Capillary ElectroChromatography

BECKMAN COULTER

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Table of Contents

About the Author

Dr. Norman Smith is a lecturer at the Zeneca/SmithKline Beecham Centre for Analytical Sciences at Imperial College, London, UK and currently heads a group of 5 full-time and 2 part-time students studying Capillary ElectroChromatography. Prior to this he spent 33 years in the Pharmaceutical Industry, all with Glaxo-Wellcome, during which time he was instrumental in bringing techniques such as HPLC, SFC, CZE, and more recently Capillary ElectroChromatography, into mainstream Pharmaceutical Analysis. Particular areas of interest include the analysis of chiral compounds and the investigation of novel stationary phases. The authors introduction of strong cation exchange phases for the analysis of highly basic compounds by CEC has resulted in extremely sharp, highly focussed peaks that still defy current chromatographic theory. The understanding of this focusing effect forms the basis of post-doctoral research by one of his students. In 1995, he was awarded the Chromatographic Society Silver Medal for his contribution to Separation Science.

Acronyms and Symbols

The following acronyms and symbols are found throughout this book:

c	molar concentration
CEC	capillary electrochromatogrphy
CZE	capillary zone electrophoresis
d_c	diameter of a capillary
d_p	particle diameter
E	electric field strength
F	Faraday constant
HPLC	high performance liquid chromatography
$\frac{1}{\kappa}$	the thickness of the double layer
K	thermal conductivity
L	column length
MEKC	micellar electrokinetic chromatography
Δp	pressure drop across the column
Q	rate of heat generated for unit volume of electrolyte
R	universal gas constant
T	absolute temperature
ΔT	temperature excess at the centre of column
u	linear velocity
δ	thickness of the electrical double layer
ε	porosity of a packed bed
ε_o	permitivity of a vacuum
ε_r	dielectric constant or relative permitivity of medium
ϕ	dimension less pressure resistance factor for packed columns
η	viscosity of the solvent
λ	molar conductivity
σ	charge density
ζ	zeta potential
H	height equivalent to a theoretical plate

1. Introduction

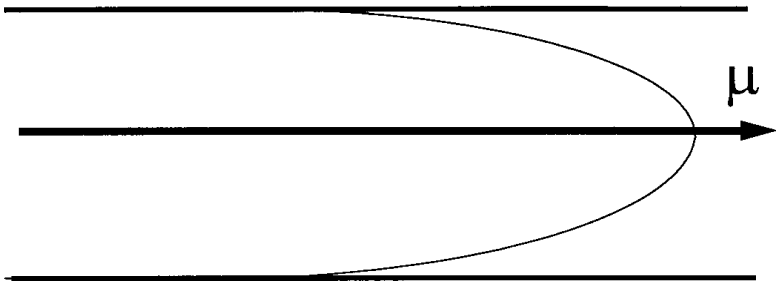
Although Capillary Zone Electrophoresis (CZE) is a high-efficiency separation technique for charged analytes, it is incapable in its native form of separating neutral molecules. This problem was elegantly resolved by Terabe [1] who introduced the concept of Micellar ElectroKinetic Chromatography (MEKC). Surfactants were added to the background electrolyte in excess of their critical micelle concentration whereby they form a pseudo-stationary phase analogous to the stationary phase in HPLC. Neutral molecules as well as ionic species can then be conveniently separated as a direct result of their solubilisation within the micelle, those that are highly solubilised being more retained than those that are excluded from the micelle. Initially the lack of suitable surfactants was a technique limiting factor but this is now being overcome with the devlopment of novel surfactants. Further details of this technique can be found in an earlier Beckman booklet entitled 'Micellar Electrokinetic Chromatography' edited by Terabe.

High Performance Liquid Chromatography (HPLC) on the other hand is the most widely used analytical technique of all and is capable of resolving a wide range of both neutral and charged analytes. Since the early 70's there has been a tremendous increase in stationary phase development leading to the current position where there are now a multitude of phases of widely differing chemistries available in particle sizes ranging from 1.5μm to 10μm.

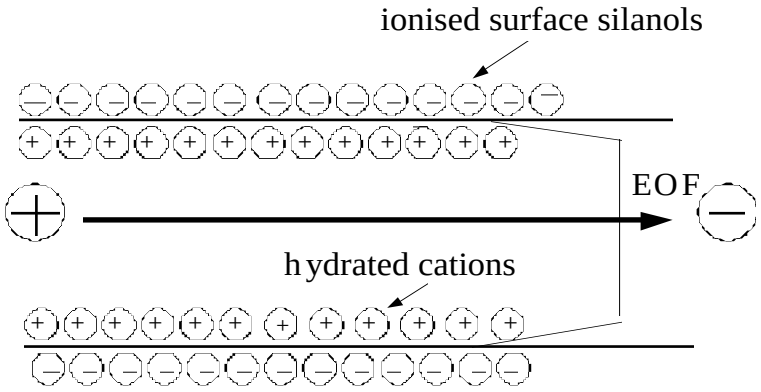
CZE is capable of routinely generating in excess of 500,000 plates per m^{-1}, much more than is achievable by conventional HPLC. However, CZE currently has limitations, for example the inability to resolve analytes with similar electrophoretic mobilities. HPLC on the other hand offers a wide range of parameters which can be manipulated in order to aid the chromatographer achieve separation, particularly the wide range of stationary phases which have been developed over the years. Although HPLC theory predicts an increase in efficiency with decreasing particle size, constraints on pumping systems limits the use of very small particles in routine HPLC.

Capillary ElectroChromatography (CEC) is a hybrid separation method that couples the high separation efficiency of CZE with HPLC, and uses an electric field rather than hydraulic pressure to propel the mobile phase through a packed bed. Since there is no back pressure it is possible to use small diameter packings and thereby achieve very high efficiencies. An additional benefit of CEC compared to HPLC is the fact that the flow profile in a pressure driven system is parabolic, whereas in an electrically driven system it is plug-like and therefore much more efficient.

FLOW PROFILE-
PRESSURE DRIVEN



PLUG FLOW-
ELEC TRICALL Y DRIVEN



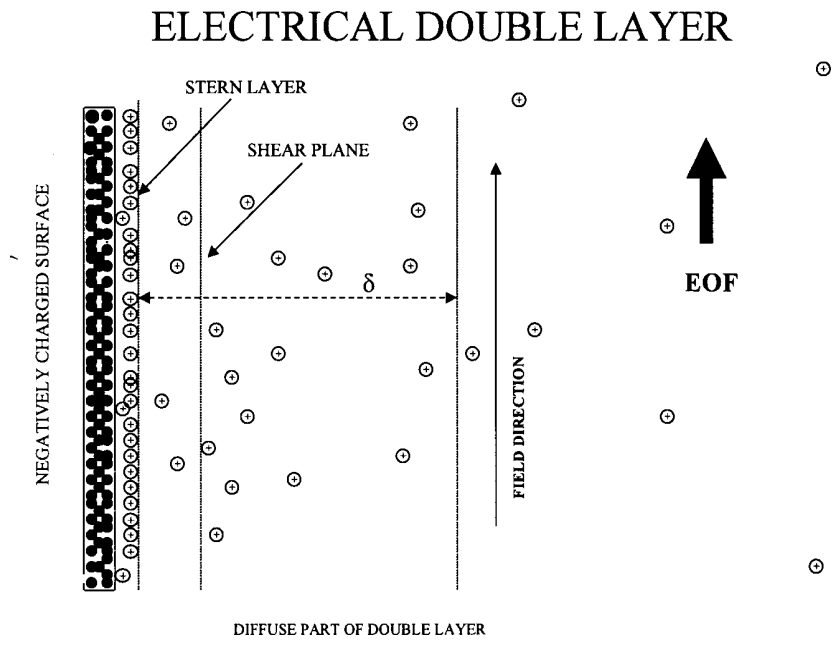
Flow Profiles in a pressure driven and electrically driven system

Although Lecoq [2] and Strain [3] discussed the use of electroosmotic flow in chromatography, Pretorius [4] first demonstrated the ability to use electroosmotic flow in order to drive a mobile phase through a chromatogaphy column. In 1981 Jorgenson and Lukacs [5] reported the separation of 9-methylanthracene from perylene on a 170μm i.d. capillary packed with 10μm reversed-phase packing material. The solvent used to drive the mobile phase through the column was acetonitrile, and although the efficiencies were relatively low (~60,000 plates m^{-1}) the principle of electrically driven chromatography using small diameter particles was proven. However, it was the pioneering theoretical and practical approach of Knox and Grant in 1987 and 1991 [6,7] that finally resurrected the interest in CEC.

2. Theory of Capillary ElectroChromatography

Electrochromatography is a term used to describe narrow bore packed column separations where the liquid mobile phase is driven not by hydraulic pressure as in HPLC but by electroosmosis. The advantages of using electroosmosis to propel liquids through a packed bed are the same as for in open capillaries i.e. reduced plate heights as a result of the plug flow profile and the ability to use smaller particles leading to higher peak efficiency than is possible in pressure driven systems (HPLC).

The driving force in electroseparation methods results from the electrical double layer that exists at the liquid - solid interface between, in the case of CZE, the bulk liquid and the capillary surface and in CEC, the packing material and mobile phase, and this is illustrated below.



Under alkaline conditions, the surface silanol groups of the fused silica will become ionised leading to a negatively charged surface. This surface will have a layer of positively charged ions in close proximity which are relatively immobilised. This layer of ions is called the Stern layer. The remainder of the excess charge, constituting the so called Goüy layer, is solvated and has the characteristics of a typical solvated ion. This layer extends into the bulk liquid and is the so called double layer. The concentration of ions in the double layer is relatively small compared to the total ionic concentration, and falls off exponentially from the capillary surface, as does the corresponding electric potential which is proportional to the charge density. The potential at the boundary between the Stern layer and the interface with the diffused double layer, is called the zeta potential, ζ, and values range from 0-100mV.

As the charge density drops off with distance from the surface, so does the zeta potential, and the distance from the immobile layer to a point in the bulk liquid at which the potential is 0.37 times the potential at the interface with the Stern layer and the diffuse layer, is defined as the thickness of the double layer and is denoted by δ.

The equation describing δ was given by Knox [6] as follows:

$$\delta=[\varepsilon_r \varepsilon_o RT / 2cF^2]^{1/2} \qquad (1)$$

where ε_r = dielectric constant or relative permitivity of medium
ε_o = permitivity of a vacuum
R = universal gas constant
T = absolute temperature
c = molar concentration
F = Faraday constant

Using the above equation with water as the solvent (ε_r = 80), Knox calculated that for a 1:1 electrolyte such as NaCl, at a concentration of 0.001M in water, the thickness of the electrical double layer would be 10nm, and at a concentration of 0.1M it would be 1nm. Electroendosmotic flow within a capillary arises when an electric field is applied longitudinally along the length of a column. When this field is applied, ions in the diffuse (Goüy) layer which are not absorbed in the Stern layer, will migrate towards the cathode and shearing will occur within this

region. Electroosmosis results because the core of liquid within this sheath will also be transported to the cathode. Since there is no charge imbalance within the sheath no shear takes place in this region. Because shearing only occurs within the diffuse layer, the resulting flow profile is plug-like and its velocity is independent of the capillary bore d_c, provided that $d_c \oplus 10\ \delta$. (usually $d_c > 20\ \delta$). If d_c approaches δ then double layer overlap occurs and the EOF is considerably reduced and assumes a parabolic flow profile. In the case of packed capillaries, the open capillary diameter is replaced by the mean channel diameter and Knox calculated that using aqueous electrolytes between 0.001M and 0.01M there would be no double layer overlap as long as the partical diameter d_p is $\oplus$ 20 δ.

The relationship between the linear velocity u and the applied electric field is given by the Smoluchowski equation as follows:

$$u = \frac{\varepsilon_o \varepsilon_r \zeta E}{\eta} \qquad (2)$$

where ε_o = the permitivity of a vacuum
ε_r = dielectric constant of the eluent
ζ = zeta potential
E = electric field strength
η = viscosity of the solvent

It can be seen from this equation that the velocity in the absence of electrical double layer overlap, is directly proportional to ζ. The zeta potential on the other hand is dependent on the charge density σ as shown in the following equation:

$$\zeta \cong \frac{\delta\, \sigma}{\varepsilon_r \varepsilon_o} \qquad (3)$$

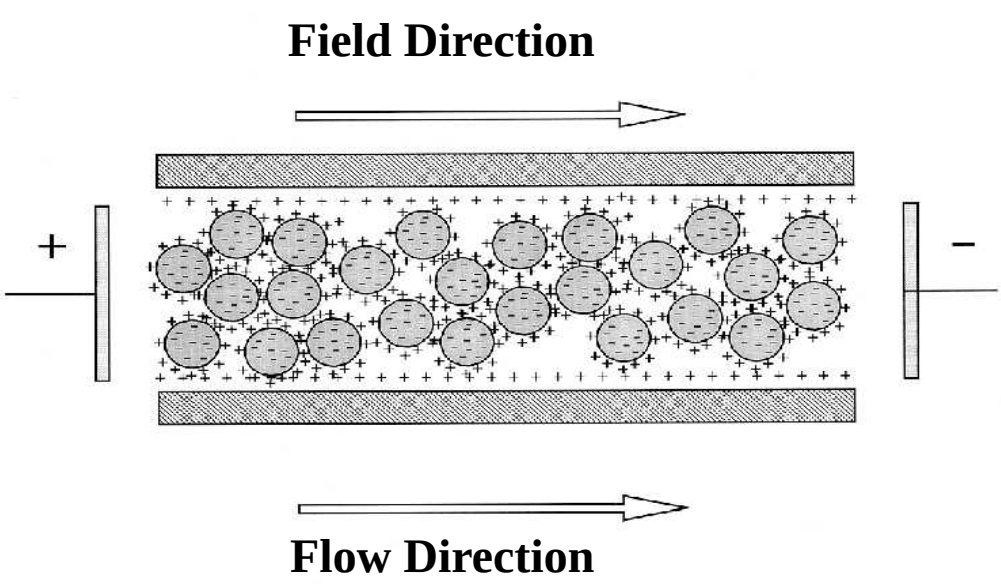
When equations 1-3 are combined we obtain the expression

$$u = \frac{1}{\kappa} \frac{\sigma E}{\eta} \qquad (4)$$

where $\frac{1}{\kappa}$ = the thickness of the double layer.

