip, where ion desorption takes place. Thus no transfer line between the CZE capillary and the

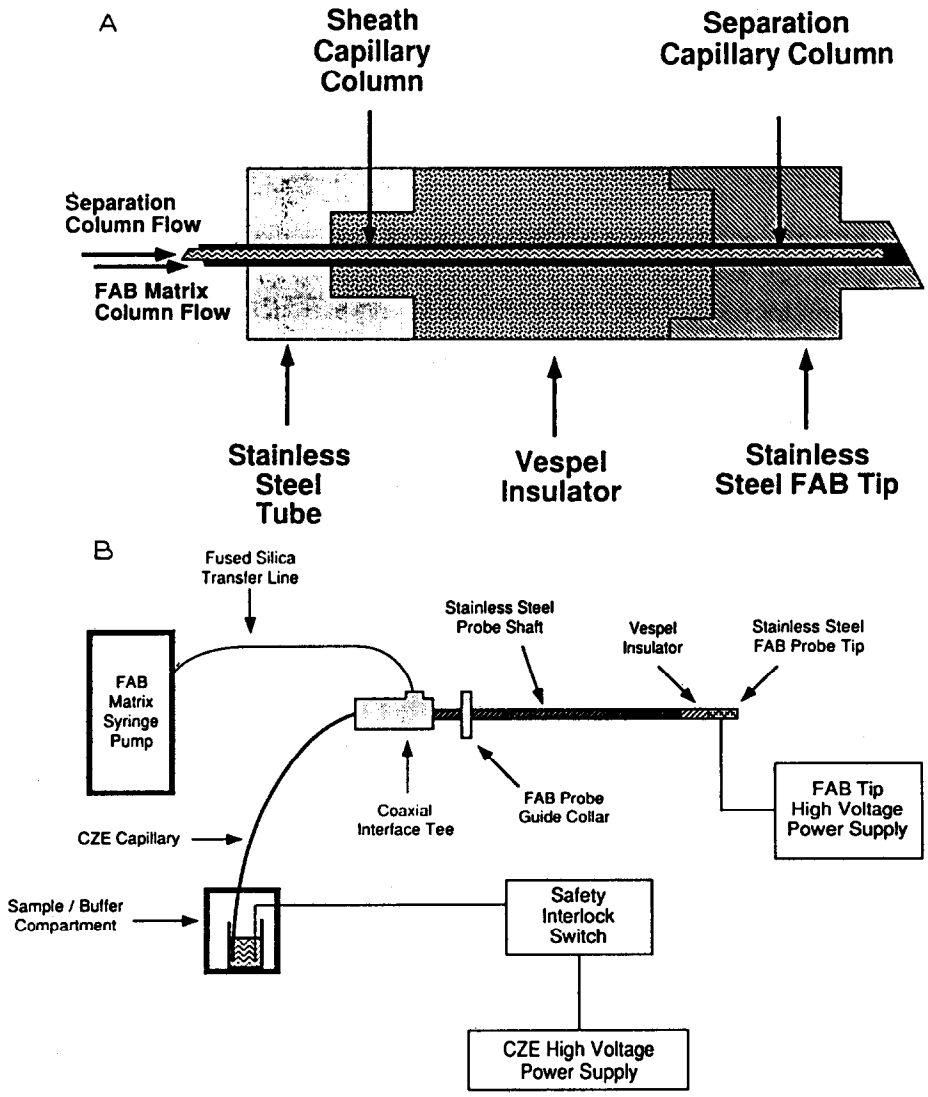

Fig. 17. Schematic representations of (A) the coaxial continuous-flow FAB tip and (B) on-line coaxial continuous CZE–FAB-MS system (from ref. 104, with permission).

FAB probe tip is needed and post-column band broadening is eliminated. The CZE high-voltage supply delivers 30 kV to the anodic end of the capillary, while the detection end of the capillary (the FAB probe tip) is maintained at 8 kV, thus yielding a 22-kV potential drop across the CZE capillary column. The FAB matrix used in this CZE–FAB-MS system was 25% glycerol in 0.0005 *M* heptafluorobutyric acid, which served to provide ions for electrical contact between

the FAB tip and he CZE column effluent. Concomitantly it acidified the FAB probe tip and helped to increase the production of protonated molecular ions.

Caprioli *et al.* [106] described another type of CZE–continuous-flow FAB interface. This consists of a Plexiglas block (Fig. 18) containing two perpendicular intersecting passages. The effluent (cathode) end of the CZE capillary column meets the intake of the continuous-flow FAB capillary in a short segment of PTFE tubing placed in the left arm of the horizontal passageway. In the upper part of the vertical passage a flow-through stainless-steel electrode attached to a syringe is placed. The lower part of the vertical passageway is used for introducing the continuous flow FAB carrier (5% glycerol with 3% acetonitrile).

The flow-through electrode in the upper part allows for periodic flushing of the compartment with the FAB solvent to remove occasional bubbles formed at the interface. Simultaneously, the syringe attached provides a means of introducing the sample at the anodic end of the capillary. By sucking a constant volume and using methylene blue as visual indicator, reproducible amounts of the sample are introduced into the capillary. Regarding the limit of detection for this sytem, with 75 fmol of angiotensin a peak having a signal-to-noise ratio of 2.5:1 was recorded (the mass spectrometer was scanned over a 4 mass units wide window centred on m/z 1046, the $[M + H]^+$ ion). For peptides having m/z between 500 and 3000 the

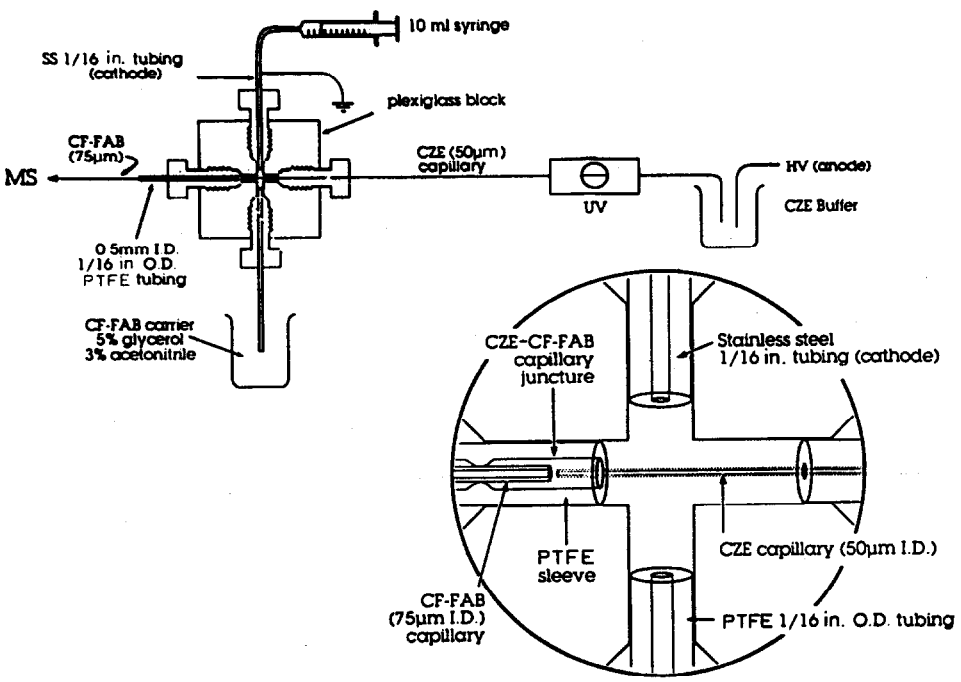

Fig. 18. Schematic representation of the CZE–continuous-flow FAB instrument arrangement and interface. See text for details. SS = stainless steel; HV = high voltage (from ref. 106, with permission).

amount of sample needed to obtain reasonable signal-to-noise ratios has to be in high femtomole or low picomole range.

5. SPECIAL ARRANGEMENTS OF CAPILLARY ZONE ELECTROPHORESIS

5.1. Chiral separations

In micellar electrokinetic chromatography, an ionic surfactant is used as a buffer solution at a concentration higher than the critical micelle concentration. The idea of chiral separations in CZE is to use a chiral micelle. The ionic micelle moves in the electrophoretic system with a different speed to the surrounding aqueous medium and the injected solute distributes between the micellar and aqueous phases.

One of the first attempts in this respect was published recently by Dobashi *et al.* [113]. The procedure is based on a micellar electrokinetic separation mechanism. In this system, separation is effected through the distribution of a solute between the micellar and aqueous phases. Thus micellar (electrokinetic) chromatography should reveal the essential hydrophobic binding affinity of micelles towards a solute and provide a versatile means of obtaining micellar selectivity towards different analytes. Given an effective charge through the micelle, even uncharged analytes can be separated by endoosmotic flow in the capillary.

The aim in enantioselective separations is to make the micelle chirally specific, which can be realized by introducing a chiral functionality into the micelle. This can be achieved by micellization of N-dodecanoyl-L-amino acid sodium salts. These chiral media differ from the structural chiral cavity of cyclodextrin with which a solute binds hydrophobically with the formation of an inclusion complex. The chiral micelles are ordered under the influence of the surrounding aqueous solution and the interfacial phase adjacent to the bonded phase. Consequently, micellar phases are unlikely to allow a tight fit of the stereospecific molecule as is believed to occur in inclusion complexes. Instead, they possess different binding affinities toward enantiomeric solutes. Consequently, it is postulated that enantioselectivity in these micellar chiral surfactants is observed only when the solute to be separated binds through hydrogen bonding with the amide functionality in the micellar interior core. Typically, racemic N-3,5-dinitrobenzoylamino acid isopropyl esters can be separated as shown in Fig. 19. For the amino acid derivatives, the D-enantiomer is eluted faster than the L-enantiomer, indicating a preference of the micelle to bind the L-enantiomer. Obviously this is due to the fact that both the L-enantiomer and the micellar phase have the same configuration.

Among the different amino acid derivatives investigated, the alanine derivatives were the least strongly retained and the phenylalanine derivatives were the most retained. It was concluded that the elution order of each series of amino acid derivatives reflects the increase in hydrophobicity of the amino acid side-chain followed by entanglement with the micellar interior core. It is worth noting that

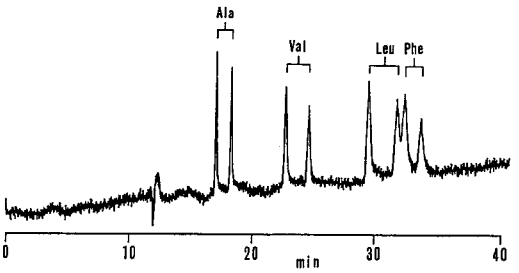

Fig. 19. Optical resolution of a mixture containing four enantiomeric pairs of amino acids as N-(3,5-dinitrobenzoyl)-O-isopropyl ester derivatives by electrokinetic capillary chromatography. Chromatographic conditions: column, fused-silica tubing (Scientific Glass Engineering), 50 cm × 50 μm I.D., for effecting the separation; micellar solution, 0.025 *M* sodium N-dodecanoyl-L-valinate (SDVal) in 0.025 *M* borate–0.05 *M* phosphate buffer (pH 7.0); total applied voltage, *ca.* 10 kV; current, 26 μA; detection UV at 230 nm; temperature, ambient (*ca.* 20°C) (from ref. 113, with permission).

esterification of N-acylated amino acids was found to be essential as unesterified racemic 3,5-dinitrobenzoyl amino acids were insensitive to the chiral micelle separations and they were generally less retained than the corresponding isopropyl ester derivatives.

