Review

Overview of the status and applications of capillary electrophoresis to the analysis of small molecules

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Abstract

The status of capillary electrophoresis (CE) in the analysis of small molecules is reviewed and summarised with the illustrative use of recent literature references. Examples are cited in this review which demonstrate that CE is now a recognised and established technique in many industries, law courts and government regulatory agencies. Each of the principal areas of CE application in small molecule analysis are covered in sections which highlight the recent developments and possibilities within that area. Application areas include the analysis of pharmaceuticals, agrochemicals, chiral separations, and forensics is covered. This is an update to a previous review article [J. Chromatogr. A 856 (1999) 443] and covers papers published between 1999 and 2002. Technical developments and improvements, such as the advent of capillary array instrumentation for increased sample throughput, and improved detection options are described. Overall it is concluded that CE has become a recognised and established technique in many areas and is still within a period of development of both instrumentation and application which will continue to expand usage.
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1. Introduction

In recent years capillary electrophoresis (CE) has expanded its scope and range of both instrumentation and application. CE was seen as a fast moving area of analytical chemistry research in the late 1990s but still represents an area where significant improvements and developments are continually being reported. Equally significantly, routine CE methods are being applied in a number of industrial environments and CE has become recognised as an acceptable and reliable alternative to traditional analytical methods. The acceptability of CE has been endorsed by numerous government agencies and academic bodies. For example the International Union for Pure and Applied Chemistry (IUPAC) have established [1] definitions for CE terminology.

Details of registered CE methods are now included in pharmacopieas [2] and routine CE methods have been successfully submitted to regulatory authorities, such as the US Food and Drug Administration (FDA). CE is an acceptable analytical method in court, and it is employed in clinical arenas for diagnostic and assay purposes. The use of CE to perform environmental monitoring is established and a Environmental Protection Agency (EPA) method has been published [3] for the determination of inorganic anions in waste water. CE is also routinely used in a number of forensic applications and is a standard tool in the characterisation of products, such as proteins/peptides from the biotechnology industry.

The relatively simple mechanism of separation in CE, migration speed based on solute size and charge under the influence of applied voltage, allows very successful minaturisation to be achieved on chip based devices. The simple mechanisms involved in enantiomeric separations in CE has also prompted a marked change in approaches to this activity and CE is often applied in preference to HPLC. The ability to effectively separate and characterise DNA portions, largely based on size, has lead to many advances. Most notably the ability of CE to separate DNA was heavily utilised in the Human Genome Project where multi-capillary instrumentation was used to dramatically increase sample profiling throughput. It is estimated that CE determinations accounted for approximately 80% of the raw data in the Human Genome Project. The multi-capillary array instruments allowed simultaneous separation of 96 samples/standards, which has significantly impacted on analytic speed and throughput.

The early research efforts in CE led to development of stable method conditions which have been commercialised as kits for the determination of species as diverse as inorganic ions, proteins and DNA. The inorganic ions (such as metal ions, chloride, sulphate, etc.) employ indirect UV detection and pre-prepared buffer reagent are sold as application kits. The use of kits is also employed in microchip CE where the chips are primed with suitable reagents prior to their rapid separation and on-chip detection/quantitation.

The weaknesses of CE were frequently highlighted during the infancy of the technique, where continous comparisons with the highly established technique of HPLC showed deficiencies. Instrument manufacturers and both academic and industrial scientists have worked on improving system performance and developing chemical solutions to separation problems. For example, the sensitivity and range of detectors have increased and the repeatability/robustness of methods has improved as scientists have developed a more thorough understanding of the parameters that require attention during routine operation. New chiral selectors have been developed and increasing attention has been paid to the use of non-aqueous solvents. Generally the use of free solution CE (FSCE) in which simple electrolytes, such as phosphate or borate are employed with no additives is sufficient to achieve most separations.

It was thought by many that CE would rapidly replace HPLC, but considerable previous investment in HPLC equipment purchases and training has created an analytical inertia that CE has found a difficult to change. CE often offered only an alternative to HPLC, not an improvement, and therefore CE was not widely implemented. CE was well adopted in niche areas, such as chiral separations and indirect UV absorbance determinations of inorganic anions and metal ions. Technological developments have continued to occur in CE due to its suitability for multiplexing and miniaturisation. The commercial availability of array and microchip CE systems has and will continue to offer key operating advantages over HPLC, such as speed/simplicity of analysis and huge sample throughput. It is the authors belief that these factors will serve to progress CE more into the forefront of analytical chemistry and that CE methods will start to replace HPLC methods and dominate separation science applications.

Readers are also referred to a recent review [4] from H.J. Issaq entitled "thirty-five years of capillary electrophoresis: advances and perspectives". Readers with internet access and an interest in the background to CE are referred to http://www.ceandcec.com.

2. Pharmaceutical analysis

CE is well established [5] as an analytical technique for assessment of both small molecule pharmaceuticals and biopharmaceuticals. Methods have been validated and successfully submitted to many regulatory authorities. Descriptions of CE and specific CE methods have been published in most Pharmacopeias. Application areas include determination of drug content in drug substance and formulations, chiral separations, counter-ion determination, determination of drug properties, such as pK_a and solubility, and clinical monitoring of drug levels. There has also been a strong recent emphasis on forensic determinations of drugs in a variety of applications.

CE methods and monographs have begun to appear in the United States Pharmacopiea (USP) and EP (European Pharmacopiea). A paper was recently published [6]

by workers from the European Directorate of Quality of Medicines (EDQM), Strasbourg (advisors to the EP). They tested CE and HPLC methods for determining ephedrine enantiomers. The DL for CE was 0.1% for (+) ephedrine with R.S.D. values of less than 20% for trace level enantiomer determinations and good linearity 0.1–1%. The CE method was cheaper, more robust and with better sensitivity than the HPLC method using an alphagylcoprotein (AGP) column, due in no small part to poor peak shape obtained on the LC column. The authors concluded [6] that CE method could replace current optical rotation method in the EP. An extensive assessment and reproducibility study on the performance of a CE method for the determination of the isoform distribution of erthyropoietin (EPO) has been [7] performed. The CE method for EPO was successfully repeated in 15 industrial and regulatory laboratories worldwide. A new USP chapter ⟨1047⟩ describes the use of various modes of CE to characterise biotechnology derived pharmaceutical products. A further USP chapter on the Analysis of Glycans will include use of CE and CE–MS for characterisation and determinations.

Numerous fully validated CE methods have been reported in recent years with validation conducted according to the International Conference on Harmonisation (ICH) guidelines. These reports represent only a small fraction of the routine CE work being undertaken in the pharmaceutical industry given the strict confidentially of many of the studies undertaken. A selected number of validated CE methods are discussed below.

A fully validated method has been reported [8] for the assay of ximelagatran and related substances in both drug substance and tablets. A low pH phosphate buffer containing both acetonitrile and hydroxypropyl-β-cyclodextrin was employed to achieve the required selectivity for related substances. Benzamidine hydrochloride was used as an internal standard in quantification. Performance of the validated method offers equivalent and complementary information, in terms of selectivity, sensitivity, accuracy, linearity and precision, to that of an established gradient LC method employed for similar purposes. Robustness of the method was investigated by experimental design and evaluated using multivariate calculations.

