The Analyst

Tutorial Review

Capillary electrochromatography

Maria G. Cikalo[a], Keith D. Bartle*[a], Mark M. Robson[a], Peter Myers[a] and Melvin R. Euerby[b]

[a] *School of Chemistry, University of Leeds, Leeds, UK LS2 9JT*
[b] *Pharmaceutical and Analytical R and D, Astra Charnwood, Bakewell Road, Loughborough, Leicestershire,UK LE11 5RH*

Capillary electrochromatography (CEC) has seen a resurgence of interest during the 1990s, despite having origins in the 1970s. The technique combines the desirable features of both high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE): the separation process is based on differential interactions between the stationary and mobile phases, whilst the electroosmotic flow transports the mobile phase through the capillary. Thus, it has demonstrated advantages over both HPLC and CE, which are yet to be fully exploited over a wide field of application; already the popularity of CEC is on the increase, as reflected in the number of scientific publications and seminars held. The aim of this tutorial review is to increase awareness and understanding of both theoretical and practical aspects of CEC. Whilst it does not provide an in-depth account of CEC, it does attempt to cover the more important, relevant work available in the open literature: only major advancements associated with CEC applications are highlighted. Material presented in the review was typically obtained by literature searches involving *Analytical Abstracts, Chemical Abstracts* and 'BIDS' (for academic use only).

Keywords: *Review; capillary electrochromatography; microseparations*

Maria Cikalo graduated from Kingston Polytechnic where she studied for a GRSC part time whilst working in formulation development in the pharmaceutical industry. Following a year travelling abroad, in which she obtained experience of working in quality control, she briefly returned to the same field before embarking on an MSc in Analytical Science at the University of Hull. Moving slightly north, she obtained her DPhil from the University of York where her research was chiefly concerned with the use of indirect detection in capillary electrophoresis. Since then, she has maintained her interest in electroseparation science and is currently undertaking research in the field of capillary electrochromatography at the University of Leeds.

Introduction

Capillary electrochromatography (CEC) is a recently developed (Table 1) variant of high-performance liquid chromatography (HPLC) in which the flow of mobile phase is driven through the column by an electric field, rather than by applied pressure. This electroosmotic flow (EOF) is generated by applying a large voltage across the column; positive ions of the added electrolyte accumulate in the electrical double layer of particles of column packing, move towards the cathode and drag the liquid mobile phase with them. As in capillary electrophoresis (CE) and micellar electrokinetic chromatography (MEKC), small internal diameter (50–100 μm) columns with favourable surface area-to-volume ratios are employed to minimise thermal gradients from ohmic heating, which can have an adverse effect on bandwidths. CEC differs crucially from CE and MEKC, however, in that the separating principle is partition between the liquid and solid phases (Table 2).

Avoiding the use of pressure results in a number of important advantages for CEC over conventional HPLC. First, the pressure driven flow rate through a packed bed depends (Table 3) directly on the square of the particle diameter and inversely on column length; for practical pressures, generally used particle diameters are seldom less than 3 μm, with column lengths restricted to approximately 25 cm. By contrast (Table 3), the electrically driven flow rate is independent of particle diameter and column length so that, in principle, smaller particles and longer columns can be used. It follows that considerably higher efficiencies can be generated in CEC than in HPLC. A second consequence of employing electrodrive is that the flow–velocity profile in EOF reduces dispersion of the band of solute as it passes through the column, further increasing column efficiency. The combined effect of reduced particle diameter, increased column length and plug flow leads to CEC efficiencies of typically 200 000 plates per metre and substantially improved resolution. Thus the two tipredane

Table 1 Landmarks in CEC

First report of use of EOF in chromatography	Strain	1939
Separation of polysaccharides using EOF through a colloidal membrane	Mould and Synge	1954
Use of EOF in column chromatography	Pretorius	1974
Electroosmosis in capillaries	Jorgenson and Lukacs	1981
CEC in open-tubular columns	Tsuda	1986
Theory of CEC and technique development	Knox and Grant	1987, 1991
Analysis of pharmaceutical compounds by CEC	Smith and Evans	1994

diastereoisomers, which were very difficult to separate by conventional HPLC, were readily resolved by CEC (see Applications).

Voltages up to 30 kV are supplied to generate the electric field usually for solutions of 1–50 mM buffers in aqueous reversed-phase mobile phases; non-aqueous CEC has also been carried out with ammonium acetate buffer.[1] The dependence of EOF flow rate on solvent dielectric constant has been confirmed, but the electrical potential (the zeta potential) of the boundary between the fixed and diffuse layers (the double layer) of positive ions at the stationary phase wall (Fig. 1) is less well understood. The conclusion of a theoretical study by Rice and Whitehead which suggested that flat EOF profiles in a capillary of diameter d would result if d were considerably greater than the double layer thickness, δ, has been confirmed by experiment; for channels between particles, however, the influence of δ is less clear. Current indications are that it should be possible to use monodisperse particles with diameters down to 0.5 μm. Pores sizes of commonly used HPLC particles are too small to give rise to EOF, but larger pore packings show promise. Although CEC has been demonstrated for stationary phases bonded to the walls of open tubes, and in sol–gel derived phases, most work has been carried out on columns packed with HPLC stationary phases; a new generation of packings custom synthesised for CEC is, however, now beginning to make an impact.

Principles of electroosmotic flow

Electroosmosis is best described as the movement of liquid relative to a stationary charged surface under an applied electric field.[2] Substances tend to acquire a surface charge as a result of ionization of the surface and/or by interaction with ionic species. In a fused silica capillary, the ionization of silanol groups gives rise to a negatively charged surface, which affects the distribution of nearby ions in solution. Ions of opposite charge (counter-ions) are attracted to the surface to maintain the charge balance whilst ions of like charge (co-ions) are repelled. The double layer of electric charge thus formed (see Fig. 1) is generally explained by a revised version of the Gouy–Chapman model, which is covered extensively in the literature.[2–4] Essentially the counter-ions are arranged in two layers, fixed and diffuse, with a surface of shear at just beyond the interface. The voltage drop between the wall and this surface of shear is known as the zeta potential, ζ. In the diffuse layer, the potential falls exponentially to zero, and the distance over which it falls by e^{-1} is known as the double layer thickness, δ. When the voltage is applied, the solvated cations in the diffuse layer migrate towards the cathode, dragging the solvent molecules along with them.

The linear velocity of the EOF, u_{eo}, is best described by the equation shown in Table 3, which shows how the EOF is governed by changes in the dielectric constant and viscosity of the electrolyte and the zeta potential; ζ, itself, depends on the charge density and δ, which is inversely related to the ionic strength of the electrolyte. The flow profile is assumed to be near-plug-like as essentially it originates from the capillary wall, but in reality it depends on the capillary internal diameter, d, and δ. Theoretical studies by Rice and Whitehead[5] proposed that u_{eo} is only independent of the capillary diameter when $d \gg \delta$. As d approaches δ, double layer overlap occurs with a simultaneous reduction in flow velocity, until finally a parabolic flow profile is obtained when d and δ are similar. It has been proposed[6] that the EOF velocity is acceptable when $d \geq 10\delta$. In CE, however, double layer overlap is unlikely to be a problem: for a salt concentration of 1 mM in water, the double layer thickness is calculated to be 10 nm.[6] The use of microscope optics to image flow profiles in narrow capillaries has produced conflicting results. Taylor and Yeung[7] have observed the plug

Table 3 Equations of note in microchromatography

Pressure drive: $$u = \frac{d_p^2 \Delta P}{\phi \eta L}$$

Electrodrive: $$u_{eo} = \frac{\varepsilon_o \varepsilon_r \zeta E}{\eta}$$

Resolution: $$R_s = \frac{N^{\frac{1}{2}}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k}{1+k}\right)$$

where d_p = particle diameter, ΔP = pressure drop across column, ϕ = column resistance factor, η = mobile phase viscosity, L = column length, ε_o = permittivity of a vaccum, ε_r = mobile phase permittivity, ζ = zeta potential, E = electric field strength, N = number of theoretical plates, α = selectivity, k = retention factor

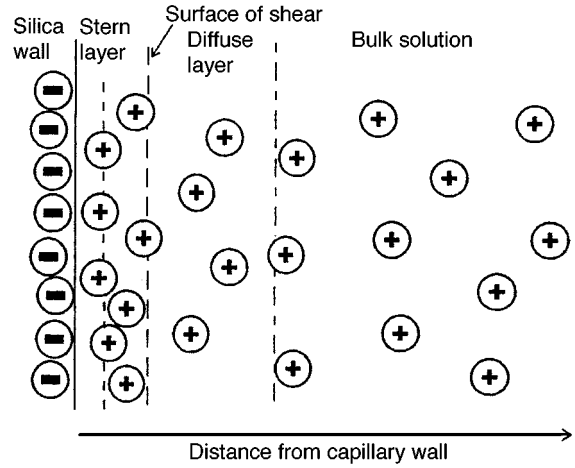

Fig. 1 Double-layer structure at a silica wall. Reprinted with permission from ref. 15.

Table 2 Comparison of electrically driven separation methods

	CE	MEKC	CEC
Separation principle	Different mobilities of ions in electric field	Partition between bulk solution and micelle moving in opposite direction to analyte	Partition between solid stationary phase and mobile phase
Column diameter/μm	50–100	50–100	50–100
Stationary phase	None	None	Silica or cellulose particles with bonded groups; bonded or imprinted polymeric matrices
Mobile phase	Electrolyte solution	Electrolyte solution	Electrolyte solution
Sample type	Charged species	Neutrals	Neutrals and charged species

flow profile predicted from theory,[6,8] whereas Tsuda *et al.*[9] have not: they found the EOF at the capillary wall to be greater than that at the centre of the capillary. The importance of the EOF profile in CEC necessitates further research in this area.

The EOF in CEC

The current CEC development has much to owe to the theoretical and experimental papers published by Knox and co-workers[6,10–12] over the last 10 years. More recently, CEC has been reviewed by Colón and co-workers,[13,14] Robson *et al.*[15] and Kowalczyk.[16] Crego *et al.*[17] focused on the fundamental principles of CEC, whilst Dittman *et al.*[18] gave an overview of the theory and practice of CEC and Rathore and Horváth[19] compared HPLC, CE and CEC. With the exception of Ståhlberg,[20] who considered the migration of charged species in CEC, theoretical treatments have mainly focused on neutral species.

In packed CEC, both the capillary wall and column packing carry surface charges that are capable of supporting EOF. To date, most of the work carried out suggests that the column packing is responsible for the generation of EOF;[21,22] there is a greater number of free silanol groups present since the solid packing has a far larger surface area compared with that of the internal silica wall. If the column is assumed to consist of a closely packed array of non-porous spherical particles, then the EOF arises from the channels between the particles. The average interparticle channel is estimated to be one-quarter to one-fifth the particle diameter.[6,23] Knox and Grant[6] subsequently suggested that, on the basis of the Rice and Whitehead treatment,[5] the particle diameter should be no less than 40δ if double layer overlap and subsequent loss of plug flow are to be avoided. With the ionic strengths typically used in CEC, namely 1–10 mM, this means that particle sizes as small as 0.4 μm can be utilised with little loss of EOF velocity. Since the particle sizes routinely used in CEC are typically 3 μm, there is considerable scope for the use of smaller particle diameters before double layer overlap becomes a problem. However, this is not the case for porous materials, where EOF generation can occur within the pores. Li and Remcho[24] have studied the role of pore size in CEC using materials with pores ranging from 6 to 400 nm. Packing materials of large channel diameter (> 200 nm) were found to be capable of supporting through-particle (perfusive) EOF. In addition, a significant increase in efficiency was observed.

The EOF velocity in a CEC column is most likely to be reduced compared with that in an open tube, on account of the tortuosity and porosity of the packed bed. Although there does not appear to be any adverse effect as a result of packing irregularities,[25] further investigations are now being made on packing structure using electrical conductivity measurements.[26] The results have been promising in that the electrical conductivities obtained for open and packed capillaries can give an indication of the flow permeability of the column. However, a factor often overlooked in CEC is the contribution of the open capillary present: most packed capillaries have an open section for detection purposes. Choudhary and Horváth[27] discussed both theoretical and practical aspects of having open and packed sections of capillary of differing conductivities, across which the voltage gradients, and hence electric field strengths, will vary. Their study found that having identical charge on both the capillary wall and packing material always resulted in a reduced EOF, which could not be explained readily. In conclusion, they suggested that the characterization of the individual column segments is necessary if a better understanding of the EOF in CEC is to be obtained.