It is now apparent that the electroosmotic flow depends upon the surface charge density, the field strength, the thickness of the electrical double layer, and the viscosity of the separation medium which in turn is dependent upon the temperature.

Silica gels used in the manufacture of stationary phases possess different properties depending on the method of preparation. As a result of this it is not surprising that such phases can have different charge densities depending on the surface area of the silica and the acidity of the surface silanols groups. Assuming a zeta potential of 100mV in an aqueous medium, electroosmotic flow velocities in open tubes of 0.8mm/s at 100V/cm and 3.2mm/s at 400V/cm are expected. Since HPLC columns packed with 3-5μm diameter reversed-phase particles require linear velocities in the region of 0.5 - 3.0mm/s for optimum separation, then it seems reasonable to assume that sufficient electroosmotic flow could be generated in capillaries packed with similar diameter particles to achieve efficient separation. Since silica based reversed-phase packing materials also contain silanol groups, it is possible to generate electroosmotic flow in capillaries packed with silica gel and/or chemically modified silicas as illustrated below:



In fact, because the surface areas of microparticulate silica based packing materials are much greater than that of the capillary walls, most of the electro-osmotic flow is generated by the surface silanol groups of the stationary phase. Clearly this would not be expected to apply in the case of totally base deactivated materials, such as Hypersil BDS or Spherisorb ODS-B which have minimal levels of residual silanol groups.

The linear flow in a pressure driven system is given by the following equation described by Knox and Grant [6]:

$$u = \frac{d^2 \Delta p}{\phi \eta L} \qquad (5)$$

where u = linear velocity
d = particle diameter
ϕ = pressure resistance factor for packed columns
Δp = pressure drop across the column
L = column length
η = viscosity of the solvent

whereas the linear velocity through a packed capillary under an applied electric field is given by the Smoluchowski equation (2). What is noticeable when comparing equations (2) and (5) is that the linear velocity u is proportional to d^2 in a pressure driven system whilst it is independent of the particle diameter in an electrically driven system. Since plate values are generally lowered as a result of using small diameter particles, it should be possible using an electrically driven system to use very small diameter packing materials and still maintain high linear velocities to yield rapid and very efficient separations, provided the particle diameter $d_p > 20\ \delta$, so that no double layer overlap occurs.

Knox [6] calculated that particles as little as 0.4μm could be used with electrolytes in the concentration range of 0.001M-0.01M without introducing a significant loss in electroendosmotic flow. Theoretical calculations show that it should be possible using particles of $d_p = 0.5\mu m$ to generate 870,000 plates m^{-1}, clearly beyond the capability of a pressure driven system.

An important consideration in CEC, is the relationship between the linear velocity and the concentration of electrolyte. Since u is directly proportional to the zeta potential ζ, which itself decreases with increasing electrolyte

concentration, it is an important variable to consider during method development. Knox [7] measured the effect of electrolyte concentration on the zeta potential using 5μm Hypersil ODS. He plotted plate height and linear velocity against concentration for NaH_2PO_4 and found that the reduced plate height was lowest at 10^{-3}M and the linear velocity altered little over the range 4 x 10^{-5}M to 2 x 10^{-2}M. It was therefore concluded that the best overall performance, i.e. low values of H at high electroosmotic velocities, would be achieved at electrolyte concentrations of ~ 0.002M. Although buffer concentrations used in CEC tend to be low, this might not always be the case especially if charged molecules are analysed when it may be necessary to increase the ionic strength.

2.1 Self-Heating in CEC

Knox and Grant [6] studied the effect of self-heating in both pressure driven and electrically driven chromatography and showed that the heat generated per unit volume of electrolyte (Q) in a packed capillary is given by the following equation:

$$Q = E^2 \lambda c \varepsilon \qquad (6)$$

where E = the electric field strength
λ = molar conductivity
c = molar concentration of electrolyte
ε = porosity of the packed bed

They also showed that the excess temperature at the centre of the tube is given by equation (7).

$$\Delta T = E^2 \lambda c \varepsilon d_c^2 / 16K = Q d_c^2 / 16K \qquad (7)$$

Where d_c = the diameter of the capillary
K = the thermal conductivity of the electrolyte

Knox and Grant were then able to calculate that the heat generated per unit volume in an electrically driven system was ~ 1500 times greater than in a

pressure-driven system, thus emphasising the need to use low concentrations of low-conductivity electrolytes, and to reduce the diameter of the capillary since self-heating is proportional to the square of the capillary diameter.

Although these theoretical considerations lead to the belief that only packed capillaries with diameters <100μm can be used in CEC, in fact due to the very low currents produced in an electrically driven system, capillaries up to 320μm have been used successfully. However, under conditions where self-heating does occur, serious problems can arise resulting in out-gassing within the capillary followed by a breakdown in the current. This problem is often compounded when high concentrations of organic solvent are present resulting in a lowering of the effective boiling point of the solvent. The suppression of bubble formation when operating at high voltages with high concentrations of organic solvents and buffers, therefore requires that pressurisation of the mobile phase is necessary if CEC is to be performed over long periods, free from bubble formation.

2.2 Theory of Band Broadening in CEC

Because electrochromatography involves the use of a stationary phase, the principles of band broadening are similar to those that occur in conventional liquid chromatography. Therefore the plate height can be expressed using a modified Van Deemter plate height equation:

$$H = Ad_p (ud_p / D_m)^{1/3} + B / u + Cud_p^2 / D_m \qquad (8)$$

where A, B and C are constants, and

u = linear velocity
d_p = particle diameter
D_m = diffusion coefficient

The A term in the above plate height equation relates to the diffusion arising from different flow paths that solute molecules can take through the packed bed. Because molecules in the wider paths can move faster than those in narrow paths, band broadening occurs, and this is a function of the so-called tortuosity of the packed bed. This so called "eddy diffusion" assumes that the solute

molecules are fixed in a given flow channel, but in fact they are able to diffuse laterally and change channels and in order to take account of this convection i.e. the effect on band broadening resulting from the exchange of solute molecules between streams moving at different velocities, it is necessary to couple the eddy diffusion term with the mobile phase mass transfer term. This is more significant in HPLC where the mean flow rate will vary from channel to channel, but in capillary electrochromatography, because the velocities between the channels will be identical, then the contribution to band broadening would be expected to be significantly lower than in conventional chromatography for a given particle size of packing. It is clear from equation (8) that a reduction in the particle diameter will lead to more densely packed and uniform columns and in turn a lower A term. The B-term in the equation is the contribution to the plate height resulting from longitudinal diffusion (molecular diffusion in the axial direction) and arises from the tendency of the band to diffuse away from the band centre as it moves down a column and is proportional to the time that the sample spends in the column and also to its diffusion coefficient in the mobile phase. The longer a solute spends in the column the greater the extent of diffusion and therefore the B-term only becomes significant at low flow rates. The C-term reflects band broadening due to slow equilibration (resistance to mass transfer) between the mobile and stationary phases and is increased as the mobile phase velocity increases because less time is available for equilibration. The contribution to band broadening from the C-term also can be reduced by the use of small diameter packing materials. For example Grant [8] calculated that if the particle diameter was to be reduced to 0.5μm then the contribution to the plate height from the A-term (eddy diffusion) would be ~ 0.5μm and the C-term 0.025μm thus the major contribution to the plate height (2 μm) would be from axial molecular diffusion i.e. the B-term in the Van Deemter equation. If the particle size is reduced significantly, for example to < 1μm then the A and C-terms will be reduced to such a level that the dominant contribution to band broadening will be the B-term i.e. only axial molecular diffusion is a contributing factor to band broadening and now H is given by the well known plate height equation used in CZE:

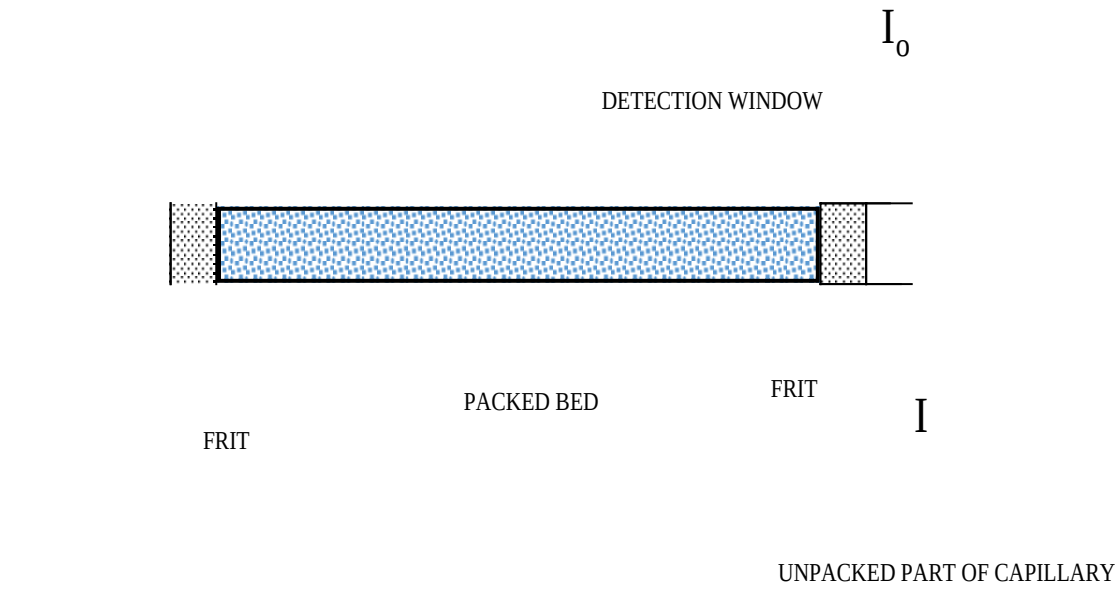
$$H = \frac{2D_m}{u} \qquad (9)$$

3. Instrumentation

As mentioned earlier in this document, although with careful degassing it is possible to perform CEC on conventional CZE instruments, this usually restricts the operating conditions to < 20kV at ambient temperatures. Smith [9] described the use of a modified CZE instrument that allowed electrochromatography to be performed with up to 90% organic solvent at 30kV and 40°C with no interference due to bubble formation. Bubble formation was originally believed to be due to Joule heating but subsequent investigation has led most researchers to now accept that bubbles are in fact formed at the frit adjacent to the detection window and occurs as a result of the change in electroosmotic flow as the solvent passes from the packed bed into the unpacked region of the capillary through this frit.

3.1 Packed Capillaries

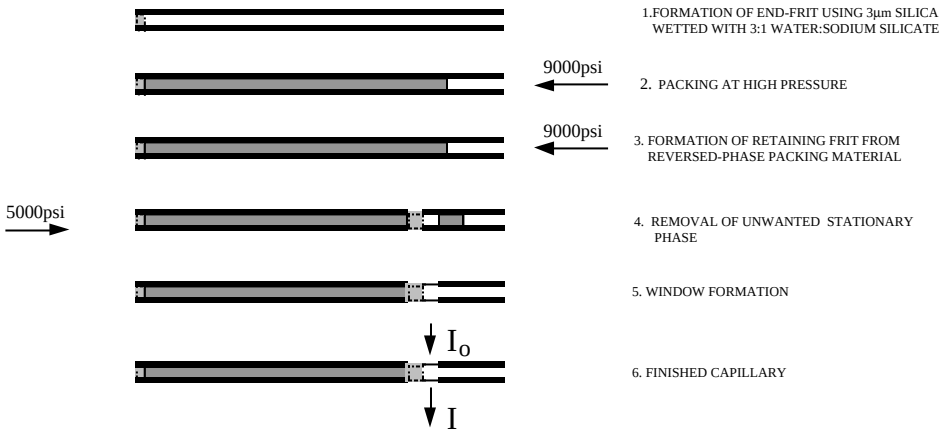
Capillaries are usually packed with between 3µm and 5µm C_{18} or C_8 stationary phases held in place with two retaining frits. The detection window is immediately adjacent to one of these frits thereby allowing on-column detection with very little if any extra-column band broadening. A schematic of a packed capillary is shown below:



3.2 Packing Procedure

Capillaries are packed by the method outlined below. Basically a retaining frit is made from silica and a slurry of the stationary phase is then pumped into the capillary at high pressure. Once packed, a second retaining frit is burnt in place and excess packing material removed by reversing the direction of flow. Once removed, a detection window is formed and the capillary is then ready for use. These capillaries are now available from several commercial manufacturers in a range of dimensions and stationary phases.

Schematic of capillary - Packing procedure



3.3 Conditioning and Testing of Capillaries

Capillaries will usually be supplied from the manufacturer with a test certificate and will have been shipped in the mobile phase used to test the performance. It is highly recommended that the performance of a new capillary is checked against that of the manufacturer, especially retention time and efficiency data. In order to make the comparisons easier, it is sensible if the same chromatography conditions and test mixture to those of the manufacturer are used. Good capillaries packed with 3mm materials should generate at least 150,000 plates per metre and anything less should be considered as underperforming.

3.4 Operating Guidelines

In order to perform CEC routinely it is necessary to follow certain guidelines, some of which are listed below:

(i) Always filter samples prior to injection. This can prevent undissolved material blocking the inlet frit causing a breakdown in current and consequently, flow.

(ii) Samples should be dissolved in the mobile phase or a weaker solvent.

(iii) Conditioning of columns depends very much on the mobile phase. Low pH buffers will take longer to equilibrate since the electroosmotic flow will be lower.

(iv) When possible, try to use “biological buffers” since these produce much lower currents thus allowing the use of higher concentrations.