A CE assay using 50 mM phosphate buffer, pH 2.5 has been [9] developed and validated for the quantitation and determination of common impurities in a potassium channel blocker (3,4-diaminopyridine and 4-aminopyridine). Specificity, linearity range, detection limits, operating range, limits of quantitation and detection, precision and robustness were determined. All known related substances could be detected at the 0.05% level.

A CE method was developed and validated [10] for the determination of metacycline in the presence of its related substances. Experimental designs were used to develop the optimised electrolyte of 160 mM sodium carbonate and 1 mM EDTA (pH 10.35)/methanol (89:13, v/v). The method showed good selectivity, repeatability, linearity, and

limits of detection and quantitation were 0.02 and 0.06%, respectively for the related substances.

A low pH, CE method was optimised and validated [11] for the simultaneous determination of several angiotensin-II-receptor antagonists; candesartan, eprosartan, irbesartan, losartan potassium, telmisartan, and valsartan.

Separation and quantitation of drug enantiomers (Section 3) remains one of the key applications of CE in pharmaceutical analysis and has been recently [12] reviewed. The rapid method development offered by CE coupled to the robustness of the methods ensures that CE is often viewed as a superior technique compared to HPLC in the area of chiral separations. Numerous examples of chiral separations of drugs showing detection levels of 0.1% or lower for the trace enantiomeric impurity have been reported.

A chiral CE separation of galantamine hydrobromide enantiomers was obtained [13] using alpha cyclodextrin in pH 3 phosphate buffer, A limit of detection of 0.04% (w/w) was reported and the method was successfully included in an new drug application (NDA) by Janssen Pharmaceuticals The method was fully validated to ICH guidelines and was successfully transferred to laboratories in Europe, US, Japan and China.

In a report from workers at Merck a detection limit of 0.05% for the undesired enantiomer was reported [14] for both HPLC and CE. The CE method using sulphated cyclodextrin proved more robust than the AGP column based HPLC method. The HPLC method varied appreciably between columns. The CE method was therefore routinely employed.

The use of CE methods for the determination of ionic drug counter-ions is well documented. Typically, this type of determination is achieved by use of indirect UV absorbance based methods (Section 5). For example, the trifluoroactetate (TFA) salt of an opioid peptide, biphalin, was determined [15] by CE using indirect UV absorbance detection. More unusually the potassium counter ion and inorganic cationic impurities of an acidic drug have been determined [16] by CE with conductivity detection.

Simple free solution CE separates solutes based on their charge and size. The charge is related to the pK_a of the solute. It is therefore possible to easily determine the $pK_a(s)$ of a test solute by sequentially varying the pH of the separation [17] and measuring the migration of the solute compared to a neutral solute which is used to measure the electroendosmotic flow (EOF). Rapid pK_a screening of solutes can be achieved by injecting samples at the end of the capillary nearest the detector and simultaneously applying pressure and voltage during the separation process. The pressure application improves analysis times especially at low pH where the EOF is minimal and migration times would otherwise be excessive.

The affinity for solutes to a substrate or additive is described by the binding constant. The determination of the binding constant of drug candidates is of interest in drug

development. CE can be used [18] to measure binding constants. The solute is initially separated in the electrolyte containing no additive and the migration time is determined. The additive, such as a protein or cyclodextrin is then added at increasing concentrations and the solute mobilities determined. The increase in migration times is directly related to the solute binding to the additive (after adjustment of the measurements for any viscosity increases due to presence of the additive). The partitioning of a solute into a micelle or microemulsion droplet is related to its solubility. Solute migration times in both micellar and microemulsion electrokinetic chromatography can therefore be correlated [19] to solute solubility.

Clinical determinations of pharmaceuticals in biological fluids by CE offers great possibilities as CE can offer high separation efficiencies and the biological fluids can often by directly injected into the capillary, or after only minor pretreatment. The main problem to the widespread implementation of CE into this area has been the lack of detection sensitivity. The strategies to improve the sensitivity of CE for the analysis of drugs in biological fluids has recently been [20] reviewed. Laser-induced fluorescence (LIF) detection or mass spectroscopy (MS), can be applied to maximise sensitivity. However, LIF detection can only be used for fluorescent analytes and the current equipment for CE–MS coupling provides only small improvements in sensitivity compared to UV detection. The detection window for UV detection can be enhanced using capillaries with an extended light path (bubble cell), Z-shaped capillaries or extended lightpath flowcell (Fig. 1). Injection techniques like sample stacking can also be applied to achieve limits of quantification in the lower µg/l range.

Several applications of clinical drug determinations have been published. For example carvedilol enantiomers have been determined [21] in serum using a CE method with cyclodextrins incorporated into the buffer. An internal standard was employed to ensure acceptable injection precision. A validated routine method for the bioanalytical analysis of moxifloxacin in plasma and microdialysate has been [22] reported with He–Cd LIF. Samples were injected directly into the capillary from very small volume of microdialysate and plasma. Fifty injections were possible from only 10 µl of analyte. The method's dynamic range covered three orders of magnitude (plasma: 2.5–5000 µg/l; microdialysate: 5–5000 µg/l). The method was validated to ICH guidelines and was used routinely.

The use of multi-capillary systems with charge-coupled device cameras and frequency doubled Ar-ion laser ($\lambda = 257$ nm) for laser induced fluorescence has been [23] demonstrated to offer the possibility of highly sensitive, high sample throughput clinical analysis applications.

Demonstrations of quality assurance (QA) is an important aspect in therapeutic drug monitoring (TDM). The method under QA test is used to generate results on spiked reference samples. The test results obtained are then statistically compared to the known concentrations. CE assays
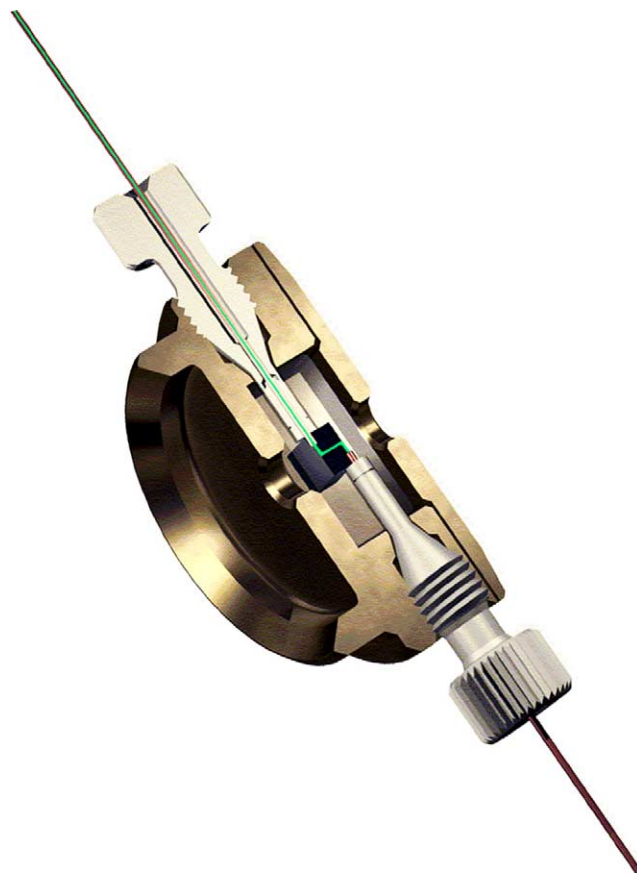