A number of other factors were also investigated. Thus, for instance, the tested enantiomeric solutes were the more difficult to separate the more the chiral micellar phase was diluted with achiral sodium dodecyl sulphate (SDS) and at a concentration of chiral phase about 33% the separation ceased completely. Investigation of the influence of the amino acid side-chain in the chiral surfactant indicated that, *e.g.*, the presence of alanine in the chiral surfactant provided smaller separation factors than if valine was incorporated into the chiral micelle. This has been related to the fact that in the presence of alanine the critical micellar concentration was much higher than in the presence of valine and to the much smaller perturbation of the micellar system by alanine, as indicated by spectroscopic measurements [113].

The resolution of enantiomeric mixtures was improved by adding methanol to the co-micellar solution at a level of 5–10% (v/v). The retention of solutes became shorter with increasing methanol concentration. Differences in the elution order in the co-micellar systems are ascribed to the differences in the interactions of the inner capillary wall with the micellar phase.

Cohen *et al.* [114] described the use of a micellar system of SDS and N,N-didecyl-L-alanine in the presence of copper(II) for chiral separations of some amino acids. The chiral separations were considered to be based on differential metal chelate complexation on the surface of the micelle; in other words, the mechanism involved is similar to the chiral separation with an amino acid–copper (II) complex [115]. However,the N,N-didecylamino acid derivative had to be used, as, *e.g.*, alanine itself does not form a micelle.

The above approaches are based on SDS perhaps because it is the most com-

monly used surfactant in micellar electrokinetic chromatography (micellar CZE), although other surfactants such as cetyltrimethylammonium bromide and sodium N-lauroyl-N-methyltaurate have also been successfully used.

Terabe *et al.* [116] described chiral separations in CZE with bile salt chiral surfactants. In order to act as the micellar phase, the surfactant must be ionic and therefore some commercially available bile salts were used such as sodium cholate, sodium taurocholate and sodium taurodeoxycholate. Typically 50 m*M* phosphate (pH 7) was used as the background buffer. Amino acids tested as enantiomeric solutes had to be derivatized to facilitate interaction with the micellar phase. In this particular case they were dansylated. At the specified pH and with taurocholate as the micellar component, all the dansylamino acids migrated towards the cathode. With decreasing pH (to 3), not only was the electroosmotic flow reduced considerably but also the migration direction of the solutes was reversed. The order of elution then generally reflects the hydrophobicity of the solute; hence a more hydrophobic solute that is more easily incorporated into the micelle will migrate faster than a less hydrophobic solute. The best results reported by Terabe *et al.* [116] for chiral separations of dansylamino acids were accomplished with 50 m*M* taurodeoxycholate using 50 m*M* phosphate (pH 3) as the background buffer at 40°C.

New horizons were opened up by using SDS as the micellar mobile phase with a suitable chiral additive such as N,N-didecylvaline in the presence of copper, digitonin or cyclodextrin [10]. Cyclodextrin derivatives can be used also directly (*e.g.*, for the separation of enantiomeric amino acids) as they have both chirality and an ionic group in their molecule [52]. Recently Fanali and Bocek [117] reported the separation of D- and L-tryptophan using α-cyclodextrin as a chiral active component in the background electrolyte. They were also able to demonstrate the separation of (+)- and (−)-epinephrine by supplementing the background electrolyte with heptakis(2,6-di-O-methyl-β-cyclodextrin). As a practical applicaton, the separation of epinephrine enantiomers in pharmaceutical adrenaline preparations was shown.

Enantiomeric amino acids have served as test solutes for chiral separations frequently in the past. A chiral copper(II)–histidine and a chiral copper(II)–aspartame complex have been successfully applied (as a pseudo-phase) [115].

As with chromatographic separations there is also the possibility of derivatizing the enantiomeric solutes to diastereomers and separating them in this derivatized form. Thus D,L-amino acids can be converted into the respective diastereomers, *e.g.*, by reaction with 2,3,4,6-tetra-O-acetyl-β-D-glycopyranosyl isothiocyanate or Marfey's reagent. The separation itself is then carried out in SDS solution [118].

5.2. Two-dimentional separations by means of LC–CZE coupling

Although the separation potential of CZE is high, it would be useful to increase it further. Some prospects lie in the application of sophisticated detection

systems as discussed earlier. The other possibility is to use two-dimensional separations (LC–CZE coupling).

Yamamoto *et al.* [119] reported on the construction of a new type of apparatus which combines gel permeation chromatography with CZE. Proteins were used as test solutes. They were separated by gel permeation chromatography in the first step followed by their separation according to their effective charge in CZE.

A schematic representation of the apparatus is shown in Fig. 20. The first separation (chromatographic) step was realized in a polyethylene microbore tube packed with Sephadex G-50 (fine). A microperistaltic pump was employed to introduce the column effluent into the sample injection port of the electrophoresis

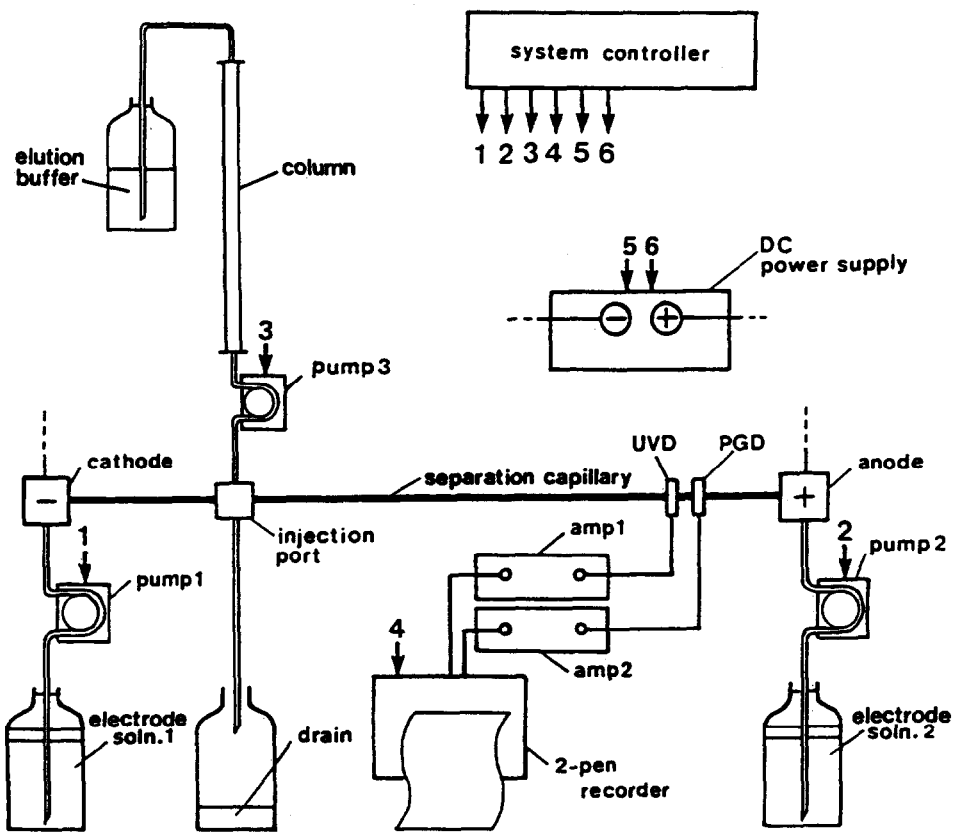

Fig. 20. Schematic representation of a combined apparatus for chromatography and capillary electrophoresis. The terminating electrolyte (soln. 1) and the leading electrolyte (soln. 2) are pumped by peristaltic pumps (pumps 1 and 2) to wash the electrodes and separation capillary. The effluent from the column is loaded with the microperistaltic pump (pump 3) through the sample injection port. High-voltage d.c. is applied between the electrodes and the protein zones are detected with the potential gradient detector (PGD) and UV detector (VVD) during the run. The numbered arrows indicate the output lines connected from the system controller to the individual subassemblies (from ref. 119, with permission).

apparatus. One perfluorinated ethylene–propylene (PFEP) tube connected the column outlet to the silicone-rubber tubing of the peristaltic pump, and another PFEP tube was used to connect the micropump outlet with a glass capillary tube

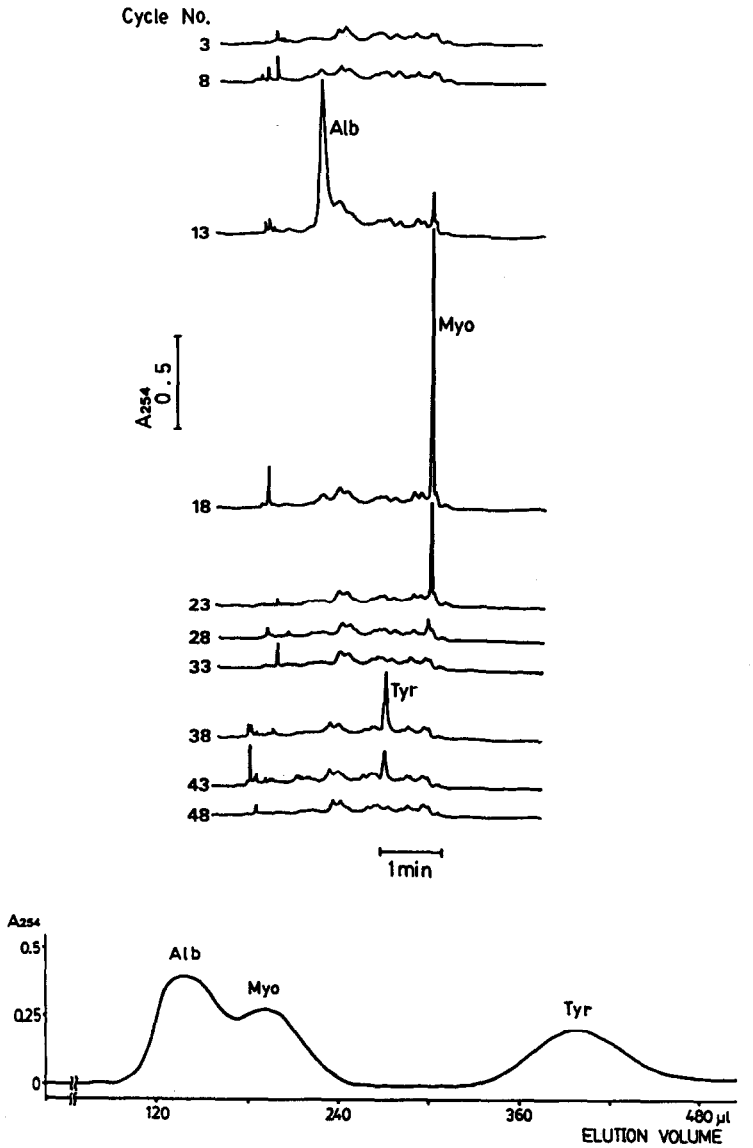

Fig. 21. Separation of albumin, myoglobin and tyrosine with the combined apparatus. The UV patterns of the electropherograms were traced every five cycles and the separation of the samples was demonstrated. In the lower part the result of gel permeation chromatography is shown (adapted from ref. 119, with permission).