The use of open tubular columns in CEC has several advantages: the approach to understanding and generating the EOF is less complex, their fabrication is easier and they are far more robust. Columns have been prepared for reverse, normal and chiral separations, by applying the general procedures used in open tubular liquid chromatography (OTLC).[28–30] Several groups have studied the role of surface modifications in open tubular electrochromatography (OTEC), in terms of both the EOF generated and the separations obtained. The EOF has been found to vary dramatically between capillaries ranging from untreated to those etched and coated with octadecylsilanol groups.[30] Since the EOF arises from both the surface coating and residual silica, it is likely to be reduced compared with untreated capillaries as a consequence of effective shielding of the silanol groups.[31] Although an enhanced EOF and improved peak shape could be obtained in polymer coated capillaries when using surfactants in the buffer,[32] Francotte and Jung[33] reported that these parameters were most likely dependent on the coating thickness. In contrast, Tan and Remcho[34] demonstrated that the flow velocity does not exhibit an obvious trend with monomer and/or cross-linker concentration. However, they showed that the selectivity could be controlled by careful adjustment of the monomer and cross-linker concentrations, and by incorporation of other functional groups into the polymer matrix. A recent comparison of open tubular liquid chromatography and open tubular electrochromatography for chiral separations[35] indicated that although OTEC exhibits greater efficiency and resolution due to the plug flow, OTLC remains the faster technique.

How does CEC improve on HPLC?

If existing HPLC analyses are to be replaced by CEC methods, the practising analyst must perceive substantial advantages along with at least equivalent performance in quantitative analysis. The first question likely to be asked is whether the undoubted increased efficiency, actual and potential, discussed above is relevant to a given analysis. For comparatively simple mixtures, increased theoretical plate numbers, N, may not always be required; many HPLC separations are achieved on the basis of selectivity, α, which along with N is the major factor influencing resolution, R_s (Table 3). Probably more relevant is the peak capacity, the number of peaks in a chromatogram between realistic retention factor limits. Clearly, CEC will offer substantial advantages here, and for very complex mixtures of, for example, biological compounds separation by CEC may become the method of choice. It has to be said, of course, that CE now separates many such mixtures with high resolution. Nonetheless, there is probably an analogy here with the progress of gas chromatography, where the advent of fused silica column technology offered the resolution necessary to make routine the analysis of complex fuel and environmental mixtures.

Pressure drop across an HPLC column restricts the mobile phase flow rate, but in any case the well known rising graph of plate height, h, (*i.e.*, decreasing N) against mobile phase velocity, u, is a considerable disincentive to shortening analysis times in this way. On the other hand, plug flow in CEC means that the plate height increases much less with increasing u_{eo}, so that in principle shorter analysis times are possible without loss of resolution. It may be, however, that higher applied voltages than are currently commonly used may be necessary to achieve very high flow rates, and Choudhary and Horváth[27] pioneered experiments with voltages up to 60 kV. An influential factor in CEC development is that charged analytes may be subject to separation by both electrophoresis and chromatography.

The miniaturisation of HPLC has been driven by the necessity of analysing very small amounts (picomoles) of substance available, for example, in small volumes of body fluids or in the products of single-bead combinatorial chemistry. If small amounts are to be analysed, micro-HPLC (μHPLC) is carried out on packed capillary columns with diameter of 300 μm or less. It is comparisons of CEC with μHPLC that are

probably most meaningful, and the necessary development for μHPLC of robust, easily installed columns with reliable injection procedures and available gradient elution methods parallels the practical requirements of CEC for future routine use.

The great test of CEC will be whether regulatory authorities will specify its use in analysis, especially of pharmaceuticals. Here, precision, accuracy, trace analysis and repeatability are vital, and promising results have already been obtained. Figures of merit for repeat analyses of a mixture of test compounds that are not dissimilar from those observed in HPLC analysis have been reported. Robson *et al.*[36] showed that with both unpressurised and pressurised systems, highly repeatable separations can be obtained; for a series of injections of a test mixture, relative standard deviations were less than 1% for retention time and, typically, 1–3% for peak height and corrected peak area. In addition, retention time, column efficiency and retention factor have been demonstrated to remain essentially constant for at least 200 repeat injections on the same column.[18,36,37]

Still lacking, however, are convincing demonstrations of trace analysis for, say, impurities at the 0.1% level. Longer light-path flow cells are becoming available, and it may be that current experiments with low (or even zero!) electrolyte concentrations, and hence reduced ohmic heating, will permit larger column diameters to be utilised.

Packing materials used in CEC

Working from the proposition that the support material must have a large zeta potential, the materials used have been, and are mostly, HPLC supports that are not 'end capped'. Traditionally, this has meant that the bonded phase is reacted in non-stoichiometric quantities on to the support; thus, in the case of a silica support, there are unreacted silanol groups left on the surface (Fig. 2) which are capable of generating EOF. An example of this type of material is the octadecylsilane ODS1 class of bonded phases that were developed in the late 1970s as the first HPLC phases. End capping is a process performed after the phase has been attached to the surface, to minimise the number of these residual silanol groups. For CEC, however, although the particle size is normally 3 μm, the silica itself, in terms of its physical characteristics and particle size distribution, is still the same as that developed for HPLC. The pore size of the supports is commonly of the order of 8–10 nm. This means that EOF flow will only occur on the outside of the particles as double layer overlap will occur in the pores.[11]

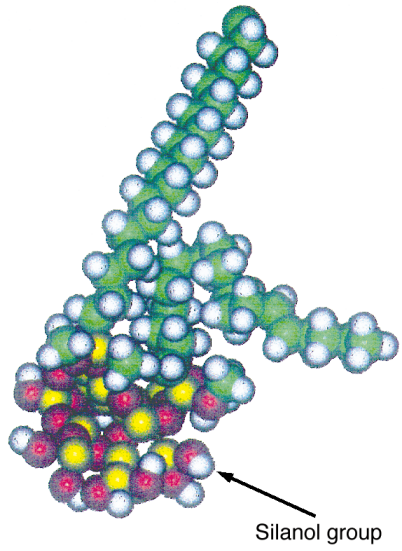

Fig. 2 Molecular model of the surface of a silica support that is not 'end capped'. Key: yellow, silicon; white, hydrogen; red, oxygen; green, carbon.

A variety of results have been reported from these HPLC derived phases: Table 4 outlines published results on the separation of PAHs using isocratic CEC. The differences shown, although not normalised in any way, are far greater than one would expect from HPLC comparisons and may result from the packing of these materials into narrow-bore(50–200 mm id) capillaries. Although they may be listed as 3 μm material, all the packings will have unique particle size distributions. In addition, in a manner analogous to the molecular size distribution of polymers, the distribution will vary according to how it is measured; currently there are three ways of characterising particle size distribution, namely number, area and volume. Manufacturers typically do not stipulate which method was used to characterise a particular stationary phase, and thus a nominal 3 μm material may vary from company to company. However, extremely noticeable in all of the number distributions is the presence of fine material below 2 μm (see Fig. 3), which is thought to impede the packing process. This material is very difficult to remove *via* the normal air classification used by manufacturers to produce different particle sizes. However, work by some manufacturers has led to new particle size distributions that are optimised for the packing procedures used in the packing of 50–200 μm fused silica capillaries. Monodisperse solid silicas are now available for use in CEC,[42] but as yet there are insufficient data to compare these with porous silicas.

The first specially manufactured phases for CEC were prepared by Myers and reported in papers by Smith and Evans.[43] These were based on a new 3 μm particle size distribution silica and bonded with propylsulfonic acid. Efficiencies from this phase have been reported in terms of millions

Table 4 Efficiencies obtained for isocratic CEC of PAHs using HPLC stationary phases

Stationary phase	Range of efficiencies (plates per metre)	Ref.
3 μm Spherisorb ODS1	200 000–240 000	38
3 μm Nucleosil 100 C_{18}	91 000–147 000	39
3 μm Spherisorb C_{18} PAH	Up to 260 000*	36
3 μm Synchrom	102 000–138 000	40
3 μm Vydac C_{18}	> 160 000	41
3 μm CEC Hypersil	240 000–280 000	21

* Calculated for a 50 μm id column of length 280 mm, d_p 3 μm and a minimum reduced plate height of 1.3.

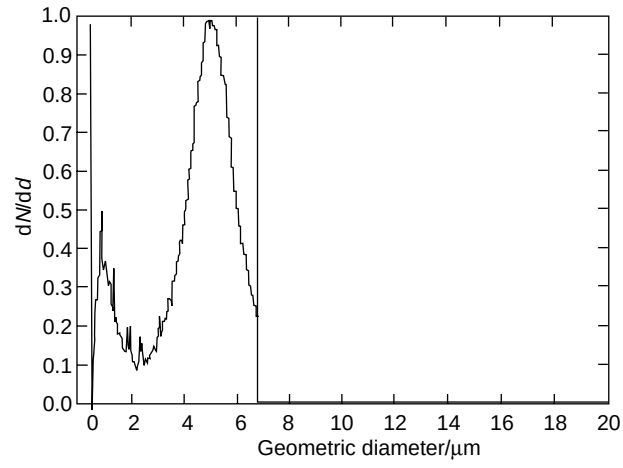

Fig. 3 Number distribution of a 5 μm CEC silica.

of plates for the analysis of basic drugs.[43,44] However, the reproducibility of these very high efficiencies is extremely poor and, because of the low carbon load, very short retention times are obtained for neutral compounds. At present, we are attempting to increase the hydrophobic nature of the support by bonding octadecyl groups on to the silica with the sulfonic acid (Fig. 4). These materials have yet to show the same high efficiencies on basic compounds, but promising results have been obtained for neutrals. New phases are also being developed, by adjusting the carbon chain length and the ratio of the alkane chain to the sulfonic acid, in an attempt to obtain a silica based phase that provides a good EOF over a wide pH range and provides good selectivity for neutrals. Other work on the effect of the particle size and the bonding of the stationary phase is being undertaken in the hope that the focusing effect can be understood and controlled, thus permitting the production of purpose made supports for CEC.

Work has been reported on the use of wide pore material with pore sizes up to 400 nm.[24] In these systems it has been shown that above 200 nm the materials are capable of supporting through-particle electroosmosis, which in turn results in a significant increase in efficiency. New CEC columns have been manufactured by polymerisation of either silica or polymers inside the column to produce a monolithic bead, which is then derivatised with the stationary phase.[45] This technique removes the problem of the inlet and outlet frits that are required for producing particle columns, and is a more readily available technique to researchers who may not have access to the small particle size silicas produced by speciality manufacturers. Other monolithic capillary columns have been produced in a single step copolymerisation process,[46] which allows fine control of the porous properties of the final column. The EOF through the column is dependent on the monolith pore size and the proportion of charged groups on the surface. The use of macroporous polyacrylamide–poly(ethylene glycol) gels in CEC has also been reported;[47] in these gels the EOF is generated by a sulfonic acid group as opposed to the silanol on silica.

Practical variables in CEC

Since CEC is essentially a hybrid of CE and HPLC, there appear to be a large number of variables to consider before attempting any separations; the selection of the most appropriate conditions for an application is not for the fainthearted. It helps to go back to the basics of analytical chemistry and define the analytical problem, *i.e.*, the nature of the sample, the end use of the results, the species to be separated and what information is required. Established HPLC or CE methods can provide a good starting point, but may not be ideal for a particular requirement. In addition, it is a good idea to have available such sample information as pK_a values and solubility data, since these are often overlooked and may have significant implications for the analysis. The general theory of HPLC and CE can be found described in a number of texts,[48–51] so only more practical considerations will be covered here.

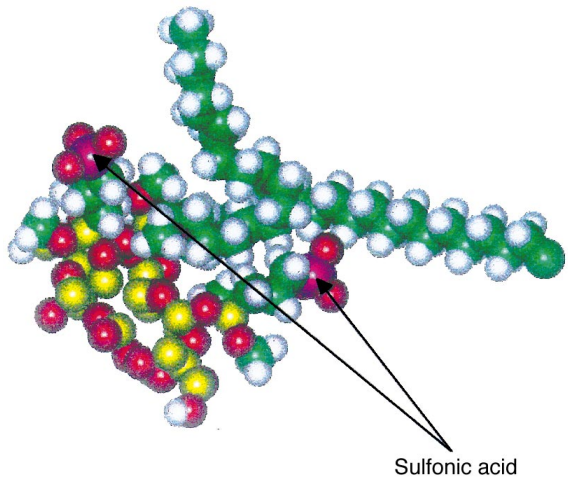

Fig. 4 Molecular model of sulfonic acid on a silica support. Key: yellow, silicon; white, hydrogen; red, oxygen; green, carbon; pink, sulfur.