Fig. 1. CE detector flow cell. Reproduced with permission from http://www.Agilent.com.

for the determination of (i) ethosuximide via direct injection of serum or plasma, (ii) lamotrigine after protein precipitation by acetonitrile and analysis of an aliquot of the acidified supernatant, and (iii) carbamazepine and carbamazepine-10,11-epoxide after solute extraction followed by analysis of the reconstituted extract have been [24] characterized via analysis of a large number of commercial quality control sera containing up to 14 analytes (nine of which were anti-convulsants) in sub-therapeutic, therapeutic and toxicological levels. CE data obtained in single determinations are shown to compare well with the theoretical values and the mean of data determined in other laboratories. The available data reveal the effectiveness of assay assessment via analysis of quality control sera and confirm the robustness of the assays for TDM in a routine setting.

Routine CE methods have been adopted in many biopharmaceutical companies with methods being used to characterise and assay protein-based products in particular (see Section 15 for more examples). Authors from Genetech have reported [25] implementation of routine QC methods into their QA laboratories for batch release. Specific application include [25] protein content, hetrogeneity studies, peptide digest analysis as a characterisation tool and carbohydrate determinations. Workers from Medimmune reported

[26] the optimization, validation, and use of capillary gel electrophoresis for quality control testing of Synagis (R), a monoclonal antibody. The method was successfully [26] approved by FDA and is in routine use.

The use of CE in the forensic determination of drugs has received considerable attention in recent years as researchers have appreciated the ability of a single CE method to simultaneously separate and monitor a range of drugs. For example, a buffer of 0.1 M phosphoric acid adjusted to pH 3.0 with triethanolamine has been used [27] to resolved amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylene-dioxymethamphetamine (MDMA), 3,4-methylenedioxy-ethamphetamine (MDMEA), N-methyl-1-(1,3-benzodioxol-5-yl)-2-butamine and ephedrine within 8 min and without interference from the common adulterants usually found in illicit powders. Section 12 demonstrates that DNA profiling by CE has considerable use in forensic examinations.

A commercial buffering/capillary coating solution system was used [28] to separate amphetamine, methamphetamine, MDA, MDMA, and an internal standard (n-butylamphetamine) in <5 min. The run-to-run migration time percentage R.S.D.s and peak area percentage R.S.D.s were typically <0.3% and <2.1%, respectively. The percentage R.S.D.s of the relative migration times compared with the internal standard on a day-to-day and capillary-to-capillary basis were <0.2 and <0.06%, respectively. The linear dynamic range using peak area, ranged from 0.003 to 0.10 mg/ml. The correlation coefficients were less than 0.9998, with all calibration curves passing through, or near the origin. The method allowed screening of basic, acidic, and neutral adulterants in drug seizures; identification was facilitated by the use of automated UV library searches.

3. Chiral separations

The resolution of enantiomers remains one of the most prominent and frequently cited applications of CE. Method development can be rapid and the methods tend to be more robust than their HPLC counterparts. Chiral separations are based on the formation of diastereomeric complexes between the enantiomeric analytes and a chiral selector added to the electrolyte solution. Recent reviews [29–32], and an entire volume of J. Chromatogr. A [33] have comprehensively covered the background theory and applicable equations required for optimization of selectivity coefficients, resolution and analysis time in the zone electrophoretic mode.

Enantiomeric separations of various compounds, e.g., pharmaceuticals, drugs and related metabolites in biological fluids, amino acids, di- and tri-peptides, pesticides and fungicides, have been performed using different chiral selectors. Native and derivatized cyclodextrins continue to be the most widely used chiral selectors. Other chiral selectors, such as natural and synthetic chiral micelles, crown

ethers, chiral ligands, proteins, oligo- and polysaccharides, and macrocyclic antibiotics have also been applied to chiral CE separations. There is a marked increase in the number of communications on the utilization of electrophoresis for practical chiral separations within recent years which suggests that the basic period of intensive research in the field is concluded and reports are becoming more routine. In recent years the development and commercialisation of highly purified suphated cyclodextrins have allowed analysts to rapidly develop and optimize chiral separations. These methods have been shown to be capable of quantifying trace level enantiomers and have found considerable utility in pharmaceutical analytical laboratories.

The determination and modelling of the chiral selector-analyte and interactions in chiral CE separations has proven to be of considerable academic interest. Studies have been performed [34] using nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS) and X-ray crystallography to obtain a better understanding of the chiral recognition mechanisms in CE.

The commercialisation of highly purified sulphated cyclodextrins has lead to many industrial applications [35] being developed. Sulphated cyclodextrins (Fig. 2) can achieve a high degree of enantioselectivity resulting in sufficiently large resolutions to allow their use for determining the trace level enantiomer in single isomer drugs. The sulphated cyclodextrins are especially useful in the separation of basic drugs as the resolution is achieved by a combination of enantioselective inclusion and ion-pairing. A systematic method development approach for resolving chiral basic drugs has been reported [36] which involves modifying specified method parameters. These include concentration of the chiral selectors, buffer pH, type of organic modifiers, buffer type, temperature and applied voltage. Many practical aspects were also discussed via several specific examples in order to demonstrate how to develop and validate a precise, sensitive, accurate and rugged separation.

Numerous CE methods have been shown [37] to be suitable for stereospecifically evaluating the enantiomers of a drug in the quality control of enantiomerically pure drugs (e.g. determination of the enantiomeric excess, EE). Typically, a limit of detection of 0.1 percent is necessary to fulfil the requirements of the ICH guidelines. Optimisation of a number of factors is necessary in order to obtain a sufficient separation of mixtures of a minor and a major isomer to determining the EE. These parameters include the nature and concentration of the cyclodextrin derivative, the composition of the background electrolyte, the pH value, the organic modifier, and the type and conditioning of the capillary.