(50 mm × 240 mm I.D.) inserted in the injection port of the electrophoresis apparatus. The time schedule of the automated apparatus was as follows. First buffers were pumped by the peristaltic micropump to rinse (separately) both the anodic and cathodic jars; when rinsing the cathode, at the same time the capillary was filled with the buffer solution. After this, the chromatographic column effluent was introduced directly into the sampling part of the electrophoresis apparatus and separation was run at a constant current of 150 μA. After the run was completed, the high d.c. voltage and the recorder were switched off and the procedure was repeated from the beginning. The time needed for one cycle was 18 min. However, only the part during the last 9 min was recorded. The electropherograms were traced every five cycles. The separation of a test mixture consisting of albumin, myoglobin and tyrosine is shown in Fig. 21.

6. APPLICATIONS

6.1. Amino acids

The determination of minute amounts of amino acids is a frequently occurring problem in biological chemistry. It may be argued that current HPLC techniques meet the demands of amino acid analysis in most biochemical laboratories and therefore no additional techniques are necessary. However, in natural samples the determination of extremely small amounts of amino acids is needed, particularly if the sample itself is already very small. This is the area in which CZE may find numerous applications.

The first problem to be solved in this respect is the method of detection. To date most of the sensitive amino acid assays have exploited fluorescence derivatization reagents. Low- to subfemtomole amino acid analyses have been reported using laser-induced fluorescence detection of 9-fluorenylmethyl chloroformate [120], 5-dimethylaminonaphthalenesulphonyl chloride [74] and naphthalenedialdehyde derivatives [121], and also CBQCA (see Section 4.2 and Fig. 8). Some absorbance derivatization reagents exhibit comparable results, *e.g.*, phenyl isothiocyanate and 4-(dimethylamino)azobenzene-4′-sulphonyl chloride [122,123].

In early attempts at CZE separations, frequently mixtures of amino acids were used as test solutes (*e.g.*, refs. 2 and 31). However, systematic reports are limited and make use mainly of 4-(dimethylamino)azobenzene-4′-sulphonyl (DABSYL) amino acids.

With respect to DABSYL amino acids, Yu and Dovichi [124] were able to separate attomole amounts by CZE using a mixed acetonitrile–aqueous buffer system. Detection was performed with an on-column thermooptical absorbance detector based on a pumped argon ion laser. Detection limits achieved were $5 \cdot 10^{-8}$ *M* for methionine and $5 \cdot 10^{-7}$ *M* for aspartic acid. Only 37 amol of methionine and 450 amol of aspartic acid were present in the injected sample. It

was emphasized that these detection limits are four times better than those obtained with fluorescence detection in chromatographic separations. The practicability of this technique was shown with subnanolitre samples of human urine; it was demonstrated that the amounts present in these samples were three orders of magnitude higher than the detection limit of the procedure. A list of detection limits for six naturally occurring amino acids is presented in Table 1.

The separation was carried out in a 1:1 mixture of acetonitrile and 20 m*M* phosphate buffer (pH 7) which contained 5 m*M* SDS. A typical electrophoretic run is shown in Fig. 22.

It may seem surprising that absorbance detection gives similar or even better results in comparison with fluorescence measurement. However, it must be borne in mind that fluorescence measurement in narrow-bore tubes (needed in CZE separations) results in the formation of a fare of scattered laser light in a plane perpendicular to the tube which represents a substantial part of the background noise. In thermooptical measurements scattered light is easily eliminated, thus helping significantly to increase the difference between the signal and background noise.

Fluorescein isothiocyanate- and phenyl isothiocyanate-labelled amino acids represent two other types of derivatives suitable for CZE separations, as demonstrated in Fig. 23 [31,81].

Another promising detection technique that appears to be applicable to amino acid analysis is indirect fluorescence detection. In this instance the fluorophore is used as the principal component of the electrophoretic buffer, allowing the visualization of non-fluorescing ions through charge displacement of the fluorophore [125,126]. Naturally this type of detection can be used with many other types of solutes besides amino acids, such as nucleotides, nucleosides and proteins, and

TABLE 1

DETECTION LIMITS FOR SELECTED AMINO ACIDS WITH THERMOOPTICAL DETECTION AND STATE-OF-THE-ART HPLC WITH FLUORESCENCE DETECTION

From ref. 115, with permission.

Amino acid	Detection limit[a] (amol)	
	Thermooptical detection	Fluorescence detection
Methionine	37	420
Glycine	42	240
Alanine	58	300
Tryptophan	71	540
Glutamic acid	81	240
Aspartic acid	421	240

[a] Detection limits correspond to the amount of reagent injected onto the column that produces a signal that is three times larger than the standard deviation of the background signal.

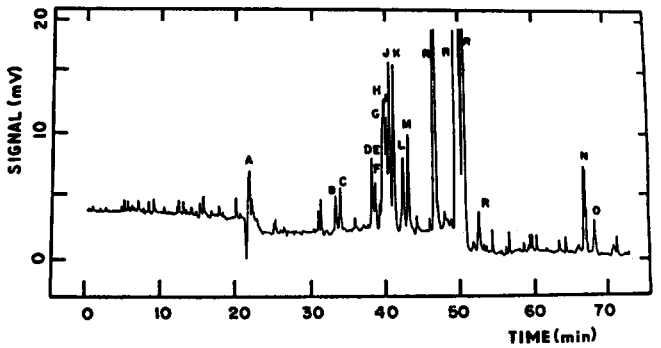

Fig. 22. Electropherogram of eighteen DABSYL amino acids, $2.5 \cdot 10^{-6}$ *M* injected. Peaks: A = arginine; B = histidine; C = lysine; D = cysteine and tyrosine; E = tryptophan; F = proline; G = phenylalanine; H = leucine; I = methionine; J = isoleucine and valine; K = tyrosine and serine; L = alanine, M = glycine; R = reagent peaks; N = glutamic acid; O = aspartic acid (from ref. 124, with permission).

limits of detection up to 50–100 amol of the injected samples can be achieved. A description of the separation of amino acid enantiomers was presented at the end of Section 5.1.

Recently a thorough study was published on CZE and laser-based detection of fluorescein thiohydantoin and dimethylaminoazobenzene thiohydantoin derivatives of amino acids [127]. The detection limits achieved were 10^{-21} mol for the fluorescein derivative and 10^{-16} mol for the dimethylaminoazobenzene derivative. The separation itself was carried out in a 1 m × 50 μm capillary with 10 m*M* phosphate buffer (pH 7.0) at 30 kV. The resulting separation of fluorescein thiohydantoin derivatives is presented in Fig. 24.

6.2. Proteins and peptides

The high efficiency of CZE together with the small samples needed for analysis and easy automation of the separation procedure represent a challenge to many biochemists. Compared with gel electrophoretic techniques, the advantage of CZE is seen mainly in the possibility of direct quantification of eluted peaks. The main problem that has to be overcome here is the high affinity of proteins for silica surface, which results in irregular peaks and poor reproducibility of results.

6.2.1. Peptides

In peptide analysis, much emphasis is currently placed on predicting the electromigration properties of these analytes. The possibility of simple and selective determination of posttranslational modifications and the identification of genetic variants are among the most important tasks. In the past LC has been exploited for this purpose. There is no doubt that CE will augment or complete the possibilities offered by LC so far, particularly because, owing to the difference in separation mechanisms, there is no correlation between CE migration times and LC

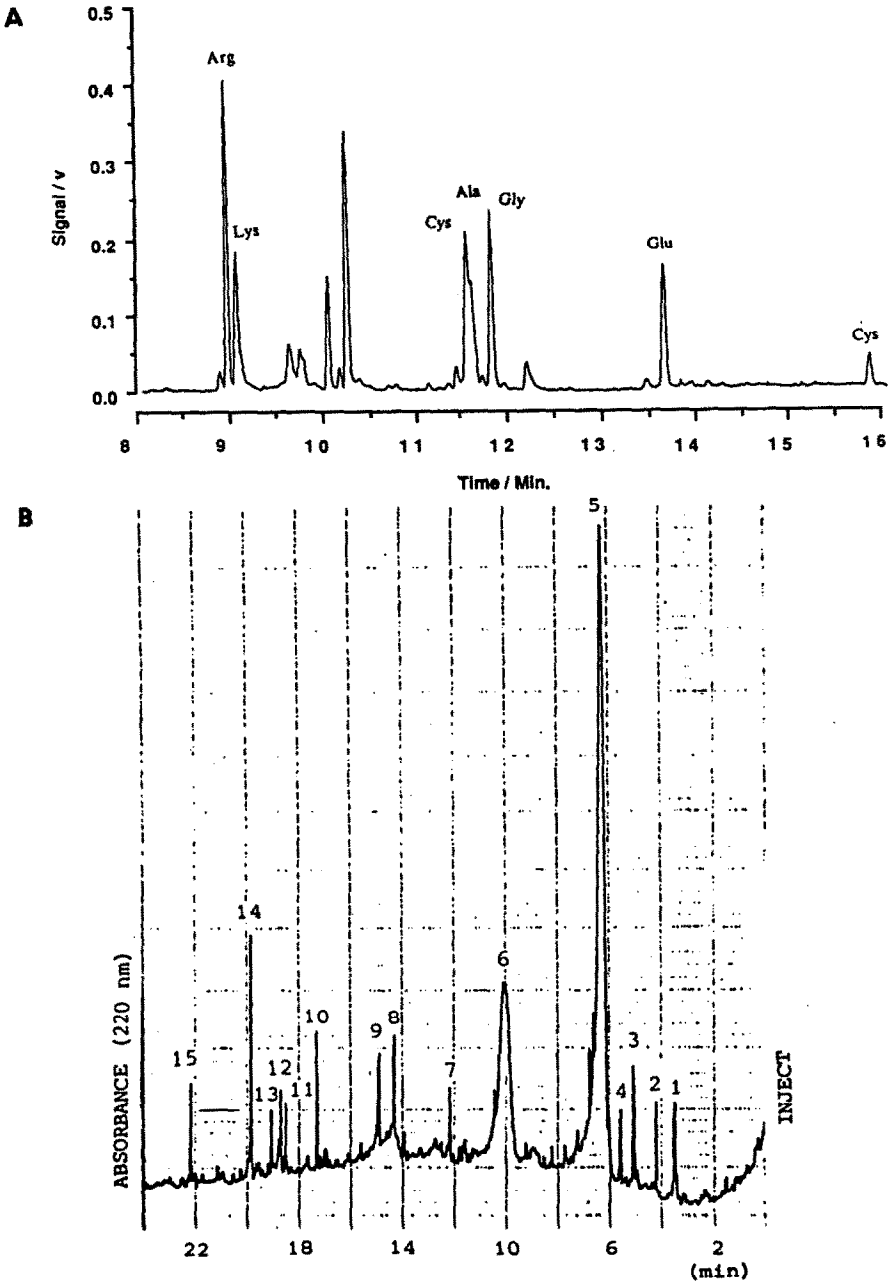

Fig. 23. (A) Separation of fluorescein isothiocyanate-labelled amino acids. The concentrations of arginine, lysine, alanine, glycine and glutamic acid were $1.14 \cdot 10^{-9}$ *M* and that of cysteine was $3.42 \cdot 10^{-9}$ *M*; 1 m × 50 μm I.D. fused-silica capillary, 30 kV; pH 10 aqueous carbonate buffer (from ref. 81, with permission). (B) Electrophoretic separation of standard amino acid mixture as phenylthiocarbamyl derivatives: 2.5 pmol of each amino acid, separation from the right to the left; 5 m*M* borate buffer (pH 9.6), untreated silica capillary, 50 cm long to the detector × 100 mm I.D., 20 kV per capillary, 25 μA. Peaks: 1 = arginine; 2 = lysine; 3 = leucine; 4 = isoleucine; 5 and 6 = reagent peaks; 7 = combined peak of phenylalanine and histidine; 8 = combined peak of valine and proline; 9 = threonine; 10 = serine; 11 = alanine; 12 = glycine; 13 = tyrosine; 14 = glutamic acid; 15 = aspartic acid (from ref. 31, with permission).

