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Journal of Chromatography A, 892 (2000) 219–255

JOURNAL OF CHROMATOGRAPHY A

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Review

Electrochromatography

N.W. Smith[a,*], A.S. Carter-Finch[b]

[a]*Imperial College of Science, Technology and Medicine, Zeneca/SKB Centre for Analytical Sciences, Department of Chemistry, South Kensington, London SW7 2AY, UK*

[b]*Innovatech Ltd., Unit 5K/L, Business and Technology Centre, Bessemer Drive, Stevenage, Herts. SG1 2DX, UK*

Abstract

Although Mould and Synge were credited with the first demonstration of the use of electroosmotic flow to drive a liquid in a thin-layer chromatography system, it was the work of Knox and Grant that extensively detailed much of both the theoretical and practical implications of capillary electrochromatography. This review is aimed at giving a very broad outline of the progression of this technique since the late 1980s up to the present time, including some of the current limitations. Examples will be given of the analysis of a wide variety of materials including neutral, acidic, basic and chiral pharmaceutical compounds, along with some of the instrumentation used for this technique. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Electrochromatography; Chiral stationary phases, electrochromatography; Electroosmotic flow; Non-aqueous capillary electrochromatography

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1. Introduction

Capillary electrochromatography (CEC) is a technique that combines the advantages of capillary zone electrophoresis (CZE) with those of high-performance liquid chromatography (HPLC). Electroosmotic flow (EOF) is used to propel the mobile phase through a packed capillary, typically 50–150 μm internal diameter. Although Mould and Synge [1,2] were credited with the first demonstration of the use

*Corresponding author. Tel.: +44-171-5945-841; fax: +44-171-5945-833.

E-mail address: n.w.smith@ic.ac.uk (N.W. Smith).

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PII: S0021-9673(00)00470-2

of electroosmotic flow to drive a liquid in a thin-layer chromatography system, it was Pretorius et al. [3] who demonstrated the possibility of electrically pumping a mobile phase through a column packed with an adsorbent. In 1981, Jorgenson and Lukacs [4] demonstrated the separation of 9-methylanthracene from perylene using a 170 μm I.D. capillary packed with 10 μm reversed-phase packing material, and achieved efficiencies of 31 000 plates for a packed bed of 58 cm. The ability to use small particles and still generate sufficient electroosmotic flow was demonstrated by the pioneering work of Knox and Grant [5], who obtained efficiencies using 5 μm Hypersil ODS of 69 000 plates on a drawn packed capillary, 60 cm long.

The linear flow through a packed capillary under the influence of an electric field is given by the Helmholtz–Smoluchowski equation as shown in Eq. (1):

$$u = \frac{\epsilon_0 \epsilon_r E \zeta}{\eta} \quad (1)$$

where ϵ_r and ϵ_0 are the relative and vacuum permitivities, respectively and: E = applied electric field; ζ = the zeta potential and η = the viscosity of the solvent.

The important feature of this equation is that it implies that the flow through a packed capillary has no dependence on particle size, therefore paving the way for the use of very small particles. The only limitation to the use of small particles in CEC is the avoidance of double layer overlap as described by Knox and Grant [5,6]. They were able to demonstrate that there was no reduction in electroosmotic flow using particles down to 1.5 μm in diameter. Using both 3 and 5 μm material, they were able to demonstrate that electrically driven chromatography produced superior efficiencies when compared to the pressure driven alternative. Dittmann et al. [7] showed that a reduction in Eddy diffusion in CEC compared to high-performance liquid chromatography (HPLC), produced a lower minima in the van Deemter plot resulting in smaller height equivalent to a theoretical plate (HETP) values. Although the theoretical understanding of CEC increased tremendously during the late 1980s and early 1990s, all reports on CEC used 3–10 μm stationary phases, almost exclusively of the type used in HPLC. The dependence of the EOF on the properties of the stationary phase is given by the following equation:

$$u = \frac{1}{\kappa} \cdot \frac{\sigma E}{\eta} \quad (2)$$

where $1/\kappa$ = the thickness of the double layer and σ = charge density.

This equation highlights the fact that the EOF has a dependence on the surface charge density which for most common HPLC stationary phases will depend upon the number of silanol groups present, their degree of ionisation, the surface area of the particles and possibly, the nature of the ligand bonded to these particles.

This review will illustrate some of the major developments in the progress of CEC from the very early reports through to the present time, showing how as expertise grew, highly efficient separations have been performed for a wide variety of compounds using a whole range of stationary phases and conditions, including non-aqueous and gradient elution.

2. Experimental

Much of the data produced in this report were produced by the authors using the following equipment: a modified ABI 270A CZE instrument (Foster City, CA, USA), a modified Unicam Lauerlabs Prince CZE instrument (Cambridge, UK) and a Hewlett-Packard HP^{3D} CE instrument (Waldbronn, Germany).

In their paper [6], Knox and Grant pointed out that bubble formation due to self-heating could lead to catastrophic effects, in particular a breakdown in current due to dry spots in the packed bed. However, subsequent research in our laboratory and others [7,10,12] has shown that the formation of bubbles by self-heating is unlikely under typical CEC conditions, and that they in fact formed in the outlet section of capillary. This is due to the fact that there is an increase in EOF on going from the packed region into the open part of the capillary, and this is readily observed under a microscope. Therefore, in

order to perform routine CEC, it is necessary to solve the problem of bubble formation, and in the same paper, Knox recommended that the whole column should be operated under pressure. A schematic of the layout of the modification performed in our laboratory on the ABI and Unicam Instruments is shown in Fig. 1.

The components of the pressurisation vessels are shown in Fig. 2 and comprise of two Omnifit preparative chromatography glass columns, 17 mm I.D. Although the pressure relief valve in Fig. 1 was designed to open at 500 p.s.i., an operating pressure of 100–200 p.s.i. was found to be sufficient to prevent bubble formation (1 p.s.i. = 6894.76 Pa).

The Hewlett-Packard instrument was capable of running at pressures up to 12 bar and had a 48-position autosampler.

Capillaries were either packed in the laboratory or purchased from Innovatech (Stevenage, UK) and were packed with stationary phases supplied by Phase Separations (Clwyd, UK). HPLC-grade methanol and acetonitrile (ACN) were purchased from BDH (Poole, UK). The components of the neutral test mixture, thiourea, dimethyl phthalate, anisole and naphthalene were also purchased from BDH. Amitriptyline, clomipramine, imipramine and nortriptyline were purchased from Sigma (Poole, UK), whilst the compounds carbovir, ranitidine, ondansetron, fluticasone propionate and other Glaxo Research compounds were supplied by GlaxoWellcome Research and Development (Stevenage, UK). If no pH adjustment was necessary, mobile phases were prepared simply by mixing the appropriate volumes of organic solvent and buffer. When pH adjustment was required, the pH of the buffers was altered before mixing with organic solvent.

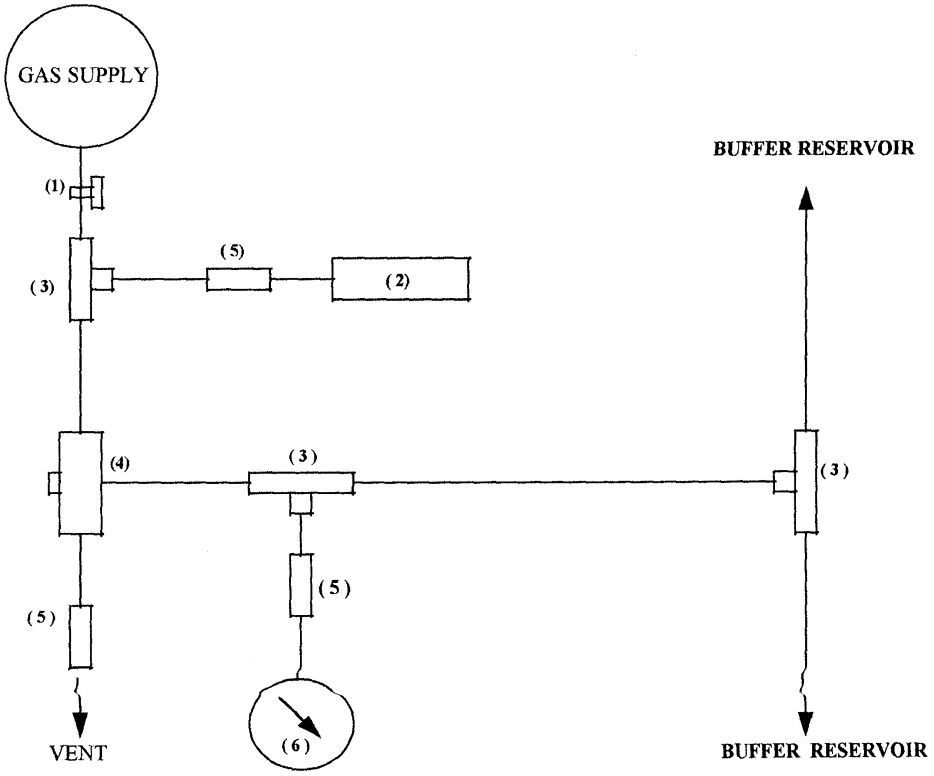


Fig. 1. Schematic layout of pressurisation system. 1 = Shut-off valve; 2 = pressure relief valve; 3 = 1/8 in. tees; 4 = three-way valve; 5 = adapters; 6 = pressure gauge.

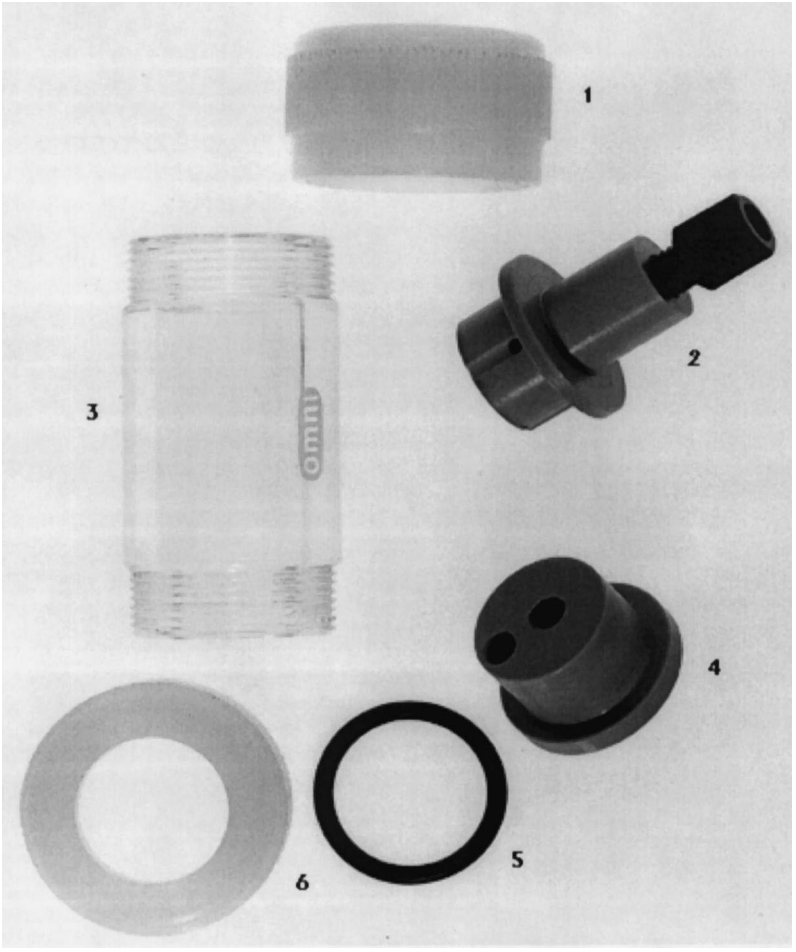


Fig. 2. Pressurisation vessel. Components: 1=Tefzel end cap (top); 2=Kel-F bottom fitting; 3=glass column (50 mm×17 mm I.D.); 4=Kel-F top fitting; 5=O-ring; 6=Tefzel end cap (bottom).