(v) If working at low pH, use a mixed-mode phase which will provide a much better eof than conventional ODS type phases.

(vi) Because capillaries are fragile, users are advised if possible to store capillaries in their cell/cassette. It is removal of the capillary from the cell that is most likely to lead to breakage.

(vii) In CEC, the overall buffer concentrations used are low, resulting in operating currents much lower than those experienced in CZE, rarely exceeding 10uA. However, if higher buffer concentrations are required to aid separation, then it is recommended that biological buffers are used since these tend to produce much lower currents than their non-biological counterparts. Where the buffer concentration or type leads to high currents and potentially Joule heating, then this can be removed by highly efficient capillary cooling systems as found in commercial instruments such as the Beckman P/ACE and MDQ.

(viii) Should the current break down, this is usually as a result of bubble formation in the capillary. This can normally be rectified in two ways. Either by pressurizing one side of the capillary whilst applying a moderate voltage e.g.10-15kV or by attaching the capillary to a HPLC pump and passing either water or mobile phase through until the bubbles have been swept from the capillary. This normally takes less than 10 minutes. Care should be taken if taking the latter option to connect the capillary to the pump at the outlet end and not the end containing the inlet frit as this can easily be broken! If water is the solvent used to sweep the capillary, there is no need to then introduce the mobile phase in order to continue electrochromatography, since the mobile phase will still be electrophoresed into the capillary on application of a voltage, even though the capillary contains water.

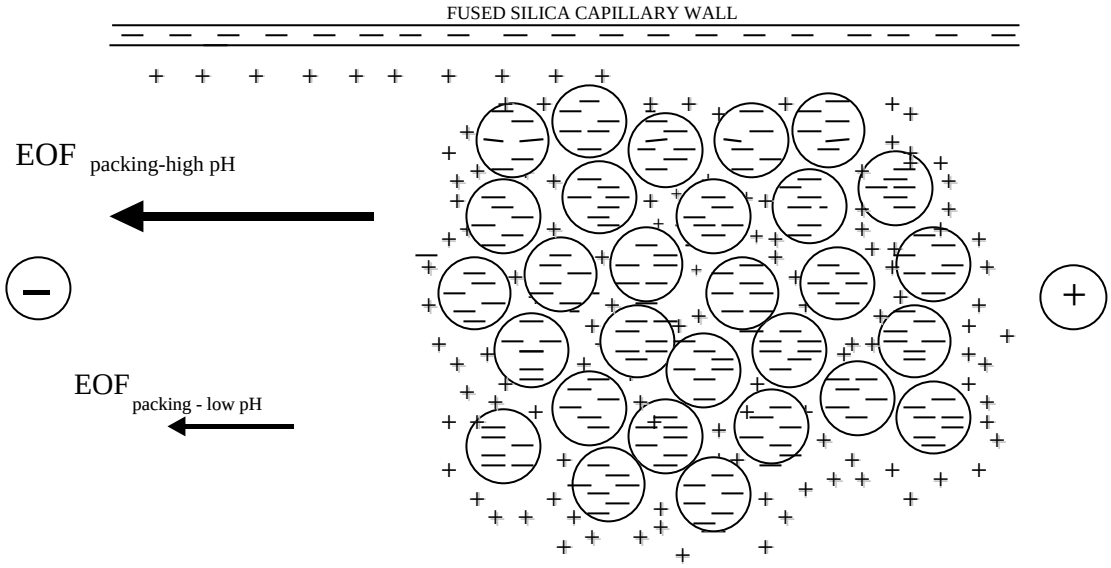
(ix) It is recommended that the capillary be pressurised at both ends during each run to suppress bubble formation within the capillary.

4. Method Development

The driving force in CEC is the electroosmotic flow and this is highly dependent on pH, the buffer concentration, the organic modifier and the type of stationary phase. The chemistry used to prepare the stationary phase can have a dramatic effect not only on the separation but also the speed of analysis, since the concentration of silanol groups present under the operating conditions largely determine the EOF. For conventional silica based stationary phases, the electroosmotic flow drops off almost linearly between pH10 and pH 2 often by as much as a factor of 3, and therefore most CEC is performed above pH 8.0. The following figure illustrates the contribution to the total EOF in a capillary packed with typical silica based 3µm C_{18} or C_8 stationary phases.

ELECTRO-OSMOTIC FLOW AT HIGH and LOW pH

STANDARD HPLC PHASES (e.g. C_{18} C_8)



The major contribution to the EOF is from the packing material at high pH, with little or no contribution from the capillary surface, however, as soon as the pH drops, then the surface silanol groups on the packing become protonated with a resultant decrease in the mobile phase velocity. The degree to which this reduction in EOF occurs is very dependent on the silica used for the preparation of the

stationary phase and also the type of ligand bonded in order to produce that phase. By far the most common stationary phase used in CEC is 3 µm C_{18} and depending on the manufacturer, the silica back bone can be very acidic and therefore promote EOF even at very low pH. The following charts shows a plot of EOF *vs* pH for two stationary phases, 3 µm Spherisorb ODS-1 and 3 µm Spherisorb mixed mode. This latter phase contains both a C_6 and a sulphonic acid group attached to the same silica particle, and is not a mechanical mixture.

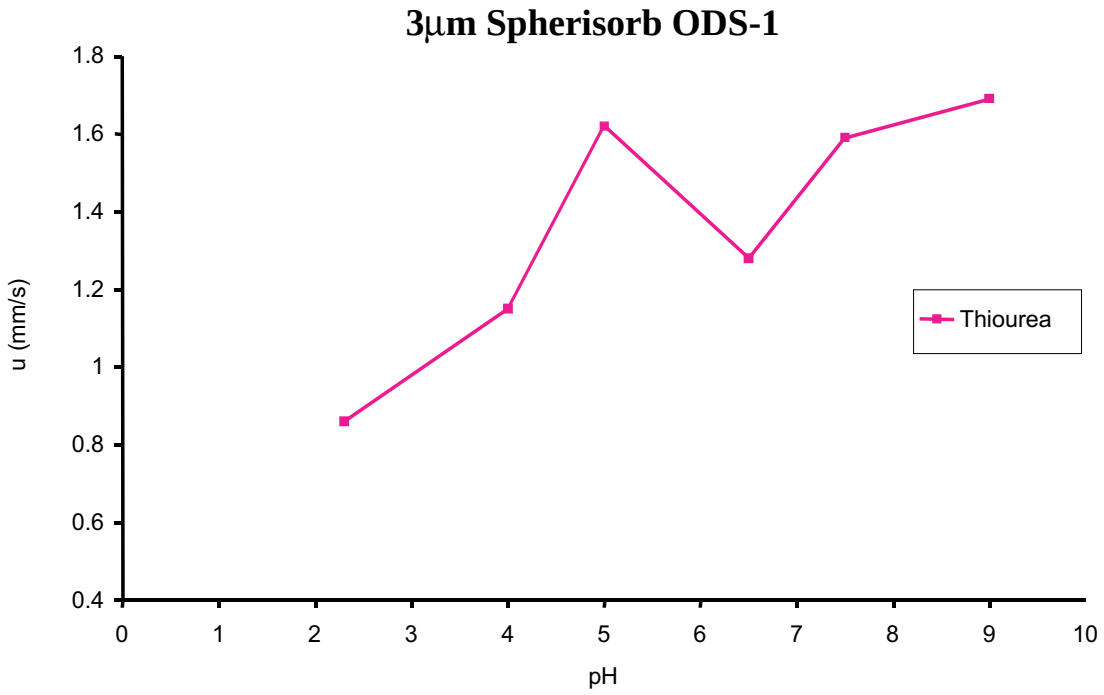


Chart 1. *Reproduced by permission of N.W. Smith*

The buffers used to produce the EOF/pH chart are as follows:

70% Acetonitrile 30% 20mM $Na_2B_40_7$ pH 9.0
70% Acetonitrile 30% 20mM Na_2HPO_4 pH 7.5
70% Acetonitrile 30% 20mM Na_2HPO_4 pH 6.5
70% Acetonitrile 30% 20mM Na Citrate pH 5.0
70% Acetonitrile 30% 20mM Na Citrate pH 4.0
70% Acetonitrile 30% 20mM NaH_2PO_4 pH 2.3

In this example, although the pH shows a downward trend, there is still a considerable EOF even at pH 2 and this is because Spherisorb silica is highly acidic and will therefore have a significant number of ionised silanol groups even at low pH.

With the C_6/SCX mixed mode phase, the EOF is much less dependent on the pH because of the presence of $-SO_3H$ groups attached to the silica matrix. As a result of these, there will always be a sizeable contribution to the EOF even at low pH since these groups are strong acids and therefore always ionised.

3μm Spherisorb C_6/SCX

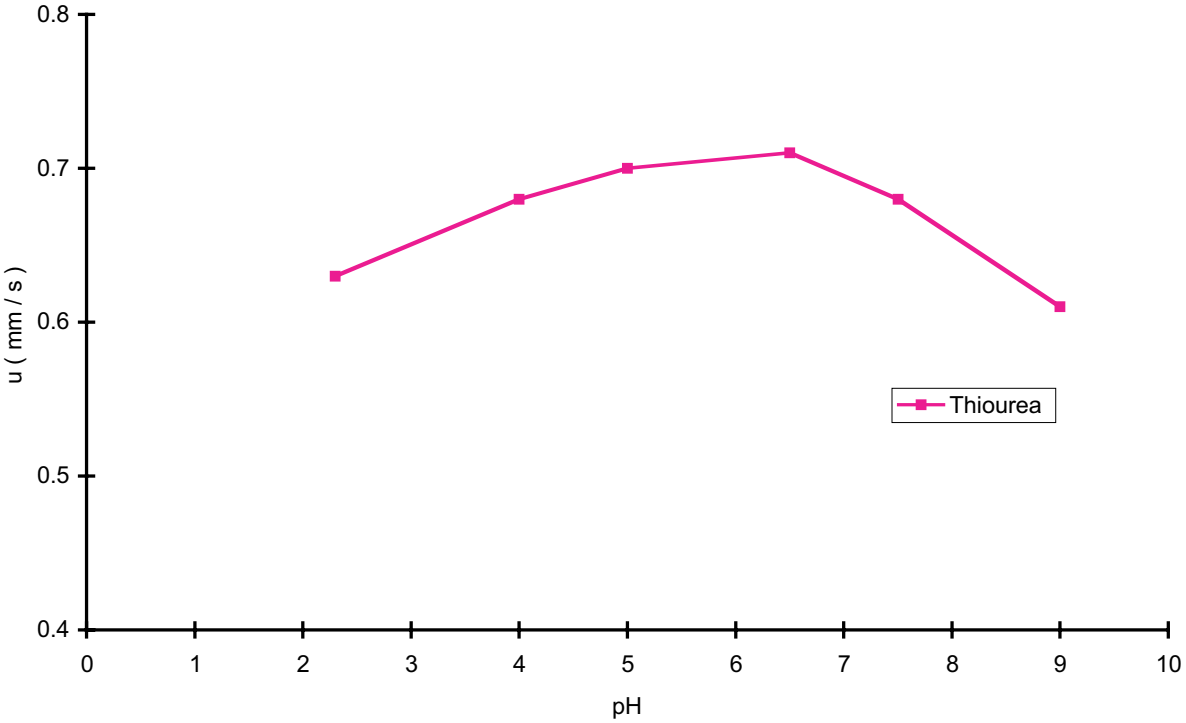


Chart 2. *Reproduced by permission of N.W. Smith*

The test solute often used as an EOF marker in CEC is thiourea, and the linear flow is measured using the following equation:

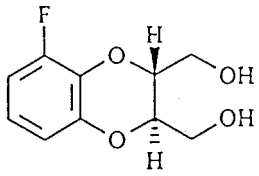
$$u = \frac{L_d}{t} \qquad (10)$$

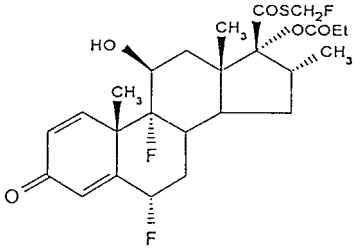
Where u = the linear velocity
L_d = the length of the packed bed
t = the elution time

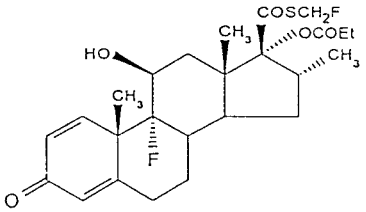
It is desirable when evaluating a new stationary phase to test both the EOF and the chromatographic performance and this is done using a suitable test mixture. Such a test mixture is shown below.

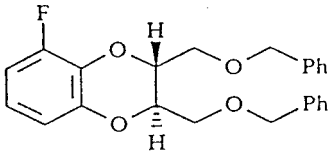
Test Mixture

Peak 1 Thiourea $NH_2.CS.NH_2$

Peak 2 GR57888X [+ -] Trans 

Peak 3 Fluticasone Proprionate 

Peak 4 Des 6 α-fluoro analogue 

Peak 5 GR 57994X [+ -] Trans 

This particular test mixture is constructed to give an indication of the EOF by the inclusion of thiourea, and also an indication of the hydrophobic character by the presence of the two compounds with widely differing polarities, components 2 and 5. Finally the resolving power of the chromatographic phase is determined by the two very closely related components 3 and 4. Figures 1 and 2 show the separation of this test mixture on a Spherisorb ODS-1 stationary phase at a pH of 2.3 and ~8.