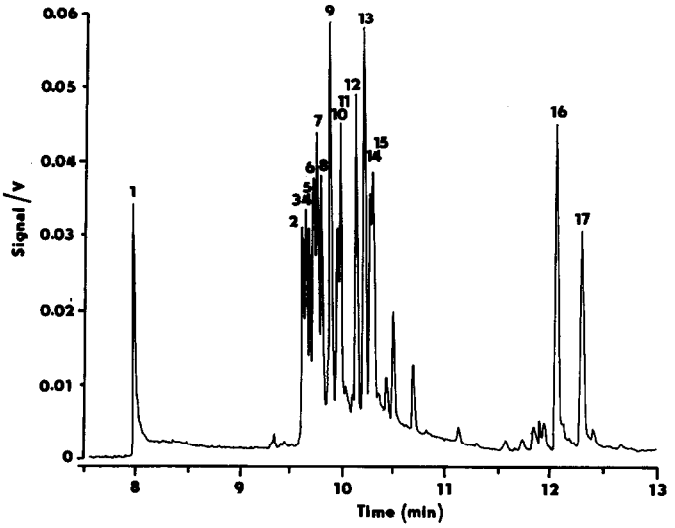

Fig. 24. Separation of fluorescein thiohydantoin derivatives of amino acids. Peaks: 1 = Arg; 2 = His; 3 = Leu; 4 = Ileu; 5 = Tyr; 6 = Met and Thr; 7 = Val; 8 = Hyp; 9 = Pro; 10 = Thr and Ser; 11 = Ala; 12 = Gly; 13 = Cys and Ser; 14 = reagent; 15 = Gln; 16 = Glu; 17 = Asp. Concentrations $1.5 \cdot 10^{-8}$ *M* for serine and $1 \cdot 10^{-9}$ *M* for other amino acids injected (from ref. 127, with permission).

retention times [128]. Consequently, CE and LC are ideally suited as complementary techniques in peptide analysis.

Much effort was put into using mobile phase additives to enhance the quality of separations or to separate a particular category of peptide. This concerns not only chiral additives and chiral separations (for more details see Section 5.1), but also other situations. Thus, Stover *et al.* [129] used putrescine as a cationic additive and zinc perchlorate as a complexation reagent to increase the separation selectivity of histidine-containing peptides. Cyclodextrins were used as additives by Novotny *et al.* [52]; they not only improve the separation efficiency because of the host–guest interactions but also increase the fluorescence intensity, thus making the solutes more easily detectable.

The propensity of many metal ions to coordinate atoms of nitrogen, oxygen and sulphur means that these ions will interact with a number of sites on proteins and peptides. This property can be exploited for better (or amino acid-specific) separations of particularly small peptides [130]. The impact of not only Cu^{II} but also Zn^{II} salts in electrophoretic buffers clearly affects the electrophoretic behaviour of histidine-containing dipeptides. Interaction with a metal ion differentially decreases the electrophoretic mobilities of peptides that co-migrate in the absence of the metal ions, thus causing their separation. Such effects are obtained at low pH values where the large net charge of the sample results in short analysis times. It should be emphasized that these effects are not limited to peptides. Karger and co-workers [10,64] have shown that metal ions enhance the resolution

of nucleic acids and amino acids in buffers containing a micellar phase (see also section 5.1).

Micelle-forming additions bring the CE separations close to reversed-phase chromatography, creating pseudo-stationary phases migrating against the direction of the endoosmotic flow. In other words, micellar electrokinetic chromatography, aimed originally at the separation of lipophilic compounds (see Section 6.4), can be usefully applied also to charged solutes, thus giving the CZE separations a new dimension. Novotny *et al.* [84] reported, *e.g.*, an improved separation of a group of angiotensin-related peptides in a cationic micelle system (cetyltrimethylammonium bromide) (Fig. 25).

The generally applied UV detection of peptides is suitable for larger than microgram amounts of peptides. Enhancement of the detection sensitivity is generally achieved by using precolumn fluorescence labelling (see also Section 6.1). Jorgenson and Lukacs [3] used fluorescamine for this purpose (see also ref. 52). *o*-Phthalaldehyde or naphthalene-2,3-dicarboxaldehyde [52,82] represent alternative possibilities. However, they seem suitable particularly for small peptides (three to ten amino acids) and for both conventional and laser-induced measurements. Incomplete derivatization and formation of multiple peaks from a single peptide represent the main problems here. Derivatization with 3-(4-

Fig. 25. Separation of angiotensin analogues in the presence of a cationic detergent (cetyltrimethylammonium bromide), (A) below and (B) above the critical micelle concentration. Separation was run in 10 m*M* Tris–10 m*M* Na_2HPO_4 (pH 7.05). Concentration of detergent: (A) $2.0 \cdot 10^{-3}$ *M*; (B) 0.05 *M* (from ref. 84, with permission).

carboxybenzoyl)-2-quinoline carboxaldehyde appears to have a wider applicability as far as the size of the peptide analysed is concerned.

6.2.2. Proteins

There are basically two approaches to hindering protein absorption on the capillary: modification of the capillary wall (static approach) or carrying out the separation under conditions under which these effects are minimized (dynamic approach).

In the static approach, Hjertén [55] used non-cross-linked polyacrylamide or methylcellulose to prevent absorption. Jorgenson and Lukacs [2,131] used compounds such as 3-(glycidoxypropyl)trimethoxysilane, Zelec DX, Pluronic polymers, Carbowax and OV-17 vinyl. Covering of the inner capillary wall with polyacrylamide is also gaining popularity [55].

Bruin *et al.* [132] used carbohydrate moieties and epoxydiol coatings. The diol-type coating prepared by bonding γ-glycidoxypropyltrimethoxysilane to the capillary wall followed by acid hydrolysis leads to similar results to those obtained with polyethylene glycol. Generally, however, the diol-type coating is worse than polyethylene glycol modification. Maltose coating appears to shield the silica surface well up to pH 7; the shielding efficiency is smaller, however, than with other modifications. In addition, the maltose coating exhibits good stability only if antimicrobial agents are added to the buffer (see also ref. 133).

As most of the work on capillary coating has been derived from previous work in LC and gas chromatography (GC), it is perhaps not surprising that silylation was preferred at the beginning. Already in the early experiments of Jorgenson's group with γ-glycidoxypropyltrimethoxysilane, although some improvement in peak widths and shape was observed, the coating was obviously unstable under CZE conditions. Silylation was also used as an additional coverage with capillaries covered with methylcellulose and non-cross-linked polyacrylamide [55]. McCormick [134] used an organosilane coating (spacer) to prepare polyvinylpyrrolidone-coated capillaries. These capillaries have proved useful for peptide and protein separations at very low pH values. The risk of structural alterations under such extreme conditions, however, precludes this approach from general applicability. The most serious problem common to all silylation procedures is the instability of the coating at higher pH values. Cobb *et al.* [135] attempted to eliminate this stability problem by using an alternative bonding chemistry, as shown in Fig. 26.

In fact, the attachment of the vinyl moiety through the Grignard reagent allowing for final reaction with the polyacrylamide monolayer is a variation of the original approach of Hjertén. The difference is, however, that in this instance the polyacrylamide monolayer is attached through Si–C bonds rather than an Si–O–Si–C linkage. This modified type of coating is claimed to be stable over the pH range 2–10.5 with a considerable decrease in uncontrolled protein adsorption to the capillary walls. Additional advantages claimed are improved reproducibil-

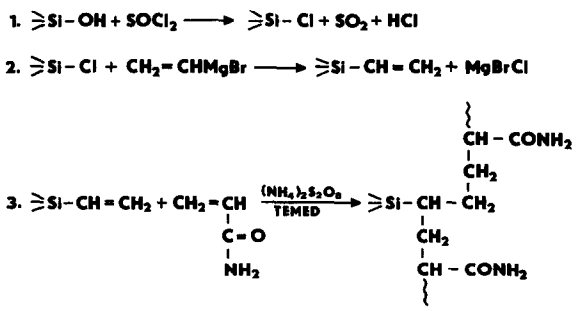

Fig. 26. Reaction scheme for the vinyl-bound polyacrylamide coated capillaries (from ref. 135, with permission).

ity of migration times (at both low and high pH) and improved reproducibility in capillary preparations.

Although capillary surface covering appears to be a reasonable approach, the current problems involved can be specified as follows. First, it is still difficult to obtain a set of capillaries with identical performance. Second, the capillaries with a modified surface tend to deteriorate with time. Third, the efficiency of coated capillaries is considerably lower than that of capillaries without a coating, perhaps with the exception of Cobb's treatment [135]. Finally, it must be emphasized that capillary surface deactivation results in the elimination of or at least a decrease in the endoosmotic flow. Depending on the particular system used, this may be either an advantage or a disadvantage.

In the dynamic approach two methods of realization are possible. First, it is possible to run the separation at a pH value that is higher than the isoelectric point of any protein present in the mixture. This renders proteins negatively charged and results in mutual repulsion of the separated protein and the capillary wall.

Several drawbacks of this approach were foreseen. Possibly the most important is the dissolution of silica at high pH. Possible deterioration of enzymic activity and partial protein hydrolysis were also discussed. As far as the enzymic activity is concerned, this is not a strong argument because in, *e.g.*, SDS electrophoresis, the enzymic activity is likewise lost but still the procedure is extremely popular. In our experiments with a wide selection of diverse proteins, we have not seen any indications of protein hydrolysis, so we believe that in spite of these potential dangers this approach is acceptable.

The other type of the dynamic approach, aimed at the elimination of protein adsorption to the capillary surface, considers their adsorption as an ion-exchange process and tries to block it by increasing the ionic strength through the addition of salts to the running buffer [54,133]. Under such conditions metal ions are preferred in the competition for available adsorption binding sites. 2-(N-Cyclohexylamino)ethanesulphonic acid (CHES) with 0.25 *M* potassium sulphate and

0.01 *M* EDTA proved suitable for this purpose. A high salt concentration in the running buffer creates several obstacles that must be considered. Addition of salt naturally increases the conductivity, which sets a limit to the potential that can be applied. In part this may be overcome by using small-bore capillaries; however, this solution has another limitation, namely high demands upon detection. Addition of salts also decreases the endoosmotic flow by changing the dielectric constant of the medium. The consequence is an increased separation time, which is a drawback (see also the static approach). The unfortunate combination is hindered endoosmotic flow and lower potentials available for practical use, both of which increase the separation time. Moreover, with the widely used UV spectrophotometric detectors the demands upon optical purity of the salts employed are high. A typical example of this category of separations is presented in Fig. 27.