3. Results and discussion

3.1. Neutral compounds

In 1992, Yamamoto et al. [8] showed the high-resolution separation of benzene derivatives on 3 μm Hypersil ODS, achieving reduced plate heights of between 1.8 and 2.2 for thiourea and benzene derivatives, and this is shown in Fig. 3.

They observed that plate numbers decreased as the EOF increased and this was attributed to band broadening caused by heating. This was evident when they plotted the linear velocity against applied voltage and plate number and found that efficiency began to decrease at linear velocities above 3 mm/s, i.e., >30 kV. In 1994, Smith and Evans [9] published their results and described the packing procedure used to produce highly stable and efficient CEC capillaries. When the steroid fluticasone propionate was analysed by gradient HPLC using ACN–water as the mobile phase, the chromatogram shown in Fig. 4 was obtained. It is important to note that all four related components are neutral and are incapable of becoming charged.

When the same samples were analysed by CEC on the same batch of ODS packing material, the highly efficient separation of the steroid fluticasone propionate from three related impurities was obtained with a

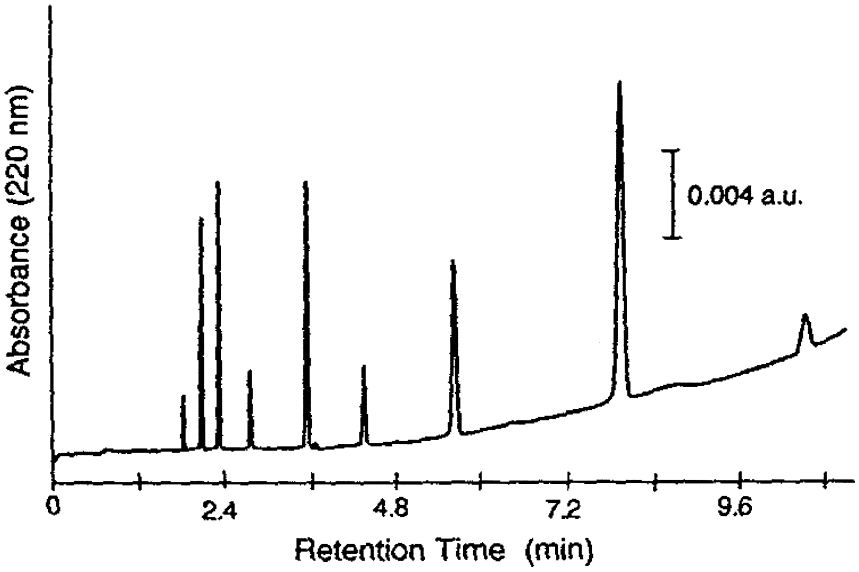


Fig. 3. High-resolution analysis of benzene derivatives. Peaks, from left to right: thiourea [number of plates, $N=46\,000$, reduced plate height (h)=2.0], benzyl alcohol ($N=54\,000$, $h=1.8$), benzaldehyde ($N=56\,000$, $h=1.7$), benzene ($N=47\,000$, $h=2.0$), 1,2-dichlorobenzene ($N=54\,000$, $h=1.8$), 1,2,3-trichlorobenzene ($N=52\,000$, $h=1.8$), 1,2,3,4-tetrachlorobenzene ($N=49\,000$, $h=2.0$), pentachlorobenzene ($N=43\,000$, $h=2.2$), and hexachlorobenzene ($N=43\,000$, $h=2.2$). Capillary, 285 mm; packing, Hypersil ODS (3 μm); mobile phase, 2 m*M* sodium tetraborate–acetonitrile (20:80, v/v); applied voltage, 45 kV (current 2.0 μA); sampling, 2.5 kV for 5 s. F_{OS} (electroosmotic flow)=2.6 mm/s. From Ref. [8] with permission ©.

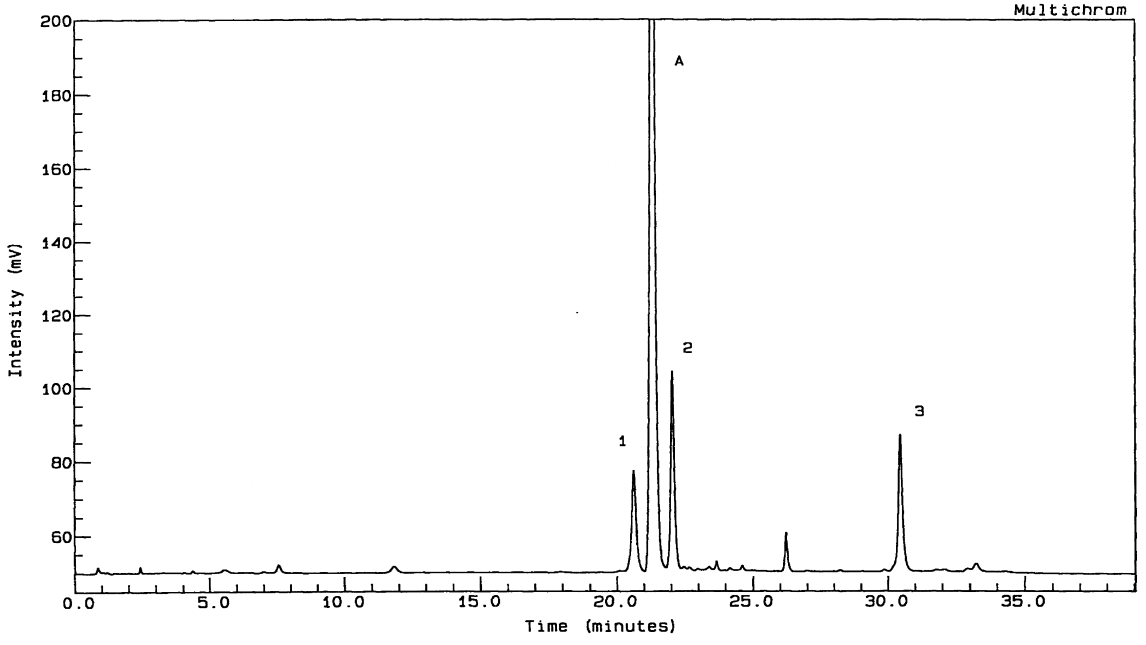


Fig. 4. Gradient HPLC separation. One, two and three closely-related impurities of the steroid fluticasone propionate (A). Column: 15 cm×4.6 mm I.D. packed with 3 μm Spherisorb ODS-1, batch 93/071. Flow: 1.0 ml/min, detection at 238 nm, range: 0.05 AUFS. Gradient: ACN–water (40:60)→ACN–water (70:30) in 20 min, exponent 5.

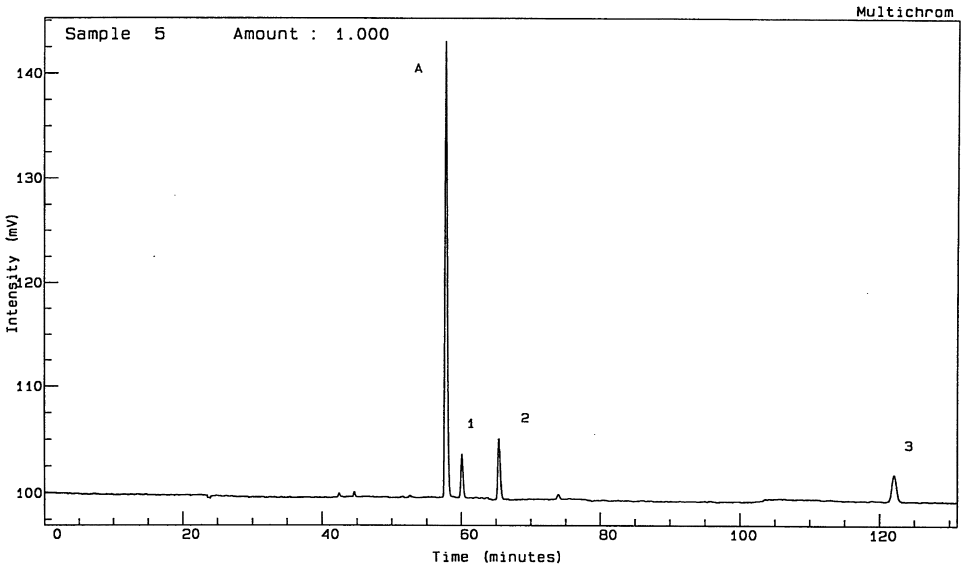


Fig. 5. Instrument: Unicam Prince 40 cm×50 μm I.D. capillary packed with 3 μm Spherisorb ODS-1, batch 93/071. Detection at 238 nm, range 0.05 AUFS. Mobile phase: ACN–water, 2 m*M* Na_2HPO_4 (70:30, v/v) adjusted to pH 8.3. Peak A fluticasone propionate peaks 1, 2 and 3 related impurities. From Ref. [9] with permission ©.

reduced plate height of 0.9, and this is shown in Fig. 5.

It is apparent that there has been a change in the elution order for peaks 1 and 2 despite the fact that these components are neutral and would therefore not be expected to be influenced by any electrophoretic effect. In order to try and resolve this issue, a new test mixture containing only components 1 and

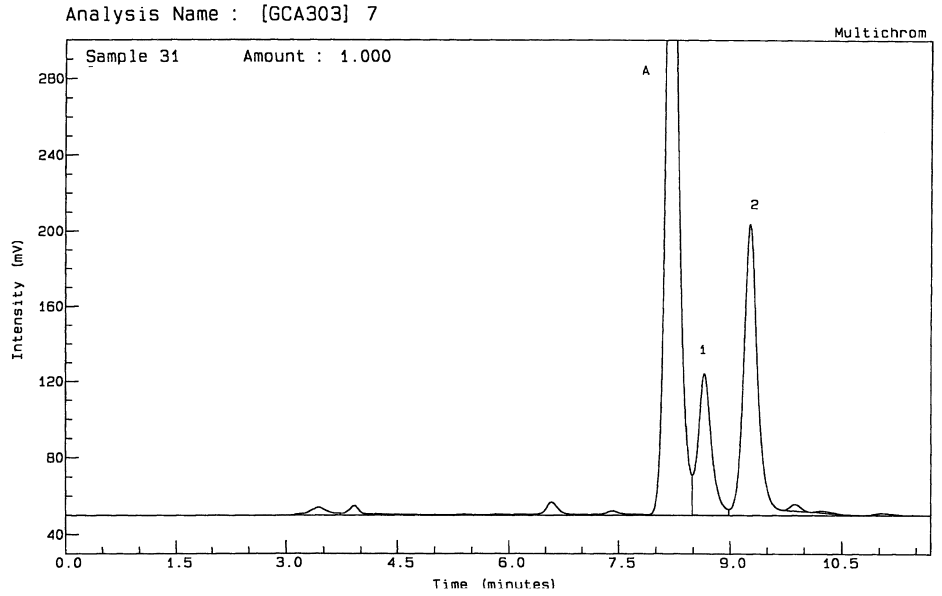


Fig. 6. Modified HPLC method. Column: 15 cm×4.6 mm I.D. packed with 3 μm Spherisorb ODS-1, batch 93/071. Flow: 0.2 ml/min, detection at 238 nm, range: 0.05 AUFS. Mobile phase: ACN–water, 2 m*M* Na_2HPO_4 (70:30, v/v) adjusted to pH 7.0. Peak A fluticasone propionate peaks 1 and 2 related impurities.