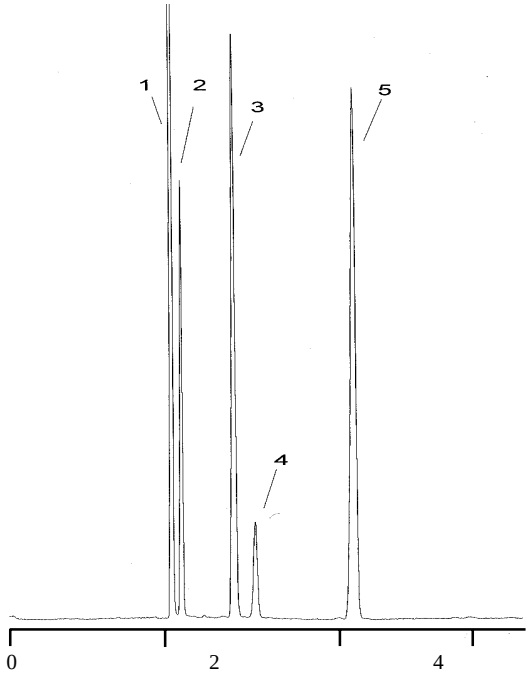


Figure 1. *3µm Spherisorb ODS-1, Mobile phase: 70% Acetonitrile / 30% 0.01M Na_2HPO_4 , Detection 210 nm, Applied voltage: 30kV, Temperature 30°C, Injection: 10kV/5 seconds, Peak identity: 1= Thiourea 2=GR57888X 3=Fluticasone propionate 4=Des flouro analogue 5=GR57794X. Reproduced by permission of N.W. Smith.*

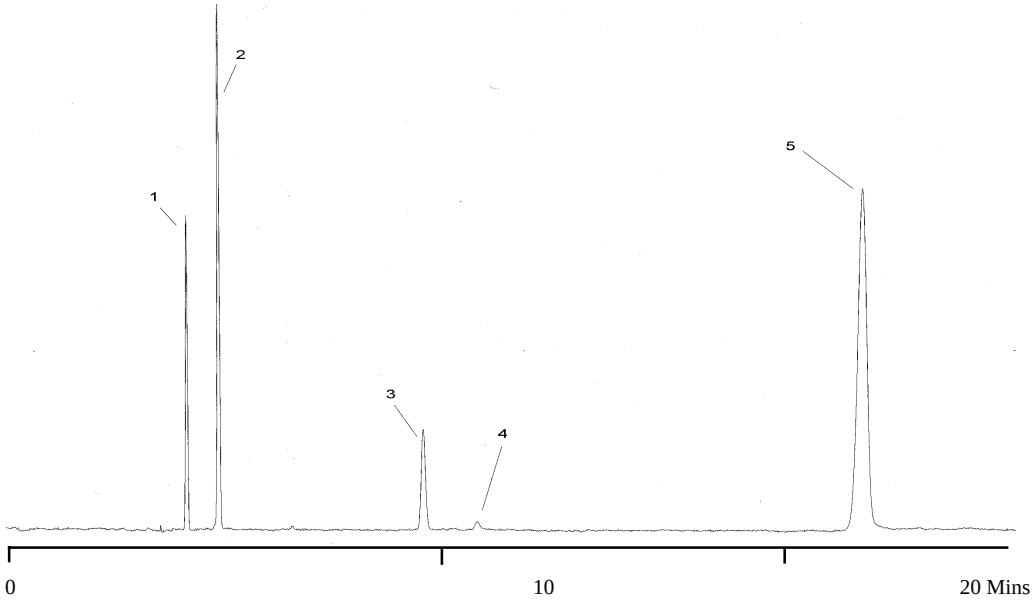


Figure 2. *3µm Spherisorb ODS-1, Mobile phase: 70% Acetonitrile / 30 (20mM NaH_2PO_4 pH 2.3), Detection 210nm, Applied voltage: 30kV, Temperature 30°C, Injection: 5kV/10 seconds, Peak identity: 1= Thiourea 2=GR57888X 3=Fluticasone propionate 4=Des flouro analogue 5=GR57794X. Reproduced by permission of N.W. Smith.*

Because the packed length and overall length of these two capillaries are identical, it is possible to make a direct comparison of performance since the field strength and column bed length are the same. Quite apparent is the fact that the EOF has decreased dramatically between pH 8 and 2.3 with the resulting analysis time increasing from approximately 5 minutes to over 20 minutes at the lower pH. When the same test mixture is run on the C_6/SCX mixed-mode phase there is practically no increase in analysis time on going from high to low pH, and just as important, no compromise on chromatographic performance and this is particularly important during method development. Figures 3 and 4 show the results of running the test mixture on this phase at the extremes of pH.

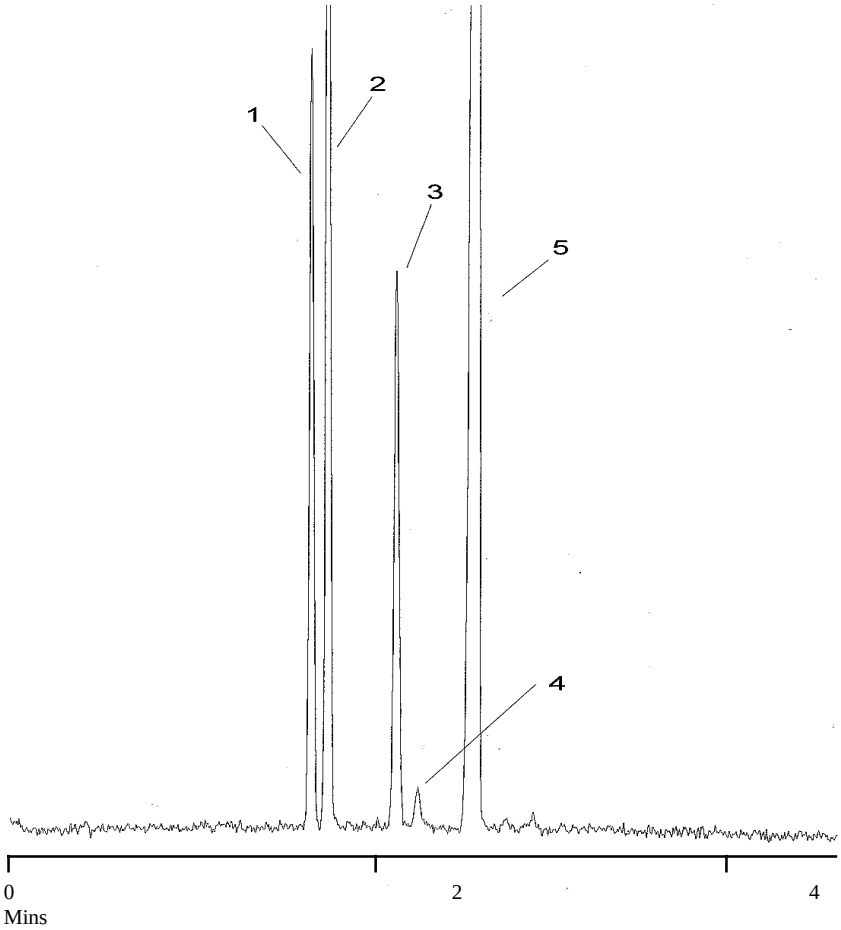


Figure 3. *3µm Spherisorb C_6/SCX, Mobile phase: 70% Acetonitrile / 30% 0.01M Na_2HPO_4 , Detection at 210 nm, Applied voltage: 30kV, Temperature 30°C , Injection: 5kV/10 seconds, Peak identity: 1= Thiourea 2=GR57888X 3=Fluticasone propionate, 4=Des flouro analogue 5=GR57794X. Reproduced by permission of N.W. Smith.*

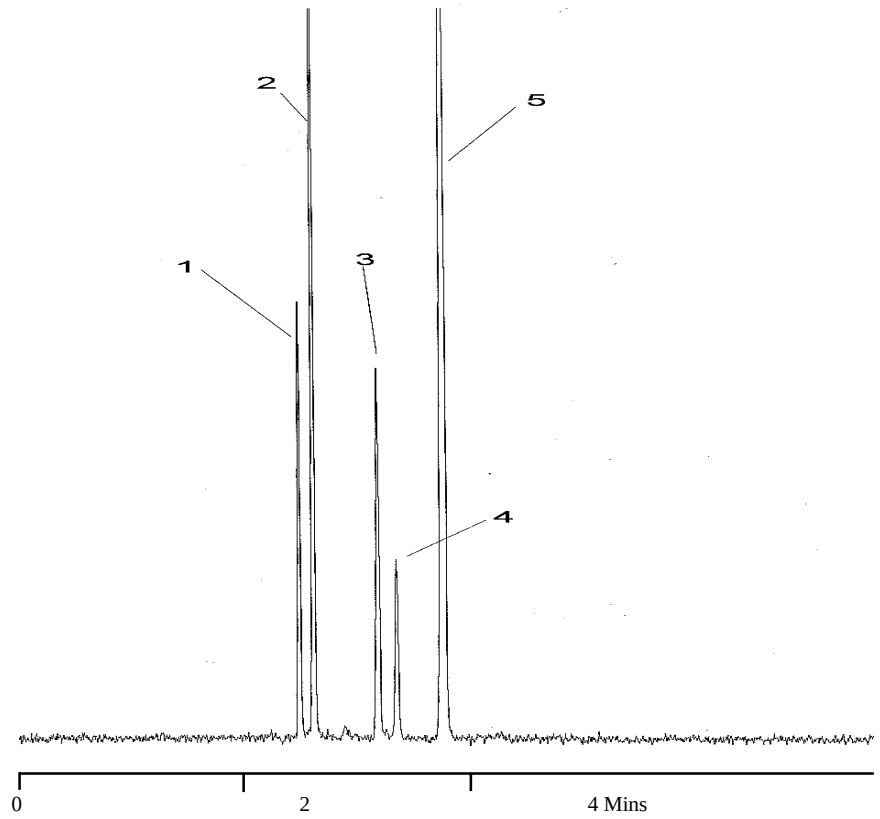


Figure 4. *3µm Spherisorb C_6/SCX, Mobile phase: 70% Acetonitrile / (30% 0.05M NaH_2PO_4 pH 3.5), Detection at 210 nm, Applied voltage: 30kV Temperature 30°C, Injection: 10kV/5 seconds, Peak identity: 1= Thiourea 2=GR57888X 3=Fluticasone propionate 4=Des flouro analogue 5=GR57794X. Reproduced by permission of N.W. Smith.*

The type of organic modifier has a dramatic effect on the separation in CEC. It has been shown [10-12] that with acetonitrile as the organic modifier, the EOF increases almost linearly with increasing acetonitrile concentration but passes through a minimum with methanol. It has also been observed [10] that iso-elutropic mobile phases containing methanol and acetonitrile produce widely differing EOF's and therefore much slower analyses in the methanol system. This is an important consideration in CEC method development.

The effect of electrolyte concentration on both EOF and efficiency has been discussed by Knox and Grant [6,7] who found that the electrolyte concentration had little effect on EOF over the range from 4×10^{-5}M to 2×10^{-2}M. They did however conclude that efficiency increased significantly with electrolyte concentration over

the same range. Although higher electrolyte concentrations tend to yield higher efficiencies, two possible problems could materialise as a result. Firstly, higher electrolyte concentrations can limit the percentage of organic modifier used, which can severely hamper method development. This is a result of the low solubility of many common inorganic buffers in organic solvents. Secondly, high electrolyte concentrations give rise to high currents when conventional buffers are used, and this can give rise to Joule heating followed by loss of current as a result of bubble formation. However, both of these obstacles can be overcome by use of the so-called "biological buffers" such as TRIS and CAPS. These are much more soluble in organic solvents than their inorganic counterparts and also generate much lower currents at similar concentrations. Therefore, their use in method development is highly recommended.

5. Applications

The vast majority of examples of capillary electrochromatography to date have been performed on either C_8 or C_{18} stationary phases and this is not surprising considering the prolific amount of data available on these phases from the HPLC literature.

Figure 5 shows the gradient HPLC separation of the steroid fluticasone propionate from the des flouro analogue, a closely related impurity and another very hydrophobic impurity. The analysis is carried out on a 15cm column packed with 3µm Spherisorb ODS-1 packing material. When the same analysis is carried out using the same batch of 3µm Spherisorb ODS-1 but this time using isocratic CEC [9], the chromatogram shown in Figure 6 is obtained.