In an attempt to circumvent the problems arising from high salt concentrations, Bushey and Jorgenson [136] used zwitterions instead of ionic salts. The idea is that zwitterions are unlikely to contribute to the conductivity of the running buffer but should be able to compensate for the negatively charged inner surface of the capillary and also the negatively charged protein sites. Because zwitterions

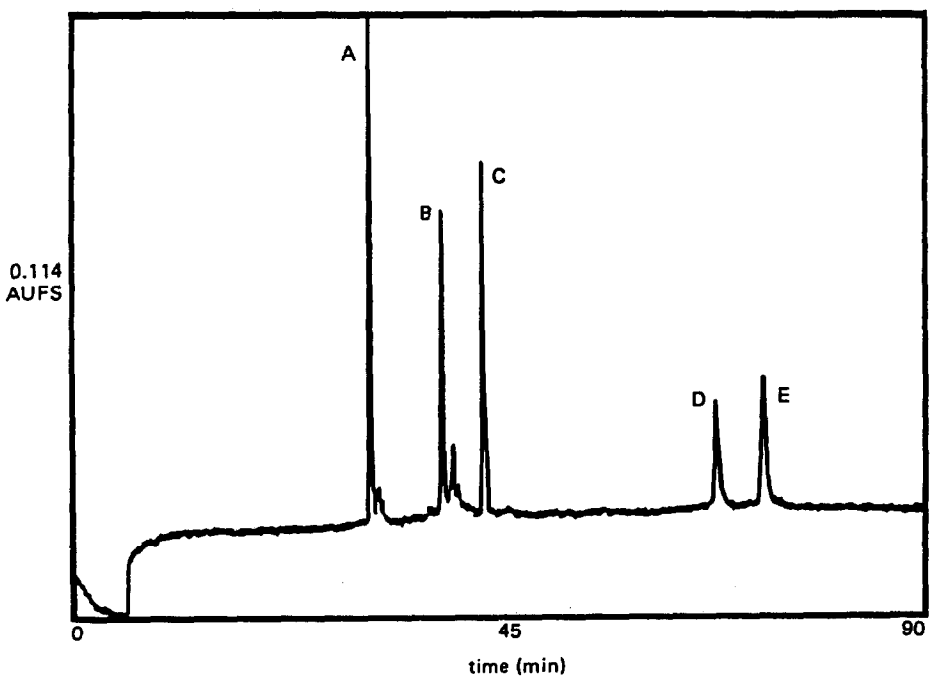

Fig. 27. Capillary electrophoretic separation of proteins in a high elecrolyte concentration buffer. Peaks: A = hen egg lysozyme; B = bovine pancreatic trypsinogen; C = horse heart myoglobin; D = bovine milk β-lactoglobulin B; E = bovine milk β-lactoglobulin A. 0.001 *M* EDTA–0.25 *M* K_2SO_4–0.1 *M* cyclohexylaminoethanesulphonic acid (pH 9.0); 40 cm × 25 μm I.D. capillary, 5 kV (from ref. 54, with permission).

do not contribute to the buffer conductivity, higher voltages can be used and shorter separation times are needed. The results obtained are encouraging, but only data on lysozyme and α-chymotrypsinogen A are currently available.

Finally, lowering of the pH of the running buffer below the isoelectric point of the separated proteins results in positively charged solutes. At the same time some of the negative charge of the capillary wall will be titrated off and, consequently, the coulombic interactions between the capillary surface and the separated proteins wil be lowered. This is applicable with proteins exhibiting high isolectric points and high stability at low pH. This approach was successfully applied by Vinther *et al.* [137] in experiments with aprotinin.

Wiktrowicz and Colburn [138] proposed dynamic surface charge reversal as a suitable means of abolishing stacking of basic proteins to the capillary wall. The method is suitable for proteins that are composed of non-covalently bound subunits (protein clusters) sensitive to separation pH, proteolytic digests of basic proteins and highly basic proteins with minor post-translational modifications. Charge reversal was achieved by flushing a 0.01% solution of the coating agent (not specified) for 5–10 min. Longer flushing resulted in higher endoosmotic flows. After the flushing period, the excess of the coating agent was removed with the running buffer (without the coating polymer). Charge reversal was monitored by running an uncharged marker solute (4-methyl-3-penten-2-one; mesityl oxide) with reversed polarity of the capillary surface; polarity reversal of the system was needed in order to make the marker pass the detection window. Capillaries 90 cm long were used with 400 V/cm (4 μA) and 5 m*M* sodium phosphate buffer (pH 7.0). The capillary was of 50 μm I.D. and during the run it was thermostated at 30°C. It was stated that the amphipathic nature of the positively charged polymer allows charge reversal by first neutralizing the negative capillary surface charge. The surface-bound, neutralized polymer binds free polymer through hydrophobic interactions, resulting in a more heavily positively charged capillary surface. It appears that the positive charge is due to amino groups. From the data presented, it appears that the surface-bound modifier survives at least several separations as there is no comment stating that the addition of a capillary modifier is needed for the running buffer. Also, no data are available on the stability of the coating, for practical applicability an interesting problem indeed. The procedure was tested with LDH isozymes, histone H4 from cuttlefish and trypsinogen.

Let us now turn from the capillary wall adsorption to the process itself. Recently, based on well developed theoretical considerations, Hjertén [20] proposed a new approach to increase further the separation possibilities in CZE. This can be briefly described as introducing multi-buffer systems. The basic idea was to create a sharp starting zone in some analogy with electrophoretic separations carried out in polyacrylamide gel. Zone sharpening in this instance is accomplished by a conductivity difference between the buffer and the sample, which is created automatically by introducing the displacement electrophoresis step. The transition from displacement electrophoresis to zone electrophoresis is obtained

by using a leading buffer with a higher pH than the succeeding buffer (terminating buffer). The mobility of the terminating buffer (mostly glycinate) will increase on entering the region with a higher pH value. When this mobility overcomes that of the sample ions, the latter will destack and will leave the boundary between the leading and terminating ion and will migrate in the terminating electrolyte as in conventional zone electrophoresis. Although more distinct in polyacrylamide gels, the principle is also applicable for CZE (for more details, see ref. 20).

There are still practical problems that have to be solved before putting this idea into practice, namely the choice of the terminating electrolyte: first, the unavoidable increase in molecular mass must not be so large that it would not entirely compensate for the increase in mobility caused by the increase in the negative charge, and second, the terminating compound must be water-soluble even at pH values where its net charge is low. This is because just at this pH it will function as a terminator. Such compounds are to be sought in the category of diaminodicarboxylic (sulphonic) acids; 2,6-diaminopimelic acid may serve as a typical example. The practical applicability of this approach is shown in Fig. 28; 0.06 *M* diaminopimelic acid adjusted to pH 7.7 with Tris served as the terminator. The proteins to be separated are dissolved in the stacking buffer diluted 1:10 with water. The composition of this buffer is, of course, different to that of the

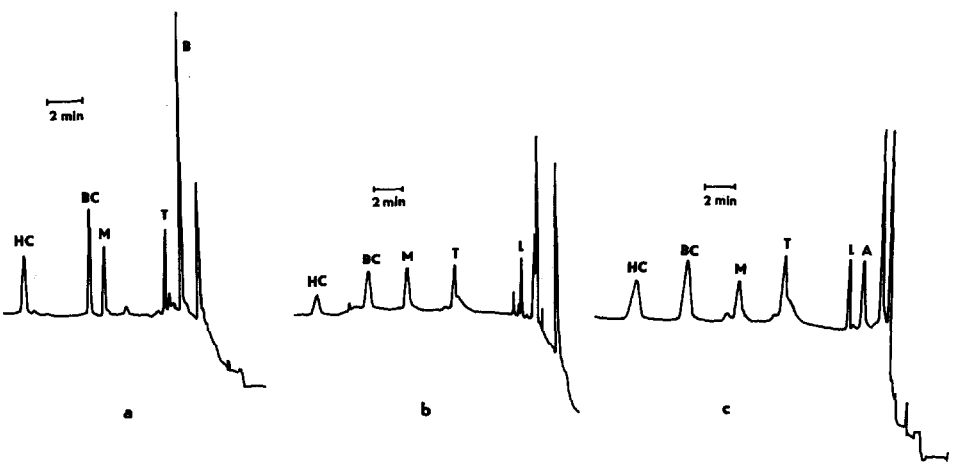

Fig. 28. High-performance free zone electrophoresis of model proteins in a multi-buffer system according to Hjertén [20]. Peaks: A = human serum albumin; L = β-lactoglobulin; M = equine myoglobin; BC = bovine carbonic anhydrase; HC = human carbonic anhydrase. The protein concentration was 0.2 mg/ml except for transferrin (0.4 mg/ml). The proteins were dissolved in the stacking buffer diluted 1:10 with water. Sample length, 2–3 mm. Electrophoretic capillary, 140 mm × 0.075 mm I.D. Terminator: 0.06 *M* 2,6-diaminopimelic acid adjusted to pH 7.7 with Tris (a–c). Leading buffers: (a) 0.06 *M* Tris–HCl (pH 8.9), (the concentration refers to HCl); (b) 0.06 *M* Tris–HCl (pH 9.6); (c) 0.12 *M* Tris–HCl (pH 9.6). Stacking solutions: (a,b) 0.06 *M* Tris–HCl (pH 6.7); (c) 0.12 *M* Tris–HCl (pH 6.7). Length of stacking solution, *ca.* 15 mm; running potential, 4–5 kV; detection at 220 nm. Increasing (b) pH and (c) ionic strength of the same leading buffer helps the separation of the fast proteins.

leading buffer: three different leading buffers were used by Hjertén [20], namely (a) 0.06 *M* Tris–HCl (pH 8.9); (b) 0.06 *M* Tris–HCl pH 9.6 and (c) 0.12 *M* HCl (pH 9.6); the respective stacking solutions were 0.06 *M* Tris–HCl (pH 6.7) (a and b) and 0.12 *M* Tris–HCl (pH 9.6). Separation was carried out in a 140 mm × 0.075 mm I.D. capillary with a sample length of 2–3 mm at 4–5 kV and with absorbance detection at 220 nm. When the leading electrolyte was 0.06 *M* Tris–HCl (pH 8.9), only four of the six proteins in the mixture were separated; the remaining two were stacked to the boundary. On increasing the pH to 9.6 an additional protein was unstacked and, finally, all the proteins were separated when the concentrations of the leading electrolyte was doubled.

It is usually a large step from artificial mixtures to naturally occurring mixtures. Keeping this in mind, Hjertén [20] specified the conditions for the separation of, *e.g.*, human serum proteins: leading buffer 0.75 *M* Tris–HCl (pH 9.7), terminating electrolyte 0.08 *M* 2,6-diaminopimelic acid (pH 8.4), stacking solution 0.25 *M* Tris–H_2SO_4 (pH 6.8); the capillary was the same as described above except that the tube was closed with a gel plug to eliminate hydrodynamic flow. The voltage applied was 3 kV. The overall arrangement of the system is shown in Fig. 29.

In spite of the methodological problems and in spite of the fact that the methodology of CZE protein separations has not yet been firmly established, several areas of practical applications of protein separations by means of CZE have emerged. The fundamental fact to be emphasized in this respect is that compared with chromatography the small diffusion coefficient of a protein results in band broadening and a decrease in separation efficiency. In contrast, in CZE the small diffusion coefficient of proteins results in better separations [3].

An area that is now widely exploited is separations of glycoproteins; this relates to the fact that many of the biotechnologically produced proteins of commercial importance are glycoproteins. A typical example is ribonucleases. The applicability of CZE in this respect is documented in Fig. 30: here ribonucleases A, B1 and B2 are successfully separated at 250 V/cm (5 μA) using 20 m*M* (cyclohexylamino)propanesulphonic acid (pH 11.0) as a running buffer in a 100 mm × 50 μm I.D. capillary at 30°C. Fig. 30 documents well what a mixture one is working with using a commercially available ribonuclease preparation [139]. Another example may be the separation of immuno-complexes such as anti-hGH and immunoglobulin G.