Enantioselective capillary electrophoresis methods have been [38] developed and validated for two Astra Zeneca drug substances. A novel direct thrombin inhibitor melagatran and its oral prodrug, ximelagatran. This work was undertaken as chiral liquid chromatography had failed to achieve sufficient selectivity and sensitivity. A robust and sensitive chiral CE method was optimised using a low pH phosphate
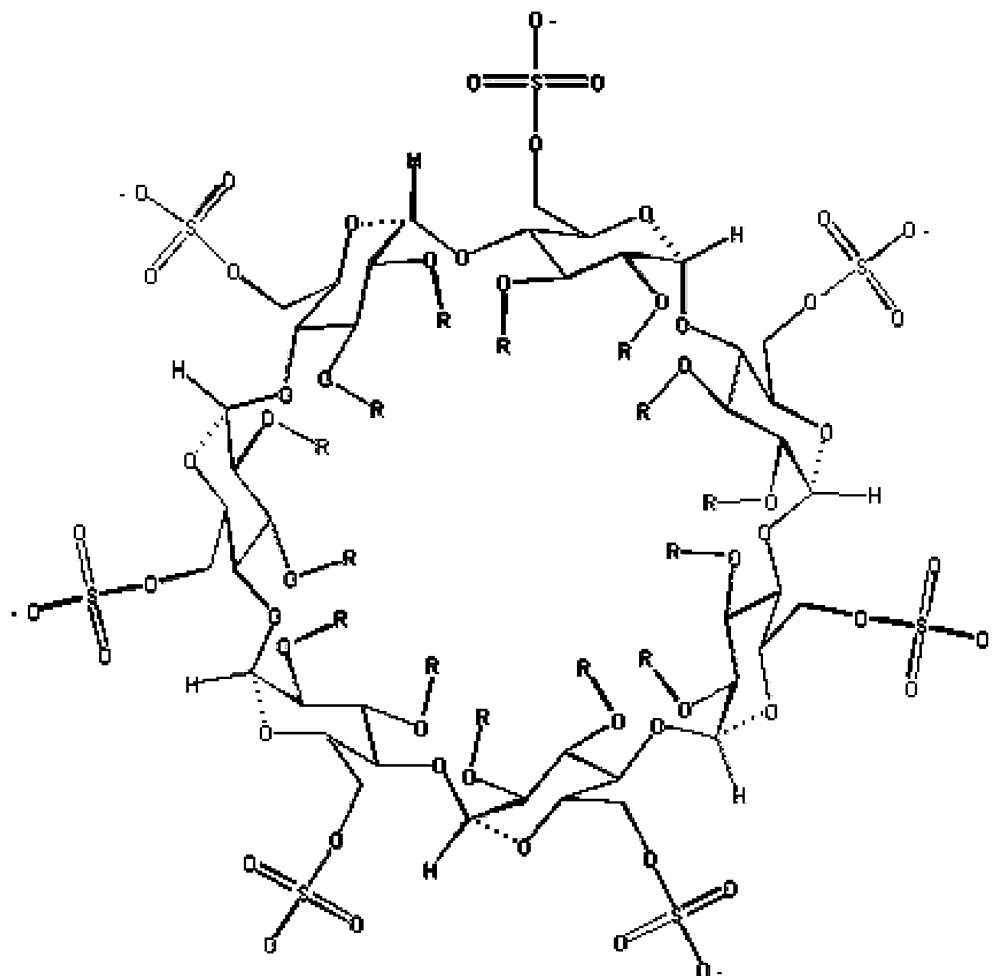
Fig. 2. Sulphated cyclodextrin.

buffer with heptakis (2,6-di-*O*-methyl)-beta-cyclodextrin with additional organic modifier. Quantitation limits of 0.05% (w/w) were demonstrated as part of a full validation package, according to ICH guidelines. Other validation aspects demonstrated included selectivity, linearity, accuracy, precision, stability of analytical solutions, robustness, detection and quantitation limits. Cyclodextrin batch variations were amongst the parameters verified for method robustness.

Simultaneous enantioseparation of a basic drug compound (M3) and its acidic intermediate (MA) was obtained [39] by CE using a single-isomer cyclodextrin, octakis (2,3-diacetyl-6-sulfo)-γ-cyclodextrin (ODAS-γ-cyclodextrin). The optimal method was validated in terms of linearity, sensitivity, precision, ruggedness, and specificity. A mixture of enantiomers of M3 and MA were spiked into the reaction matrix used for the synthetic step and the resulting solution was evaluated by the optimized CE-chiral method. The results indicate both pairs of M3 and MA enantiomers were free from interference from the reaction matrix. The feasibility of utilizing the method for in-process monitoring of possible racemization during the synthetic process was demonstrated.

Chirally selective CE methods can be [40] interfaced to mass-spectrometer detectors. However, the methods using charged cyclodextrins or surfactants require the use of a partial-fill technique to avoid the selectors entering the mass-spectrometer. The partial-fill technique generally involves filling the detector end of the capillary with the chiral selector solution and injecting the sample solution at the opposite end of the capillary. Separation conditions are selected such that the chiral selector migrates away from the detector, whilst the solute migrates towards the detector. The chiral separation occurs where the zones overlap.

Finally, recent developments in preparative enantioseparation in continuous free-flow system [41] utilizing isoelectric membranes have been reviewed.

4. Agrochemical analysis

The applications of CE for the analysis of agrochemicals are similar [42,43] to those areas where CE is applied in pharmaceutical analysis. These include assay, impurity profiling, and chiral separations. Many pesticides and

herbicides, such as phenoxy acids are acidic in nature and are ideal separation candidates for CE analysis with high pH buffers. Quaternary ammonium agrochemicals, such as paraquat are very basic in nature and can be analysed successfully [44] at low pH, often with addition of organic solvents to the buffer.

Environmental monitoring (see Section 6) of agrochemicals is an important activity and a number of reports [45] have shown the successful application of CE to the determination of herbicides, fungicides, insecticides, acaricides in water, soils and foodstuffs. This comprehensive review article contains a listing of the pesticides, the separation conditions, the detection mode, and detection limits. A variety of preconcentration methods, such as pressurized liquid extraction and solid-phase extraction were employed and are also listed.

A recent chiral separation example is the development of a chiral CE method using a cyclodextrin which allowed [46] chiral resolution of 18 aromatic aminophosphonic acid enantiomers. This separation was successfully used for routine aminophosphonic acids enantiopurity determination.

5. Determination of metal ions and inorganic anions by indirect UV

Separation and determination of both metal ions and inorganic anions by CE have been the focus of considerable attention in recent years. A recent review by Timerbaev [47] covered advances and trends in capillary electrophoresis of inorganic ions and cited 267 references. Detection is generally achieved by indirect UV following the addition of a UV absorbing species into the electrolyte used for separation. Commercial kits are available and frequent used and therefore the methods are simple to operate and can have analysis times in the range of 2–10 min which compares favourably with ion exchange chromatography (IEC) counterparts. IEC columns are expensive and require regeneration. The range of metal ions that can be simultaneously analysed in CE include alkaline, alkaline earth and transition metals which require multiple IEC methods to cover the same analyte range.

Metal ions can be determined following complexation with some chelating species, such as EDTA or 1,10-phenanthroline [48] within the electrolyte. The metal ions rapidly chelate with the additive and the complex thus formed is detected by direct UV absorbance.

Speciation of metals ions is possible by CE and was recently reviewed [49]. Various important aspects of speciation, such as sample pretreatment, metal ion complexation, detection, detection limit, choice of electrolytes were described for an number of examples. Speciation of anions is also possible, for example speciation of sulphur containing anions has been reported [50]. Detection was achieved by the addition of pyromellitate ion into the pH 10 electrolyte.