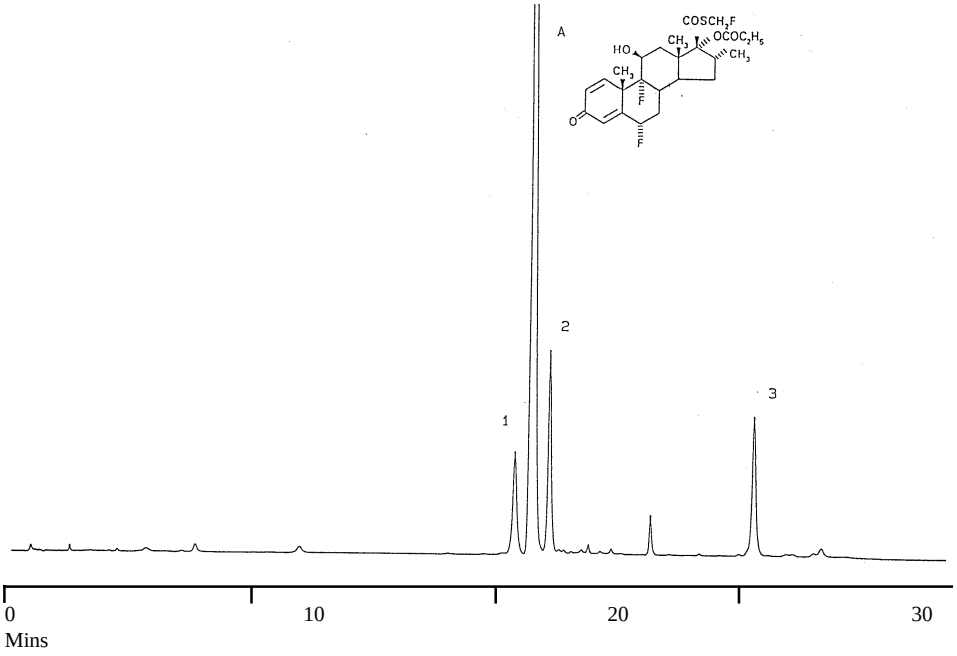


Figure 5. *3µm Spherisorb ODS-1 15cm x 4.6mm i.d., Gradient: 40% Acetonitrile/H_2O to 70% Acetonitrile/H_2O in 20 minutes (EXP 5), Detection at 238nm, Flow: 1.0ml/min, Peak Identity: A=Fluticasone propionate 1=closely related impurity 2=Des flouro analogue 3=hydrophobic impurity. Reproduced by permission of N.W. Smith.*

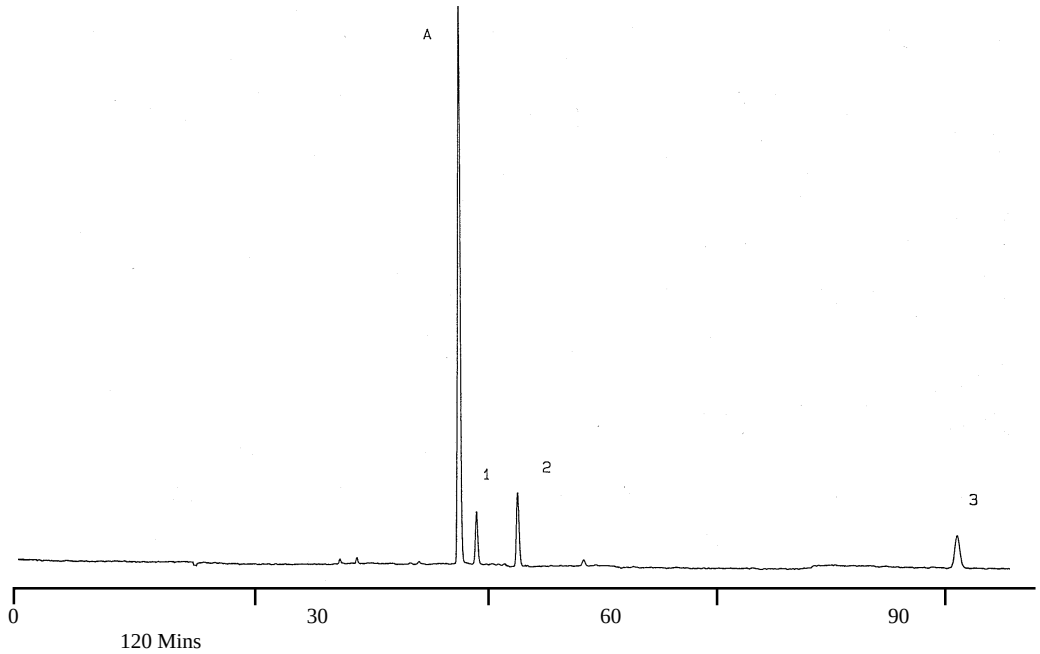


Figure 6. *3µm Spherisorb ODS-1 40cm packed, 55cm total length, Mobile phase: 75% Acetonitrile / H_2O + 2mM Na_2HPO_4 , Detection at 238nm, Applied voltage: 30kV , Temperature 30°C , Injection: 20 kV/10 seconds, Peak identity: A=fluticasone propionate 1=closely related impurity 2=Des flouro analogue 3=hydrophobic impurity. Reproduced by permission of publishers, reference 9.*

Despite the long analysis time by CEC, component 4 eluting at a k' of ~ 6 has almost 400.000 plates per metre giving a reduced plate height h = 0.9.

A comparison of the two chromatograms highlights certain differences. Firstly, despite the fact that all components of this sample are neutral, there is a change in selectivity with impurity 1 now eluting after the main peak in the CEC mode. Secondly, in view of the capillary length and the fact that the CEC analysis is isocratic, there is a tremendous difference in the analysis time. The discrepancy in the elution order is in fact due to the difference in the mobile phases used for the two analyses. If the mobile phase used for the CEC method is used for the HPLC analysis then the elution orders are the same, suggesting that the high pH used for the CEC separation is effecting the silica surface characteristics. The analysis time can be dramatically improved by increasing both the organic solvent concentration and the pH whilst trying to maintain a reasonable electrolyte concentration in order to improve column efficiency. Figure 7 shows the highly efficient rapid analysis of the steroid sample with all components resolved in 10 minutes.

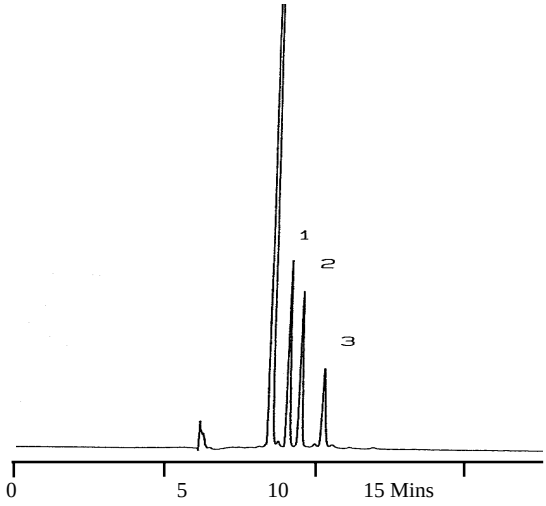


Figure 7. *3µm Spherisorb ODS-1 40cm packed, 55cm total length, Mobile phase: 90% Acetonitrile / 10% 0.1M Tris, Detection at 238nm, Applied voltage: 30kV , Temperature 30°C, Injection: 10 kV/30 seconds, Peak identity: A=Fluticasone propionate 1=closely related impurity 2=Des flouro analogue 3=hydrophobic impurity. Reproduced by permission of publishers, reference 35.*

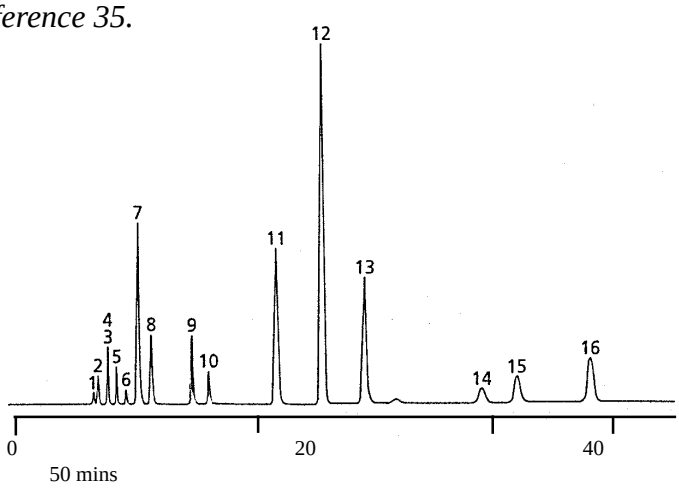


Figure 8. *3µm Porous ODS 33cm packed, Mobile phase: 80% Acetonitrile / 4mM Sodium Tetraborate, Applied voltage: 15kV , Injection: 5kV/5 seconds, Peak identity: 1=napthalene 2=acenaphthylene 3=acenaphthene 4=fluorene 5= phenanthrene 6= anthracene 7= fluoranthene, 8= pyrene 9=benz[a]anthracene 10= chrysene 11= benzo[b] flluoranthene 12= benzo[k] flluoranthene 13= benzo[a] pyrene 14= dibenz[a,h] anthracene 15= benzo[ghi] perylene 16= indeno[1,2,3-cd] pyrene. Reproduced by permission of publishers. First published in LC-GC International, Vol. 10, Number 3 (1997).*

The analysis of polyaromatic hydrocarbons is a common analysis carried out by HPLC and an example of the analysis of a mixture containing 16 components by CEC performed by Yan *et al* [13] is shown in Figure 8, where the samples are detected using a laser induced fluorescence detector. Analysis was carried out on a 3µm porous ODS packing with efficiencies approaching 180,000 plates per metre.

Lubman *et al* [14] reported the analysis of a protein digest using pressurised CEC with detection by reflectron time of flight mass detector. Because peptides have very close capacity factors and are therefore eluted within a very narrow solvent band, gradient elution is usually employed when HPLC is the separating mechanism. However, because in CEC the electrophoretic migration of the peptides themselves provide an additional separation mechanism, it was shown to be relatively easy to resolve peptide mixtures using isocratic CEC. The separation of bovine cytochrome *c* digest on a 6cm packed capillary is shown in Figure 9. A comparison with the gradient HPLC separation on the same capillary is made and it is clear that the CEC separation with a supplementary pressure of 70 bar gives a superior separation.

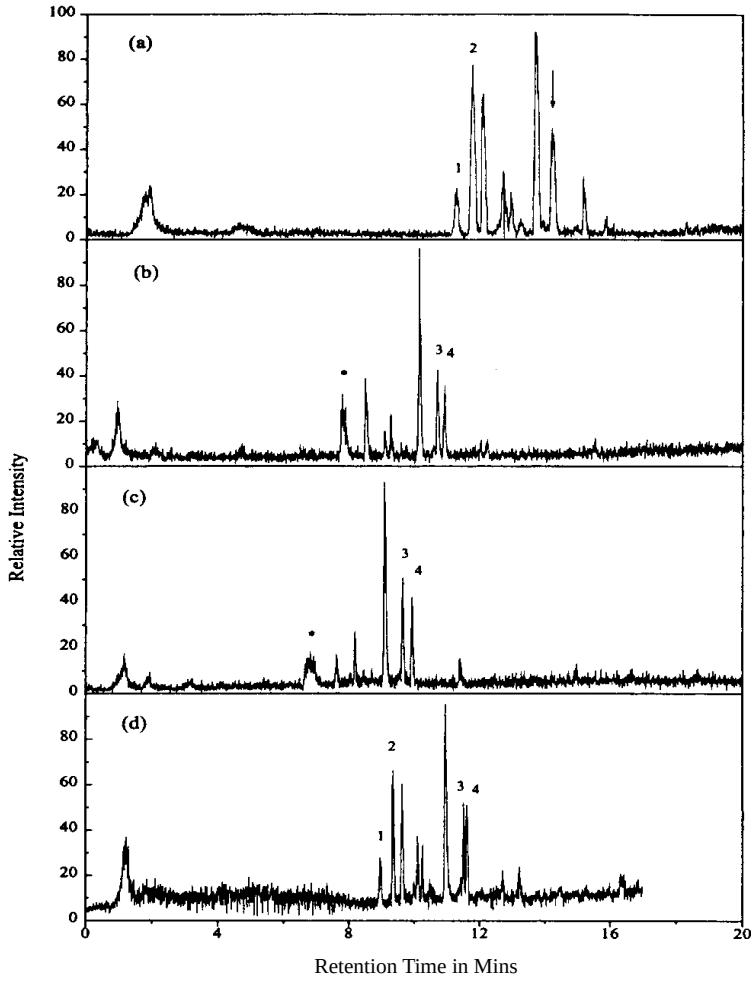


Figure 9. *Separation of Bovine cytochrome c digest using a 20min, 0-50% acetonitrile gradient with 8pmole sample injections, column length 6.0cms. Operating conditions: a)HPLC mode with a back pressure of 90 bar; b)1000V applied voltage with 50 bar pressure; c)1400V applied voltage with 50 bar pressure; d)600V applied voltage with 70 bar pressure. Reproduced by permission of publishers, reference 14.*

Smith and Evans [9] were able to baseline separate the diastereoisomers of a Cephalosporin antibiotic and a related impurity using a 3µm Spherisorb ODS-1 phase, Figure 10. However, in order to obtain adequate resolution, a capillary of packed length 40cm was required. To perform this analysis by HPLC would be impracticable on this length column since the pressure requirements would be outside the capabilities of existing HPLC pumps.

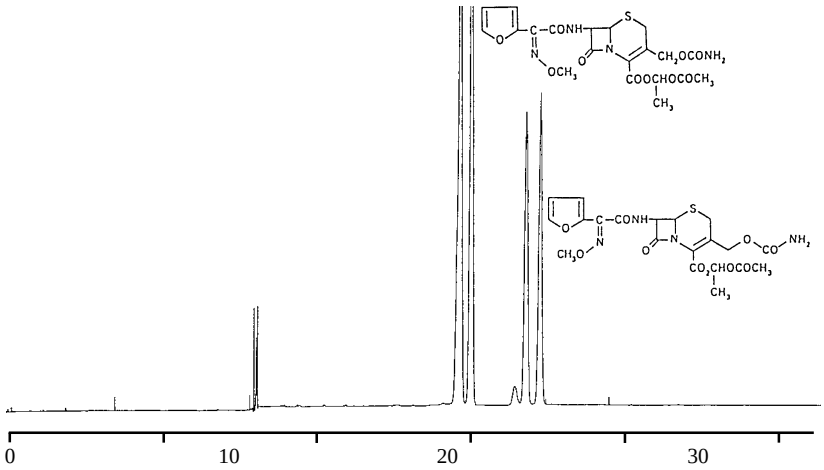


Figure 10. *3µm Spherisorb ODS-1 40cm packed, 55cm total length, Mobile phase: 50% Acetonitrile / 50% 0.01M Na_2HPO_4 , Applied voltage: 30kV, Temperature 30°C, Injection: 20kV/0.2min. Reproduced by permission of the publishers, Ref. 9.*

Also separated on a 3µm Spherisorb ODS-1 phase was a crude Prostaglandin and this is shown in Figure 11. This is a highly efficient separation with the peak at ~37 minutes exhibiting almost 300,000 plates per metre.