CZE separations are becoming popular also in protein purity determinations, in particular in situations when the protein (or large peptide) is prepared by the techniques of genetic engineering. Analyses of biosynthetically prepared human

Fig. 29. Schematic arrangement of the multi-buffer system according to Hjertén [20]. G = Gel plug of 1% agarose in leading buffer; L = leading buffer; St = stacking solution; Sa = sample; T = terminator. T or L can serve as the anolyte. The anode is on the left-hand side (from ref. 20, with permission).

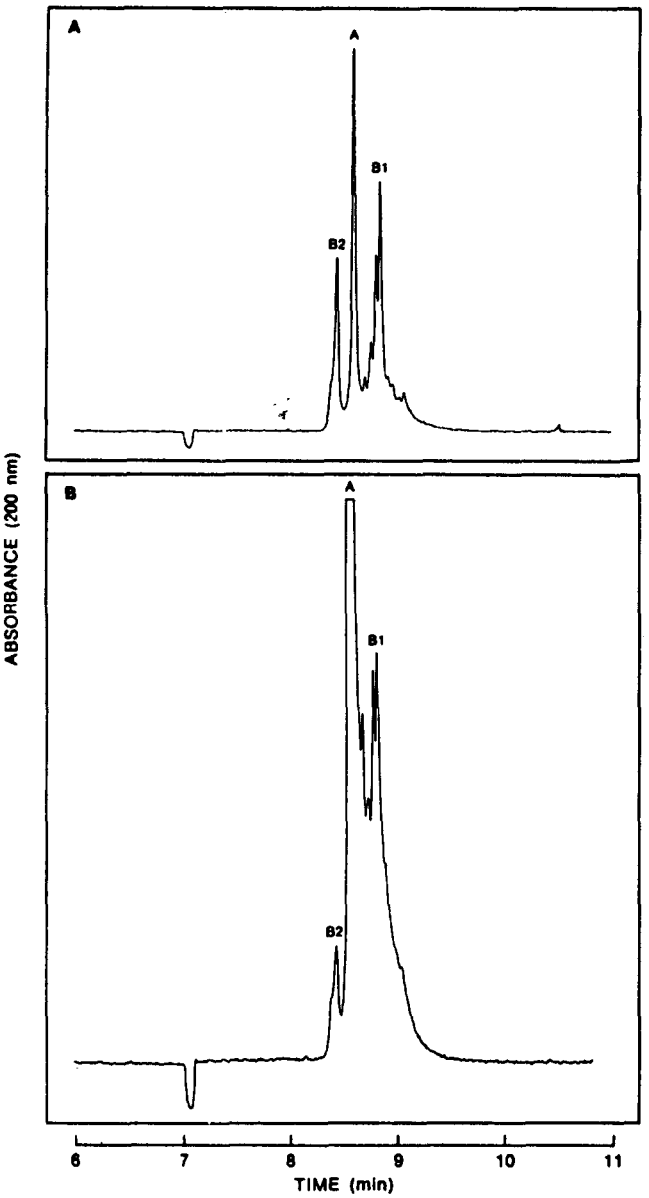

Fig. 30. Capillary electrophoretic separation of ribonucleases A, B1 and B2. 120 cm × 50 μm I.D. capillary, 250 V/cm, 5 μA; temperature, 30°C; buffer, (cyclohexylamino)propanesulphonic acid (CAPS), 20 m*M*, (pH 11.0). (A) Artificial mixture; (B) commercial preparation (from ref. 139, with permission).

insulin or human growth hormone can be mentioned [128,139,140]. Other reported applications refer to the separation of transferrin [141] and collagen isoforms [69].

The reason why CZE is attaining such popularity in protein separations is based on the fact that many proteins cannot be successfully separated by reversed-phase chromatography because of size or solubility limitations. On the other hand, slab gel electrophoresis cannot be routinely used as it cannot be easily quantified. Not negligible also is the fact that CZE and HPLC are complementary techniques, as will be mentioned later.

At least a few words should be said about the limits of detection: with a UV detector set to 220 nm we were able to detect reliably 10–15 amol of collagens injected into the CZE apparatus [7]. The limit of detection for impurities is determined by the maximum sample load and minimum detectable peak area. The maximum sample load is limited by its solubility and field inhomogeneity introduced by the sample. It has to be borne in mind that if the conductivity of the main sample component is considerably different from that of the running electrolyte, the electric field is considerably distorted and poor separations are obtained. It should be stressed that generally one is operating with dilute running buffers which have a low buffering capacity (mostly 20 m*M*). In addition, there are constraints regarding the non-linearity of the detector response at high sample loads. In general, the limits for applying a sample that is to be checked for impurities lie between 0.05 and 25 mg [128].

The sample volumes applied are exceedingly small. Probably less than 5 μl are required for a well designed sample injector; of this amount only a few nanolitre are actually injected. On the other hand, the samples applied have to be fairly concentrated: 0.05–2 mg/ml appears the most suitable concentration for UV detection. For laser-based fluorescence detection the limits are considerably less [33,80,83,85].

So far we have discussed the problems of protein separation as such, and the determination of protein impurities. There is still another field that should be briefly mentioned, namely the nature of proteins separated. It is well known that by digestion of a protein into smaller peptides it is easy to detect subtle structural differences and an identical fingerprint is a definitive proof of protein identity. Nielsen *et al.* [140] applied CZE for the separation of nineteen peptide fragments arising from the enzymatic digestion of human growth hormone. Almost all the fragments were separated under non-reducing conditions by CZE and a marked difference in selectivity between CZE and reversed-phase HPLC was demonstrated (Fig. 31). CZE was thus shown to be a powerful complement to reversed-phase chromatography for the routine investigation of protein digests.

At the end of this section let us emphasize some general rules related to protein and peptide separations by CZE. Relative retention times of proteins and peptides in CZE can be correlated with p*I*, molecular mass and the Offord parameter $M^{2/3}/Z$ (M = molecular mass; Z = apparent charge). Recently it was demonstrated [70] that for a large number of proteins varying considerably in their relative molecular mass an almost linear relationship is obtained on plotting relative retention times *versus* p*I*. This relationship applies for a wide pH range

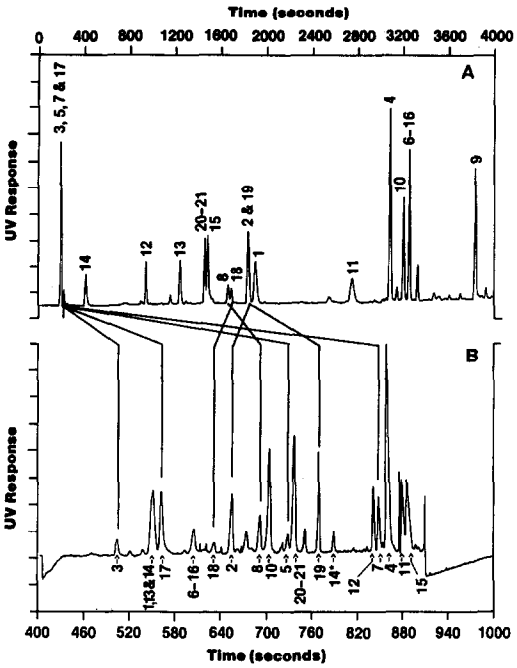

Fig. 31. Differences in peptide mapping by (A) reversed-phase column liquid chromatography and (B) CZE. CZE conditions: 0.01 *M* tricine–0.02 *M* NaCl–0.045 M morpholine (pH 8); 95 cm × 50 mm I.D. capillary made from fused silica, polyimide coated, 30 kV, 40 μA; detection by UV absorbance at 200 nm (from ref. 140, with permission).

(6.86–10.5) and for untreated capillaries. Very large proteins show a clearly linear relationship with relative molecular mass. Further separation according to charge differences was observed also with these very high-molecular-mass proteins; however, this may reflect some specific features of the solutes tested (collagen polymers). On the other hand, cyanogen bromide cleavage of collagen polypeptide chains abolishes the relationship between the relative retention time and molecular mass and a linear dependence on the Offord parameter was observed.

6.2.3. Protein structure investigation

Structure investigations of proteins involve basically two approaches: specific or non-specific cleavage of proteins resulting in peptide maps and their comparison and sequence analysis of the fragments formed. For peptide separation methodology, see Section 6.2.1. Problems of enzymatic cleavage of small amounts of protein in dilute samples can be overcome by using small columns of immobilized trypsin for cleavage as shown by Novotny *et al.* [84].

MS had long been considered a promising tool for protein sequencing, but only recently has the technology of mass analysis approached the stage where this method is practically applicable to structure studies [142,143]. In recent years the increased sensitivity of MS equipment has allowed the coupling of small capillary

columns to FAB equipment. For more detailed information regarding CZE–MS, coupling see Section 4.4.4 and a recent review [144]. CE–FAB-MS coupling can evaluate peptides at the 250 fmol level at a column efficiency of 600 000 plates. Protonated ions are generated and molecular mass information about the major components of a tryptic map appears marginally feasible [104].

ESI in connection with CZE has also attained much interest, particularly because of the inherent capability of promoting multiple charged species that can be subsequently detected [145]. Proteins larger than 100 000 relative molecular mass yield spectral profiles that can be used for molecular mass determination [105] (Fig. 32).

In addition to the problem of determining the molecular masses of proteins present in complex mixtures, and protein characterization through peptide maps, structural investigation by tandem MS–MS is attracting interest. A preliminary assessment of the CE–MS–MS combination for model peptides was published recently by Novotny *et al.* [84]. Parent- and daughter-ion mass spectra for model peptides at femtomole levels were presented.

6.3. Nucleic acids and their constituents

The separation of nucleic acids and their fragments represents another challenging field for CZE. Dolnik *et al.* [146] carried out a thorough investigation of various parameters affecting the separation of oligonucleotides. With polycytidines as model compounds it was demonstrated that variations of pH between 5 and 8 and of ionic strength between 20 and 200 m*M* have little effect on the separation. However, if spermine is added to the background electrolyte the migration order is inverted as the migration of the larger oligonucleotides is consid-

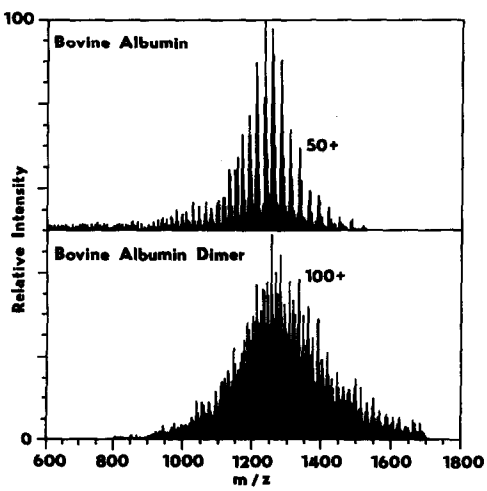

Fig. 32. Electrospray ionization mass spectra for bovine serum albumin (BSA). Top, relative molecular mass 66 300; bottom, BSA dimer, relative molecular mass 133 000 (from ref. 105, with permission).