In HPLC, chromatographic separation is the result of specific interactions between sample molecules with the stationary and mobile phases. Hence it follows that these are the most important source of variables in HPLC, with mobile phase flow rate and column temperature playing a lesser role. At the heart of the separation is essentially the chromatographic column, which can be varied in both the physical dimensions (length, internal diameter) and the characteristics of the packing material (nature and quality of the stationary phase, particle size and porosity). The various components of the mobile phase (water, organic solvent, buffer, *etc*.) are adjusted to control such factors as solvent strength and viscosity. For CE, however, where separation is primarily based on mobility in an electric field, factors that affect the charge and effective size of the analyte and the magnitude of the EOF play the dominant role. In particular, the electrolyte pH is of primary importance since it affects the degree of ionisation of both the analyte and the silanol groups on the capillary wall. The physical dimensions of the capillary have typically taken a secondary role; capillaries of larger inner diameter tend to be used in cases where increased detection is required, and smaller capillaries when ohmic heating, which can adversely affect resolution and efficiency, needs to be minimised.

In CEC, the fundamental driving force is the EOF, which is mainly influenced by parameters affecting the surface charge of the capillary column and the double layer thickness, *i.e.*, the stationary phase properties and the mobile phase composition. In practice, EOF control is achieved most readily by selecting the required stationary phase type, *e.g.*, chiral, ion-exchange, then varying mobile phase characteristics such as pH, concentration of electrolyte and proportion of organic solvent to give the EOF and separation required. Recent investigations into the influence of these parameters have been carried out by Li and Lloyd,[52] Lelièvre *et al*.[53] Dittman and Rozing,[22] Euerby *et al*.,[44] Kitagawa and Tsuda,[54] Wan,[55] Seifar *et al*.[56] and Wright *et al*.[57] For simplicity, an overview of their findings follows. The majority of CEC analyses have typically used capillaries (50–100 μm id) packed with 3 μm HPLC phases; although open tubular CEC has also been reported,[30–35] it will not be discussed here.

In CEC, the relationships which describe the variation of EOF are far less well defined than in CE. In part this can be attributed to the experimental conditions chosen; in many cases, there has been little attempt to keep all variables, except the one under investigation, constant. Subsequent results may be misleading. pH is a typical example of this: low pH buffers are often prepared by adjusting a higher pH buffer with acid, hence the decrease in EOF observed on going from high to low pH could be due to both an increased ionic strength and a decreased surface charge. In addition, the incorporation of organic solvents into the electrolyte can alter the ionisation equilibrium and analyte solvation. Adding organic solvents to the electrolyte generally shifts the pK_a values of the surface silanol groups to higher values; this has been demonstrated in CE by Schwer and Kenndler[58] and in CEC by Kitagawa and Tsuda.[54] As expected, at high pH values (> 9), where all the surface silanols should be dissociated, the EOF exhibits very little change. At low pH, however, substantial EOF has been demonstrated despite surface silanol groups being predominantly non-ionised.[21,44] The pH dependence of the solute must not be ignored; CEC

permits the separation of both charged and uncharged species. Often it is beneficial to work with the analytes in their non-ionised forms (subsequently referred to as the ion-suppressed mode), for example to minimise ionic interactions with the packing, or in the case where the analytes are negatively charged and would migrate away from the detector.

Most of the work reported in CEC has employed low concentration buffer solutions in order to avoid ohmic heating effects; typical concentrations are 1–10 mM for inorganic salts such as phosphate and borate. For applied voltages in the range 5–30 kV, we have observed a virtually linear relationship between EOF velocity and electric field strength, implying that the heat generated is negligible at low electrolyte concentration. However, poor migration time reproducibility and ion depletion may occur as a result of the low buffering capability.[59] The use of higher concentrations of low-conductivity zwitterionic buffers such as 2-morpholinoethanesulfonic acid (MES) and TRIS, is therefore to be recommended. In accordance with theory, the EOF in open capillaries was found to decrease as the buffer concentration was raised from 0.04 to 1 mM:[55] the double layer thickness, and subsequently ζ, are reduced with increasing ionic strength. In contrast, the EOF in packed columns remained relatively constant as the electrolyte concentration was decreased, a factor attributed to double layer overlap. The EOF velocity was, however, seen to be dependent on the concentration of a salt (sodium chloride) added to the buffer to increase the ionic strength.[60] Although the effect of changing the buffer and salt concentrations was different, this was hardly surprising since the concentration of sodium chloride added ranged from 0.01 to 0.45 M, concentrations rarely used in CE, let alone CEC. As we have observed in our work, the nature of the anion or cation influences the EOF rate; in this case the phosphate exerted a greater influence on EOF than did the chloride. In addition, tetrabutylammonium bromide had a significant effect on EOF whilst sodium dodecyl sulfate (SDS) did not. SDS is a surfactant which, although typically used to form micelles, can also be used as a dynamic surface modifier like tetrabutylammonium bromide. In CEC, SDS has been found to be effective in not only controlling but also stabilising EOF.[56] The EOF was found to increase with increasing concentration of SDS, and was attributed to changes in zeta potential due to adsorbed SDS molecules. This behaviour can be explained more simply by considering the structure of the SDS molecule: the tails of the SDS molecules will interact with the stationary phase, thus leaving the negatively charged heads to impart more negative character to the surface, hence increasing EOF. The reversal of EOF in CE by the use of triethylammonium acetate (TEA) has also been demonstrated in CEC.[52]

Studies in open capillaries have shown that increasing the organic component in a buffer solution up to around 80% results in a decrease in the EOF[58] for a variety of solvents; the overall reduction was found to be least for acetonitrile, and greatest for ethanol and propan-2-ol. A number of papers have demonstrated similar findings in CEC for mobile phases containing an added electrolyte,[22,61] but only Wright *et al.*[57] have reported behaviour in solvents without supporting electrolyte. They confirmed these results but showed that as the organic content approaches 100%, the EOF is further increased; the EOF typically passed through a minimum corresponding to an organic proportion of around 70–80%. Although the variation of the ε/η ratio with solvent composition follows a similar pattern, the change in EOF velocity cannot be attributed to this alone. The extremely high EOF observed for CE separations in 100% acetonitrile, namely 17×10^{-8} m^2 V^{-1} s^{-1}, is partially explained by solvatochromatic solvent polarity studies, which take into consideration hydrogen bond donor ability and polarizability. For practical purposes, these results illustrate that substantial EOFs can be generated in the absence of an electrolyte and in non-aqueous media. Euerby *et al.*[62] have demonstrated two important HPLC concepts in CEC: (i) linearity between the logarithm of the retention factor (ln k) and the percentage of acetonitrile in a mobile phase containing TRIS buffer (50 mM, pH 7.8) over the range 50–80% acetonitrile; and (ii) isoeluotropic strength. From these findings it is evident that well established theories used in HPLC method development are directly applicable to CEC, as are HPLC optimization programmes.

CEC Equipment

The equipment required for CEC is very simple. Essentially a capillary electrochromatograph (Fig. 5) can be broken down into four main components: (a) a system for either electrokinetic or pressure driven injection; (b) a column in which EOF and chromatographic processes take place; (c) a detector; and (d) a high voltage power supply. Most CEC is performed on laboratory-built or modified CE equipment which has the option to pressurise one or both ends of the capillary column; at present, only Hewlett-Packard have produced a commercial instrument for CEC which allows the column to be pressurised. Although it has been found that with proper degassing of the mobile phase (using helium sparging) column pressurisation is not necessarily required, it is extremely useful for conditioning columns on the instrument and for method development. For long automated runs, where mobile phases are likely to need further degassing, it is essential. In addition, under these circumstances there may be appreciable solvcnt evaporation from the sample and mobile phase vials; to minimise the effect of solvent losses, the vial compartment should ideally be cooled. However, owing to solubility constraints of buffers in the mobile phase some compromise may be necessary. Performing reproducible CEC requires stringent control of parameters such as temperature, voltage and pressure if used. The use of commercial CE equipment that permits automatic control of these parameters has led to significant improvements in reproducibility.

The majority of CEC analyses reported to date have used aqueous isocratic mobile phases with equipment similar to that described previously. Examples of non-aqueous isocratic CEC are far less documented despite their potential. This has been

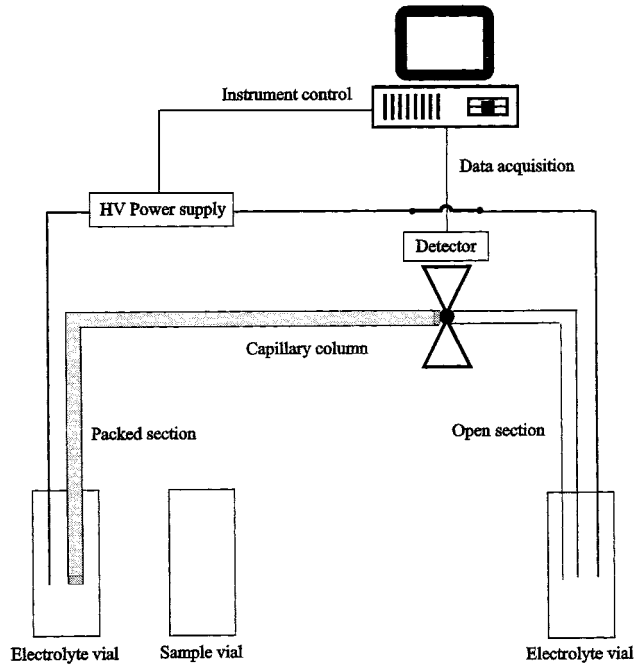

Fig. 5 Schematic diagram of a CEC instrument.

realised in a separation of PAHs and fullerenes using a mobile phase consisting of acetonitrile, methanol and tetrahydrofuran but no buffers.[41] The elution of coronene and C_{70} in approximately 11 and 170 min, respectively, demonstrated the applicability of non-aqueous CEC to a whole range of petrochemicals not normally separated by reversd phase LC.

In situations where an increased flow of mobile phase is desirable, the use of pneumatic pressure, applied at the injection end of the capillary, should be considered. The idea was developed initially by Tsuda,[63] and later by Dekkers *et al.*,[64] who combined it with electrospray mass spectrometry where an increased flow was required. This approach decreased retention times without compromising resolution, and appeared to reduce bubble formation.

The use of continuous gradient CEC techniques is increasing, despite the current lack of commercial instrumentation. Generally the systems used are of two types. In the first, a conventional gradient LC pump supplies a changing mobile phase across the CEC column inlet and the EOF drives it through the column.[65] In the second, two high-voltage power supplies are used to control the EOF in two different mobile phases which then mix and enter the column.[66] Although the use of the LC pump gives control of the mobile phase composition, it wastes mobile phase and may impose a small pressure driven flow, which will distort the plug flow profile. However, whilst no mobile phase is wasted in the EOF flow controlled system, the exact composition of the mobile phase as it enters the column is unknown. Results on both systems have been extremely encouraging. An alternative approach to continuous gradient, which can be performed on normal instrumentation, is that of the step gradient. This is achieved by changing the inlet vial during the chromatographic run with a new buffer vial containing a different mobile phase. Euerby *et al.*[67] applied this technique to the analysis of a mixture of six diuretics of widely differing lipophilicity; the rapid separation they obtained demonstrated the advantage of step gradient over isocratic conditions.

Column packing techniques

Fused silica capillary columns are mainly packed using either supercritical CO_2[36] or an organic solvent[11,59] at pressures of up to 600 bar. One end of the fused silica capillary is connected to a packing reservoir containing packing material (approximately 200 mg) and the other is connected to a 1/16 in union containing a sintered metal frit. Alternatively, a retaining frit made at one end of the capillary may be substituted for the union when packing with organic solvents. The reservoir is then connected to a high-pressure pump by means of a high-pressure valve that allows the introduction of the solvent into the packing reservoir, thus transporting the packing material into the column. After the column has been packed to the required length, as seen under a microscope, the column is disconnected from the reservoir and flushed with distilled water. Two other methods that have been used to pack capillary columns are centrifugal and electrokinetic packing, but they have not gained widespread use.

It must be mentioned, however, that there are several commercial suppliers of packed capillaries for CEC. These offer a variety of column dimensions to suit different instrument configurations, and a wide selection of packing materials and particle sizes. In addition, many companies offer a service in which they will instal the capillary column into the instrument cartridge sent to them, albeit at a cost.