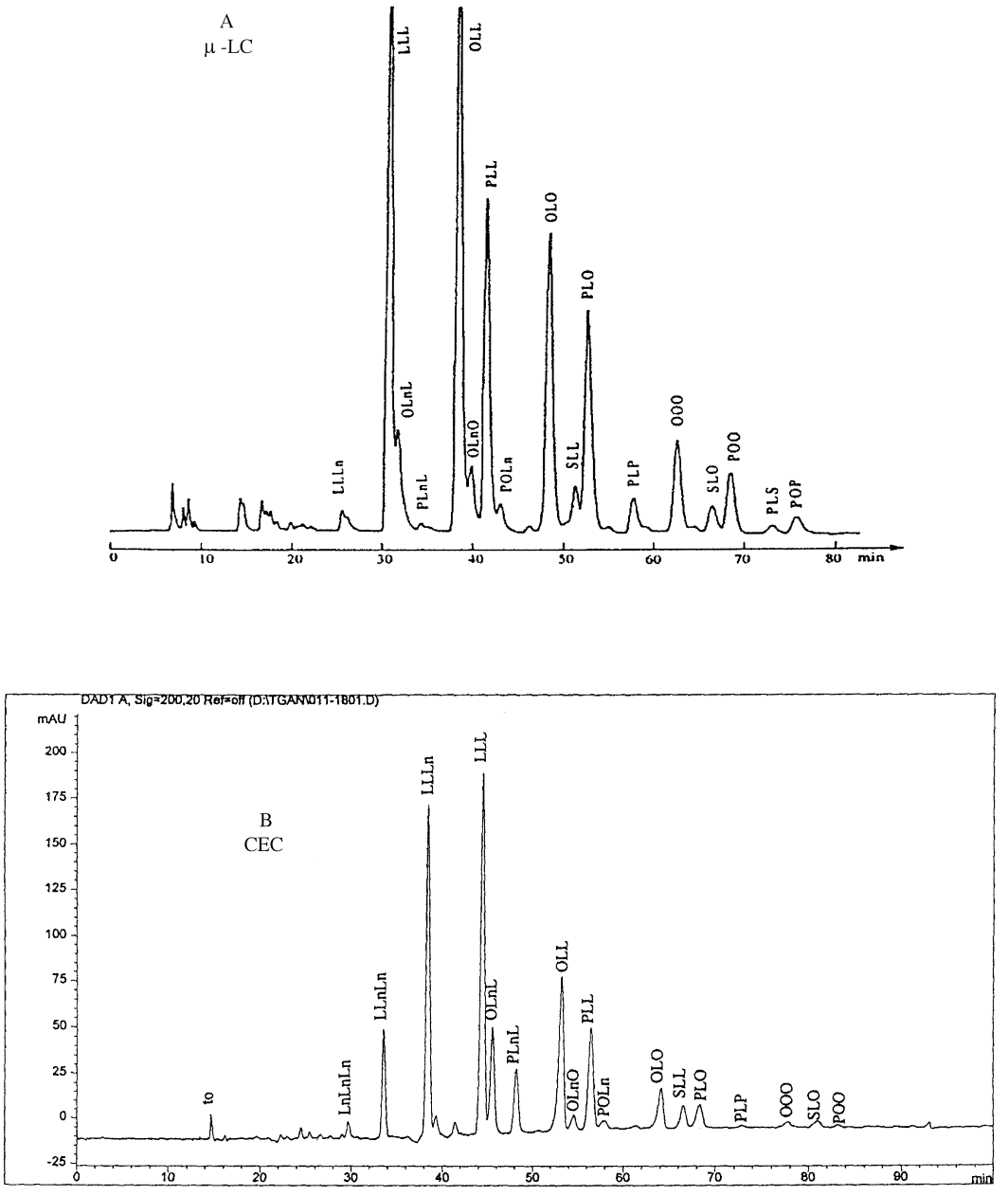


Fig. 7. (A) Triglyceride analysis of corn oil by μ-HPLC. Column: 2×25 cm×1 mm I.D. stainless steel Biosil C_{18} HL, 5 μm. Mobile phase: ACN–IPA–*n*-hexane (57:38:5, v/v) at 55 μl/min. Detection: evaporative light-scattering detection (ELSD) (Sedex 45) at 50°C, 1.8 bar nitrogen range 4. Temperature 20°C. (B) Triglyceride analysis of corn oil by CEC. Column: 40 cm×0.1 mm I.D. fused-silica open tubular (FSOT), Hypersil ODS, 3 μm. Mobile phase: ACN–IPA–*n*-hexane (57:38:5, v/v) with 50 m*M* ammonium acetate. Detection: UV at 200 nm. Temperature 20°C. Voltage 30 kV. Injection 10 kV during 3 s. From Ref. [10] with permission ©.

2 was prepared, and this was electrically injected into a capillary packed with the same batch of ODS material, and then eluted under pressure using the same mobile phase as was used for the CEC experiment. Peaks 1 and 2 now eluted in the same order, suggesting that the change in selectivity is probably due to the different mobile phases used for HPLC and CEC. When the mobile phase used for CEC was used as the solvent in the HPLC experiment, the results obtained confirmed that the mobile phase appeared to be having an effect on the ionisation of the silanol groups which in turn effects the selectivity as shown in Fig. 6.

A comparison of Figs. 5 and 6 highlights the increase in efficiency that can be gained as a result of the plug flow profile generated by the electrochromatography system compared to the parabolic flow profile of the pressure-driven system.

Sandra et al. [10] showed the highly efficient separation of triglycerides in vegetable oils, and found CEC to give far better resolution than LC. In their study, CEC was performed simply by adding 50 m*M* ammonium acetate to the mobile phase used for the LC separation, and this was claimed to be the first practical application using a non-aqueous mobile phase for CEC. Using a mobile phase containing ACN–isopropanol (IPA)–*n*-hexane (57:38:5) with 50 m*M* ammonium acetate, they were able to separate the constituents of corn oil with a reduced plate height of 1.88 for the triglyceride POP. A comparison of the separation by micro-LC (μ-LC) and CEC is shown in Figs. 7A and B.

Roed et al. [11] reported the CEC separation of retinyl esters using a 180 μm I.D. capillary. Because the esters are very hydrophobic in nature, it was necessary to use non-aqueous conditions. Lithium acetate was found to give the highest EOF. Acetonitrile was abandoned as the mobile phase because of problems with the capillary coating. Methanol however gave significantly lower efficiencies and because retinyl esters have a very limited solubility, was also ruled out as a mobile phase. *N*,*N*-dimethylacetamide (DMA) and *N*,*N*-dimethylformamide (DMF) both gave significantly higher efficiencies

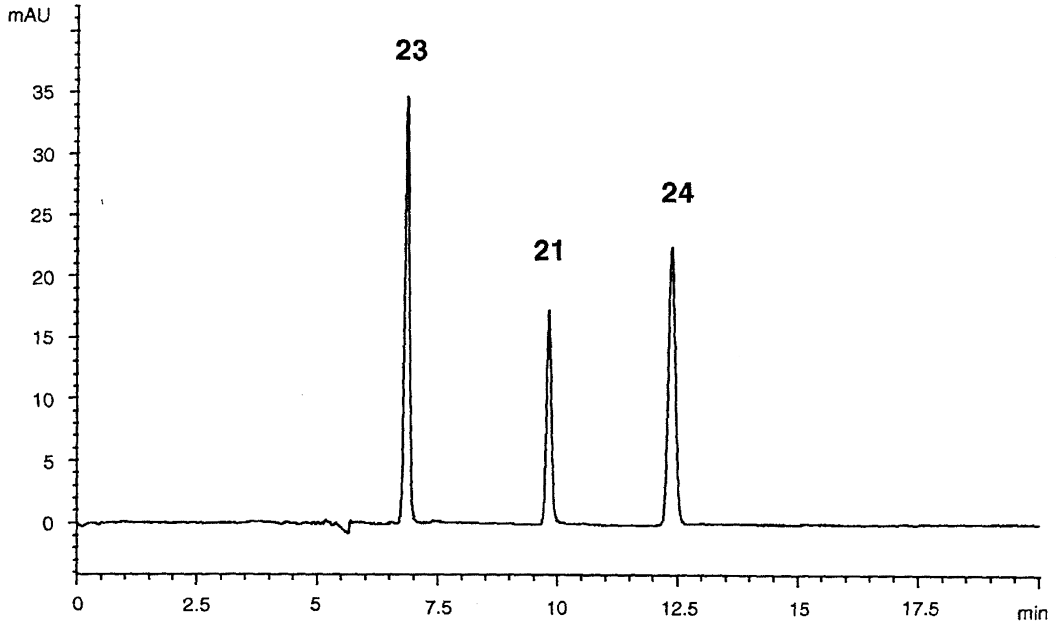


Fig. 8. Separation of acidic test mixture at pH 2.5. Efficiency values of 97 000 (*p*-hydroxybenzoic acid, 23), 174 000 (bumetanide, 21), and 155 000 (flurbiprofen, 24) plates per meter were obtained. Detection wavelength 230 nm with a 10 nm bandwidth and a 1 s rise time. Electrochromatography was performed on a pressurised CE system using a 230 mm×50 μm I.D., 3 μm, CEC Hypersil C_{18} packed capillary (kindly supplied by Hypersil). ACN–Na_2HPO_4 (50 mmol/l, pH 2.5) buffer–water (40:20:40, v/v), applied voltage of 30 kV, capillary temperature of 15°C, and an electrokinetic injection of 5 kV/15 s. Analyte concentration 0.2 mg/ml in acetonitrile–water (50:50, v/v). From Ref. [12] with permission ©.

than methanol, but because the retention factors were too low in DMA to obtain satisfactory resolution, this was substituted for DMF. Although retinyl esters were soluble in DMF, they had limited solubility in methanol. However, the addition of small amounts of methanol to the mobile phase was necessary when lithium acetate was used as electrolyte. However, the concentration of electrolyte had to be reduced to 2.5 m*M* when using a 180 μm I.D. capillary due to unacceptable Joule heating. The best performance was obtained using a 180 μm I.D. capillary packed with 3 μm Hypersil ODS using a mobile phase of 2.5 m*M* lithium acetate in DMF–methanol (99:1, v/v). Using these conditions at 40°C, they were able to analyse liver extracts from arctic polar foxes and seals and found that whereas the seals contained the

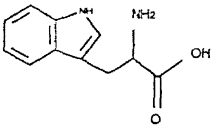
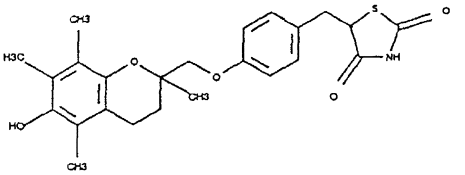
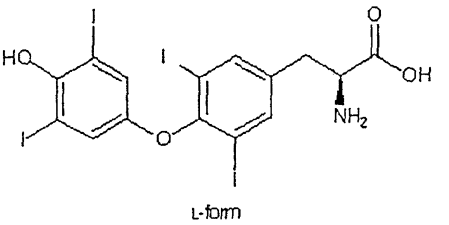
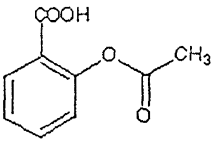
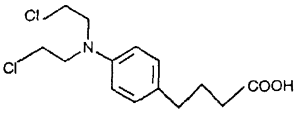
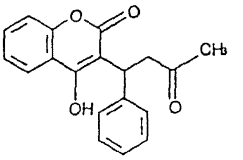
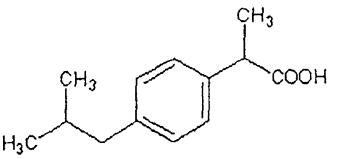
Pharmaceutical	pKa	Use	Structure
Tryptophan	pK_1 2.83 pK_2 9.39	Treatment of neuropsychiatric disorders	
Troglitazone	pK_1 6.1 pK_2 12	Anti-diabetic	
Thyroxine acid	n/d	Thyroid hormone	 L-form
Acetylsalicylic acid (aspirin)	pKa 3.49	Analgesic, anti-inflammatory	
Chorambucil	n/d	Antineoplastic	
Warfarin	n/d	Anti-coagulant	
Ibuprofen	n/d	Analgesic, anti-inflammatory	

Fig. 9. Structures of acidic pharmaceuticals. From Ref. [13] with permission ©.

retinyl ester RC_{16} as the main component, it was only a minor component in the polar foxes.

3.2. Acidic compounds

Because the EOF for silica-based packings tends to drop at low pH, CEC is usually performed at high pH in order to maximise the EOF. However, under these conditions anionic compounds will migrate towards the anode and will therefore not be detected. Euerby et al. [12] confirmed that when an acidic test mixture was analysed under conditions of high EOF, i.e., ACN–Tris buffer, pH 7.8–water (70:20:10, v/v), these compounds were not eluted since their mobilities were sufficiently great at pH 7.8 to cause them to migrate towards the anode. However, using ion-suppression, they were able to elute all three acidic compounds by running in a pH 2.5 buffer whereby all of the analytes were present in their protonated forms. Because these compounds are now neutral, they eluted with the EOF, which was surprisingly high at such a low pH. The resulting chromatogram is shown in Fig. 8.