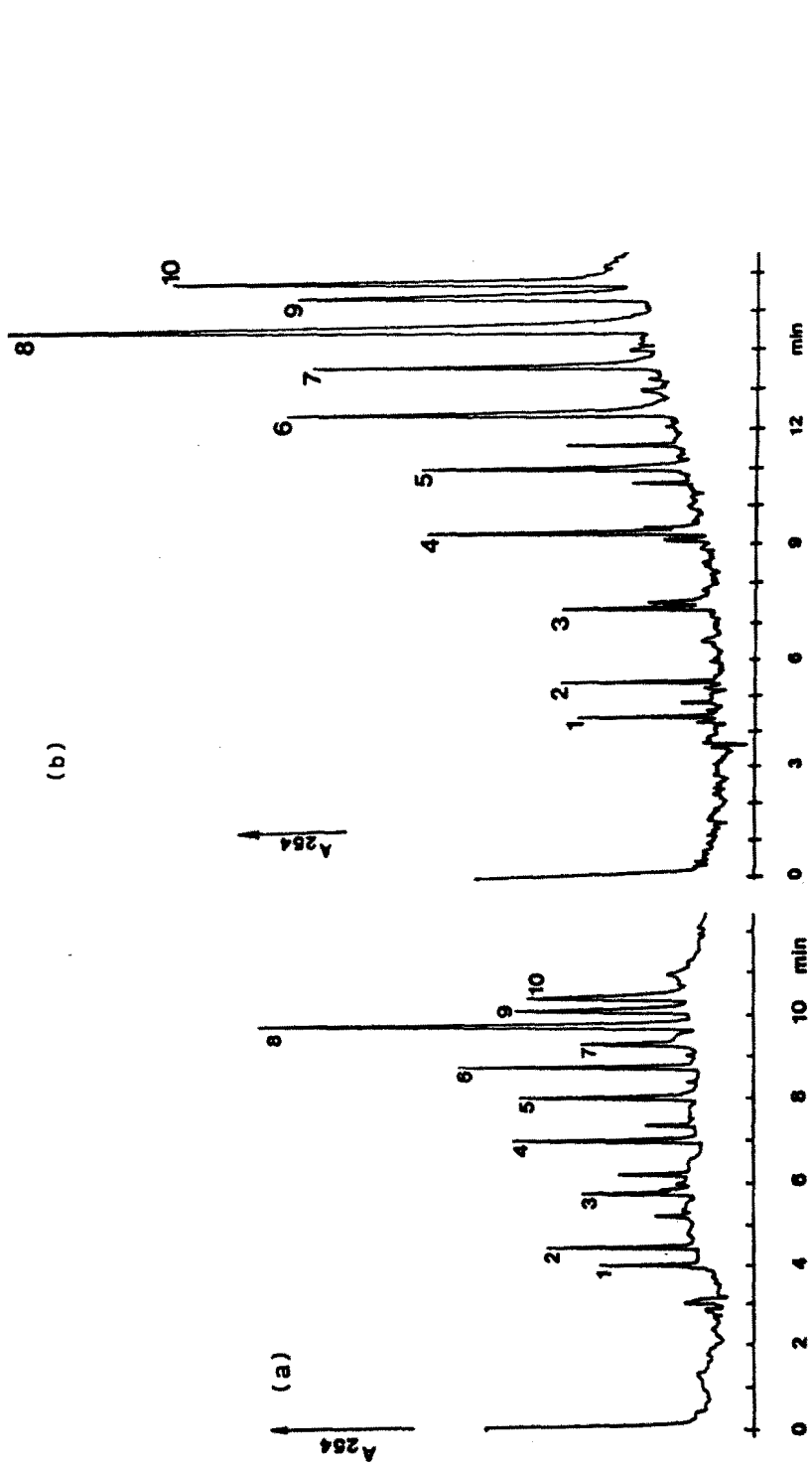

Fig. 33. Separation of polycytidines in the presence of SDS at pH 6. Background electrolyte: (a) 60 m*M* histidine–30 m*M* glutamic acid–50 m*M* SDS; (b) as (a), with the addition of 3 m*M* spermine. Capillary, 45 cm × 50 *μm* I.D., *uncoated*, 25 kV (from ref. 146, with permission).

erably slowed. If the background electrolyte contains not only spermine but also SDS, then the separation order is also considerably affected. Reportedly the best separations were achieved with an electrolyte containing 60 m*M* SDS and 3 m*M* spermine (Fig. 33).

On the other hand, Yamamoto *et al.* [119] claimed that nucleic acids with a wide range of molecular size (from mononucleotides to calf thymus DNA) can be separated under conditions similar to those for the isotachophoretic separation of proteins with ampholine added to the background buffer. It was also shown that the chain length under these conditions was not the major factor with regard to the mobility of individual solutes. Polynucleotides containing more than 10^3 bases appeared as sharp peaks, but this sharpness was lost after DNAse treatment, indicating heterogeneity and incomplete separation of the products. In this respect Heiger *et al.* [147] attempted high-performance capillary electrophoretic (HPCE) separations using gel and open-tube capillary columns of fluorescence-labelled single-stranded oligonucleotides for DNA sequencing. There are indications that HPCE polyacrylamide gel-filled capillary columns are probably better suited for DNA sequencing than CZE separations [148]. Similar results were also obtained independently by Drossman *et al.* [149].

In this context also, Swerdlow *et al.* [150] used capillary polyacrylamide gel separation technology for the resolution of deoxycytidine-terminated DNA fragments. A post-column laser-induced fluorescence detector was used to minimize background noise due to light scattered from the gel-filled capillary. A detection limit of 10^{-20} mol of fluorescein-labelled DNA was achieved. Separations were run using 108 g/l Tris–55 g/l boric acid–40 ml/l 0.5 *M* aqueous EDTA in 50-cm-long capillaries. These were filled with 6% T, 5% C acrylamide/bisacrylamide, 8 *M* urea, 0.07% (w/v) ammonium persulphate and 0.07% (v/v) TEMED dissolved in a 1:10 diluted Tris buffer described above. A typical separation is shown in Fig. 34.

Chin and Colburn [151] introduced a new technique called counter migration capillary electrophoresis, which offers the advantage of long separation paths and high field strength while the capillary length remains relatively short. Both double- and single-stranded DNAs can be separated in this way. Single-base resolution of poly(A) nucleotides ranging from 19- to 60-mers can be achieved within 30 min. Double-stranded fragments between 75 and 5000 base pairs were baseline separated within 13 min. The migration rates of the individual fragments were purposefully altered by the addition of polysaccharides.

Agarose-filled capillaries should be useful for the separation of large nucleic acid species, typically viruses, DNA and large protein complexes. The problems with agarose emerge from the presence of residual carboxyl and sulphate groups resulting in an electroosmotic flow and a low melting temperature of 60°C.

Polyacrylamide gels with smaller pore size than agarose are well suited for separating samples within the mass range 10 000–35 000. Small polynucleotides were separated successfully on such gels by Karger's group [147,148]. A deeper

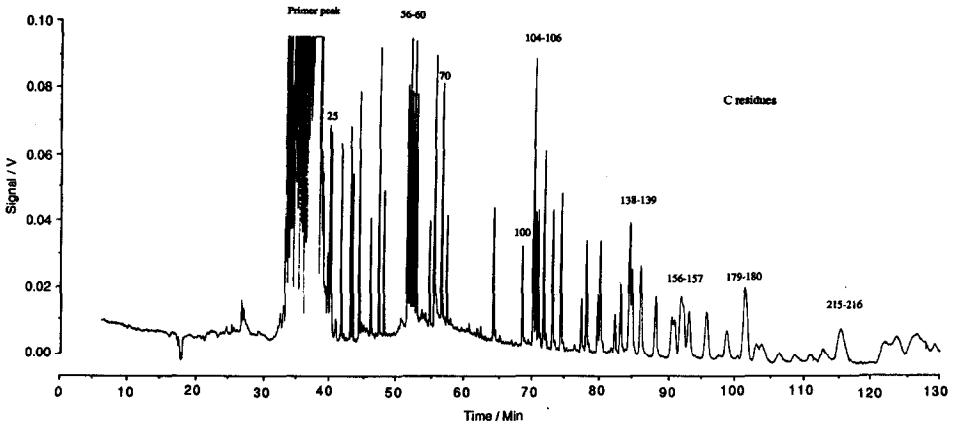

Fig. 34. Capillary gel electropherogram of a DNA sequencing reaction terminated with dideoxycytidine triphosphate. Each peak beyond No. 25 corresponds one-to-one to a C-residue in the known DNA sequence. Peak numbering corresponds to total length of oligonucleotide fragments. Capillary, 50 cm × 200 μm I.D., filled with 6% T, 5% C acrylamide/bisacrylamide, 8 *M* urea, 0.07% (w/v) ammonium persulphate, 0.07% (v/v) TEMED; 30 kV (from ref. 150, with permission).

insight into the overall performance and reproducibility of such systems was recently presented by Paulus *et al.* [152]. Polyacrylamide gel-filled columns were tested with homopolymeric standard samples. Plots of migration time *versus* molecular size over a range of 30–160 bases were reported. Using 2.5–4% T and 3.3% C gels, good resolutions over the specified mass range were obtained with peak half-widths of 3–6 s at 200–400 V/cm. Heteropolymeric samples separated in polyacrylamide-filled capillaries exhibited a linear migration time *versus* base number relationship that was identical for three different oligonucleotide samples. Consequently, it was possible to conclude that gel-filled capillary separations can be applied to molecular mass determinations. A typical separation is depicted in Fig. 35.

6.4. Drugs

The prospects of CZE for drug separations are promising, but have not been exploited very much so far. The subject was covered recently in a review by Nishi and Terabe [153]. There are generally three areas in this field that have been investigated so far, namely separation of non-ionic drugs, purity determination of pharmaceuticals and attempts to assay drugs by direct plasma injection.

Non-ionic drugs are not accessible to direct CZE analysis because of obvious solubility problems. Equally, SDS electrokinetic chromatography does not represent a solution as the solubilization effect of the micelle is too strong. On the other hand, the use of bile salt micelles (long-chain allyl anionic surfactants) led to a successful separation of corticosteroids and diltiazem analogues. A typical elec-

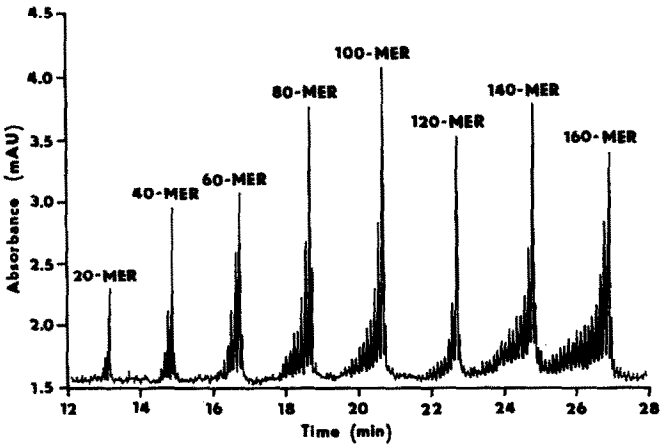

Fig. 35. Example of the separation of oligothymidylic acids pd(T) 20–60; capillary, 50 cm to the detector × 100 μm I.D. gel, 2.5% T, 3.3% C polyacrylamide; buffer, 89 m*M* Tris–69 m*M* boric acid–7 *M* urea–2 m*M* EDTA (pH 8.6); 400 V/cm; 40 μA. Detection at 260 nm. Injection, 10 kV per capillary, 10 s (according to ref. 152, with permission).

tropherogram of such a separation is shown in Fig. 36. It was also demonstrated that deoxy-type bile salts exhibit a larger solubilization effect than others, which was ascribed to the increased lipophilicity reflecting the absence of the hydroxy groups. Micellar electrokinetic chromatography with taurine-conjugated bile salts was identical with that achieved with non-conjugated bile salts, indicating that the ionic interaction was not significantly involved in solubilizing the non-ionic solutes. Also, it was proved that dehydroxycholate lacks the solubilization effect.

p-Hydroxybenzoates can be separated by using a variety of surface-active agents such as SDS, N-lauroyl-N-methyltaurate and bile salts; however, sodium dehydroxycholate was ineffective. The migration order was shown to reflect the lipophilicity of the separated solutes and was identical with the elution order in reversed-phase LC. The analogy between micellar electrokinetic separations and reversed-phase chromatography can even be quantified; the capacity factor depends linearly on the number of repeating groups, known in chromatography as Martin's rule (at least this is valid for the seven parabens separated [153]).