Frit manufacture and bubble formation

There are three principal methods which have been used to form frits: the reaction of sodium silicate solution with formamide to form a porous silica plug[41,68] and the use of either an electrical heating element[27,59] or micro torch[27,40] to fuse the stationary phase. The electrical heating element is the preferred method owing to its ease of use and the reproducibility of the frits formed. The heating element is made from a few turns of resistance wire mounted on a thermocouple plug, and can be powered by simply a battery or a more sophisticated power supply, with time and current control. This technique relies on the stationary phase having a high sodium content, *e.g.*, Spherisorb material which contains approximately 1500 ppm Na, which may not be found in the newer types of silica manufactured from tetraethoxysilicate. The frits are made by heating the silica stationary phase to a temperature sufficient to form a porous sodium silicate plug. Columns produced using this sintering process for the frits are generally based on variations of the following procedure.

The capillary column is connected to a HPLC pump (Fig. 6) and flushed with water at approximately 100 bar for about 60 min. With the HPLC pump still on, a frit is formed near the column outlet. The second frit is formed at a distance back from the first frit according to the dimensions set by the CEC instrument and required detection mode, and the pump switched off. Detection can be made either through the frit, through a packed section of capillary or just below the outlet frit on an unpacked part of capillary; in the last case any excess packing material must be removed by flushing. Prior to use the column is conditioned with the required CEC mobile phase (degassed with helium or by vacuum) for at least 60 min and until no bubbles are observed leaving the column.

The problem of bubble formation in a CEC column is undesirable since it may lead to the breakdown of current and subsequent loss of EOF. In addition, it may cause the column to dry out, although this can be rectified by reflushing the column with mobile phase at high pressure. Bubbles are thought to arise within the packed section of the capillary or the frits as a result of either ohmic heating in the column, or a change in the EOF velocity on moving from the packed bed through the frit into the open capillary. Bubble formation is typically observed when using non-pressurised systems but can be minimised by proper degassing of mobile phases (helium sparge) and careful selection of buffers. The use of low conductivity buffers, such as TRIS and MES, is to be recommended since they are zwitterionic in nature and thus can be utilised at higher concentrations without contributing significantly to ohmic heating. It has also been suggested that a higher proportion of organic solvent in the mobile phase could reduce self-heating and bubble formation.[53]

Column coupling

Conventional CEC capillary columns are relatively fragile and prone to breakage owing to having a frit close to the detection window. A technique that overcomes these limitations is to use a separate detection cell from the chromatographic column. This consists of a conventional chromatographic column that is sealed with frits at each end, but is coupled to the detector cell

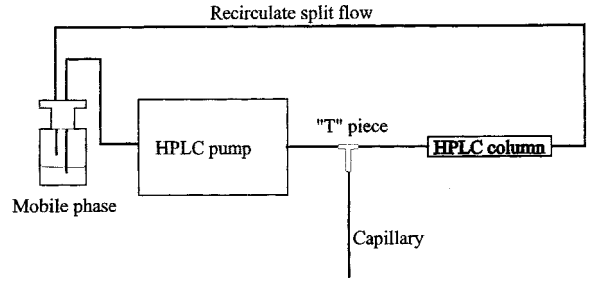

Fig. 6 Schematic representation of apparatus used for conditioning capillaries.

capillary by a PTFE connector. The connector is manufactured from 1/16 in PTFE tubing 10–15 mm long with an interference fit hole for the fused silica capillary. With both the packed capillary and detector cell each pressed half way into the PTFE sleeve, it is possible to operate the PTFE connector at pressures up to 6 bar. Alternatively, 340 mm id Teflon tubing is commercially available.

CEC detectors

Detection in CEC is principally UV/VIS detection through the unpacked part of the capillary where the polyimide coating has been removed. This has several limitations: the small pathlength of the cell, the fragility imposed by the removal of the polyimide coating and the fact that detection takes place after the frit. Extended light path cells, such as the 'bubble' and 'Z' type cells developed by Hewlett-Packard for capillary electrophoresis, are available and do give increased sensitivity; their use in CEC is illustrated in the Applications section. Detection through the packed column is possible but we have found that baselines are typically more noisy owing to scattered light. One cannot, however, ignore the data collection system; with the high efficiencies observed, *e.g.*, for bases on SCX stationary phases, it is imperative to use the correct sampling and detector data capture rates if the loss of valuable peak information is to be avoided. Euerby *et al.*[69] have found the peak efficiency values to be highly dependent on the detector rise time employed.

The use of fluorescence detection for PAH determinations has been reported by Rebscher and Pyell[39,70] for capillaries ranging from 50 to 150 μm id and by Yan *et al.*[40] In the latter case, laser-induced fluorescence was used to evaluate both on- and off-column detection methods. Limits of detection were10^{-9}–10^{-10} M, with efficiencies of up to 400 000 and 150 000 plates per metre being obtained for on- and off-column detection, respectively.

Since the first demonstrations of CEC–mass spectrometry (CEC–MS),[71] the technique has developed rapidly into a powerful analytical tool. The extremely low flow rates (< 1 μl min^{-1}) encountered in CEC can be utilised readily since an electrospray source typically requires a liquid make-up flow of 0.75–500 μl min^{-1}, and have allowed the direct coupling of electrospray (ESI) and atmospheric pressure ionisation (API) MS sources. This approach has been used successfully for the determination of sulfonamides,[64] fluticasone propionate and cefuroxime axetil[72,73] and non-ionic disperse textile dyes.[74] Mixtures of benzodiazepines, corticosteroids and thiazide diuretics have been separated by gradient CEC with UV absorbance and ESI-MS detection.[75] Gordon *et al.*[76] have reported on the use of a FAB probe for the separation of steroids; the detection of $[M + Na]^+$ peaks did not represent a problem even when a sodium-containing buffer was used. However, a loss of chromatographic resolution was observed on account of dispersion in the unpacked section of capillary used for coupling to the mass spectrometer. A novel solution to this problem has been designed and evaluated by Lane *et al.*[73] Their CEC–ESI-MS interface incorporated a working CEC within the interface probe, thus allowing short columns and high electric field strengths to be employed. A significant improvement on previous CEC–MS systems has also been demonstrated by Schmeer *et al.*[42] In their system, which uses a Sciex API mass spectrometer, the CEC column is placed directly in the MS source, thus eliminating the need for extra make-up flows or connecting capillary.

CEC application review

Since the re-emergence of CEC in the early 1990s, workers have mainly concentrated on establishing reliable column packing technologies[36,43,59,77,78] and investigating the theory and mechanism underpinning CEC.[6,11,18,21,38] Only in the last 3 years have we seen a rapid increase in the number of presentations and publications relating to CEC. Despite there being numerous separation techniques which can be classified under the general term of electrochromatography, this review of applications will be restricted to those involving EOF driven separations in packed capillaries only. The review will not cover pseudo-electrochromatography[71,79] in which the hydrodynamic flow is augmented by the application of positive or negative voltage along the column, or open tubular CEC, in which a thin film is coated on the internal surface of the capillary.[80] In addition, this review will only highlight the major advances associated with CEC applications and will not report on conference presentations, details of which are often not available in the open literature.

After a brief introduction to the current scope and applicability of analysing various chemical functionalities, the review will be divided into chemical classes/application fields and, finally, cover the more specialised areas of chiral analyses and the analysis of compounds from various matrices.

Scope and applicability of CEC

Neutrals

To date, most of the reported applications of CEC have been devoted to the analysis of neutral species of widely differing structures. Neutral species are particularly amenable to CEC because they can be chromatographed over the pH range 2–9. The pH of the mobile phase has been typically > 7 in order to promote a high EOF due to increased silanol ionisation. Older type HPLC stationary phases have generally been employed as they are 'unendcapped' and possess a large number of acidic silanol groups. However, certain manufacturers have now begun to market various reversed phases specifically for CEC use.

Acids

Acidic analytes which are separated in their ionised form tend to migrate towards the anode, *i.e.*, against the EOF, and are either not loaded on the column during electrokinetic injection or are not swept towards the detector. Hence they may not be detected. In order to chromatograph acids successfully by CEC, a mobile phase pH must be employed which will allow the separation of the acids in their ion suppressed mode, *i.e.*, as neutral species. As a consequence of using acidic mobile phases, the EOF and hence linear velocities are reduced, *e.g.*, linear velocities obtained are typically in the region of 0.75 mm s^{-1} compared with 1.5 mm s^{-1} at pH 7.8. However, successful and rapid separations of acidic diuretics (anti-inflammatory arylpropionic acids) are still possible.[44] Recently we have demonstrated the rapid separation of acids in their ion suppressed mode at acidic pH by the use of mixed mode phases which possess a *C*-alkyl and a sulfonic acid group bound to the same silica particle. The presence of the sulfonic acid group, which is ionised at all workable pH values, generates a good EOF over a wide pH range, thus enabling extremely rapid analyses to be performed without sacrificing the partitioning capacity of the phase.[69]

Bases

The separation of basic analytes by CEC is problematic since, in order to generate a good EOF, a silica which is acidic in nature is required. This, however, causes severe peak tailing of basic analytes due to strong secondary interactions of the base with the ionised silanol groups.[43] We have recently reported that, in an analogous manner to HPLC, these interactions can be minimised by the incorporation of triethylamine (which competes with the analyte for the silanol groups) in the mobile phase

(see Fig. 7 and 8). In addition, we have shown that certain basic drugs can be successfully chromatographed on a *C*-phenyl phase (see Fig. 7) when an unacceptable peak shape is obtained with a C_{18} bonded phase under similar conditions. It is suspected that the way in which the phenyl and C_{18} are bonded to the silica (proprietary information) restricts access of certain bases to the surface silanols in the case of the phenyl phase; this may shed new light on the way in which phases should be designed. Another alternative, depending on the pK_a of the base, is to run them in their ion suppressed mode. Recently we have had reasonable success with analysing a basic drug candidate with a pK_a of 8 at a pH of 9.3 on a C_{18} type bonded phase developed for CEC. Bases, not surprisingly, have been found to behave in a similar fashion on both the mixed mode phases and traditional C_{18} materials.

Smith and Evans[43] reported a possible solution to the analysis of basic drugs by using a strong cation-exchange stationary phase. An 'on-column focusing' phenomenon of the bases produced efficiencies of up to 8×10^6 plates per metre for the separation of tricyclic antidepressants, whereas concomitant application to neutral species only produced efficiencies comparable to those seen in reversed-phase CEC. These staggering efficiencies have also been obtained by other workers for a range of structurally diverse bases. However, all workers have experienced severe irreproducibility of the phase in that severe tailing and fronting have been unexpectedly observed in the middle of successful runs.[44]

Maruska and Pyell[81] recently described a new cellulose based stationary phase (C_{18} Granocel-14Sh) which gave a good peak shape for the basic analyte pyridine. In comparison, under identical conditions using a silica based C_{18} phase, pyridine exhibited severe peak tailing due to the interaction with residual silanol groups. This indicated that the cellulose phase may be useful for the separation of bases, although in comparison with silica based phases low peak efficiencies were observed.

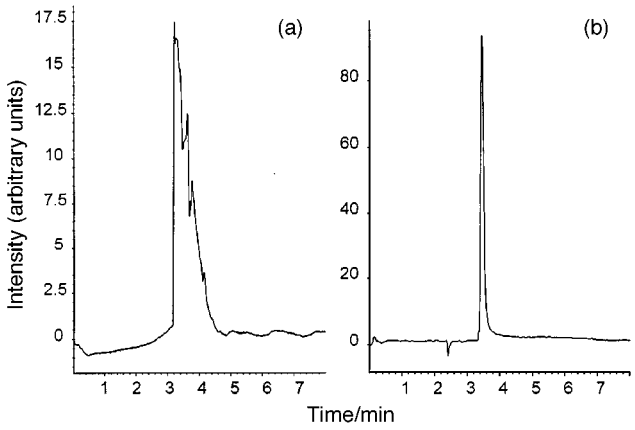

Fig. 7 CEC of basic drug candidate (I) using (a) 60 + 20 + 20 acetonitrile–TRIS (50 mM, pH 7.8)–water, 30 kV, CEC Hypersil C_{18} column (250 mm × 100 μm id); (b) 60 + 20 + 20 acetonitrile–NaH_2PO_4 (50 mM, pH 2.3)–water, 30 kV, Hypersil C-Phenyl column (250 mm × 100 μm id).