Using the ion-suppressed mode with a short length packed capillary (7 cm) Altria et al. [13] were able to resolve eight acidic drugs using a mobile phase consisting of ACN–MeOH–20 m*M* NaH_2PO_4, pH 1.5 (50:30:20, v/v/v). At this pH, all of the analytes were protonated since this was well below their pK_a values. Even at pH 1.5, 3 μm Spherisorb ODS-1 still has sufficient EOF to elute these materials in under 10 min. Fig. 9 shows the structures of these compounds, while Fig. 10 shows a typical separation of these acids at an applied voltage of only 7 kV.

In another report [14], Euerby et al. reported the effect of stationary phase selectivity on the separation of some weakly acidic barbiturate derivatives, the structures of which are shown in Fig. 11 along with their pK_a values. They observed that the EOF was unaffected by the nature of the bonded phase, but showed that the Hypersil C_8 phase was the most retentive. However, there was little difference in the selectivity between the Hypersil C_8 and Hypersil C_{18} phases. The phenyl phase did however show minor differences in selectivity and this is illustrated in Fig. 12.

When the separation was optimised on all three phases, it was shown that the elution order for all phases was identical. It was also shown that the best chromatography was obtained when the analytes were not in an ionic state since their electrophoretic mobilities opposed the direction of EOF. An example of the optimised separation of the barbiturates in the ion-suppressed mode is shown in Fig. 13.

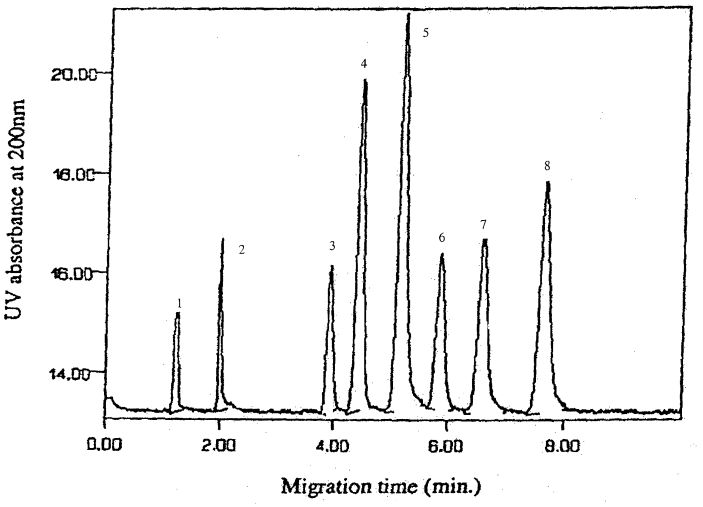
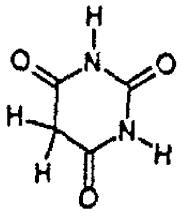
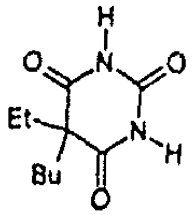


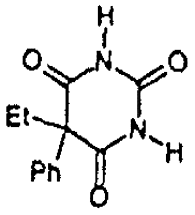
Fig. 10. Separation of eight acidic pharmaceuticals at low pH. Separation conditions: column dimensions 7 cm×50 μm packed length (total length 27 cm), 3 μm ODS, supplied by Capital HPLC, injection: 3 kV for 2 s, separation 7 kV for 15 min at 200 nm, mobile phase ACN–MeOH–phosphate buffer (20 m*M*, pH 1.5 with phosphoric acid) (50:20:30, v/v). Sample dissolved in the mobile phase. 1=Tryptophan; 2=thyroxine acid; 3=uracil; 4=aspirin; 5=salicylic acid; 6=warfarin; 7=chlorambucil; 8=troglitazone. From Ref. [13] with permission ©.



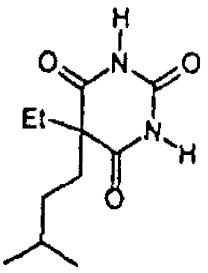
1. Barbital pKa =7.6



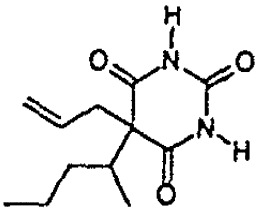
2. Butethal pKa=8.0



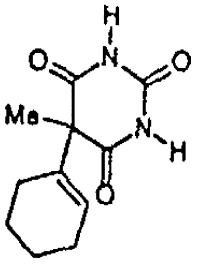
3. Phenobarbital pKa=7.4



4. Amylobarbital pKa=8.0



5. Secobarbital pKa=8.0



6. Hexobarbital pKa=7.6

Fig. 11. Chemical structures and pK_a values of the substituted barbiturates (1–6). From Ref. [14] with permission ©.

3.3. Basic compounds

Basic compounds have been shown to perform poorly by CEC even with stationary phases shown to work well by HPLC. Smith and Evans [15] showed that it was only possible to elute highly basic compounds from 3 μm Spherisorb ODS-1 by lowering the pH to $<\sim 6$, whereby the compounds eluted as their cations, but with extremely poor peak shapes. The highly efficient separation of tricyclic antidepressants using a strong cation-exchange material at pH 2.3 was first reported by Smith and Evans [15], and an example is shown in Fig. 14 where these highly basic compounds are resolved in

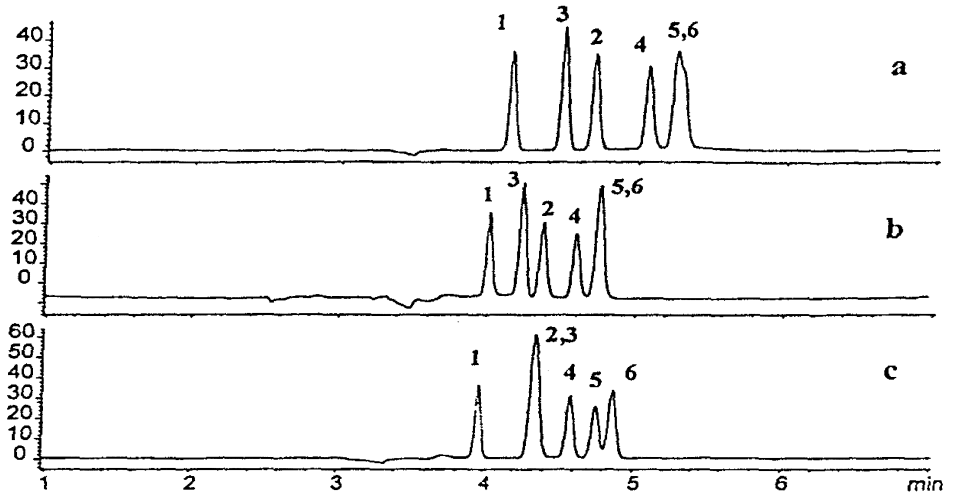


Fig. 12. Chromatograms of the separation of the barbiturates (1–6) on three different packing materials: (a) Hypersil C_8, (b) CEC Hypersil C_{18}, and (c) Hypersil phenyl. The mobile phase was acetonitrile–MES buffer (50 m*M*, pH 6.1)–water (6:2:2, v/v). From Ref. [14] with permission ©.

the presence of the neutral compound bendroflumethiazide. By comparing the EOF on the SCX phase with free solution CZE, it was found that EOF in the packed capillary was ~2.5 times that in the open capillary. However, the tortuosity effect of the packed bed would be expected to reduce the EOF. Since the linear velocity actually increased, this was strong evidence for a considerable EOF at low pH resulting from the $-SO_3H$ group on the cation-exchange phase. A study of the peaks in Fig. 14 showed that the neutral compound bendroflumethiazide was significantly broader than the retained antidepressants despite eluting much earlier.

Clearly some form of analyte focusing or stacking is taking place. However, it was widely accepted that the behaviour of this phase was highly unpredictable and non-reproducible and until a better understanding of the separation mechanism is found, this is

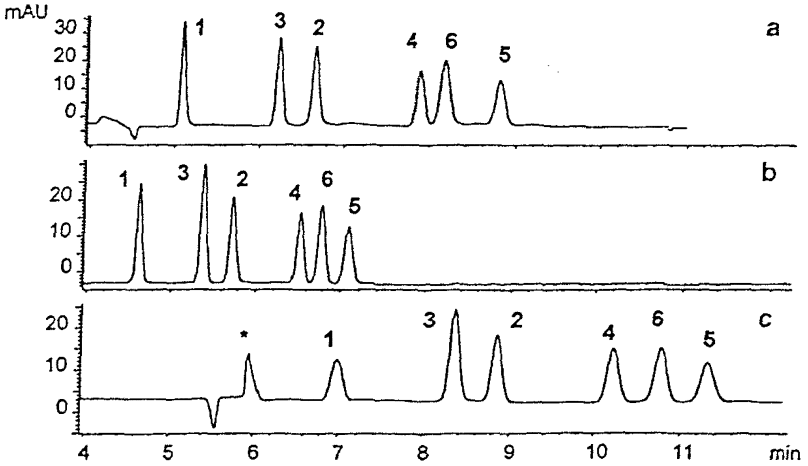


Fig. 13. Chromatograms of the optimised separation of the barbiturates (1–6) on three different packing materials: (a) Hypersil C_8, acetonitrile–MES buffer (50 m*M*, pH 6.1)–water (50:20:30, v/v), 15°C, (b) CEC Hypersil C_{18}, acetonitrile–MES buffer (50 m*M*, pH 6.1)–water (40:20:40, v/v), 15°C and (c) Hypersil phenyl, methanol–phosphate buffer (50 m*M*, pH 4.5)–water (50:20:30, v/v), 60°C. (*) Corresponds to an artefact in the mobile phase. From Ref. [14] with permission ©.

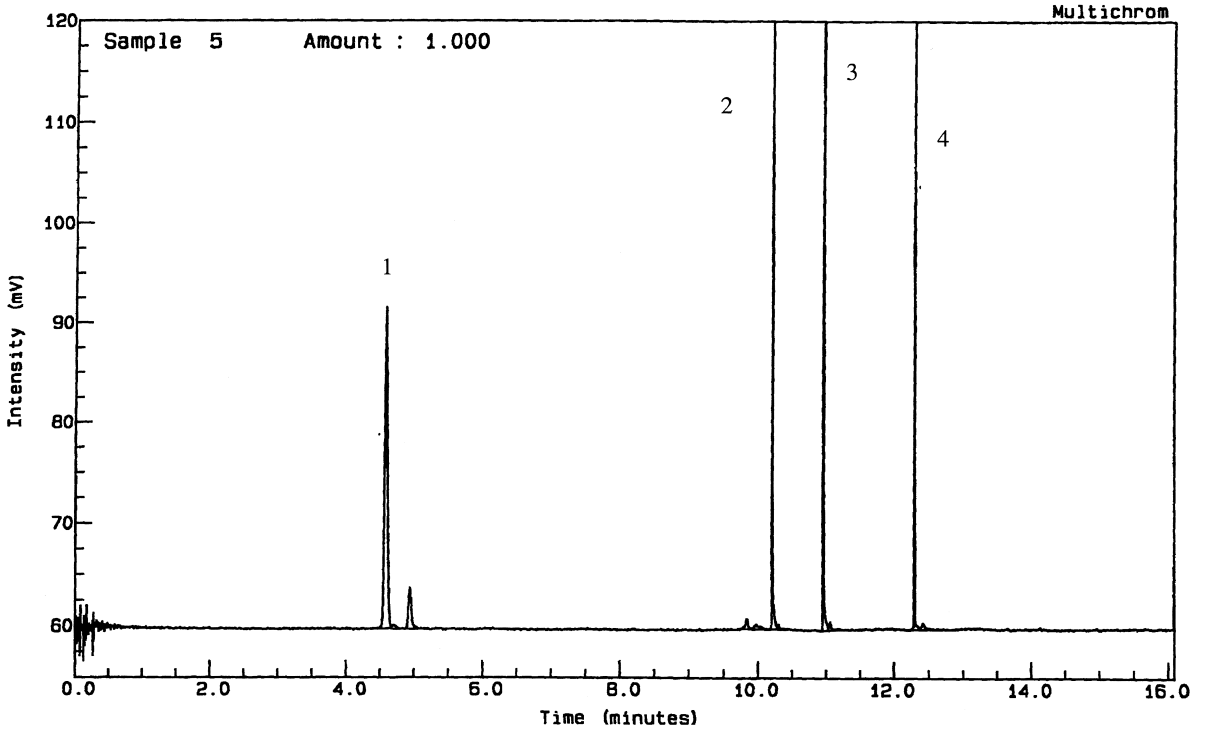


Fig. 14. Instrument: Modified ABI 270A. 3 μm Waters Spherisorb SCX, packed length 26 cm; total length 50 cm; internal diameter 50 μm. Detection at 210 nm, range 0.04 AUFS, applied voltage 30 kV, temperature 30°C, injection 2 kV for 0.5 min. Mobile phase; ACN–0.05 mol/l NaH_2PO_4, pH 2.3 (70:30, v/v). Peaks: 1=bendroflumethiazide; 2=nortriptyline; 3=clomipramine; 4=methdilazine. From Ref. [15] with permission ©.