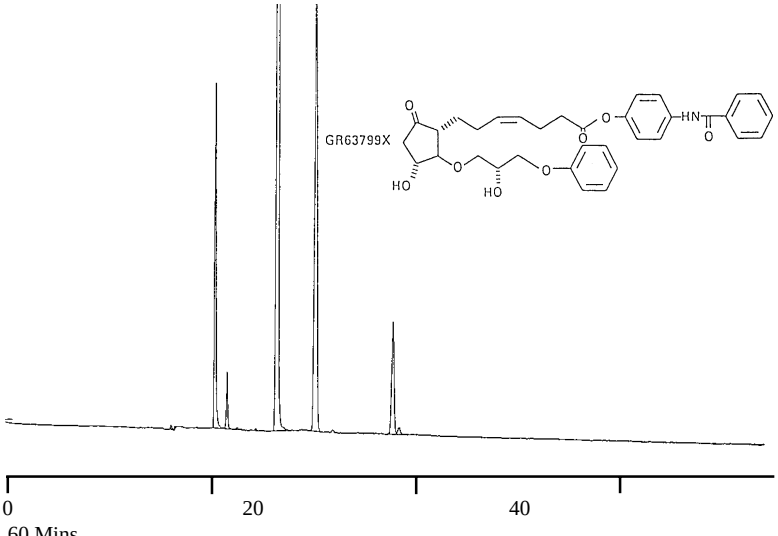


Figure 11. *3µm Spherisorb ODS-1 60cm packed, 82cm total length, Mobile phase: 70% Acetonitrile / 30% 0.01M Na_2HPO_4 , Applied voltage: 30kV, Temperature 35°C, Injection: 30kV/0.4 min. Reproduced by permission of N.W. Smith.*

Oligosaccharides are an important class of compound, and Figure 12 illustrates the highly selective separation of a dextran ladder where the constituents have been derivatised with 4-aminoacridone. Because the selectivity is so good, the "short end" of the capillary was packed in order to reduce the packed bed length and also increase the field strength, thereby reducing the overall analysis time. Using a packed bed of ~8cm, Smith [15] was able to resolve the components of the dextran ladder but was restricted by detector response. Although these derivatives are fluorescent, lack of a fluorescence detector at the time restricted detection to the much less sensitive UV.

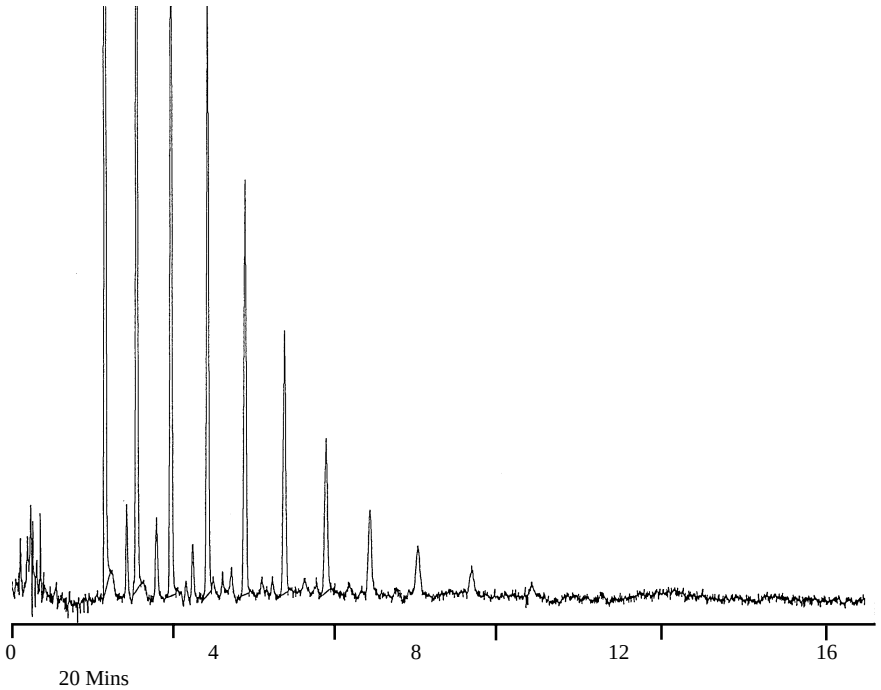


Figure 12. *3µm Spherisorb SCX , Mobile phase: 70% Acetonitrile / (30% 0.05M NaH_2PO_4 pH2.3), Detection at 254 nm, Applied voltage: -30kV, Temperature 30°C, Injection: -5kV/5secs. Reproduced by permission of N.W. Smith.*

6. CEC Mass Spectrometry

The advantages of using miniaturised HPLC when coupling to electrospray mass spectrometers (ESI) have been recognised for many years. As a concentration detector, changing from a 4.6mm i.d. column to 0.1mm i.d. provides significant theoretical gains in sensitivity. In practice, this is not achievable because of technical difficulties, however the drive for developing appropriate methodology to achieve these theoretical goals has steadily grown as scientists strive to obtain highly specific, sensitive, high resolution methods for the analysis of compounds in sample limited situations. Electrically driven separation methods offer solutions to many of these technical difficulties. Numerous papers have been published [16] on the coupling of capillary electrophoresis to mass spectrometers (CE/MS) but the technique is still not widely accepted for routine use because of the limitation on the volumes that can be analysed without compromising separation efficiency, producing poor concentration detection limits. Also, micellar electrokinetic capillary electrochromatography (MEKC) is non-compatible with mass spectrometry.

Verheij *et al* [17] and Hugener *et al* [18] have independently reported the use of mass spectrometry/pseudo electrochromatography (PEC), a combination of both pressure driven and electroosmotically driven chromatography whereby the flow profile approaches that of a pressure driven system. Gordon *et al* have reported the true CEC/MS coupling to both continuos flow fast atom bombardment [19] and electrospray mass spectrometry (ESI) [20] but noted a loss of resolution as a result of post detection window dispersion in the length of unpacked capillary necessary for coupling the commercial CE instrument to the mass spectrometer.

Lane and workers [21] demonstrated the use of fully packed capillaries interfaced to the mass spectrometer whereby chromatographic efficiency could be upheld. CEC/ESIMS separations of steroid mixtures and diastereomeric antibiotics on 95cm long capillaries were shown with excellent efficiencies and mass spec data but at the expense of long analysis times (70minutes) due to the diluted field strength across the long capillary. The separations were performed using non-volatile buffers at high pH which is necessary in order to generate sufficient electroosmotic flow on the C_{18} stationary phase that was used for the separation. Lane pointed out that the separations were not optimal for CEC/MS, but were dictated largely by instrumental constraints in coupling the CE instrument to the mass spectrometer. Also, problems arise due to the lack of suitable CEC specific stationary phases that promote high eof at low pH using the volatile buffers that are commonly used in MS. In order to circumvent the problem with long capillaries, Lane *et al* [22] also demonstrated the

use of a prototype integral CEC/MS injection-separation interface designed and built to facilitate short CEC capillaries (43cm). Using this system Lane was able to separate the same steroid test mixture in 11 minutes compared to 70 minutes on the previous capillary.

7. Non-Aqueous CEC

Jorgenson and Lukacs [5] first employed non-aqueous mobile phases in capillary electrochromatography. They used a mobile phase consisting of 100% acetonitrile which they electrically pumped through a capillary packed with 10µm Partisil ODS-2 using a voltage of 30kV. Using this system they were able to resolve 9-methylanthracene from perylene with an efficiency of 31,000 theoretical plates for the 9-methylanthracene peak and 23,000 for the perylene peak. Detection was on column using a fluorescence detector at a distance of 58cm from the inlet. The resulting chromatogram is shown in Figure 13 below:

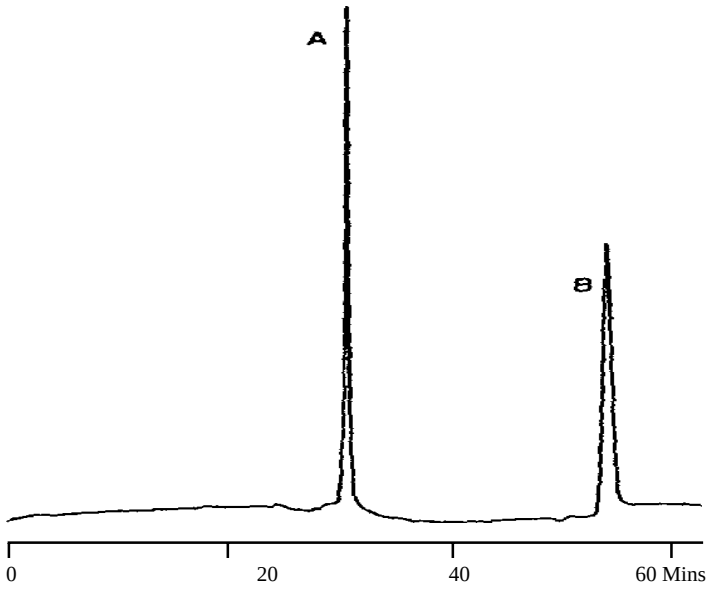


Figure 13. *Partisil-10-ODS-2 (170µm i.d. capillary) Packed length 58cm, Applied voltage: 30kV, Peak A=9-methylanthracene B=perylene. Reprinted from Journal of Chromatography, 218 (J.W. Jorgenson and K.D. Lukacs) High-Resolution Separations based on Electrophoresis and Electroosmosis, 209-216 (1981) with kind permission of Elservier Science-NL, Sara Burgerhartsraat 25, 1055 KV Amsterdam, The Netherlands.*

Sepaniak [23] described the separation of large polycyclic aromatic hydrocarbons and fullerenes using non-aqueous CEC. They found that efficiencies of 160,000 plates per metre could be generated and that large amounts of the less polar modifiers such as methylene chloride and tetrahydrofuran produced predictable decreases in the k' values for the non-polar test compounds. However, the large volumes of modifiers used (~50%) produced a four fold decrease in flow rate with a subsequent increase in retention time despite the reduction in k' values.

This suggests that the non-aqueous mixtures have a significantly lower zeta potential than 100% acetonitrile, thus producing lower eof's. It was observed that the addition of organic salts to the mobile phase also gave rise to a large reduction in eof, in-line with theory (see equations 1-4).
Figures 14A shows the separation of four polyaromatic hydrocarbons using 80/20 acetonitrile / methylene chloride as the mobile phase while Figure 14B shows the separation of C_{60} and C_{70} fullerenes using 50/50 acetonitrile /THF as the carrier.

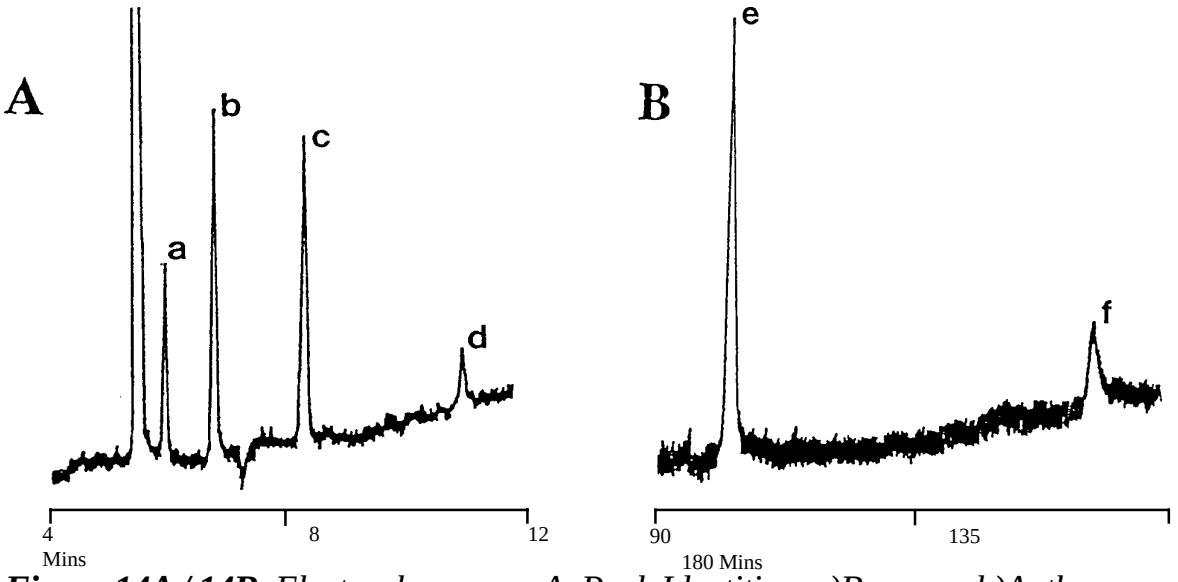


Figure 14A / 14B. *Electropherogram A, Peak Identities: a)Benzene, b)Anthracene, c)BaP, d)Coronene, 80/20 Acetonitrile/Methylene Chloride, Applied Voltage 20kV. Electrpherogram B, Peak Identities: e)C_{60}, f) C_{70}, 50/50, Acetonitrile/THF, Applied Voltage 20kV. Reproduced by permission of publishers, reference 23.*

Dorsey [24] reported on the use of non-aqueous solvents in CZE and CEC. The Smoluchowski equation (2) suggests that the linear velocity is proportional to the ratio ε_r/η and this was reported by Dorsey and also by Kendler and co.[25]. Therefore solvents with reasonable zeta potentials and ratios of ε_r/η similar to that of water, should be able to promote electroosmotic flow. Table 1 compares these figures for six solvents at 25^0C.