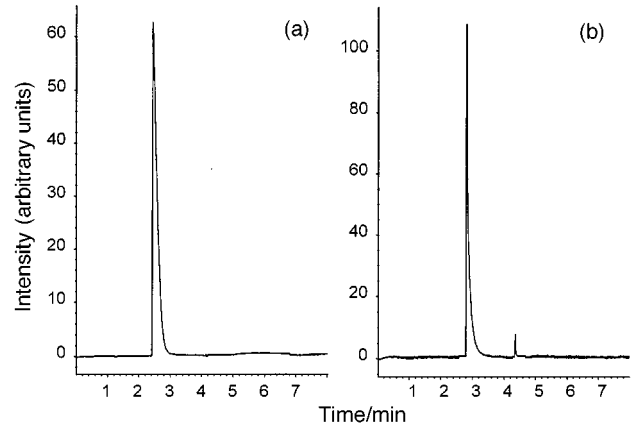

Fig. 8 CEC of basic analytes using 60 + 20 + 20 acetonitrile–NaH_2PO_4 (50 mM, pH 2.3)–water with 0.1% v/v triethylamine, 30 kV: (a) basic drug candidate (I), (b) benzylamine.

Applications

Environmental

Polyaromatic hydrocarbons (PAHs) were one of the first types of compound class to be separated by CEC and as a consequence there are many published examples.[40,82]The separation of these PAHs by CEC is typified by efficiencies 75% higher than with HPLC.[40] Recently it has been performed using non-aqueous CEC without the incorporation of electrolytes.[57, 83] The separation of 16 PAHs was attempted using a C_{18} type stationary phase and an acetonitrile (MeCN)–water composition of 80 + 20 (v/v): the addition of water was necessary to provide sufficient partitioning into the C_{18} phase. Although linear velocities comparable to those seen with conventional CEC were obtained, two pairs of co-eluting peaks still remained.

CEC has also been shown to be beneficial for the separation of a range of triazine herbicides. Using a range of stationary phase materials, the new C6/SCX mixed mode phase appeared to exhibit the best selectivity for a given mobile phase composition of 1 + 1 (v/v) MeCN–25 mM sodium acetate (pH 8).[22] In addition, polychlorinated benzene derivatives[84] and phthalates[38] have been separated.

Pharmaceutical

The pharmaceutical industry has been one of the driving forces for the development of CEC as the technique offers the potential for a separation mechanism orthogonal to the ubiquitously used HPLC. This, combined with highly efficient and rapid separations of complex mixtures, makes CEC an attractive adjunct to the conventional chromatographic methods employed. To date, most of the separations reported have not utilised the combined separation mechanisms of electromobility and partitioning; instead, workers have focused their attention on using the ion suppressed mode, thus utilising the partitioning mechanism only. This is partly due to the lack of suitable stationary phases that enable analytes to be run under conditions of high EOF whilst maintaining a satisfactory peak shape. Given these limitations, CEC has found a strong hold in the analysis of drug substances and intermediates such as cephalosporin antibiotics,[85,72] barbiturates,[69] prostaglandins,[85] diuretics,[43,44,53] steroids,[43,44,62,73,76,85] macrocyclic lactones,[73] *C*- and *N*-protected peptides,[42,44] nucleosides and purine bases.[44] Phthalates[38] and parabens[21] have also been successfully chromatographed by CEC using the standard conditions of a reversed phase column possessing a high proportion of acidic silanol groups and a mobile phase of pH ⩾6.

CEC appears to be of particular use in the early stages of drug discovery where rapid method development is essential and the demands of validation are less stringent. In nearly all cases for neutral and acidic compounds, using the ion suppressed mode where we were able to separate the components more efficiently and quickly, the development time was dramatically reduced. Two *C*- and *N*-protected tetrapeptides which differ in only the methylation of one amide function were found to be separated in less than 4 min using the standard CEC test chromatographic conditions which are employed to check the performance of our

capillaries. In comparison, the HPLC method previously used involved a 30 min gradient. Since the quantitative results from both techniques were comparable, the CEC method was therefore the obvious choice.[44]

We have shown that method development in CEC can be easily automated on the current commercially available CEC instrumentation. In addition, the chromatographic theory, central to computer optimisation in HPLC, holds in CEC for analytes using the ion suppressed mode.[44] This enables method development to progress at a rapid pace. Miyawa *et al.*[86] extended this work to show the usefulness of a modified central composite design to optimise the CEC separation of the antibacterial 3-[4-(methylsulfinyl)phenyl]-5*S*-acetamidomethyl-2-oxazolidinone from its three related *S*-oxidation products. The variables included in the investigation were applied potential, volume fraction of MeCN and buffer (TRIS) concentration. The end result was the development of a rugged CEC method for the separation of the antibacterial from its thioether, sulfone and sulfoxide diastereoisomer on a 3 μm C_{18} bonded phase in 9 min.

Steroids

Steroids seem to be particularly amenable to separation by CEC; possibly the best example is that of the separation of the corticosteroid tipredane from its diastereoisomer and five related substances.[44] As can be seen in Fig. 9, baseline separation of tipredane (14) from its diastereoisomer (15) was achieved using standard CEC conditions with no method development; in contrast, HPLC failed to achieve the baseline separation of the diastereoisomers despite extensive stationary and mobile phase optimisation. Boughtflower and Smith's groups have also demonstrated the effectiveness of CEC, in this case for the separation of the synthetic corticosteroid fluticasone.[73,85] Subsequently, many reports have augmented these findings that steroids of widely differing structure and from differing sources can be successfully separated with higher efficiencies than by HPLC, using the traditional silica reversed-phase materials with MeCN and a mobile phase pH > 6.

Lord *et al.*[87] highlighted the use of CEC in the separation of bufadienolide (bufalin, cinobufagin and cinobufatakin) and cardenolide (digoxigenin, gitoxigenin and digitoxigenin) steroids containing sugar residues and subsequently went on to couple this method with MS.

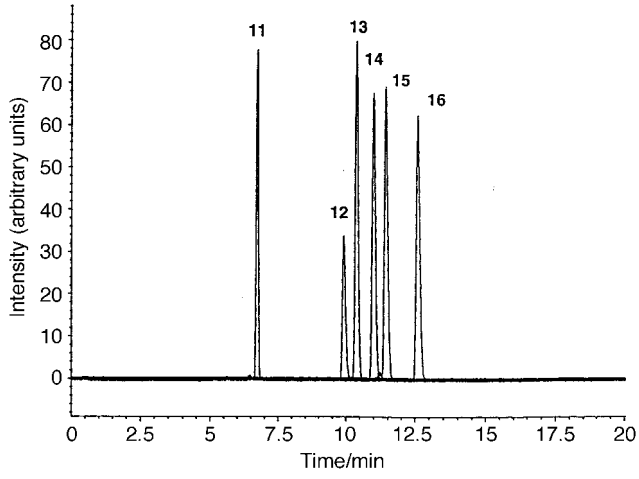

Fig. 9 Separation of tipredane (14) from its diastereoisomer (15) and structural analogues (11–13, 16). Electrochromatography was performed on an unpressurised HP3D CE system using a 3 μm Spherisorb ODS1 column (250 mm × 50 μM id), 80 + 20 acetonitrile–TRIS (50 mM, pH 7.8) buffer, 15 kV, capillary temperature 15 °C. Reprinted with permission from ref. 44.

There have been several reports on the separation of endogenous steroids such as testosterone, 17-α-methyltestosterone and progesterone[56,88] and many synthetic corticosteroids such as triamcinolone, hydrocortisone, cortisone, methylprednisolone, betamethasone, dexamethasone, adrenosterone, fluocortolone, triamcinolone and triamcinolone acetonide by CEC.[75]

We have recently reported the use of a short-end injection technique with reverse polarity to achieve rapid analysis of extremely lipophilic steroids.[89] This technique facilitated the separation of budesonide and related steroids in approximately 1 min. The attractiveness of this approach resides in the fact that most of the voltage drop occurs over the short packed capillary rather than over the entire capillary, and therefore higher EOF values are obtainable.

Diuretics

Euerby *et al.*[44] and Taylor and Teale[75] have both reported good chromatography of thiazide diuretics using CEC. However, we have found that the CEC must be performed at a pH of 2.5 to ensure that the acids are in their ion suppressed mode.[44] As can be seen in Fig. 10(a), six thiazide diuretics could be successfully separated. In view of the reduced EOF it would beneficial to

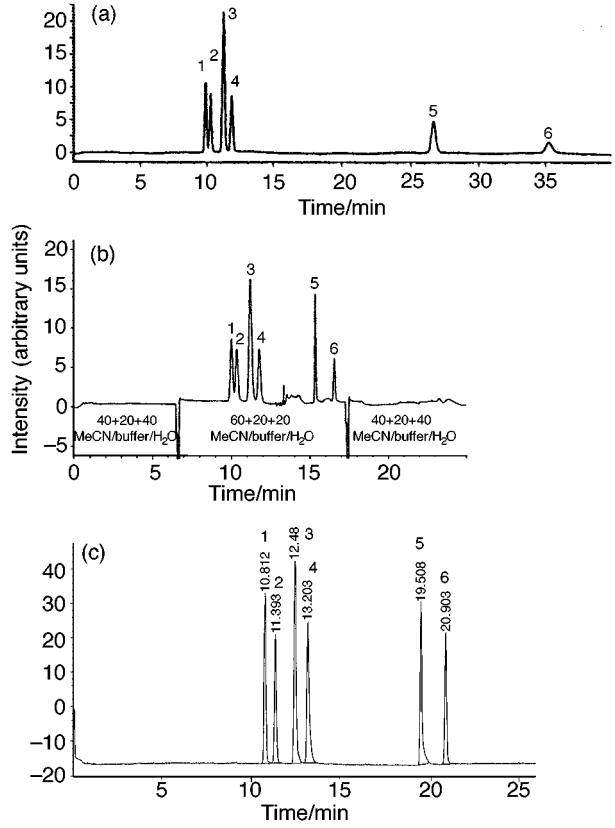

Fig. 10 CEC separation of the diuretics chlorothiazide (1), hydrochlorothiazide (2), chlorthalidone (3), hydroflumethiazide (4), bendroflumethiazide (5) and bumetanide (6). (a) Isocratic separation, CEC Hypersil C_{18} column (250 mm × 50 μm id), 40 + 20 + 40 acetonitrile–Na_2HPO_4 (50 mM, pH 2.5)–water. (b) Step gradient, column as for (a): 0–6.50 min, 40 + 20 + 40 acetonitrile–Na_2HPO_4 (50 mM, pH 2.5)–water; 6.50–17.25 min, 60 + 20 + 20; 17.25–25.00 min, 40 + 20 + 40. (c) Continuous gradient, Spherisorb ODS1 column (250 mm × 100 μm id): mobile phase A, phosphate buffer (5 mM, pH 2.3); mobile phase B, phosphate buffer (5 mM, pH 2.3)–acetonitrile (20 + 80). (a) and (b) reprinted with permission from ref. 67.

investigate the use of a mixed mode phase. In addition, it is apparent from Fig. 10(a) that the six compounds possess widely differing octanol–water partition coefficients (log *P* values) and that a gradient CEC would be preferential. Although commercial CEC systems will allow automated step gradients[67] to be performed [Fig. 10(b)], from a pharmaceutical viewpoint it would be advantageous to perform continuous gradient CEC. There have been several reports of laboratory-built gradient CEC systems in which the CEC capillary takes the changing mobile phase composition on demand;[66,75,88] since there is no pressure flow down the CEC, capillary plug flow should be maintained. Fig. 10(c) illustrates the separation of diuretics using a prototype of a commercial gradient CEC system.

Bases

Owing to the severe problem of peak tailing associated with the separation of bases using traditional reversed-phase silica materials, only limited examples have been published. Taylor and Teale,[75] however, reported the separation of two benzodiazepines (diazepam and nitrazepam) using a C_{18} type phase and an ammonium acetate–MeCN mobile phase. Under isocratic conditions the resultant peaks were broad and exhibited tailing, whereas under gradient conditions the peaks were more gaussian in appearance owing to the gradient effect on the tail of the peak. Other examples of successful separations of bases, notably those with highly efficient peaks, have been dealt with in a previous section.

Biomolecules

Amino acids

Huber *et al.*[88] have shown that reversed-phase gradient elution CEC is particularly suited to the separation of phenylthiohydantoin(PTH)-amino acids from the classical Edman degradation of peptides with phenyl isothiocyanate. Twelve PTH-amino acids were separated on a 3.5 μm C_{18} type packed capillary using a non-optimised gradient of 30–60% (v/v) MeCN with 5 mM phosphate, buffer (pH 7.55) (see Fig. 11). PTH-arginine, being positively charged, exhibited slight peak tailing due to interactions with the charged silanol groups, whereas the negatively charged PTH-aspartate and PTH-glutamate did not elute.