The determination of metal ions in water, soil and sediment samples by capillary electrophoresis is an important area of application and a recent review [51] contained 147 references on these topics.

6. Environmental monitoring applications

CE has been used [52] to monitor the environmental levels of a range of materials including phenols, surfactants, dyes, polynuclear aromatic hydrocarbons (PAHs), aromatic and aliphatic amines, drugs, aromatic acids and aromatic sulfonic acids. A range of preconcentration techniques, such as sample stacking and solid-phase extraction have been used to raise sample solution concentrations.

Sulphonated anthroquinones dyes are resistant to biodegradation and an efficient, rapid, and inexpensive CE method using a borate buffer has been developed [53] for analysis of five sulphonated anthraquinones, in the range 50–950 μM, with a simple borate buffer. The method has been used to measure environmental levels and assess a variety of wastewater treatment approaches.

The ability to utilise indirect UV detection has led to the application of CE to the determination of low molecular weight pollutants, such as carboxylic acids. A range of 14 carboxylic acids, such as malonic, oxalic, fumaric, acetic, propionic and butyric acids were determined [54] in various soil and water samples. Detection limits of 90–200 and 0.5–5 μg/l for hydrodynamic and electrokinetic injection, respectively were obtained. CE has also been used [55] extensively to monitor hydroxymethanesulfonic acid in environmental aerosol samples. Indirect UV detection was utilized by adding 2,6-naphthalenedicarboxylic acid to the electrolyte.

Drug residues (typically antibiotics) in foodstuffs have also been measured by CE. Specific examples include oxytetracycline in pig tissue [56] and enrofloxacin in chicken samples. A limit of detection of 10 μg/kg of enrofloxacin in these samples was obtained following solid phase extraction of samples. Phenol pesticide levels were monitored by laser induced fluorescence detection following preconcentration and derivatisation [57].

Chemical warfare agents (CWAs), such as sarin are often small inorganic species which have limited, or no, chromophores and are highly polar which makes their separation and detection difficult. CE using a variety of direct and indirect UV detection approaches have been used [58] to identify and quantify a broad field of classical CWAs in environmental samples and monitoring levels following controlled destruction of CWAs.

7. Clinical diagnostic applications of CE

The high separation selectivity and efficiency can often allow CE methods to be devised [59] for monitoring levels of natural substances present in biofluids. The presence of higher or lower levels of these specific marker compounds

are indicative of disease states. Many of these marker compounds are simple organic compounds, such as aliphatic acids and amino acids which have limited chromophores. The ability of CE to perform detection at low UV wavelengths can therefore often eliminate the need for sample derivatisation. The rugged nature of the capillary and the ability to vigorously rinse the capillary between injections can also allow direct injection of the sample fluid preventing the need for complex sample pretreatment. Some specific applications are discussed below.

Metabolic acidosis can result from accumulation of organic acids in the blood due to anaerobic metabolism or intestinal bacterial fermentation of undigested substrate under certain clinical conditions. Measuring fermentation products, such as short-chain fatty acids (SCFAs) and lactic acid in various biological samples is integral to the diagnosis of bacterial overgrowth. Stereospecific measurement of D- and L-lactic acid is necessary for confirmation of the origin and nature of metabolic acidosis. Separation of SCFAs and lactic acid has been achieved by a number of techniques including [60] capillary electrophoresis. Determination of urinary orotic acid levels is a useful tool for screening hereditary orotic aciduria and for differentiating other hyperammonemia disorders. CE has been used [61] for the quantitative determination of orotic acid in urine samples.

Urinary creatinine is determined as an indicator of glomerular filtration rate. Creatinine monitoring is also performed as creatine supplementation is an increasing practice amongst bodybuilders and athletes. Capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MEKC) have been used [62] to analyze urinary creatine and creatinine. These analytes have also been analyzed in serum and tissue by CE. Determination of total homocysteine content in plasma is valuable for the diagnosis and monitoring of cobalamine or folate deficiencies, and it has been accepted as an independent risk factor for premature cardiovascular disease. CE has been used [63] for this determination, as it offers a simplified approach, from a methodology perspective.

8. Carbohydrate analysis

Carbohydrates are not the most obvious candidates for analysis for CE as they are generally neutral and possess limited chromophores which precludes their detection by UV absorbance. The determination of carbohydrates is however one of the major areas of CE application. This has been achieved by means of sample derivatisation, indirect UV detection or more recently by use of MS detection. The application areas include carbohydrate determinations in simple samples, such as wine or wood extracts or more complicated assays, such as the levels of glycosolation in the characterisation of proteins. Carbohydrates are generally neutral but can be ionised at high pH values, such as pH, 10 where hydroxyl groups ionise. Alternatively a charge can

be induced by complexation with an acidic derivatisation agent.

The simplest means of determining carbohydrates is to employ indirect UV detection at high pH. For example Soga and Imaizumi [64] employed a buffer system compromising 20 mM 2,6-pyridinedicarboxylic acid (PDC) to give the background signal for detection. The method could be simultaneously applied to 206 species including inorganic and organic anions, amino acids and a variety of other negatively charged species, such as metal-EDTA complexes.

The majority of carbohydrate applications have involve solute derivatisation. For example CE was used [65] to analyse a wide range of carbohydrates (reducing saccharides, neutral monosaccharides, uronic acids) following enzymatic hydrolysis of wood and pulps. The carbohydrates were derivatised with 4-aminobenzoic acid ethyl ester and quantitatively analysed with an alkaline borate buffer with UV absorbance detection at 306 nm.

Solute derivatisation can be accomplished within the capillary by injecting plugs of the derivatising agent and sample solution. The sample and derivatisation agent solutions mix by diffusion and react to form the derivatives inside the capillary. This process can be automated and eliminates the need for any sample handling. This approach has been used [66] to derivatise reducing carbohydrates with 1-phenyl-3-methyl 5-pyrazolone (PMP). The derivatisation conditions were optimised such that quantitative derivatisation occurred within 35 min at 57 °C. Precision values of 3% R.S.D. were reported for peak area determinations.

8-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS) is a useful derivatising agent for carbohydrates as it produces [67] fluorescent charged derivatives. ANTS carbohydrate derivatives can also be determined by MS in negative ion mode. ANTS derivatization N-Linked glycans released from bovine fetuin were analysed by CE–MS. A similar approach of ANTS derivatisation and CE–MS analysis has been performed [68] for dextran analysis. CE–MS analysis of underivatised carbohydrates is also possible [69] using highly alkaline carrier CE electrolytes based on volatile organic bases, like diethylamine (DEA), combined with electrospray MS detection in the negative-ion mode.

CE has been especially useful in the analysis of the mono-, di-, and oligosaccharide constituents of glycan moieties in proteoglycans and the approaches to this analysis have been recently reviewed [70] by Lamari et al. Specific applications being the determination of the monosaccharide constituents, the disaccharide sulfation pattern, and the uronic acid distributions within glycan chains of proteoglycans.