When the electrophoretic mobility (μ_{eo}) was plotted against ε, η and the ratio ε_r/η for open tubular electrophoresis, Dorsey found the best correlation was between the μ_{eo} *vs* ε_r/η plot. Electrophoretic velocities and mobilities measured in non-aqueous systems were found to be similar to a totally aqueous media and it was therefore concluded that there were no detrimental effects on the speed of analysis by using non-aqueous solvents. In fact, 100% acetonitrile was found to have an electroosmotic flow three times faster than a 100% aqueous buffer at pH 10.9.

solvent	ε	η(cP)	ε_r/η
Acetonitrile	37.5	0.34	110
Water	80	0.89	90
Methanol	32.7	0.54	60
DMF	36.7	0.80	46
Formamide	111	3.3	34
DMSO	46.7	1.96	23.8

Table 1. *Reproduced from reference [25]*

In CZE, plots of EOF *vs* % organic modifier in a **buffered** system show a steady decrease in electroosmotic flow as the organic concentration increases, and this was confirmed by both Dorsey and Kendler. Whilst for acetonitrile the EOF tends to fall off linearly, other solvents such as methanol, ethanol and iso-propanol show a steep decrease for the first 30-40% modifier, which then slows down as the organic content is further increased. It is worth noting that Kendler only plotted EOF *vs* % organic modifier up to 80% because of solubility restrictions due to the presence of buffer. However, based on the bulk properties of η and ε, the plots would be expected to be the opposite to what was in fact observed, suggesting that the zeta potential must change as the concentration of buffer decreases. In their work however, Dorsey *et al* also plotted EOF *vs* % organic modifier in an **unbuffered,** open tubular system and found that with acetonitrile as mobile phase additive, there was little change in EOF over the concentration range 20-80%, followed by a dramatic increase in EOF at 100% acetonitrile. Using 100% acetonitrile, Dorsey [26] was able to separate highly insoluble hydrophobic dyes on a 3μm Hypersil ODS phase after the addition of TEA to act as a competing base, whereby there was a significant improvement in peak shape.

In contrast to open tubular electrophoresis, plots of EOF *vs* % organic modifier/ buffer in a packed system (CEC) show a steady increase of EOF with increasing acetonitrile concentration [10-12] and this phenomenon is used in CEC method development which will be discussed later in this article.

8. Chiral CEC

Conventional CE for Chiral Analysis has shown itself to be robust, cost effective and easy to automate the methods development process. The recent development of highly sulphonated cyclodextrins have also demonstrated dramatic results. Although there have been many reports of chiral separations using CEC [27-31] the separation efficiencies do not compare favorably with conventional CE, and this is due in part to the very low capacity of the phases used. Mayer [27] reported the separation of (+/-) 1,1'-binaphthyl-2,2'-diylhydrogenphosphate (Figure 15A) and (+/-) –1-phenylethanol (Figure 15B) on a Chirasil-Dex capillary. This is a capillary produced by immobilising the chiral cyclodextrin onto the surface of a fused silica capillary and then driving the mobile phase consisting of a phosphate– borate buffer at pH 7 through the capillary at applied voltages up to 30kV.

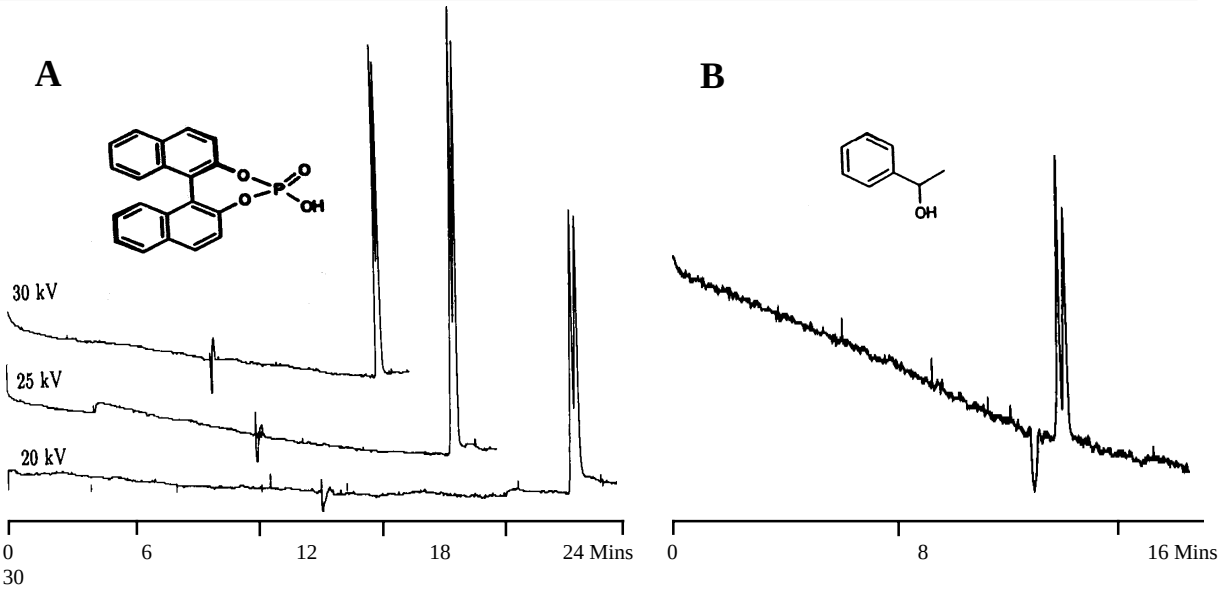


Figure 15. *Chirasil-Dex (50 μm I.D. capillary) Effetcive length 80cms, Borate-phosphate buffer, pH=7, Detection at 220nm, Temperature 20°C buffer. Electropherogram A=Applied voltages, 20,25,30kV and B=Applied Voltage of 20kV. Reproduced by permission of S. Mayer and V. Schurig, J. High Resol. Chromatogr., 15,129-131 (1992).*

In another paper [28] the same authors reported the chiral separation by open tubular electrochromatography of NSAID's on two immobilised chiral columns, one using permethylated β-cyclodextrin as the chiral selector and the other permethylated γ-cyclodextrin. Of the four anti-inflammatory drugs analysed, three were successfully resolved on the permethylated β-cyclodextrin column, however etodolac could only be resolved on the permethylated

γ-cyclodextrin phase. The influence of film thickness on retention time, enantioselectivity and efficiency was conducted with three different columns and the findings demonstrated a dramatic loss of efficiency as the film thickness increased, due partly to diastereomeric interaction of the analyte with the chiral phase and to an increase in the C-term as a result of a larger resistance to mass transfer in the mobile phase. Lloyd *et al* [29] were able to resolve a range of chiral compounds on an α_1-Acid Glycoprotein column. These included disopyramide, pentobarbital, hexobarbital, cyclophosphamide and benzoin. Regulation of retention and enantioselectivity was studied by altering the type and concentration of organic modifier, pH and electrolyte concentration. It was generally found that 2-propanol gave better results than 1-propanol, and this was reflected to some extent in the dramatic change in mobility with only a modest amount of modifier. However, this drop in retention time reflects a reduction in the interaction of the analytes with the stationary phase and consequently less enantioselectivity. Over the range pH 4.45-7.47 there was a marked increase in EOF leading to a considerable drop in retention time with an increase in pH, however the overall effect of pH on enantioselectivity was much less noticeable than in HPLC with the same phase, and this is possibly due to the fact that higher concentrations of buffer are used in the latter technique than are practicable in CEC. Although the separation efficiencies were higher than by HPLC, compared to conventional CEC with achiral stationary phases, the efficiencies were poor.

Using a β-cyclodextrin stationary phase [30] Lloyd was able to resolve the enantiomers of benzoin and hexobarbital using sodium phosphate as background electrolyte. However, because anionic species migrate against the EOF, this results in very low mobilities and prohibitively long retention times. Lloyd overcame this problem by adding triethylammonium acetate to the background electrolyte thus reversing the direction of electroosmotic flow. Now it was possible to resolve anionic dansyl and dinitrophenyl–amino acids as well as the neutral compounds benzoin and hexobarbital. Because the EOF had been reversed by inclusion of TEAA into the mobile phase, it was necessary to reverse the polarity of applied potential.

Zare and workers [31] reported the separation of the drug chlorthalidone using hydroxypropyl β-cyclodextrin as a chiral stationary phase, and as a mobile phase additive with an achiral stationary phase, 3μm Spherisorb ODS-1. The chiral stationary phase used for these experiments was available commercially as Cyclobond I 2000 RSP, which has hydroxypropyl β-cyclodextrin bonded to5μm silica. The test analyte chlorthalidone is a drug exhibiting diuretic and

hypertensive activity, but since it is neutral, its enantiomers cannot be separated by CZE using a neutral chiral selector. The enantiomers of chlorthalidone had previously been resolved by HPLC using the Cyclobond I 2000 RSP column using a mobile phase consisting of CH_3CN-70mM triethylamine (5:95 v/v) adjusted to pH 4.1 with acetic acid. Separation by CEC was achieved using a mobile phase containing CH_3CN-5mM phosphate buffer pH 6.5. The effect of acetonitrile concentration on the resolution was studied between 15–30% v/v, and the best compromise of resolution and speed of analysis was found to be at a concentration of 25% acetonitrile. The influence of acetonitrile on the separation of enantiomers is shown in Figure 16.

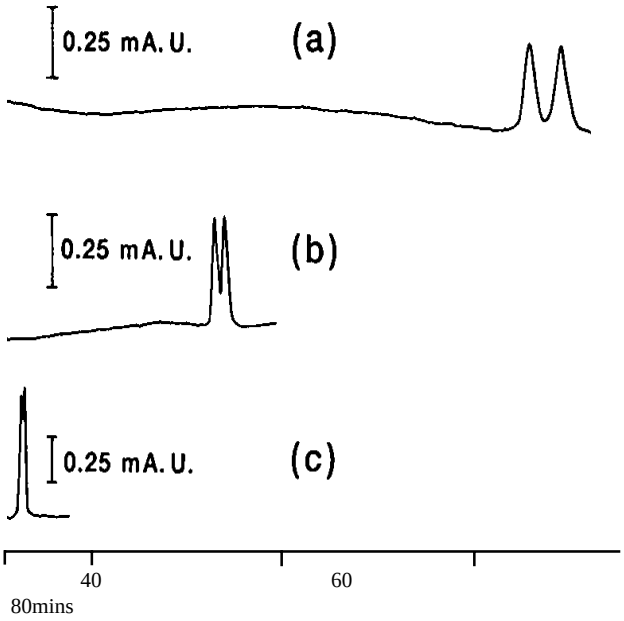


Figure 16. *3µm ODS, 50µm I.D. capillary, 25.9cms packed, 52cms total length, Electrolyte: Acetonitrile/Phosphate buffer pH=6.5, Ratio a) 15:85, b) 20:80, c) 25:75, with 10mM HPβ CD (1mM Na_2HPO_4 , adjusted to pH=6.5 with concentrated H_3PO_4), Applied Voltage 15kV, Detection at 220nm. Reprinted from Journal of Chromatography A, 723 (F. Lelièvre, C. Yan, R.N. Zare and P. Gareil) 145-156 (1996) with kind permission of Elservier Science-NL, Sara Burgerhartsraat 25, 1055 KV Amsterdam, The Netherlands.*

When chlorthalidone was analysed in an achiral environment on Spherisorb ODS-1, a mobile phase consisting of CH_3CN-1mM phosphate buffer pH 6.5 (20:80 v/v) resulted in a k' of 4.4. With this in mind, chiral resolution was attempted with 10mM hydroxypropyl β-cyclodextrin in CH_3CN-1mM phosphate buffer pH 6.5 mixtures ranging from 15-25% acetonitrile. Baseline separation of the enantiomers was

achieved with 15% acetonitrile, although the analysis time was long with the overall run time exceeding 80 minutes. Using this approach, it is important that sufficient equilibration time is allowed, which in this particular example required 15 hours before any separation was noticed and this is attributed to the slow formation of a pseudo-stationary phase whereby the chiral selector dynamically coats the ODS surface. The separations at different acetonitrile concentrations are shown in Figure 17.

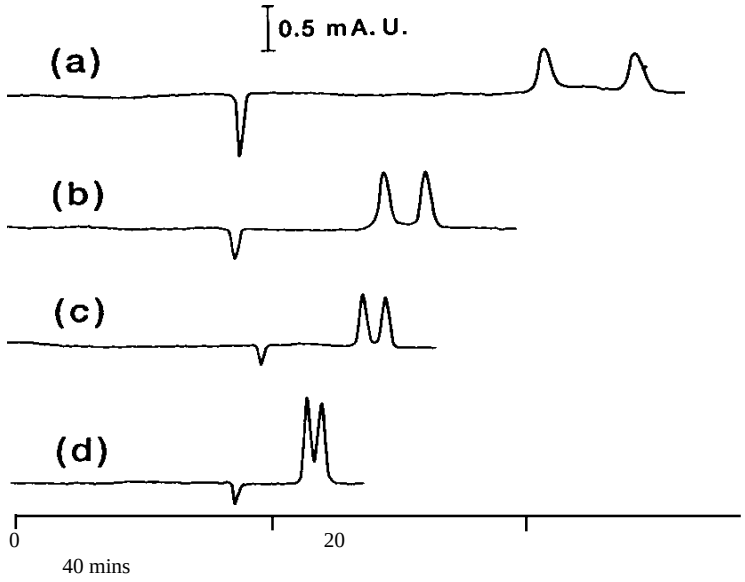


Figure 17. *5µm HPβ CD bonded silica particles, 50µm I.D. capillary, 27cms packed, 58 cms total length, Electrolyte: Acetonitrile/Phosphate buffer pH=6.5, Ratio a) 15:85, b) 20:80, c) 25:75, d) 30:70 (5mM Na_2HPO_4 adjusted to pH=6.5 with concentrated H_3PO_4 , Applied Voltage 15kV, Detection at 220nm. Reprinted from Journal of Chromatography A, 723 (F. Lelièvre, C. Yan, R.N. Zare and P. Gareil) 145-156 (1996) with kind permission of Elservier Science-NL, Sara Burgerhartsraat 25, 1055 KV Amsterdam, The Netherlands.*

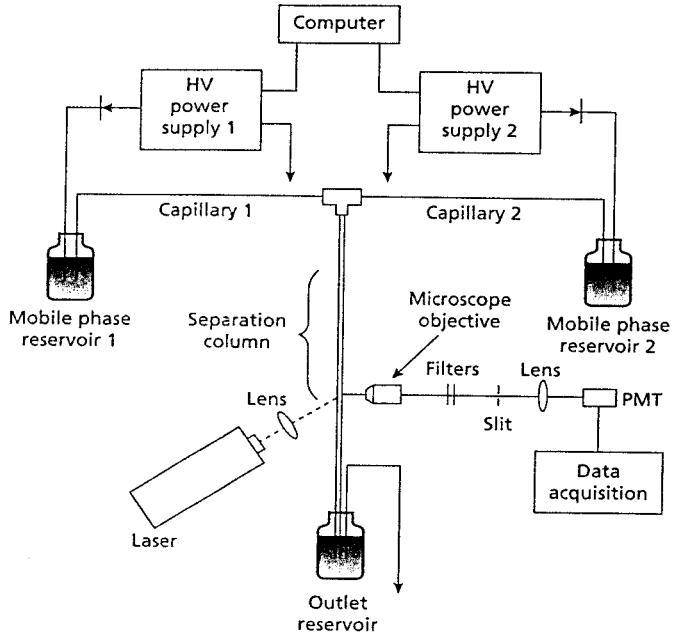
Although the chiral stationary phase produced the best resolution and lowest analysis time because of increased selectivity, the efficiency using the chiral additive was almost double that of the chiral stationary phase.