likely to remain the case. These focused peaks have been the centre of much speculation and an explanation of the likely mechanism is still awaited. Ferguson et al. [16] have made a systematic study of the parameters affecting focusing, including the volume of organic solvent in the mobile phase, buffer concentration, voltage, and the quantity of analyte injected. Similar results have been reported by other workers using different analytes to those reported by Smith and Evans. Euerby et al. [12] reported the very efficient separation of a proprietary basic drug from its synthesis impurity with efficiencies of $\sim 16 \cdot 10^6$ plates per metre. When these same compounds were analysed on a 5 μm SCX column using HPLC at a similar linear velocity, efficiencies of ~60 000 plates per metre were obtained, once again highlighting the increased efficiency of the electrically driven system.

Dittmann and Rozing [17] reported separations of tricyclic antidepressants on both a Hypersil BDS and Spherisorb SCX phases, and concluded that separations on the reversed-phase material (BDS) was achieved due to differences in electrophoretic mobilities, whilst on the SCX phase, separation involved ion-exchange chromatography.

Wei et al. [18] were able to separate seven basic compounds on bare silica with better efficiency than that obtained by CZE. Separation in CEC was reported to be multifunctional and included reversed-phase, cation-exchange as well as normal-phase chromatography. An example of the separation is shown in Fig. 15.

The effect of pH on retention showed that the k' for aniline was constant over the pH range studied since it is always neutral. When the pH was increased from 7 to 8.3, k' for the three tertiary amines increased, but decreased between pH 8.3 and 9.8. Retention of the three protonated amines increased between pH 7 and 9.98, possibly as a result of increased cation-exchange capacity of the ionised silanol groups at high pH.

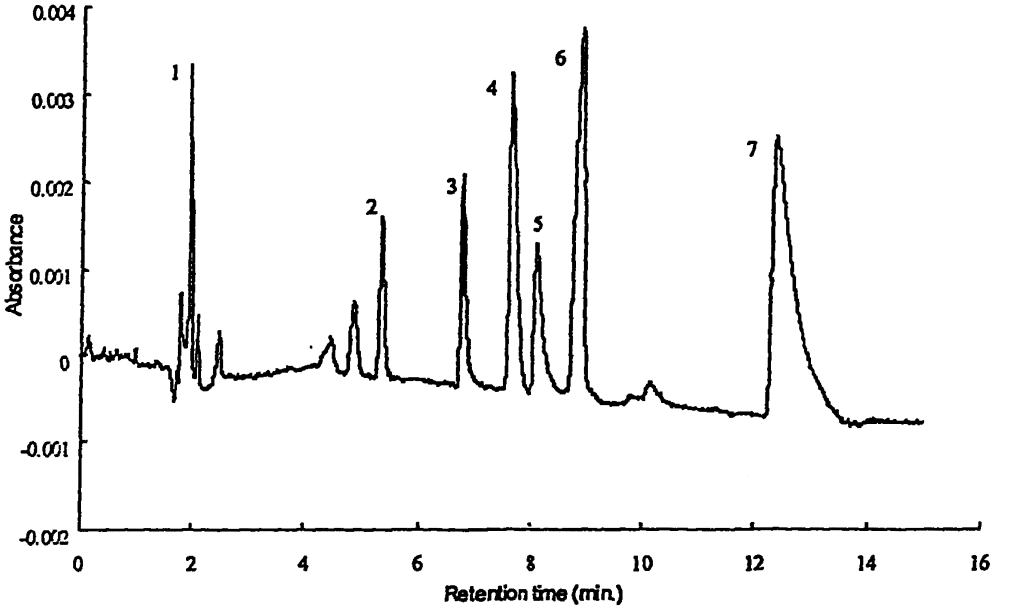


Fig. 15. Electrochromatogram of the separation of seven basic drugs on silica stationary phase. Experimental conditions, packed column: 27 cm (20 cm effective length)×75 μm I.D. Packing: 3 μm silica; mobile phase: ACN–10 m*M* buffer (pH 8.29) (80:20, v/v). Solutes: 1=aniline; 2=cocaine hydrochloride; 3=berberine hydrochloride; 4=thebaine; 5=jatrorrhizine hydrochloride; 6=ephedrine hydrochloride; 7=codeine phosphate. From Ref. [18] with permission ©.

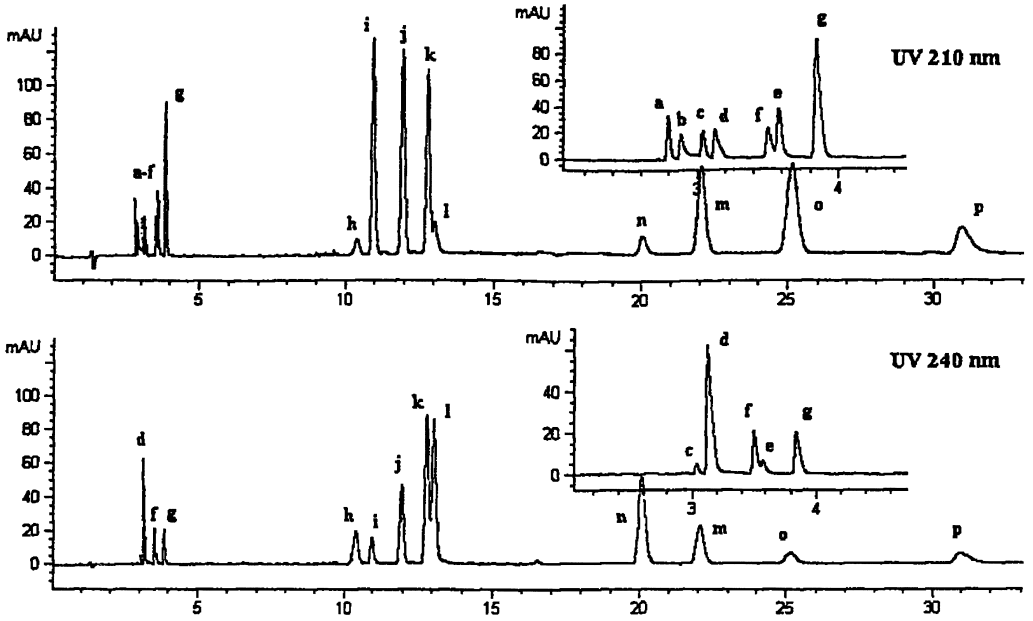


Fig. 16. Capillary: Hypersil C_8, 3 μm, 34 cm (25 cm to detector)×100 μm I.D. Mobile phase: acetonitrile–25 m*M* phosphate+2 μl/ml hexylamine (pH 2.5) 60:40 initial, 75:25 step gradient at 1 min. a=Amphetamine; b=mephamphetamine; c=procaine cocaine; e=heroin; f=quinine; g=noscapine; h=thiourea; i=phenobarbital; j=methaqualone; k=diazepam; l=testosterone; m=cannabinol; n=testosterone propionate; o=D9-tetrahydrocannabinol; p=D9-tetrahydrocannabinol acid. From Ref. [19] with permission, 1998 American Chemical Society ©.

An alternative approach to analysing basic compounds is to use ion-pairing reagents and this was reported by Lurie et al. [19] who used hexylamine as a modifier to analyse a mixture of neutral, acidic and basic compounds at low pH. Original concerns over EOF reversal as a result of hexylamine adsorption were unfounded, and the EOF as measured by thiourea was appreciable even after the addition of hexylamine. Lurie et al. concluded that separation on the Hypersil C_8 phase for strong and mildly basic drugs, was a combination of electrophoresis and chromatographic interactions. These strong and moderately strong bases eluted before the t_0 marker thiourea indicating a strong electrophoretic influence, whilst the weak bases eluted after t_0. This latter effect indicates a stronger chromatographic influence than that observed for the stronger bases. However, although the selectivity of this system was unique, good chromatography was only achieved when using low sample loading. The separation of 16 acidic, neutral and basic compounds using a step gradient is shown in Fig. 16.

As mentioned in an earlier part of this paper, stationary phases that perform well in HPLC mode for bases have been shown to perform poorly when used for CEC. Fig. 17 shows the HPLC separation of a group of bases on Develosil UG-ODS. This C_{18} material is based upon high-purity silica. Good separation is achieved at pH 10 with little evidence of peak tailing.

When a mixture of amitriptyline, nortriptyline and clomipramine is analysed by CEC on the same phase, the result is disastrous as illustrated in Fig. 18. Due to the apparent decomposition, the elution order of the three analytes is assumed to be the same as for other reversed-phase materials.

Interestingly, if these same compounds plus imipramine are analysed on the same phase in a non-aqueous mobile phase with 10 m*M* Tris, there is a significant improvement in chromatography as illustrated in Fig. 19. However, in the absence of a buffer, the chromatography is extremely poor.

Peak 1 in this chromatogram was shown by a separate experiment to be the main degradation product of nortriptyline which when injected fresh, elutes with a retention time of ~12 min under the operating conditions.

J'Sphere ODS M80 is another phase based on high-purity silica and this has also been shown to

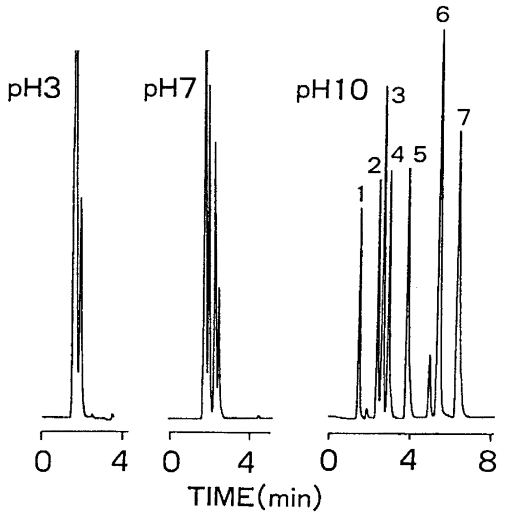


Fig. 17. Basic compounds on Develosil UG ODS by HPLC. Column; 15 cm×4.6 mm. Mobile phase: acetonitrile–0.02 *M* phosphate buffer (40:60, v/v). Flow: 1 ml/min; temperature: 30°C; detection at 254 nm. 1=Pyridoxine; 2=pyridine; 3=procainamide; 4=benzylamine; 5=*N*-methylbenzylamine; 6=metoclopramide; 7=*N*,*N*-dimethylbenzylamine. From Develosil Company literature ©.

give good chromatography for basic analytes under HPLC conditions. Under CEC conditions, the best separation for a mixture of tricyclic antidepressants was obtained with ACN–50 m*M* NaH_2PO_4, pH 2.3 (70:30, v/v) as shown in Fig. 20.