9. Instrumentation developments

There have been a range of instrumentation developments which are briefly discussed and described below. The most notable advance has been the commercial availability of microchip based CE instruments. However microchip CE
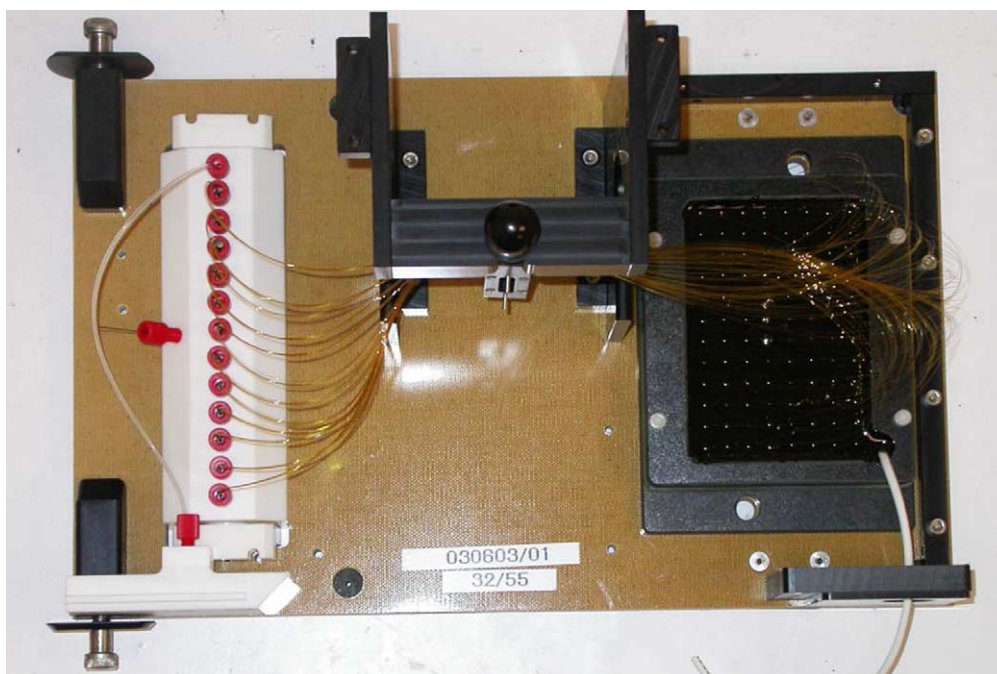
Fig. 3. UV absorbance detection 96 capillary array system (figure reproduced with permission from http://www.Combisep.com).

instruments have not been applied to the analysis of small molecules and applications have centerd on protein and DNA analysis.

9.1. Capillary array instruments

One of the principal advantages of CE is the ease of multiplexing i.e. using several capillaries simultaneously within a single instrument. In this way sample throughput can be significantly increased compared to single capillary formats. Typically, 96 capillary arrays are employed. Fig. 3 shows an internal view of UV absorbance based 96 capillary array system. A single power supply is employed to simultaneously apply the voltage across the array of capillaries. Various detection options are possible with laser induced fluorescence (LIF) detection being the most popular for the determination of DNA. It was this combination of capillary array CE and LIF which performed the majority of the determinations in the successful completion of the Human Genome project.

Capillary arrays with LIF detection can also [71] be used to perform high throughput screening of genetic mutations involving human tumor suppressor genes, oncogenes and disease-causing genes and polymorphisms. The use of the capillary array also allows rapid screening of large numbers of clinical samples.

Recent commercial capillary array instrumentation has become available that features UV absorbance detection which greatly increases the scope of the instrumentation in terms of application areas. It is expected that this type of instrument could become a routine means of performing high throughput testing driving CE into application areas that it has not significantly featured in previously. One such area

is metabolomic studies in which comprehensive analysis of metabolites and metabolite pathways in cells, and metabonomics is focused on quantitation of metabolite levels in whole organisms by analysis of biological fluids. The use of a capillary array CE instrument with UV absorbance has recently been shown [72] to have great utility in the areas of metabolomic and metabonomics due to the high sample throughput capacity. The actual application studied purine metabolites and included use of sample stacking processes to achieve detection limits of 8.0×10^{-8} M.

High-throughput screening of kinase inhibitors by multiplex capillary electrophoresis with UV absorption detection has also been [73] reported. The published results showed that multiplexed CE/UV may prove to be a straightforward and general approach for high-throughput screening of compound libraries to find potent and selective kinase inhibitors.

9.2. Detection modes

The various modes of detection in CE have been recently [74] reviewed. These include fluorescence, UV absorbance (direct or indirect), electrochemical detection. Each mode can have distinct advantages and limitations. The detection limits (ranging from single molecule to 10^{-5} M), detection scheme complexity and the particular applications dictate the selection of detection methodology in CE. Other detection approaches discussed and reviewed [74] include Laser-induced fluorescence, refractive-index detector, and pulsed amperometric detection.

Capillary electrophoresis (CE) mass spectrometry (MS), with its ability to separate compounds present in extremely small volume samples rapidly, with high separation

efficiency, and with compound identification capability based on molecular weight, is a valuable analytical technique [75] for the analysis of complex biological mixtures. The highest sensitivities and separation efficiencies are usually achieved by using narrow capillaries (5–50 μm) and by using sheathless CE–MS interfaces. Several CE–MS interfacing techniques have recently been introduced. While electrospray ionization is the most commonly used ionization technique for interfacing CE–MS, matrix assisted laser desorption ionization (MALDI) has also been used, using both on-line and off-line techniques. Moreover, the high concentration detection limit of CE has been addressed by development of several sample concentration and sample focusing methods. In addition, a wide variety of techniques, such as capillary zone electrophoresis (CZE), capillary isoelectric focusing, and on-column transient isotachophoresis have now been interfaced to MS. CE–MS has now been successfully applied to the analysis of a wide variety of compounds including amino acids, protein digests, protein mixtures, single cells, oligonucleotides, and various small molecules relevant to the pharmaceutical industry.

Chemiluminescence-based detectors have been developed [76] in order to increase sensitivity in capillary electrophoresis detection. These on-line ultrasensitive chemiluminescence detectors have achieved detection limit of nM or mass detection limits in the order of amol. The low background signal of these detectors allow massive amplification of the output signal. Additives, such as luminol can be added to the electrolyte to promote chemiluminescence activity of solutes.

Electrochemical detectors based on amperometric and voltammetric detection have been utilised [77–79] in conventional capillary electrophoresis and microchip CE [80] systems. The range of applicable classes of analytes to this detection mode include amino acids, carbohydrates, neurotransmitters and peptides.

Near-infrared laser-induced fluorescence detection has also been employed [81] in capillary electrophoresis and number of labeling dyes, such as tricarbocyanine and heptamethine cyanine dyes are available for sample tagging especially for DNA sequencing and fragment analysis.

The basic and practical aspects of interfacing capillary electrophoresis to inductively coupled plasma-mass spectrometry (CE–ICP–MS) have been recently [82] reviewed. This combination has a particular utility for elemental speciation analysis. Interfacing of CE to ICP–MS is achieved including several devices and nebulizers.