9. Gradient Elution

The use of capillary electrochromatography with gradient elution has been reported by several workers [31-33]. Beyer and Behnke [32] described the coupling of a microbore gradient HPLC system to a modular capillary electrophoresis system. A six-port rotary valve was used for injection and the eluent from the micro-pump was passed through a stainless steel T-piece in order to split the flow. As a precaution, this splitter was earthed to prevent any voltage leaking back and causing damage to the equipment. According to the authors, pressurisation provided stability and reproducibility of the separations and also prevented bubble formation. Among the examples shown was the high efficiency separation of oligonucleotides (dC_3-dC_{11}) using a gradient of 100% 10mM ammonium acetate pH 8 to 10% acetonitrile:10mM ammonium acetate pH8 over 30 minutes. The separation gave a dramatic improvement over the corresponding micro-HPLC separation.

Taylor *et al* [33] described the analysis of drugs in equine biofluids using gradient elution CEC with esi mass-spec detection. The CEC sampling interface was constructed from a 1/16" i.d. stainless steel tee-piece connected to the power supply of the electrophoresis instrument with a 50kV rated cable. The CEC capillary was then aligned with a loading capillary that was connected to the injection valve of the HPLC instrument. This alignment capillary was cut to the necessary length depending on how much excess liquid was required to pass coaxially past the CEC capillary onto the restriction capillary, which served to keep the interface pressurised. Examples analysed included a mixture of seven thiazide diuretics and equine plasma samples spiked with hydrocortisone. The mobile phase used consisted of 5mM ammonium acetate in acetonitrile:water with an acetonitrile gradient. In another paper [34], Taylor *et al* used the same interface to analyse corticosteroids in biofluids. In order to prolong the life of capillaries, sample clean-up was essential. This clean up involved two stages, a two-stage solid phase extraction in order to purify the equine urine and thus prevent contamination of the column, followed by a dialysis method used to deproteinate equine plasma. Using this system, Taylor was able to make approximately 200 injections of urine extracts and still maintain sufficient efficiency even though there was a 40% increase in peak width at half height. Zare and co-workers [13] reported on the construction of a gradient CEC system whereby the gradient was formed by controlling the electroosmotic flow from two separate solvent reservoirs by the use of two high voltage power supplies. A schematic diagram is shown below.

Gradient System

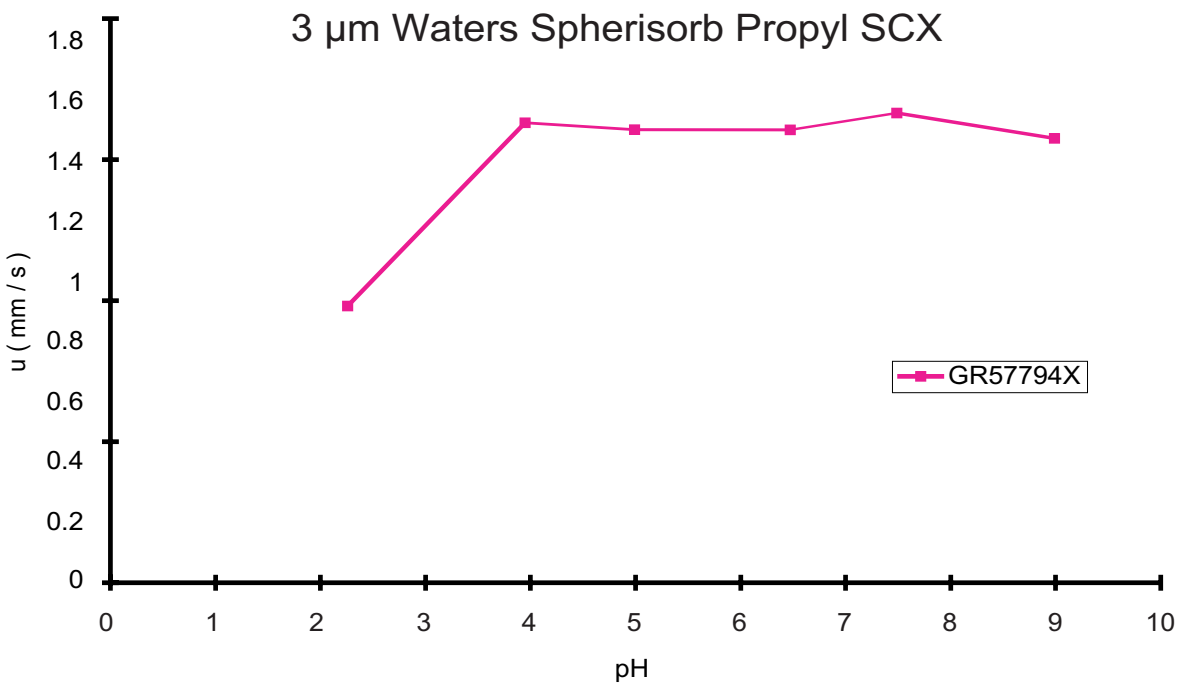


Schematic of the solvent gradient elution CEC apparatus. Reproduced by permission of publishers, reference 13. First published in LC-GC International, Vol. 10, Number 3 (1997).

These two solvents were connected to the separation capillary via a low dead volume tee, and the required gradient was formed using a computer program that controlled the voltages from the two power supplies and consequently the overall composition of the eluent. Samples were introduced by removing the packed capillary from the tee piece and placing its inlet into a sample vial, whereby the sample would be introduced into the capillary by the application of a small voltage. Using gradient elution, the author was able to resolve 16 PAH's using a gradient from 55% acetonitrile to 80% acetonitrile in aqueous buffer using a 26cm capillary packed with 3μm porous ODS stationary phase.

10. Future Developments

The biggest obstacle to the development of CEC is the inability to analyse basic compounds, since many Pharmaceutical compounds contain basic nitrogen groups. What is also important is that a stationary phase should exhibit a good EOF across a wide pH range. With this in mind, a phase was produced by Professor Peter Myers of PhaseSeparations that contained a $-SO_3H$ group attached to 3µm porous silica via a propyl linker. A plot of linear velocity u *vs* pH showed a very good profile across the pH range 4-9, dropping off by ~ 50% at pH 2, as shown below.



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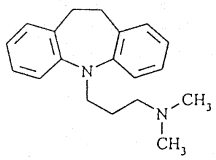
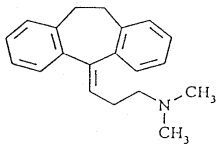
Tricyclic antidepressants are notoriously difficult to analyse by HPLC because they have very high pKA's and consequently give very poor peak shapes due to analyte: silanol interactions. It should be noted that thiourea is a poor EOF marker being strongly retained on this phase. The compound GR57994X is in fact a much better example of an EOF marker for SCX phases. The structures of these compounds are shown on the following page along with the neutral compound bendroflumethiazide.

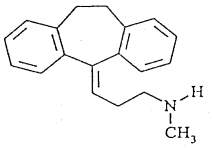
Structure of Antidepressants



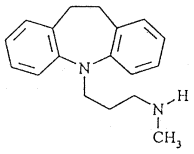
Imipramine



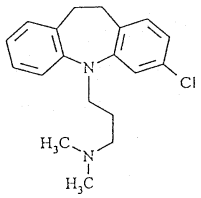
Amitrptyline



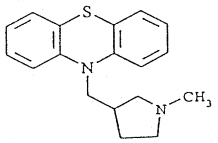
Nortriptyline



Desipramine



Clomipramine



Methdilazine

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When a test mixture containing three of these highly basic compounds were analysed along with bendroflumethiazide [35], the chromatogram shown in Figure 18 was produced.

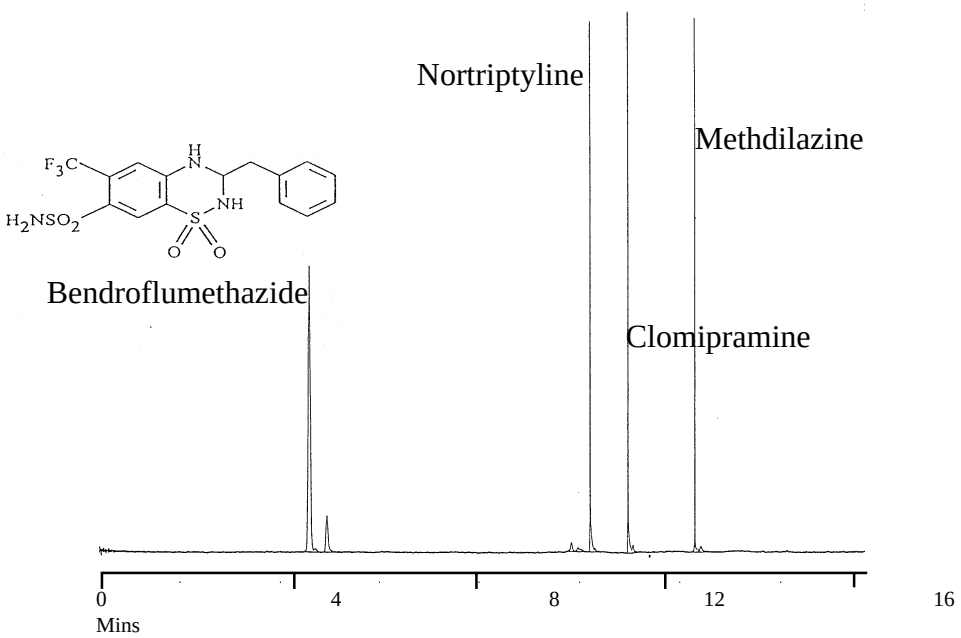

Figure 18. *3µm Spherisorb SCX , Mobile phase: 70% Acetonitrile / 30% (0.05M NaH_2PO_4 pH 2.3), Applied voltage: 30kV, Temperature 30°C, Injection: 2kV/0.5min, Detection at 220nm. Reproduced by permission of publishers, Ref. 35.*

One striking feature of this chromatogram is the amazingly sharp peaks. However, closer examination reveals the strange phenomenon of peaks that elute early actually being broader than those that are more retained, defying standard chromatographic theory. In fact the basic compounds are focused by what is yet an unexplained mechanism, and the downside to this type of stationary phase is that despite being able to separate very difficult basic compounds with amazingly sharp peaks, the overall reliability and reproducibility is poor, rendering this type of phase unsuitable for routine use in CEC. However, once a better understanding of the focusing effect is understood it should be possible to design stationary phases with the desired properties that would make them suitable for use as CEC phases, allowing the highly efficient analysis of acidic, neutral and basic compounds.

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12. Appendix

Listed below are some recommendations for the analysis of groups of compounds using a Beckman P/ACE MDQ instrument, although the operating conditions are applicable to all suitable CEC instruments.

Neutral Compounds

For the routine analysis of **neutral compounds** the following conditions offer a good starting point for method development.

Capillary dimensions:	**20cm packed; 30cm total length**
Packing material:	**3μm reversed-phase material, either C_8 or C_{18} mixed-mode**
Mobile phase:	**70% Acetonitrile/30% 10mM Tris pH 9.0**
Temperature:	**30^0C**
Applied voltage:	**30kV**
Wavelength:	**Sample dependent but 210nm is often a good compromise.**

These conditions will usually produce fast chromatography which can then be optimised in the usual chromatographic way i.e.

By altering the type and/or concentration of organic solvent.

By adjusting the pH of the buffer.

By changing the concentration and type of buffer.

By adjusting the voltage and temperature.

In order to analyse anions (often in the presence of neutral species) it is usually necessary to operate at low pH in order to suppress the ionisation of the acid. This can cause problems with some conventional HPLC phases since the eof can drop dramatically below ~ pH 5. For this reason it is advisable to perform such analyses using one of the newer stationary phases developed specifically for CEC such as the C_6/SCX or C_{18}/SCX mixed-mode phases, both of which have a good eof even as low as pH 2.0 (see page 16).

Anionic Compounds
(in the present of Neutrals)

For the routine analysis of anionic compounds (in the presence of neutral compounds) the following conditions offer a good starting point for method development.

Capillary dimensions:	**20cm packed 30cm total length**
Packing material:	**3μm reversed-phase material, either a C_6/SCX or C_{18}/SCX mixed-mode**
Mobile phase:	**70% Acetonitrile/30% 10mM NaH_2PO_4 at pH ~3.0**
Temperature:	**30^0C**
Applied voltage:	**30kV**
Wavelength:	**Sample dependent but 210nm is often a good compromise.**

Basic compounds are much more difficult to analyse by CEC. Packing materials that are quite capable of analysing bases by HPLC have usually been deactivated or synthesised in a way that shields the troublesome surface silanol groups.

Unfortunately it is these silanol groups that provide the driving force for the mobile phase and if their concentration is low, or they are unavailable, then the eof will be greatly reduced resulting in long analysis times. In addition to long analysis times, highly basic compounds have been found to tail badly on conventional HPLC phases and produce highly non-reproducible chromatography. Depending on the sample, some basic compounds have been successfully analysed on a strong cation exchanger, but in order for bases to be analysed routinely by CEC requires the development of a range of stationary phases produced specifically for electrochromatography.

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