Peptides and oligosaccharides

Horváth *et al.* have shown the worth of CEC for the separation of various peptides (tetra[Trp–Met–Asp–Phe] and pentapeptides [Trp-Gly–Gly–Phe–Met]) by the use of a 8 μm gigaporous (1000 Å) PLSCX (strong cation exchanger on highly cross-linked styrene-divinylbenzene particles) material with an MeCN–25 mM phosphate mobile phase.[27]

Palm and Novotny[47] have shown that monolithic stationary phases based on macroporous polyacrylamide and poly(ethylene glycol) are suitable for the separation of various enkephalin derivatives and 2-aminobenzamide derivatised maltose oligosaccharides (Glu 1 to Glu 7) using an MeCN–TRIS–borate buffer (pH 8.2) mobile phase (see Fig. 12).

Endogenous steroids

As seen in the previous section, steroids of endogenous origin are particularly amenable to separation by CEC.

Separation of analytes from various matrices by CEC

Bioanalysis

Until recently, the effectiveness of CEC in the analysis of biological samples has not been fully exploited. One of the major challenges for CEC is in the field of bioanalysis, where concentrations are usually low and the sample may contain varying types and amounts of endogenous interferences. Given the fact that the surface area of the packing material is small, then the likelihood of fouling the column increases. However, various workers have been able to show that CEC can be successfully used to separate a range of compounds from various biological matrices, *e.g.*, urine and plasma from differing species.

Taylor *et al.*[90] have shown that corticosteroids (adrenosterone, hydrocortisone, dexamethasone, fluocortolone) in extracted horse urine and plasma can be separated by CEC using a laboratory-built gradient elution CEC system. The separation is performed on a 50 μm capillary packed with 3 μm C_{18} bonded material, using an MeCN–5 mM ammonium acetate gradient varying from 9 to 80% MeCN. In order to prevent early deterioration of the packed capillary, the urine samples were purified by a C_8 followed by an SAX solid-phase extraction stage; in contrast, the plasma samples were purified by dialysis. The capillary was shown to be perfectly serviceable and efficient after over 200 injections of horse urine extract. A major advantage of CEC over HPLC was that the interferent, which eluted near the peaks of interest in HPLC, eluted near the EOF and well clear of the steroids in CEC, indicating that the interferences may have an amine functionality. The determination of hydrocortisone in equine urine by CEC with UV detection, after administration of tetracosactrin acetate, was shown to compare favorably with an in-house validated LC–MS method. The metabolite of hydrocortisone (20β-dihydrocortisone) was also detected (see Fig. 13). The detection levels achievable were well below that required by the regulatory bodies, and the reproducibility of the method was acceptable in terms of the precision obtained on automated runs; RSD values were typically below 2 and 7% for retention time and peak area, respectively.

Paterson *et al.*[91] elegantly illustrated the combined power of CEC and MS for the determination of a potential drug candidate

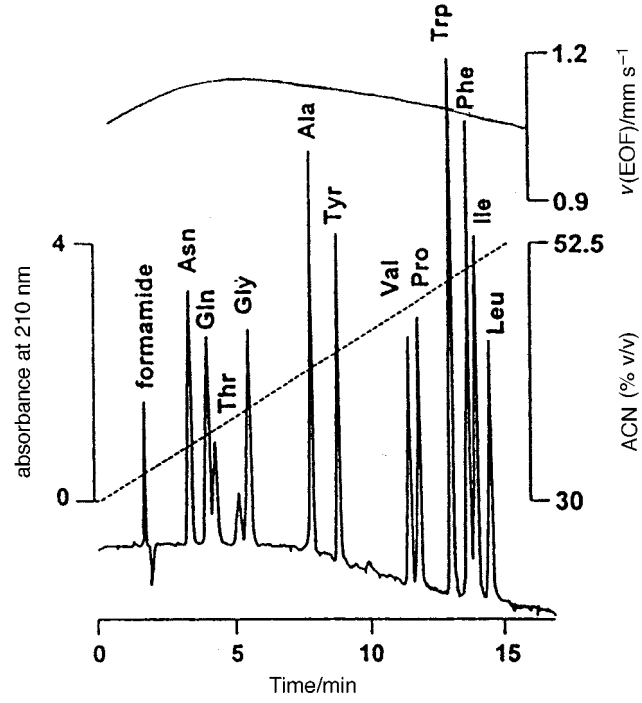

Fig. 11 Capillary electrochromatography of PTH-amino acids with gradient elution. 3.5 μm Zorbax ODS column (207/127 mm × 50 μm id): mobile phase A, phosphate buffer (5 mM, pH 7.55)–acetonitrile (70 + 30); mobile phase B, phosphate buffer (5 mM, pH 7.55)–acetonitrile (40 + 60); 0–100% B in 20 min. Reprinted with permission from ref. 88.

in extracted plasma. Using a mixed mode C_{18}–SCX phase and a mobile phase of MeCN–25 mM ammonium acetate (75 + 25) (pH 3.5), 13 structurally related compounds were separated from the parent drug candidate in 8 min. The plasma samples were purified by C_2 solid-phase extraction prior to CEC–MS and an internal standard was employed. This resulted in an RSD of 1.7% over the whole concentration range, which was excellent considering the manual injection method employed. The power of CEC–MS was illustrated by the fact that severe co-elution would have occurred if only UV detection had been employed. A detection level of 1 ng ml^{-1} could be routinely measured owing to the use of a peak stacking technique, which injected as much as a third of the column interstitial volume. This preconcentration technique in conjunction with increased pathlength detection cells should facilitate even lower detection limits (see Fig. 14).

CEC has been successfully used for the separation of complex mixtures of neutral isomeric compounds derived from the *in vitro* reaction of carcinogenic hydrocarbon (benzo[*g*]-chrysene and 5,6-dimethylchrysene) dihydrodiol epoxides with calf thymus deoxyribonucleic acid (DNA).[92] CEC demonstrated higher resolution, greater speed and lower analyte consumption than conventional HPLC. The use of a manual three-step gradient on a 3 μm C_{18}, 75 μm id capillary using various proportions of MeOH, MeCN, THF and 6 mM ammonium acetate further improved the speed of analysis. This work was further extended by coupling the method with MS for the determination of two DNA adducts of acetylaminofluorene deoxyguanosine (AAF-dG) and G4 DNA. In order to achieve the detection level required, a 7 min injection was used with concomitant peak focusing. The use of such a high loading illustrated the potential advantage of using nanospray MS coupled with CEC.[93]

These examples indicate the potential of CEC for high speed, high sensitivity multi-component analyses on very small sample volumes.

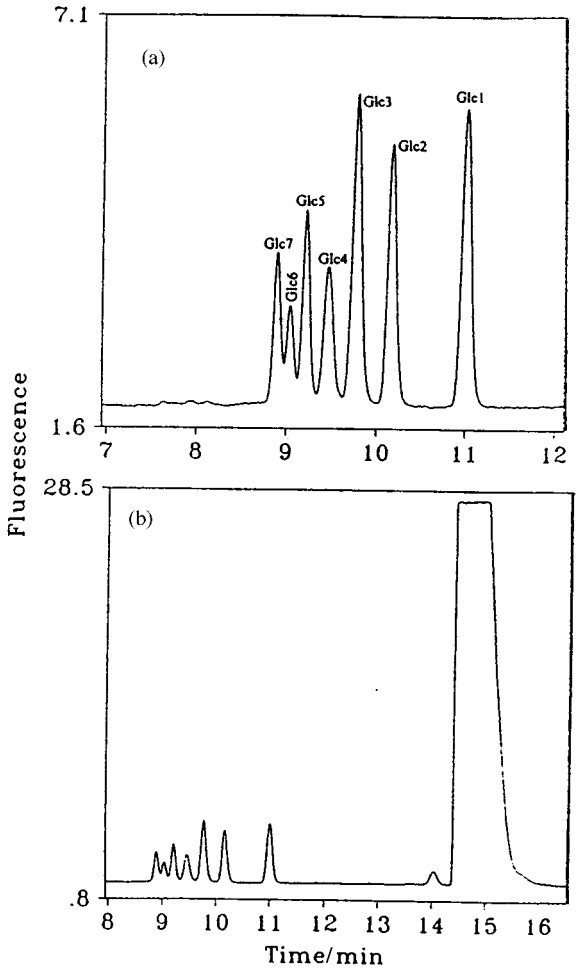

Fig. 12 (A) Isocratic CEC of maltooligosaccharides using a capillary filled with a macroporous polyacrylamide–poly(ethylene glycol) matrix, derivatized with a C_4 ligand and containing vinylsulfonic acid (effective column length 250 mm). Mobile phase 10 mM TRIS–15 mM boric acid (pH 8.2), acetonitrile content not stipulated. (B) Same analysis as (A), including the peak of the derivatization agent (14–16 min). Reprinted with permission from ref. 47.

Plant origin

The best example in this area is that of the difficult separation of triglycerides from various sources by CEC.[1] By the use of a 3 μm C_{18} type material and the novel use of MeCN–propan-2-ol–hexane (57 + 38 + 5) plus 50 mM ammonium acetate as the

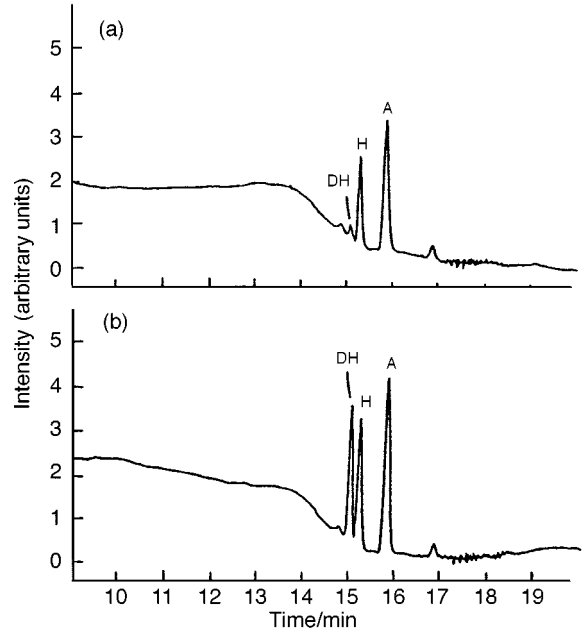

Fig. 13 Gradient CEC of equine urine samples after administration of tetracosactrin acetate and extracted by SPE. (A) 2 h post-administration; (B) 12 h post-administration. DH = 20β-dihydrocortisone; H = hydrocortisone, and A = adrenosterone (internal standard). Reprinted with permission from ref. 90.

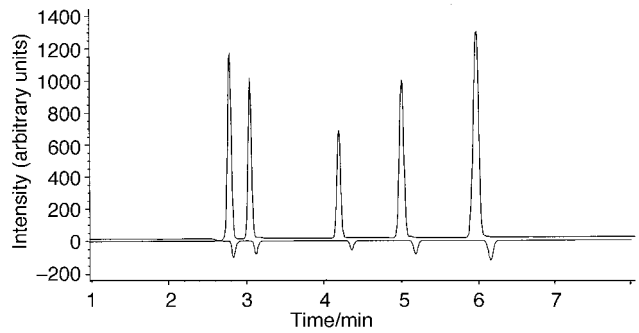

Fig. 14 Comparison of standard through capillary detection using (a) a 100 μm id fused silica capillary and (b) an extended pathlength flow cell. CEC separation of standard test mixture (thiourea, benzamide, anisole, benzophenone, and biphenyl) using a Hypersil C-Phenyl phase and standard test conditions [mobile phase composition acetonitrile–TRIS (50 mM, pH 7.8) (80 + 20)].

isocratic mobile phase, the separation of triglycerides from over 30 samples of vegetable oils, foods, soya lecithin extracts and pharmaceuticals was successfully achieved. In contrast to reversed-phase HPLC, which does not separate the triglyceride isomeric forms of OLL and LLL, CEC yielded near baseline separation. As can be seen from Fig. 15, the separation of evening primrose oil by CEC resulted in better resolution in a shorter analysis time than using HPLC. In addition, separation of testosterone esters in a formulation based on peanut oil was demonstrated.