CE instrumentation has been modified to allow on-line sampling and monitoring of chemical processes occurring in solution during production. A special sample vial was developed [83] for online coupling to a commercially available CE-instrument. In a separate development [84] an on-line capillary electrophoresis instrument with continuously flowing electrolyte solutions and sequentially applied sample introduction was reported. The system was used for the on-line determination of chloride, sulfate, and carbonate ions in process waters at a pulp mill. On-line samples from a pulping machine were taken at 6-min intervals.

9.3. Sensitivity improvements

The relatively lower concentration detection limits of CE when compared to HPLC was a frequently quoted comment during the infancy of this technique. Several approaches have been adopted [85] to overcome this difficulty. These include solute derivatisation, pre-concentration techniques, electrokinetic injection, modified capillary dimensions and use of preconcentrators.

A variety [86] of solute derivatisation schemes have been reported. These have included pre-capillary, on-line, on-capillary and post-capillary approaches. The majority of derivatisation schemes have introduced a functional grouping onto the solute to increase fluorescence and laser-induced fluorescence detectability which can increase solute sensitivity several orders of magnitude compared to UV absorbance. The most frequently reported functional groups are amines and the reducing end of (oligo)saccharides, additionally thiols, carbonyl and carboxyl groups, steroids and inorganic ions have been derivatised. Other reasons for derivatization are to enhance chiral separation, introduction of a suitable charge onto a neutral molecule (such as a saccharide) or to improve mass spectrometric detection.

Of particular interest is the ability to perform on-line derivatisation in CE due to the liquid handling abilities of the instrumentation. On-line derivatisation can be achieved by injecting the sample between two injections of the derivatising agent solution. A suitable time interval is allowed to permit the dervisation solution and sample solutions to mix by diffusion and the derivatisation process to occur. The voltage is applied after the derivatisation step is complete and the derivatised solute is separated and detected. For instance [87] in-capillary derivatisation of amino acids, biogenic amines and amino phosphonic acid-herbicides with 5-(4,6-dichloro-s-triazin-2-ylamino)fluorescein (DTAF), allowed LIF detection. Careful optimization of the electrophoretic conditions in the mixing step of this protocol allowed the determination of these solutes at concentration limits of detection at the μg/l level and with relative standard deviations from 3.5 to 5.8%.

Typically, no more than 1% of the capillary volume can be filled with sample solution before severe loss of peak efficiency and separation occurs. This limits injection volumes and therefore sensitivity. However, increased injection volumes and improved sensitivity can also be achieved using optimised separation conditions. Focussing (or stacking) of the solute within an extended sample injection volume can be achieved [88] by appropriate selection of the solute dissolving solvent and the buffer composition. This focussing process can allow the whole capillary to be filled with sample solution. Separation conditions are selected such that, after the focussing step, the sample ions migrate under the applied voltage and are concentrated into a small section

of the capillary thereby raising solute concentration inside the capillary by several magnitudes. A specific recent report utilized [89] this approach to determine peptides in the low femtomole range by capillary electrophoresis-tandem mass spectrometry for routine application in proteomic studies. A 2000-fold enhancement of detection sensitivity was reported [90] for priority phenol pollutants using sample stacking at high pH, where the phenols are ionised.

An alternative approach to stacking (known as sweeping) involves [91] the use of charged additives such as surfactants to interact with sample solutes causing a focussing inside the capillary. The interaction can be chromatographic parti-

tioning or complexation and results in the movement of all solutes molecules to the edge of the sample injection volume. Sweeping can be used to improve sensitivity for both neutral and charged solutes, whilst stacking is suitable for only charged solutes.

Typically, sample solutions are introduced into the capillary by applying a pressure differential across the capillary whilst the capillary is dipping into the sample solution. An alternative approach, electrokinetic injection, applies a voltage rather than differential pressure. In electrokinetic injection sample ions move into the capillary due to their charge. If appropriate separation conditions are selected [92] then
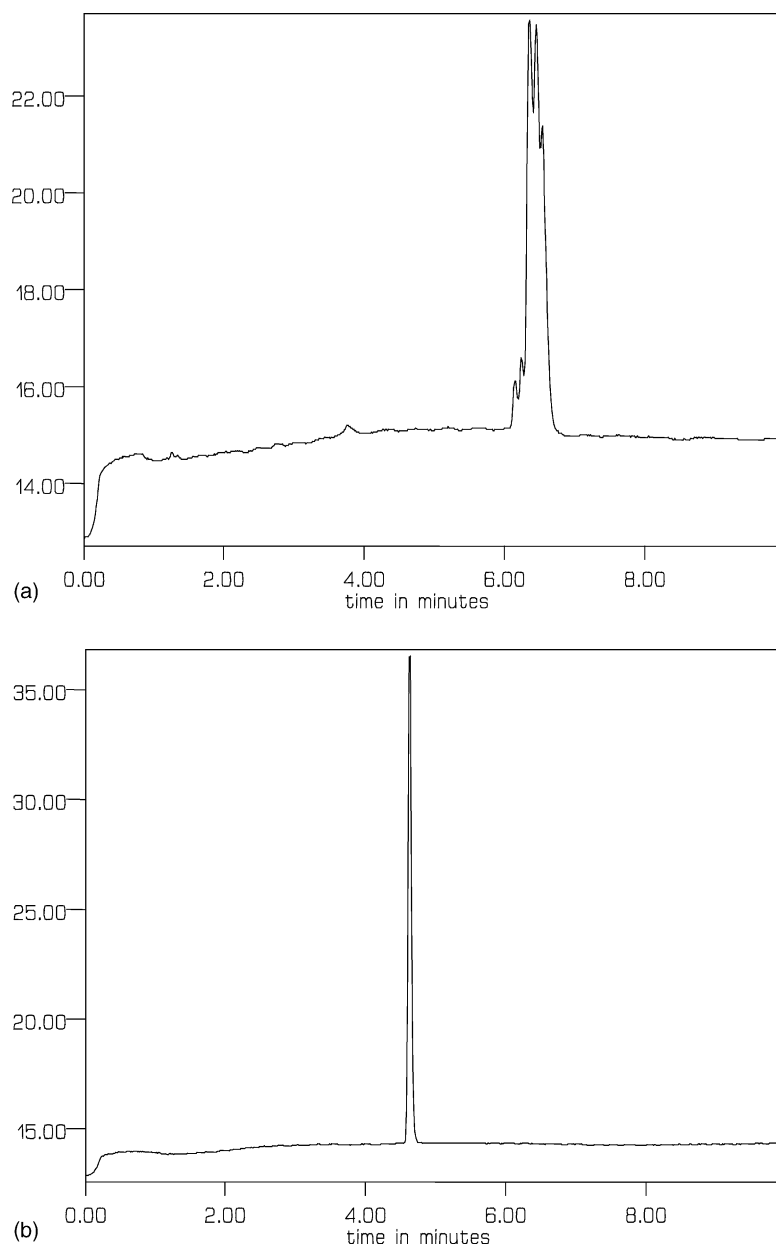

Fig. 4. Separations on a multi-bore capillary using phosphate buffer or CElixir buffer (figure reproduced with permission from reference [97]). (a) Separation using phosphate buffer 50 mM phosphate 2.5, multi-bore capillary 19 μm × 25 μm channels, 27 cm long, 130 μA, +5 kV, 30 °C, detection at 200 nm, sample salbutamol 1 mg/ml, 1 s injection. (b) Separation using CElixir buffer 50 mM phosphate 2.5, multi-bore capillary 19 μm × 25 μm channels, 27 cm long, 90 μA, +5 kV, 30 °C, detection at 200 nm, sample salbutamol 1 mg/ml, 1 s injection.

extended electrokinetic injection times can be utilised without overloading of the capillary. Sensitivity improvements are especially achieved for small, highly charged solutes such as metal ions and inorganic anions, e.g. chloride, etc.