RP Select B is a stationary phase produced following polymer coating of spherical alumina particles with polybutadiene. This material has good pH resistance and ACN be used with alkaline eluents. Because fritting of this material was extremely difficult, both inlet and outlet frits were made from silica. Fig. 21 shows the separation of neutral and basic compounds on a RP Select B column, packed length 30 cm [20]. Peak symmetry is good for all neutral compounds and amitriptyline and nortriptyline. Clomipramine however, shows signs of significant tailing.

Waters Symmetry Shield is a stationary phase designed to ''shield'' polar and basic compounds from silanol activity. The protection is accomplished by bonding an octyl carbamate ligand directly to the silica surface, and this serves to limit solute interactions with residual silanol groups by three possible mechanisms. The first of these mechanisms occurs as

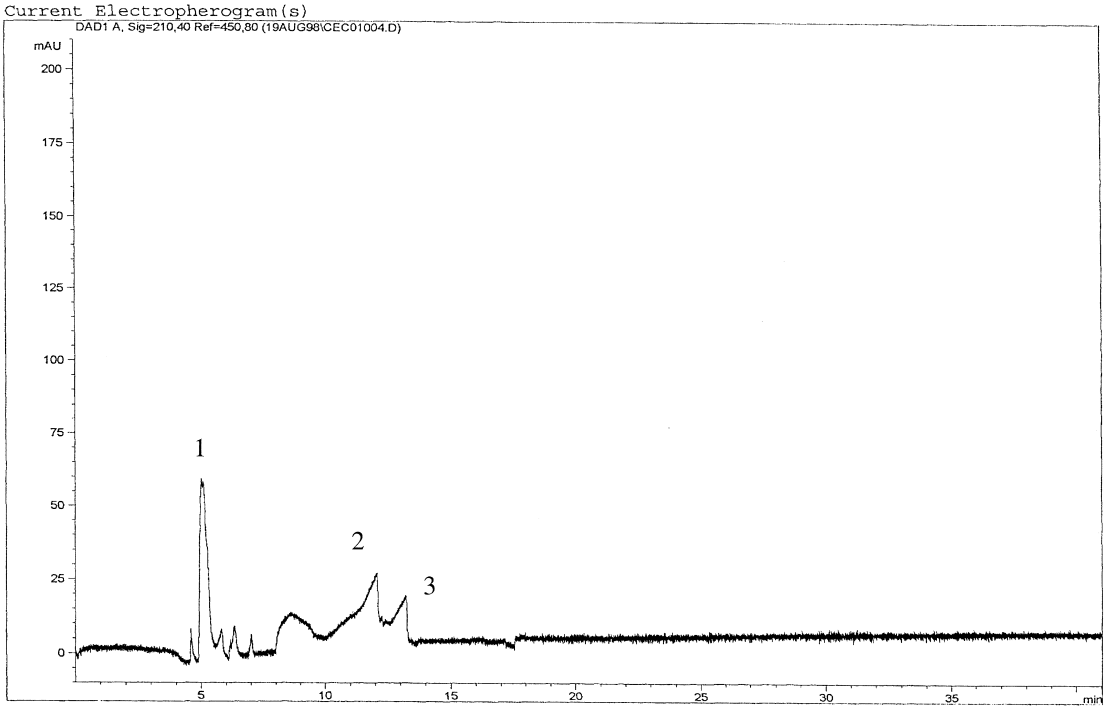


Fig. 18. Instrument: HP^{3D} CEC. Develosil UG-ODS, packed length: 40 cm; total length: 48 cm. Applied voltage: 30 kV; temperature: 30°C; detection at 210 nm; injection: 10 kV for 10 s. Mobile phase; ACN–20 m*M* Tris, pH 9.0 (80:20, v/v). 1=Nortriptyline; 2=amitriptyline; 3=clomipramine.

a result of hydrogen bonding between the analyte and the polar carbonyl group of the carbamate ligand. This interaction often accounts for a slight increase in retention for acidic compounds. The second mechanism involves hydrogen bonding between the carbonyl group of the carbamate with neighbouring silanol groups, thereby reducing the opportunities for analytes to react with these silanol groups. Finally, the embedded polar group is capable of stabilising water in the surface layer of the phase resulting in an increased concentration of water at the silica surface, accompanied by a reduction in silanol activity due to hydration compared to conventional reversed-phase packing materials. These three mechanisms are illustrated in Fig. 22.

A study of the EOF profile of this material showed that it was much more dependent on pH than other conventional stationary phases, reducing considerably below pH 5.0. Like Develosil and J'Sphere materials, this phase has also been shown to perform well with basic analytes in the HPLC mode and this is illustrated in Fig. 23. Baseline separation of all seven test components including the bases propranolol and amitriptyline is achieved in <15 min.

For comparison, the corresponding chromatogram from Symmetry C_8 is included.

Of all the stationary phases screened in our laboratory, Symmetry Shield RP_8 performed the best with basic compounds. Fig. 24 shows the structures of the components of a test mixture used to evaluate stationary phases.

When this mixture was analysed on Symmetry Shield RP_8 using ACN–100 m*M* Tris, pH 9.0 (70:30, v/v), baseline separation of all six components was achieved with good peak symmetry in ~5 min, with the stronger bases retained the most. This is consistent with a cation-exchange mechanism assuming the silanol groups of the stationary phase are in fact accessible. A typical chromatogram is shown in Fig. 25.

If a mixture of tricyclic antidepressants is run on Symmetry Shield RP_8 using non-aqueous conditions

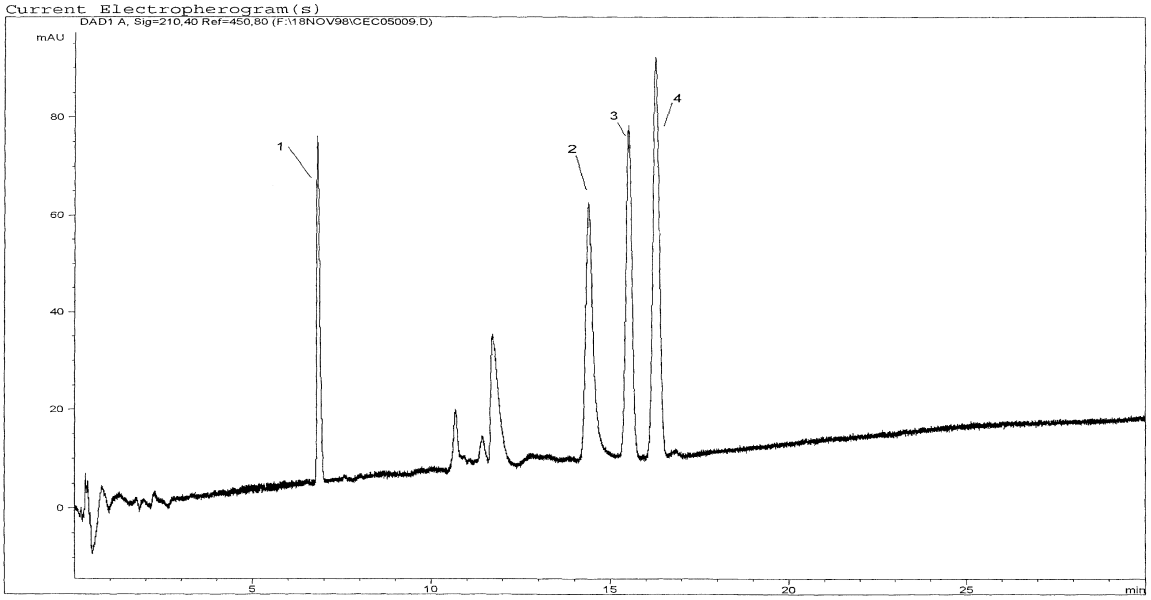


Fig. 19. Instrument: HP[3D] CEC. Develosil UG-ODS, packed length: 40 cm; total length: 48 cm. Applied voltage: 30 kV; temperature: 30°C; detection at 210 nm; injection: 10 kV for 10 s. Mobile phase: 0.01 *M* Tris, pH 9.0 in ACN–MeOH (1:1, v/v). 1 = Decomposition product of nortriptyline; 2 = imipramine; 3 = amitriptyline; 4 = clomipramine.

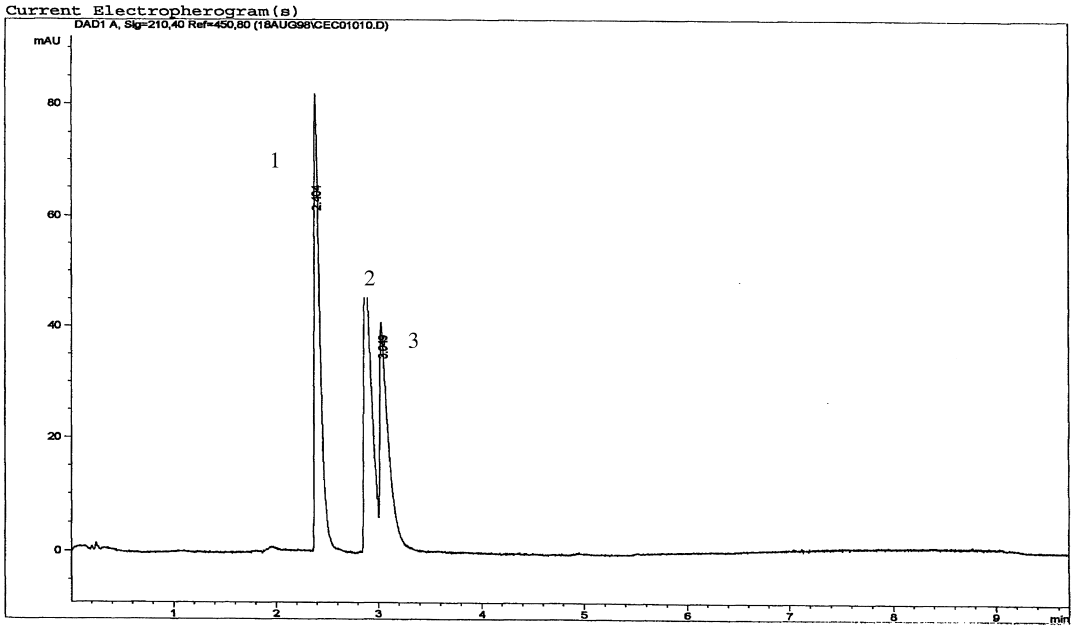


Fig. 20. Instrument: HP[3D] CEC. J'Sphere ODS M80, packed length: 24.5 cm; total length: 33 cm. Applied voltage: 30 kV; temperature: 30°C; detection at 210 nm; injection: 10 kV for 5 s. Mobile phase: ACN–0.05 *M* NaH_2PO_4 adjusted to pH 2.3 (70:30, v/v). 1 = Nortriptyline; 2 = amitriptyline; 3 = clomipramine.