The first attempts to use electrokinetic chromatography for the assay of the active ingredients in pharmaceutical preparations were made by Nishi *et al.* [154]. Using the internal standard approach, similar reproducibilities to those with reversed-phase chromatography were achieved (1% run-to-run and 3% day-to-day). The ratios of the peak area of each ingredient to the internal standard were within 5% without temperature control. Reproducibility data given by Nishi *et al.* [154] for acetaminophen, caffeine and ethenzamide are summarized in Tables 2–4. Successful separations were also obtained for diltiazem (in tablet form) and fluocinamide in a cream. Electrokinetic separation with bile salts was used for this purpose.

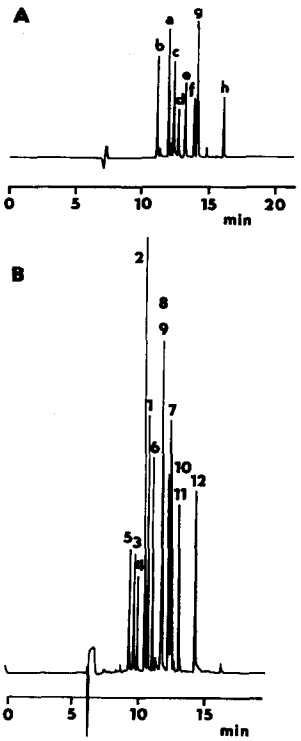

Fig. 36. Separation of (A) eight corticosteroids and (B) twelve diltiazem-related compounds by micellar electrokinetic capillary chromatography. Peaks: a = hydrocortisone; b = triamcinolone; c = betamethasone; d = hydrocortisone acetate; e = dexamethasone; f = triamcinolone acetonide; g = fluocinolone acetonide; h = fluocinonide; 1 = diltiazem; 2 = deacetyldiltiazem; 3–12 = diltiazem-related compounds. Separation of cortisone derivatives in 0.1 *M* diltiazem-related compounds in 0.05 *M* sodium cholate; 0.02 *M* phosphate–borate buffer (pH 9.0). Capillary, 500 mm to the detector × 50 mm I.D., 20 kV; detection, UV absobance at 210 nm.

Similarly to HPLC, purity determination can be also done by the peak-area percentage approach; *e.g.*, for diltiazem hydrochloride in tablets from various sources the results were in good agreement with those obtained by reversed-phase chromatography. In this particular instance it was possible to reveal about 0.22% of deacetyldiltiazem (as an impurity) with a detection limit of 0.1% at a signal-to-noise ratio of 3. With sodium taurodeoxycholate, successful optical purity determination of trimetoquinol hydrochloride was also possible. As little as 1% of the minor enantiomer was detectable at a signal-to-noise ratio of 3. Wider applicability of electrokinetic procedures is precluded at present by the lack of highly sensitive detection methods and the lack of precision microinjection equipment, together with the urgent need for process automation.

The first attempts to separate drugs by direct plasma injection by electrokinetic chromatography were reported by Nakagawa *et al.* [155]. The solutes separated were antibiotics. The technique applied was similar to micellar HPLC. In the

TABLE 2

REPRODUCIBILITY (R.S.D.) OF MIGRATION TIMES

The standard solution for the assay of Pyripan tablets was used. Conditions are given in ref. 154.

Date	*n*	R.S.D. (%)		
		Acetaminophen	Caffeine	Ethenzamide
16/6	6	0.55	0.46	0.76
17/6	6	0.59	0.69	0.68
20/6	7	0.78	0.85	0.88
23/6	5	0.69	0.76	0.76
Total (4 days)[a]		1.75	2.13	2.87

[a] R.S.D. from day-to-day.

TABLE 3

REPRODUCIBILITY (R.S.D.) OF PEAK-AREA RATIOS

The standard solutions for the assay of Novapon granules and Pyripan tablets were used. Conditions are given in ref. 154.

Ingredient	R.S.D. (%)	
	Novapon (n = 7)	Pyripan (n = 5)
Acetaminophen	1.4	1.4
Caffeine	2.3	2.0
Ethenzamide	1.0	1.5

TABLE 4

ASSAY RESULTS FOR COMMERCIAL PHARMACEUTICALS

Conditions are given in ref. 154.

Ingredient	Novapon			Pyripan		
	n	Content (%)	R.S.D. (%)	*n*	Content (%)	R.S.D. (%)
Acetaminophen	5	100.1	2.3	3	102.3	1.0
Caffeine	5	100.0	1.6	3	99.6	3.0
Ethenzamide	5	100.8	2.3	3	99.3	2.6

absence of surfactants, antibiotics co-eluted with plasma proteins. However, the separations were obscured by the adsorption of plasma proteins on the inner surface of the capillary wall and consequently alkaline washing of the capillary was essential to make it ready for the next run. Good separations using SDS micelles were obtained with, *e.g.*, cefpyramide (a cephalosporin antibiotic); the migration of plasma proteins increased with increase in surfactant concentration. It was assumed that plasma proteins were solubilized by the anionic SDS micelle, resulting in strong retardation by electrokinetic forces towards the anode and, consequently, leading to a slower migration compared with the antibiotic. Also, addition of SDS to the sample eliminated the capillary surface adsorption of the proteins, which actually made direct plasma application possible. Similar results were otained with aspoxicillin (a penicillin anbtibiotic). In this particular case the R.S.D. of the migration times was 1% and of the peak-area ratio 5%; the recovery was near 100%. This indicates the possibility of determining both the bound and unbound drugs. The calibration graphs were linear within the range 25–300 μg/ml (correlation coefficient $r = 0.999$), covering the usual clinical plasma levels of the antibiotic.

Micellar electrokinetic chromatography can also be used for the resolution of enantiomeric drugs. Optical resolution is achieved by using a chiral surfactant: so far bile salts and N-dodecanoyl-L-vanillate have been used. Apart from dansylated amino acids, these procedures have been applied to trimetoquinol hydrochloride and diltiazem hydrochloride analogues. Of the commonly used bile salts, perhaps sodium taurodeoxycholate is the best as it provides an increased solubilization capacity because of the lack of a hydroxy group at the C-7 position and because of the increased electrostatic interaction by the sulphonate group in comparison with other bile salts containing a carboxyl group.

There is also a possibility of using mixed micelles consisting of SDS and a chiral additive, *e.g.*, N,N-didecyl-L-alanine and copper(II), digitonin or cyclodextrins. The separation of DL-amino acids or 2,2,2-trifluoro-1-(9-anthryl)ethanol by adding γ-cyclodextrin to the SDS-containing system may serve as good examples. With the latter compound there is a strong indication of the formation of an inclusion complex. Additional discussion regarding the separation of amino acid enantiomers was presented in Section 5.1.

The separation of radiopharmaceuticals requires appropriate radioactivity detectors. This topic has been dealt with in Section 4.4.3.

Urine analysis for ionic substances can be considered as follows. S-Carboxymethylcysteine, a mucolytic agent employed in the treatment of pulmonary diseases, was assayed by Tanaka and Thormann [156], and some metabolites were also analysed. The analyses were performed with urine samples using on-column detection of underivatized solutes, minimum sample pretreatment and capillary columns with minimized electroosmosis. Two types of apparatus were compared, namely the Bio-Rad HPE-100 featuring coated fused-silica capillaries (25 μm I.D.) and the LKB Tachophor 2127 with PTFE capillaries of 500 μm I.D. Drug

concentrations down to 0.2 mg/ml by capillary isotachophoresis and 0.03 mg/ml by CZE were monitored.

6.5. Miscellanea

The set of applications considered above certainly do not exploit all the possibilities offered by CZE, but rather indicate the most followed areas.

Of biologically important compounds, attention was paid to low-molecular-mass carboxylic acids. This is not surprising when one considers the mass/charge differences for the individual members of this group and the work carried out with these compounds in the isotachophoresis area. Huang *et al.* [157] recently reported a CZE procedure making use of an on-column conductivity detector. The addition of 0.2–0.5 m*M* tridecyltriethylammonium bromide helped to control the electroosmotic flow so that all carboxylate anions passed through the detector.

There are a number of non-ionic compounds that can be separated in the micellar electrokinetic mode. For instance, urinary porphyrins [158] are well resolved in a 75 cm × 50 μm I.D. capillary with 100 m*M* SDS and 20 m*M* 3-(cyclohexylamino)-1-propanesulphonic acid at pH 11. Detection can be accomplished by measuring the absorbance at 400 nm or by fluorescence at 400/550 nm. The method was shown to be applicable to clinically obtainable urine specimens. Urine profiling can be done even without the micellar phase. A report on this was published recently by Guzman *et al.* [159]. The UV detection system used showed detection limits in the picogram range. The number of peaks resolved does not reach those given by LC–MS or GC–MS, and the method needs further elaboration.

In the early development stages of micellar electrokinetic chromatography, phenols were popular solutes for demonstrating its capability. As phenols represent frequently occurring pollutants, it is perhaps not surprising that attempts were made to analyse them by the capillary electrophoretic techniques. Ong *et al.* [160] used micellar solutions of SDS in phosphate–borate buffer at pH 6.6–7.5 to separate eleven substituted phenols listed by the US Environmental Protection Agency as priority pollutants. Another category of frequent pollutants is complex cyanides. Typically hexacyanoferrate(II) and -(III) ions can be separated at 25 kV in an 86 cm × 75 μm I.D. capillary in 20 m*M* phosphate buffer (pH 7.0) [161]. The experiments were successfully extended to zinc hydroxo complexes in plating solutions.

Other examples of capillary electrophoretic separations include catechols, which can be resolved in borate buffer [10 m*M* sodium monophosphate buffer (pH 7.0), 20 kV], diverse organic compounds, which are separable in acetonitrile–0.025 *M* tetrahexylammonium perchlorate (1:1) (20 kV, 75 μm I.D. capillary) [60], and Maillard reaction products, which represent very complex mixtures and are foreseen to play an important role in extracellular pigment formation and animal ageing [162].

7. CONCLUSIONS

Capillary electrophoresis (including its different variants such as micellar capillary chromatography and electrophoresis in polyacrylamide capillary gels) exhibits prospects that are at least comparable to those of high-performance chromatography. The problems with CZE lie mainly in the detection sensitivity and in the preparation of capillaries with constant properties so as not to affect separations by surface interactions. Technical problems must also be overcome; disposable capillary columns represent a goal to be reached. Combination with MS (and much work is being done in this respect) broadens the horizons even more. The possibility of working in organic solvent buffer solutions and of exploiting micellar systems sets no solubility limits on solutes to be separated. First indications have appeared showing that capillary electrophoretic separations will expand in the area of clinical chemistry and drug analysis (in the micellar mode).

The advantages of capillary zone separations (and the different variants) are seen in the extreme sensitivity and small amounts of sample needed for analysis (although the sample, no matter how small, should be concentrated for good results), the short separation times and, most important, the fact that the recorded results offer far better possibilities for quantification than planar electromigration procedures. We consider that we are entering a new era of separation technology which within the coming decade will be widely applied in the biomedical field.

8. ACKNOWLEDGEMENTS

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