The most impressive sensitivity gains can be achieved [93] using analyte preconcentrators inside the capillary. Preconcentrators are attached to the injection end of the capillary and they contain a substrate which solutes bind onto. The binding may be chemically based or biologically selective such as immunoaffinity. Several capillary volumes of the sample solution can passed through the capillary with the solute collecting onto the preconcentrator adsorbent. The solute is then released from the adsorbent by flushing with a small volume of an appropriate solution. In the case of antibody-antigen microconcentrators this approach can lead to sensitivity increases of tens of thousand fold compared simple pressure injection.

A variety of these sensitivity enhancing approaches have been utilised [94] specifically for biological samples and these have recently been reviewed.

9.4. Injection precision improvements

Injection repeatability is an issue in CE as the volume injected cannot be finitely controlled and typically precision values are worse than HPLC. The volume of the sample injected into the capillary is affected by a multitude of factors include the surface tension and viscosities of both the sample solution and electrolyte. A systematic investigation into the underlying factors affecting precision has been reported [95] and the use of internal standards and migration indexes were recommended. In-line injection has been [96] applied successfully in CE using a commercially available injection valve composed of ceramics and PEEK, which was designed for liquid chromatographic applications. Partial-loop injections from 6 to <60 nl were shown to be highly reproducible.

9.5. Capillary coatings

Capillaries can be permanently chemically coated or temporarily coated by inclusion of appropriate surface active agents into the rinse and/or electrolyte solutions. These coatings can have a range of different functions. These include reduction of undesired solute adsorption, elimination of EOF or reversal of the EOF direction. Separations of various basic and acidic proteins were performed [97] when polyethylene glycol (PEG), polyvinyl alcohol (PVA), and hydroxyethylcellulose (HEC) were employed as additives in the running buffer. Capillaries permanently coated with polyvinyl alcohol (PVA) where also compared and found to give the most efficient separations. A commercial capillary treatment system of buffers and rinse solutions has been shown [98] to improve CE repeatability as they dynamically coat the capillary with a bilayer of surface active agents ensuring that the surface coverage and EOF is consistent between injections and between capillaries. Fig. 4 highlights the consistency

of EOF when using the buffer coating system when compared to a standard phosphate buffer using a capillary that contained 19 separate channels. In Fig. 4a the peaks in the channels have different speeds and the separation obtained is poor. In Fig. 4b the EOF is consistent in each channel and the peaks all move at the same speed resulting in a single peak.

10. CE technique developments

The majority of CE reports have continued to be on the analysis of ionisable solutes using simple high or low pH electrolyte systems. CE is however a family of techniques and a range of separation possibilities exist other than use of simple aqueous based electrolytes.

Free solution CE (FSCE) is now well established with respective to buffer and sample diluent choices and developments are tending to arise following optimisation studies. A recent study, has for example, [99] specifically focussed on the sample matrix influence on the choice of background electrolyte for the analysis of basic compounds. The separation performance and resolution of the trace impurity peaks could be appreciably affected by the choice of buffer co-ion and addition of sodium chloride, 2-propanol or acetonitrile to the sample solution.

Micellar electrokinetic chromatography (MEKC) was originally designed to separate neutral solutes which were impossible to separate by free solution CE (FSCE) as they possess no charge. The separation occurs in MEKC via partitioning into surfactant micelles. The extent of partitioning is related to the solute solubility. MEKC has been considerably extended to include analysis of charged compounds which can benefit from the increase selectivity options compared to FSCE including solubility and ion-pairing factors. Optimised choice of sample diluents and electrolyte combinations can be used to increase sample online concentration by stacking or sweeping The current state of the art of MEKC has recently been reviewed [100] by Pyell. Microemulsion electrokinetic chromatography (MEEKC) has recently appeared as a complimentary technique to that of MEKC. The principal difference being that solutes interact with a oil droplet in MEEKC compared to a micelle in MEKC. The background theory, operating parameter options and application range have recently [101] been reviewed.

The use of pure non-aqueous solvents, such as methanol and acetonitrile instead of the conventional aqueous CE electrolytes is gaining increased prominence. The obvious benefit is the analysis of water-insoluble ionisable compounds. The selectivity of water-soluble compounds is often different in non-aqueous solvent when compared to aqueous solvents as the solvated size and pK_a of solutes are differentially altered in non-aqueous solvents. Riekkola has recently summarised [102] the status of non-aqueous CE and described the theoretical, methodological, technical challenges, and

applications of capillary electrophoresis in non aqueous media are demonstrated with reference to recent applications. Of note is the suitability of non-aqueous based electrolytes with the interfacing of CE–MS.

Capillary isotachophoresis (cITP) is a technique in which solutes are focussed along the capillary based on their mobility compared to "leading" and "terminating" added solutes which have fast and slow mobilities, respectively. The sample solutes focus to match the concentration of the added solutes. In this way the concentration of the solutes can be dramatically increased inside the capillary. The technique can be conducted [103] using standard CE equipment or specially designed equipment. cITP has been used to pre-concentrate sample prior to separation by conventional CE in multidimensional separations.

11. Conclusions

In summary, capillary electrophoresis and related electro-separation sciences appear in 'rude health'. CE has carved itself a niche routine applications in a number of industries which include pharmaceutical analysis, forensic determinations, clinical analysis, inorganic anions/metal ions. General usage is increasing at a rapid rate, particularly following the Human Genome initiative and related protein sequencing, where multi-capillary instrumentation have been used to increase dramatically the sample throughput. It is in this area of parallel array, multiplexing, where the greatest benefits will accrue in the future. The use of multiplexed instruments in other areas of analysis such as pharmaceutical, environmental and clinical analysis will drive increased CE usage due to the high sample throughput potential. The successful commercialisation of microchip CE instrument will also increase the application rate of CE as new applications are developed and validated. This coupled with improved detection capability for CE (e.g. CE–MS, CE–LIF, sample stacking, pre-concentrators) will address some of the historical deficiencies of the procedure versus other separation science techniques; particularly HPLC. This improved sensitivity, allied to the intrinsic selectivity, speed, cost and straightforward nature of the technique, should continue to win many new converts to the area of capillary electrophoresis.

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