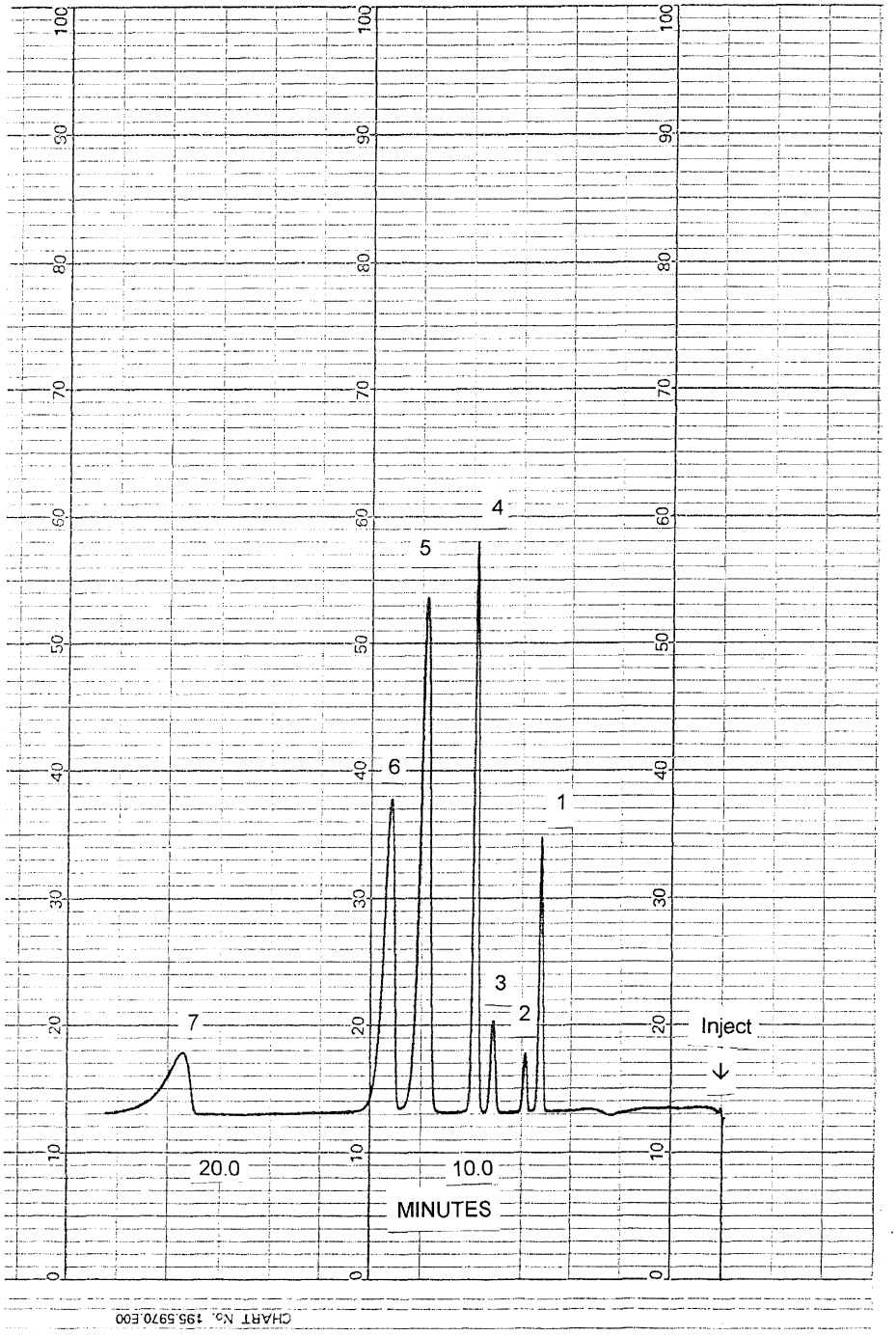


Fig. 21. Instrument: Modified ABI 270A. RP Select B, packed length: ~30 cm; total length: 50 cm. Applied voltage: 30 kV; temperature: 30°C; detection at 210 nm; injection: 20 kV for 0.2 min. Mobile phase: ACN–20 m*M* Tris, pH 9.0 (80:20, v/v). 1=Thiourea; 2=dimethyl phthalate; 3=anisole; 4=naphthalene; 5=nortiptyline; 6=amitriptyline; 7=clomipramine. From Ref. [20] with permission ©.

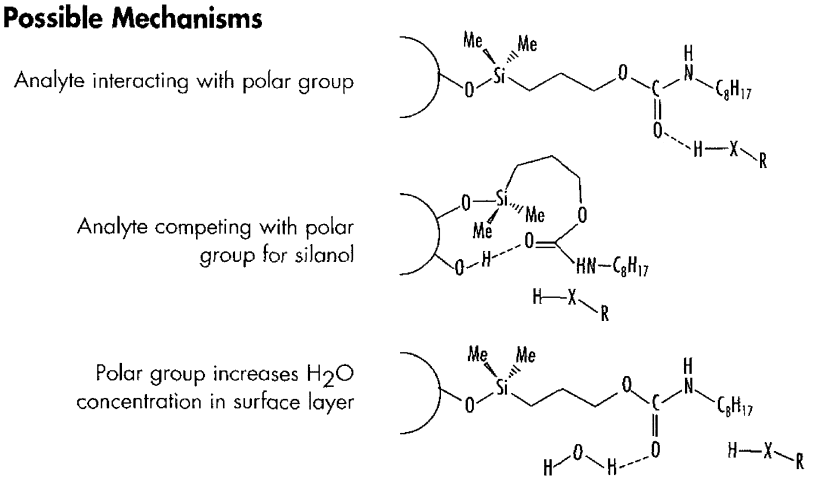


Fig. 22. Symmetry Shield RP_8 mechanisms. From Waters Company literature ©.

without the presence of a buffer, then disastrous results are obtained. However, the addition of 0.01 *M* Tris to the mobile phase brings about a remarkable improvement in peak shape and this is shown in the following chromatogram (Fig. 26).

Once again, there appears to be significant degradation of nortriptyline under these conditions.

3.4. Chiral compounds

The α_1-acid glycoprotein (AGP) stationary phase is produced by immobilising a human plasma protein onto silica. This protein consists of a single peptide chain containing 181 amino acids. Five carbohydrate units are linked to the peptide chain via the asparagine residues. The resulting phase contains many binding groups including acidic, basic and hydrogen bonding and in addition has a moderate hydrophobic character due to the hydrophobic amino acid residues that are present such as tryptophan, lysine and phenylalanine. Li and Lloyd [21] packed 50 μm capillaries with AGP stationary phase and were able

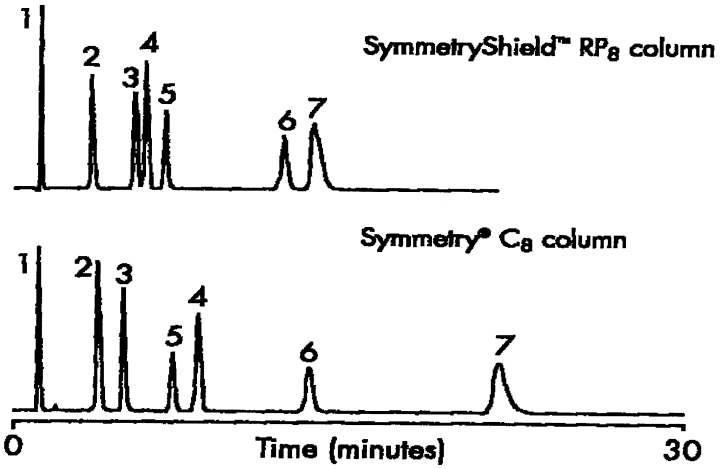


Fig. 23. Column selectivity using HPLC. Top chromatogram Symmetry Shield RP_8. Lower chromatogram Symmetry C_8. Mobile phase: methanol–20 m*M* K_2HPO_4, pH 7 (65:35, v/v). Temperature: 23°C; flow-rate: 1 ml/min. 1=Uracil; 2=propranolol; 3=butyl paraben; 4=dipropyl phthalate; 5=naphthalene; 6=acenaphthene; 7=amitriptyline. From Waters Company literature ©.

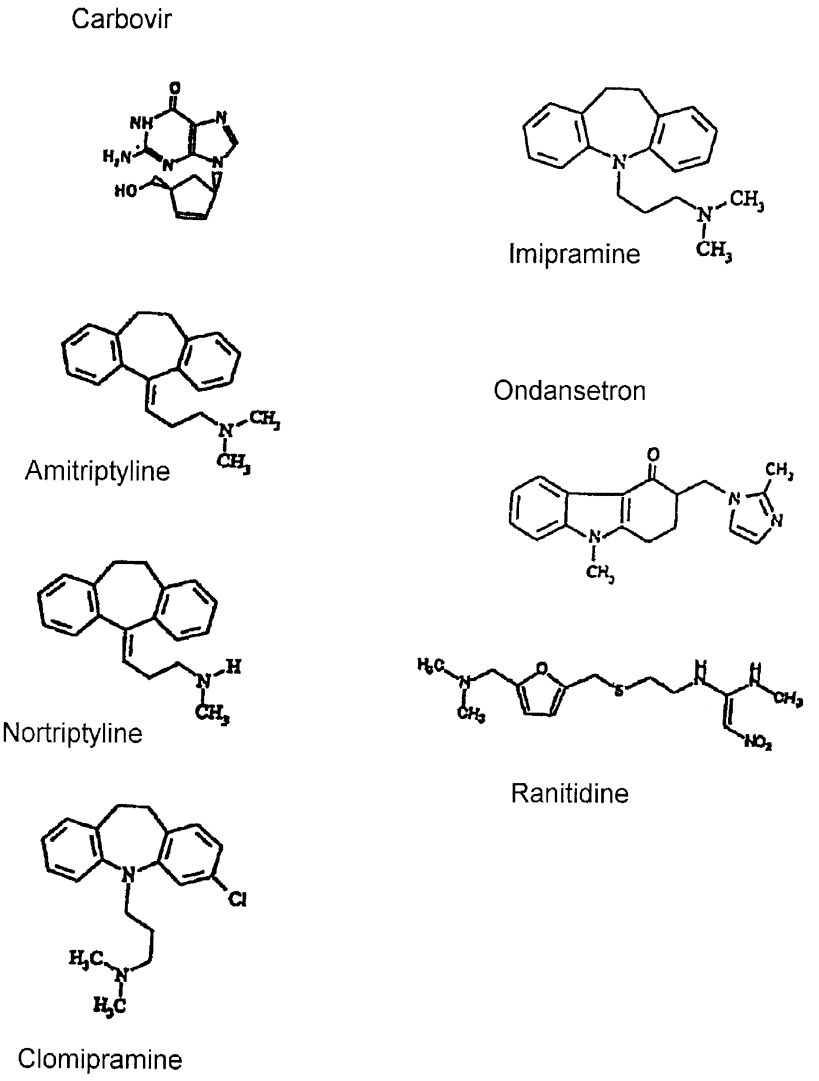


Fig. 24. Basic test compounds.

to resolve the enantiomers of a range of chiral compounds including benzoin, hexobarbital, disopyramide, cyclophosphamide, pentobarbital, prenolol, alprenolol and propranolol. Although they reported plate numbers of 5800 plates for a 17 cm packed capillary for the chiral compound benzoin, these efficiencies are poor when compared with those obtained with achiral phases such as ODS. It was concluded that low efficiency and poor peak symmetry was probably due to the properties of the AGP phase since similar results were reported when using this phase in the HPLC mode.

In another report, Li and Lloyd [21] showed the separation of a series of compounds on a capillary packed with a β-cyclodextrin stationary phase. They chose to use both phosphate and triethylammonium acetate (TEAA) as buffers and claim that the best results for the separation of benzoin were obtained using TEAA in the mobile phase. The use of TEAA in the mobile phase necessitated the reversal of the

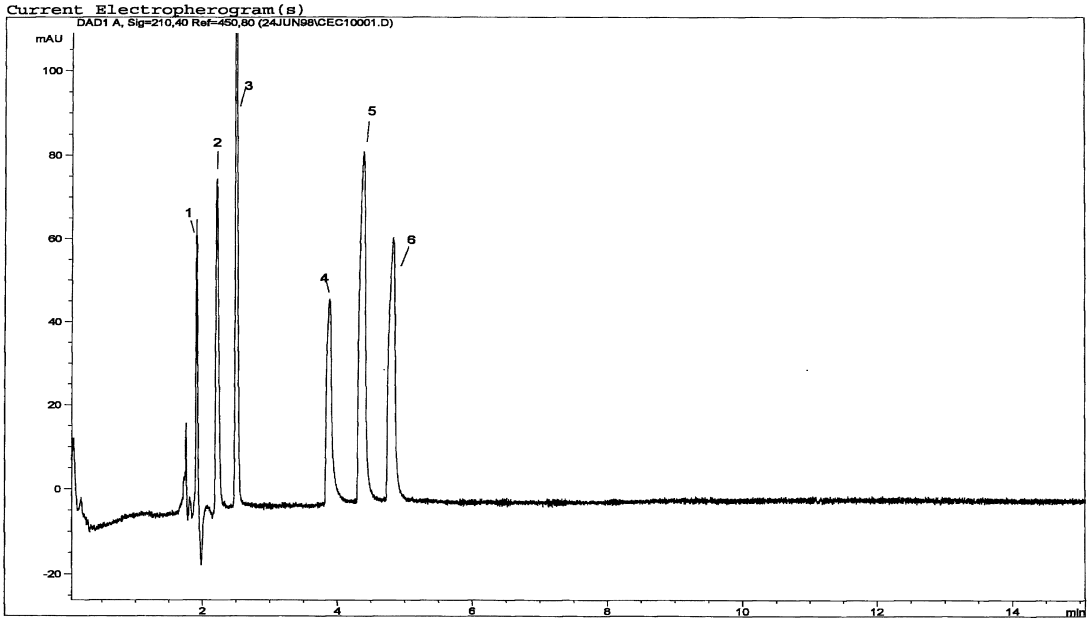


Fig. 25. Instrument: HP[3D] CEC Symmetry Shield RP_8, packed length: 24.5 cm; total length: 33 cm. Applied voltage: 30 kV; temperature: 40°C; detection at 210 nm; injection: 5 kV for 5 s. Mobile phase: ACN–100 m*M* Tris, pH 9.0 (70:30, v/v). 1=Carbovir; 2=ranitidine; 3=ondansetron; 4=imipramine; 5=amitriptyline; 6=clomipramine. From Ref. [20] with permission ©.