Miscellaneous applications

Li *et al.*[94] have reported the interesting use of ion-exchange CEC for separating iodide, iodate and perhenate ions from the Hanford nuclear site environment. A 5 μm strong anion exchanger was used in conjunction with a 5 mM phosphate buffer (pH 2.6), and since the analytes were anionic, the polarity was reversed in order to sweep them past the detector. The analytes were easily separated but eluted in a different order to that observed in CE; this change in elution was easily rationalised on the basis of ion chromatography theory. Efficiencies were shown to be much better than with either HPLC or CE and detection was 20 times better than with the latter. It is believed that the anions experience a focusing effect as a decrease in efficiency was observed for higher mass loads.

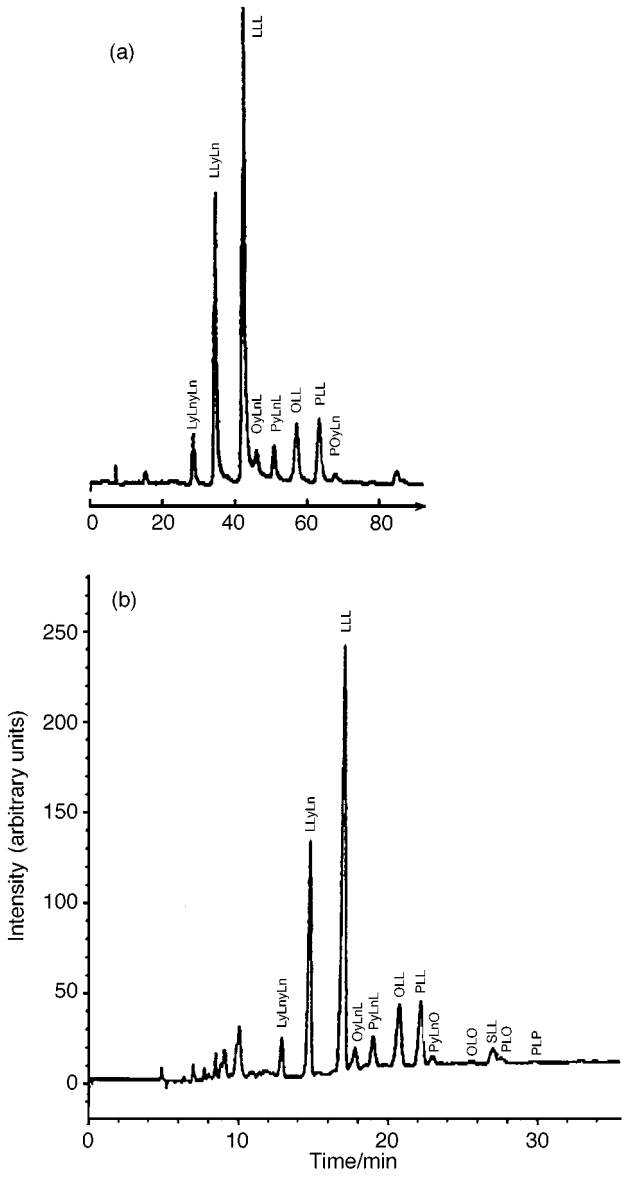

Fig. 15 Triglyceride analysis of primrose oil by (A) micro-LC and (B) CEC. (A) Column, 50 cm × 320 μm id FSOT, BioSil C-18 HL, 5 μm; mobile phase, acetonitrile–propan-2-ol–hexane (57 + 38 + 5). (B) Column, 25 cm × 100 μm id FSOT, Hypersil ODS, 3 μm; mobile phase, acetonitrile–propan-2-ol–hexane 57 + 38 + 5)–50 mM ammonium acetate. Reprinted with permission from ref. 1.

Chiral

The technique of using CEC for chiral analyses has attracted much interest; it was expected that chiral selectivity would not be so important since the high efficiency associated with CEC would compensate for any short fall in selectivity. As in HPLC, investigations into the applicability of using chiral stationary phases and chiral mobile phase additives have been pursued.

Mobile phase additives

The feasibility of this approach has been established using hydroxypropyl-β-cyclodextrin as the mobile phase additive in the chiral separation of chlorthalidone and mianserin by CEC.[53] Baseline separation was achieved but excessive analysis times were required.

Protein phases

CEC has been used with an immobilised α_1-acid glycoprotein (AGP) on a silica based stationary phase for the enantiomeric separatation of racemic hexobarbital, pentobarbital, benzoin and cyclophosphamide.[61] The separation efficiencies were slightly higher than those obtainable with chiral HPLC, but did not approach those seen with achiral CEC. The benzodiazepines temazepam and oxapam have been reported to be resolved using human serum albumin immobilised on a 7 μm silica. However, efficiencies were found to be very low and the EOF was lower than that of the AGP column.[95]

Cyclodextrin phases

Hydroxypropyl-β-cyclodextrin as a chiral stationary phase has been shown to result in the baseline separation of chlorthalidone and the partial separation of mianserin.[53] Li and Lloyd[52] successfully used the standard β-cyclodextrin chiral stationary phase to separate a range of racemic 2,4-dinitrophenylamino acid derivatives, benzoin, dansylthreonine and hexobarbital. In order to separate the anionic analytes, triethylamine was incorporated into the mobile phase and the polarity of the applied voltage was reversed. Once again the expected high efficiencies were not observed.

Pirkle type phases

Wolf *et al.*[96] have reported on the success of chiral CEC using (*S*)-naproxen derived and (3*R*,4*S*)-Whelk-O chiral stationary phases which were immobilised on 3 μm silica supports and packed into 100 μm id fused silica capillaries. Once again simple mobile phase compositions of MES (pH 6) buffer–MeCN were used to obtain efficiencies in the region of 200 000 plates per metre. Excellent enantiomeric selectivity with all of the 10 structurally diverse neutral analytes was achieved on these columns in run times of less than 10 min (see Figs. 16 and 17). Surprisingly, TRIS buffer failed to give satisfactory baseline stability.

Molecular imprinting techniques

Recently, molecular imprinting techniques have been used to produce chiral separation media used for CEC. These ap-

proaches represent an interesting complementary alternative to conventional chiral selectors.

Nilsson *et al.*[97] have described the *in situ* preparation of a monolithic phase in fused silica capillaries based on (*R*)-propanolol molecular imprinted polymers to separate several β-adrenergic antagonists into their enantiomers (see Fig. 18). As expected, the phase exhibited the best chiral selectivity for propanolol itself. However, reasonable enantiomeric separation of *rac*-prenalterol, *rac*-atenolol and *rac*-pindolol was also achieved. Efficiencies in the region of 35 000–70 000 and 5000–20 000 plates per metre for the first and last enantiomers, respectively, were obtained. In a recent communication the same group has reported chiral separation of propanolol in less than 120 s by using one of these types of phases.[98]

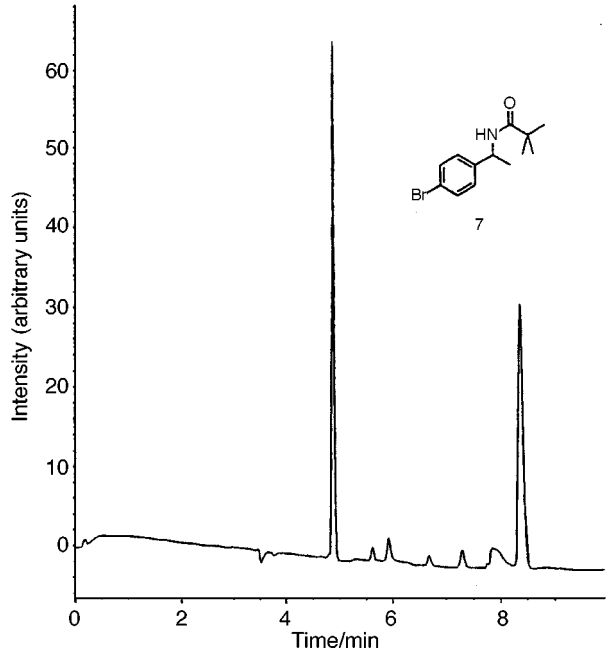

Fig. 16 CEC separation of the enantiomers of compound **7** on (3*R*, 4*S*)-Whelko-O CSP, using MES (25 mM, pH 6.0)–acetonitrile (1 + 3.5). Reprinted with permission from ref. 96.

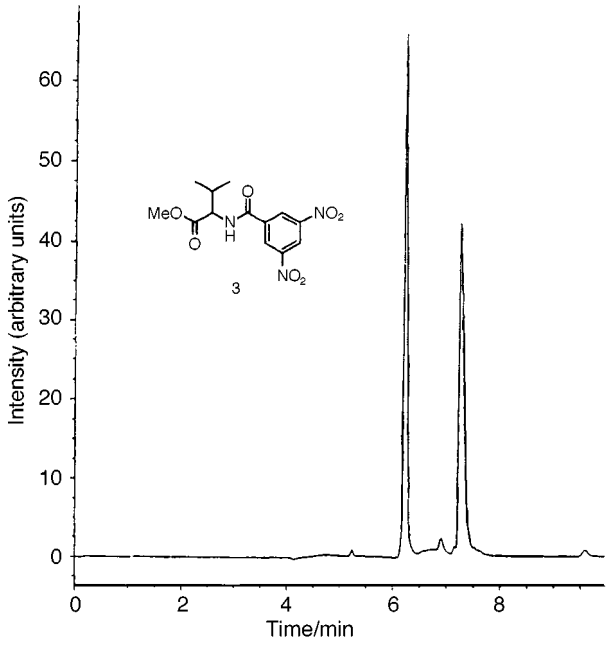

Fig. 17 CEC separation of the enantiomers of compound **3** on (*S*)-naproxen derived CSP, using MES (25 mM, pH 6.0)–acetonitrile (1 + 3.5). Reprinted with permission from ref. 96.

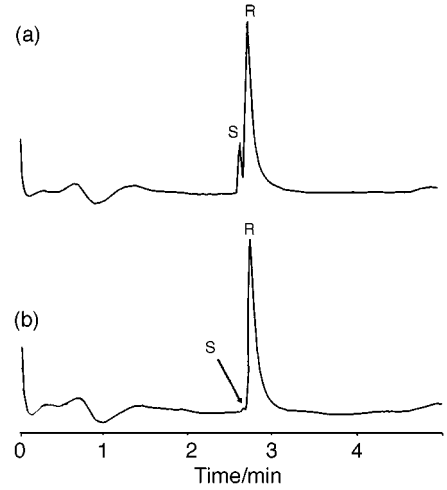

Fig. 18 Separation of non-racemic mixtures of propranolol on a capillary column containing imprints of (*R*)-propranolol, using acetonitrile–4 M acetate (pH 3.0) (80 + 20) at 60 °C. (A) 9 + 1 mixture of (*R*)-and (*S*)-propranolol; and (B) 99 + 1 mixture of (*R*)-and (*S*)-propranolol. Reprinted with permission from ref. 98.

Lin's group have reported numerous examples of using molecular imprinted polymer stationary phases, either monolithic in nature or the conventional packed type, for the enantioseparation of a range of derivatised and underivatised amino acids. The peaks obtained by CEC were much sharper than those obtained by HPLC, and thus should improve detection limits.[99–102]

Future trends

A number of applications of CEC have been described, which demonstrate the wide applicability of this relatively new technique. Progress over the last two years has been rapid; whilst much of the literature then dealt with separations of model compounds such as simple mixtures of hydrocarbons and other neutrals, it has now started to include samples of more complex nature and diversity. In the longer term, the extra peak capacity available in CEC may considerably extend its range. Already, the economic and environmental advantages of having low expenditure of solvents and stationary phases make it attractive. However, much work will still be necessary if CEC is to be recognised as an analytical technique and a viable alternative to CE and HPLC.

In particular, the future of CEC is likely to depend greatly on the nature of the column. At present, it is far from ideal; columns tend to be fragile in their present format. In addition, we have found that prolonged use of acetonitrile removes the protective polyimide coating at the capillary ends, thus rendering the frits even more susceptible to breakage. New column materials, which can provide more control over EOF and selectivity, are expected to make an appearance on the market, as are monolithic type columns. With miniaturisation being increasingly popular, a new generation of small CEC instruments on a chip with 'disposable' columns may be the way forward.

References

1 Sandra, P., Dermaux, A., Ferraz, V., Dittmann, M. M., and Rozing, G., *J. Microcol Sep.*, 1997, **9**, 409.

2 Shaw, D. J., *Electrophoresis*, Academic Press, London, 1969.
3 Foret, F., and Bocek, P., *Adv. Electrophoresis*, 1990, **3**, 272.
4 Salomon, K., Burgi, D. S., and Helmer, J. C., *J. Chromatogr.*, 1991, **559**, 69.
5 Rice, C. L., and Whitehead, R., *J. Phys. Chem.*, 1965, **69**, 4017.
6 Knox, J. H., and Grant, I. H., *Chromatographia*, 1987, **24**, 135.
7 Taylor, J. A., and Yeung, E. S., *Anal. Chem.*, 1993, **65**, 2928.
8 Pretorius, V., Hopkins, B. J., and Schiecke, J. D., *J. Chromatogr.*, 1974, **99**, 23.
9 Tsuda, T., Ikedo, M., Jones, G., Dadoo, R., and Zare, R. N., *J. Chromatogr.*, 1993, **632**, 201.
10 Knox, J. H., *Chromatographia*, 1988, **26**, 329.
11 Knox, J. H., and Grant, I. H., *Chromatographia*, 1991, **32**, 317.
12 Knox, J. H., and McCormack, K. A., *Chromatographia*, 1994, **38**, 279.
13 Colón, L. A., Yong, G., and Fermier, A., *Anal. Chem.*, 1997, **69**, A461.
14 Colón, L. A., Reynolds, K. J., Alicea-Maldonado, R., and Fermier, A., *Electrophoresis*, 1997, **18**, 2162.
15 Robson, M. M., Cikalo, M. G., Myers, P., Euerby, M. R., and Bartle, K. D., *J. Microcol., Sep.*, 1997, **9**, 357.
16 Kowalczyk, J. S., *Chem. Anal. (Warsaw)*, 1996, **41**, 157.
17 Crego, A. L., González, A., and Marina, M. L., *Crit. Rev. Anal. Chem.*, 1996, **26**, 261.
18 Dittman, M. M., Wienand, K., Bek, F. and Rozing, G. P., *LC-GC*, 1995, **13**, 800.
19 Rathore, A. S., and Horváth, C., *J. Chromatogr. A*, 1996, **743**, 231.
20 Ståhlberg, J., *Anal. Chem.*, 1997, **69**, 3812.
21 Dittman, M. M., and Rozing, G. P., *J. Chromatogr. A*, 1996, **744**, 63.
22 Dittman, M. M., and Rozing, G. P., *J. Microcol. Sep.*, 1997, **9**, 399
23 Stevens, T. S., and Cortes, H. J., *Anal. Chem.*, 1983, **55**, 1365.
24 Li, D., and Remcho, V. T., *J. Microcol., Sep.*, 1997, **9**, 389.
25 Jorgenson, J. W., and Lukacs, K. D., *J. Chromatogr.*, 1981, **208**, 209.
26 Wan, Q.-H., *J. Phys. Chem. B*, 1997, **101**, 8449.
27 Choudhary, G., and Horváth, C., *J. Chromatogr. A*, 1997, **781**, 161.
28 Bruin, J. G. M., Tock, P. P. H., Kraak, J. C., and Poppe, H., *J. Chromatogr.*, 1990, **517**, 557.
29 Guo, Y., and Colón, L. A., *Anal. Chem.*, 1995, **67**, 2511.
30 Pesek, J. J., and Matyska, M. T., *J. Chromatogr. A*, 1996, **736**, 255.
31 Mayer, S., and Schurig, V., *J. Liq. Chromatogr.*, 1993, **16**, 915.
32 Pfeffer, W. D., and Yeung, E. S., *Anal. Chem.*, 1990, **62**, 2178.
33 Francotte, E., and Jung, M., *Chromatographia*, 1996, **42**, 521.
34 Tan, Z. J., and Remcho, V. T., *J. Microcol. Sep.*, 1998, **10**, 99.
35 Jakubetz, H., Czesla, H., and Schurig, V., *J. Microcol. Sep.*, 1997, **9**, 421.
36 Robson, M. M., Roulin, S., Shariff, S. M., Raynor, M. W., Bartle, K. D., Clifford, A. A., Myers, P., Euerby, M. R., and Johnson, C. M., *Chromatographia*, 1996, **43**, 313.
37 Dulay, M. T., Yan, C., Rakestraw, D. J., and Zare, R. N., *J. Chromatogr. A*, 1996, **725**, 361.
38 Ross, G., Dittmann, M., Bek, F., and Rozing, G., *Am. Lab.*, 1996, **28**, 34.
39 Rebscher, H., and Pyell, U., *Chromatographia*, 1996, **42**, 171 .
40 Yan, C., Dadoo, R., Zhao, H., Zare, R. N., and Rakestraw, D. J., *Anal. Chem.*, 1995, **67**, 2026.
41 Whitaker, K. W., and Sepaniak M. J., *Electrophoresis*, 1994, **15,** 1341.
42 Schmeer, K., Behnke, B., and Bayer, E., *Anal. Chem.*, 1995, **67**, 3656.
43 Smith, N. W., and Evans, M. B., *Chromatographia*, 1995, **41**, 197.
44 Euerby, M. R., Gilligan, D., Johnson, C. M., Roulin, S. C. P., Myers, P., and Bartle, K. D., *J. Microcol. Sep.*, 1997, **9**, 373.
45 Fields, S. M., *Anal Chem.*, 1996, **68**, 2709.
46 Peters, E. C., Petro, M., Svec, F., and Fréchet, J. M. J., *Anal. Chem.*, 1997, **69**, 3646.
47 Palm, A., and Novotny, M. V., *Anal. Chem.*, 1997, **69**, 4499.
48 Giddings, J. C., *Unified Separation Science*, Wiley, New York, 1991.
49 Meyer, V. R., *Practical High-Performance Liquid Chromatography*, Wiley, New York, 2nd edn., 1993.
50 Li, S. F. Y., *Capillary Electrophoresis*, Elsevier, Amsterdam, 1992.
51 Heiger, D. N., *High Performance Capillary Electrophoresis*, Hewlett-Packard, Waldbronn, 1992.
52 Li, S., and Lloyd, D. K., *J. Chromatogr. A*, 1994, **666**, 321.
53 Lelièvre, F., Yan, C., Zare, R. N. and Gareil, P., *J. Chromatogr. A*, 1996, **723**, 145.
54 Kitagawa, S., and Tsuda, T., *J. Microcol. Sep.*, 1994, **6**, 91.
55 Wan, Q.-H., *J. Chromatogr. A*, 1997, **782**, 181.
56 Seifar, R. M., Kok, W. Th., Kraak, J. C., and Poppe, H., *Chromatographia*, 1997, **46**, 131.
57 Wright, P. B., Lister, A. S., and Dorsey, J. G., *Anal. Chem.*, 1997, **69**, 3251.
58 Schwer, C., and Kenndler, E., *Anal. Chem.*, 1991, **63**, 1801.
59 Boughtflower, R. J., Underwood, T., and Paterson, C. J., *Chromatographia*, 1995, **40**, 329.
60 Hanai, T., Hatano, H., Nimura, N., and Kinoshito, T., *J. High Resolut. Chromatogr.*, 1991, **14**, 481.
61 Li, S., and Lloyd, D. K., *Anal. Chem.*, 1993, **65**, 3684.
62 Euerby, M. R., Johnson, C. M., Roulin, S. C. P., Myers, P., and Bartle, K. D., *Anal. Commun.*, 1996, **33**, 403.
63 Tsuda, T., *LC-GC*, 1992, **5**, 26.
64 Dekkers, S. E. G., Tjaden, U. R., and van der Greef, J., *J. Chromatogr. A*, 1995, **712**, 201.
65 Behnke, B., and Bayer, E., *J. Chromatogr. A*, 1994, **680**, 93.
66 Yan, C., Dadoo, R., Zare, R. N., Rakestraw, D. J., and Anex, D. S., *Anal. Chem.*, 1996, **68**, 2726.
67 Euerby, M. R., Gilligan, D., Johnson, C. M., and Bartle, K. D., *Analyst*, 1997, **122**, 1087.
68 Cortes, H. J., Pfeiffer, T. S., Richter, B. C., and Stevens, T. S., *J. High Resolut.Chromatogr.*, 1987, **10**, 446.
69 Euerby, M. R., Johnson, C. M., and Bartle, K. D., *LC-GC Int.*, 1998, **11**, 39.
70 Rebscher, H., and Pyell, U., *J. Chromatogr. A*, 1996, **737**, 171 .
71 Verheij, E. R., Tjaden, U. R., Niessen, W. M. A., and van der Greef, J., *J. Chromatogr.*, 1991, **554**, 339.
72 Lane, S. J., Boughtflower, R., Paterson, C., and Underwood, T., *Rapid Commun. Mass Spectrom.*, 1995, **9**, 1283.
73 Lane, S. J., Boughtflower, R., Paterson, C., and Morris, M., *Rapid Commun. Mass Spectrom.*, 1996, **10**, 733.
74 Lord, G. A., Gordon, D. B., Tetler, L. W., and Carr, C. M., *J. Chromatogr. A*, 1995, **700**, 27.
75 Taylor, M. R., and Teale, P., *J. Chromatogr. A*, 1997, **768**, 89.
76 Gordon, D. B., Lord, G. A., and Jones, D. S., *Rapid Commun. Mass Spectrom.*, 1994, **8**, 544.
77 Yan, C., Schaufelberger, D., and Erni, F., *J. Chromatogr. A*, 1994, **670**, 15.
78 Boughtflower, R. J., Underwood, T., and Maddin, J., *Chromatographia*, 1995, **41**, 398.
79 Hugener, M., Tinke, A. P., Niessen, W. A. M., Tjaden, V. R., and van der Greef, J., *J. Chromatogr.*, 1993, **647**, 375.
80 Mayer, S., and Schurig, V., *J. High Resolut. Chromatogr.*, 1992, **15**, 129.
81 Maruska, A., and Pyell, U., *Chromatographia*, 1997, **45**, 229.
82 Dadoo, R., Yan, C., Zare, R., Deon, S., Rakestraw, D. J., and Hux, G. A., *LC-GC*, 1997, **15**, 630.
83 Lister, A. S., Dorsey, J. G., and Burton, D. E., *J. High Resolut. Chromatogr.*, 1997, **20**, 523.
84 Yamamato, H., Baumann, J., and Erni, F., *J. Chromatogr.*, 1992, **593**, 313.
85 Smith, N. W., and Evans, M. B., *Chromatographia*, 1994, **38**, 649.
86 Miyawa, J. H., Alasandro, M. S., and Riley, C. M., *J. Chromatogr. A*, 1997, **769**, 145.
87 Lord, G. A., Gordon, D. B., Myers, P., and King, B. W., *J. Chromatogr. A*, 1997, **768**, 9.
88 Huber, C., Choudhary, G., and Horváth, C., *Anal. Chem.*, 1997, **69**, 4429.
89 Euerby, M. R., Johnson, C. M., Cikalo, M. and Bartle, K. D., *Chromatographia*, 1998, **47**, 135.
90 Taylor, M. R., Teale, P., Westwood, S. A., and Perrett, D., *Anal. Chem.*, 1997, **69**, 2554.
91 Paterson, C. J., Boughtflower, R. J., Higton, D., and Palmer, E., *Chromatographia*, 1997, **46**, 599.
92 Ding, J., Szeliga, J., Dipple, A., and Vouros, P., *J. Chromatogr. A*, 1997, **781**, 327.
93 Ding, J., and Vouros, P., *Anal. Chem.*, 1997, **69**, 379.
94 Li, D., Knobel, H. H., and Remcho, V. T., *J. Chromatogr. B*, 1997, **695**, 169.

95 Lloyd, D. K., Li, S., and Ryan, P., *J. Chromatogr. A*, 1995, **694**, 285.
96 Wolf, C., Spence, P. L., Pirkle, W. H., Derrico, E. M., Cavender, D. M., and Rozing, G. P., *J. Chromatogr. A*, 1997, **782**, 175.
97 Nilsson, S., Schweitz, L., and Petersson, M., *Electrophoresis*, 1997, **18**, 884.
98 Schweitz, L., Andersson, L. I., and Nilsson, S., *Anal. Chem.*, 1997, **69**, 1179.
99 Lin, J.-M., Nakagama, T., Uchiyama, K., and Hobo, T., *J. Liq. Chromatogr.*, 1997, **20**, 1489.
100 Lin, J.-M., Nakagama, T., Uchiyama, K., and Hobo, T., *Biomed. Chromatogr.*, 1997, **11**, 298.
101 Lin, J.-M., Nakagama, T., Uchiyama, K., and Hobo, T., *J. Pharm. Biomed. Anal.*, 1997, **15**, 1351.
102 Lin, J.-M., Nakagama, T., Wu, X. Z., Uchiyama, K., and Hobo, T., *Fresenius' J. Anal. Chem.*, 1997, **357**, 130.

Paper 8/01148F
Received February 9, 1998
Accepted April 2, 1998