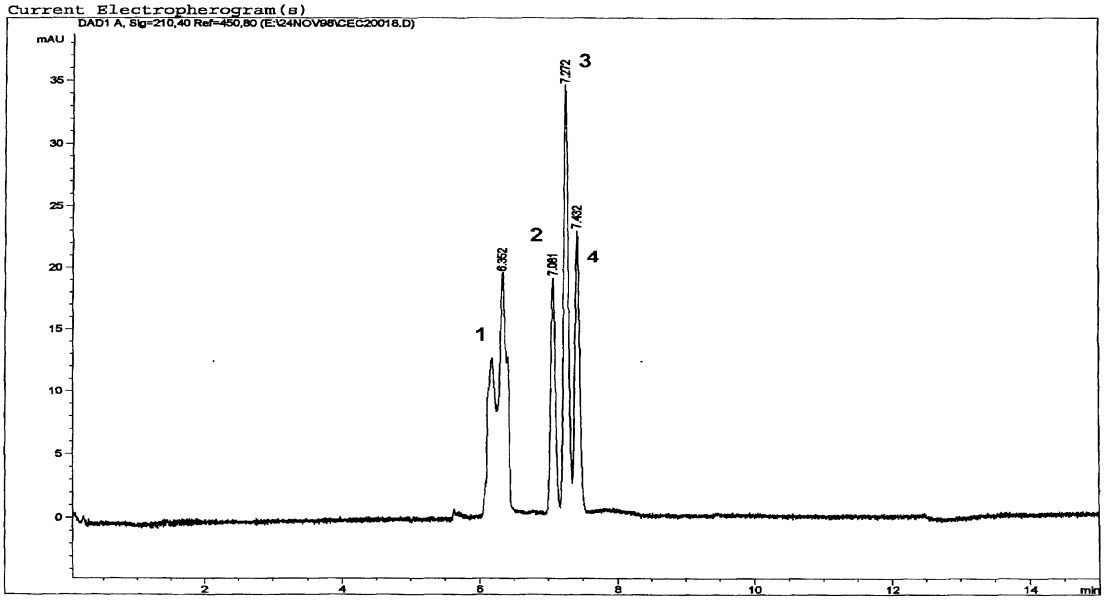


Fig. 26. Instrument: HP[3D] CEC. Symmetry Shield RP_8, packed length: 40 cm; total length: 48.5 cm. Applied voltage: 30 kV; temperature: 30°C; detection at 210 nm; injection: 10 kV for 10 s. Mobile phase; 0.01 *M* Tris in ACN–MeOH (1:1). 1=Decomposition products of nortriptyline; 2=imipramine; 3=amitriptyline; 4=clomipramine.

polarity such that the detector end was the anode. Nevertheless, the reported plate numbers for this separation were still extremely low with only 9500 plates on a 17 cm capillary. Smith [20] using a capillary (packed length 22 cm) was able to resolve the enantiomers of hexobarbital using a phosphate buffer at pH 7.1 with efficiencies of 26 151 plates/m for the second enantiomer as illustrated in Fig. 27.

Lelièvre et al. [22] reported the use of hydroxypropyl β-cyclodextrin (HPβCD) as both a mobile phase additive and a chiral stationary phase. Using hydroxypropyl β-cyclodextrin in the mobile phase, they found that equilibration took 15 h and before that time, no resolution was observed for the enantiomers of the neutral drug chlorthalidone. The HPβCD was thought to coat the stationary phase and form a pseudo chiral stationary phase. This was confirmed by Carter-Finch and Smith in a similar study [23]. The concentration of acetonitrile was important and it was found that the best separation was obtained with a mobile phase consisting of ACN–1 m*M* Na_2HPO_4, pH 6.5 (15:85, v/v) with 10 m*M* HPβCD. Complete resolution of the enantiomers was achieved in ~90 min (Fig. 28A). Using a capillary packed with 5 μm hydroxypropyl β-cyclodextrin bonded silica particles, Lelièvre et al. were also able to resolve the enantiomers of chlorthalidone using a similar mobile phase as for the additive, but with 25% ACN and an increased electrolyte concentration of 5 m*M*. Resolution (R_s) of 1.7 was achieved for an analysis time of 35 min which was considerably less than that using the chiral additive and this is shown in Fig. 28B.

Although the packed CEC method using the chiral stationary phase produced the higher resolution and shorter analysis time, the efficiency was higher for the chiral mobile phase additive, as is the case in HPLC.

Wolf et al. [24] immobilised (*S*)-naproxen and (3*R*,4*S*)-Whelk-O stationary phases onto 3 μm silica and separated a range of neutral analytes using 2-(*N*-morpholino)ethanesulfonic acid (MES)–acetonitrile mobile phases. These phases were originally developed for HPLC and were found to have a high selectivity for a range of neutral compounds. Although two mobile phase additives were studied,

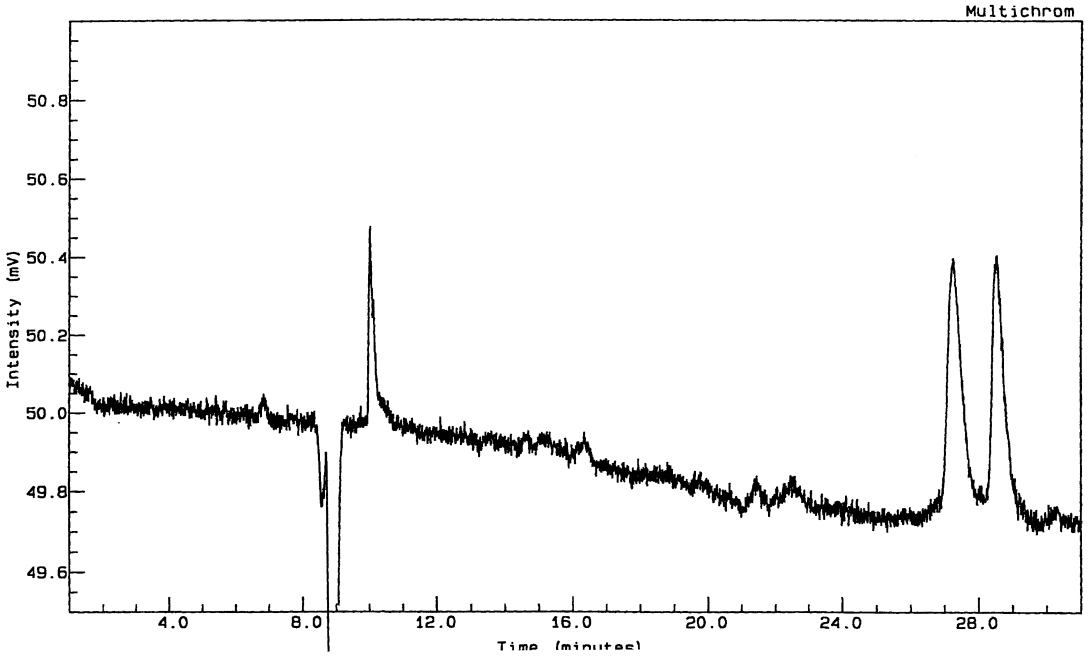


Fig. 27. Instrument: Modified ABI 270A. Cyclobond 1, packed length: 22 cm; total length: 50 cm×50 μm I.D. Mobile phase: ACN–Na_2HPO_4, pH 7.1 (5:95, v/v). Injection: 10 kV/0.3 min; applied voltage: 30 kV; temperature: 30°C; detection at 210 nm. Analyte; ±-Hexobarbital, 1 mg/ml in mobile phase.

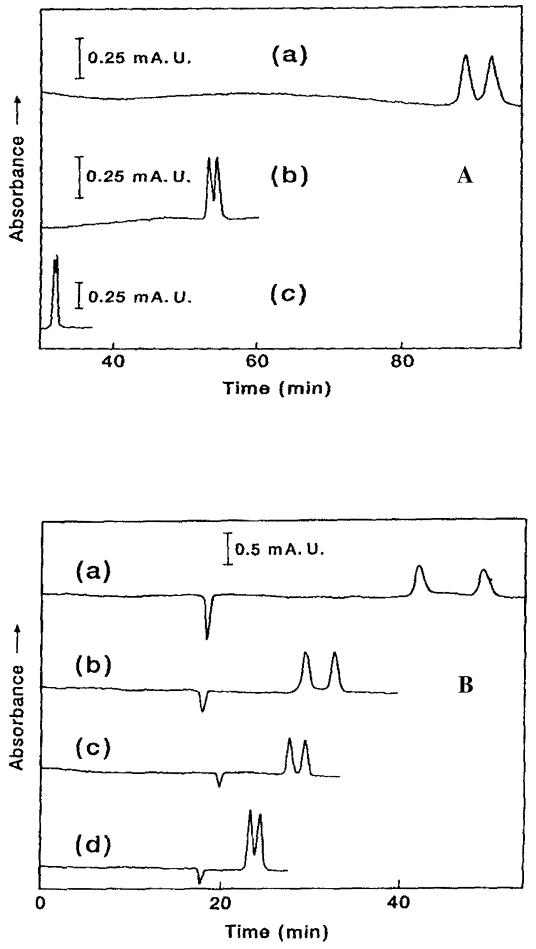


Fig. 28. The influence of the acetonitrile content on the chiral separation of chlorthalidone enantiomers by packed CEC. (A) 3 μm ODS, 52 cm (26.2 cm to detector)×50 μm I.D., 25.9 cm packed. Mobile phase: ACN–1 m*M* phosphate buffer (adjusted to pH 6.5 with concentrated H_3PO_4) containing 10 m*M* HPβCD. (a) ACN–buffer (15:85), (b) ACN–buffer (20:80), (c) ACN–buffer (25:75). Applied voltage: 15 kV; detection at 220 nm; injection: 15 kV/5 s. Chlorthalidone concentration 0.05 mg/ml. (B) 5 μm HPβCD bonded silica, 58 cm (27.2 cm to detector)×50 μm I.D., 27 cm packed. Mobile phase: ACN–5 m*M* phosphate buffer (adjusted to pH 6.5 with concentrated H_3PO_4) containing 10 m*M* HPβCD. (a) ACN–buffer (15:85), (b) ACN–buffer (20:80), (c) ACN–buffer (25:75), (d) ACN–buffer (30:70). Applied voltage: 15 kV; detection at 220 nm; injection: 15 kV/5 s. Chlorthalidone concentration, 0.05 mg/ml. From Ref. [24] with permission ©.

when working with Tris (hydroxymethyl)aminoethane (Trizma) unstable baselines were obtained even after conditioning for several days. Using MES containing mobile phases, acetonitrile was found to give less retention of the analytes and the highest EOF. Using 25 m*M* MES, pH 6.0–acetonitrile (1:3.5, v/v) all of the 10 test compounds were resolved with very high efficiency and selectivity. Using the (*S*)-naproxen stationary phase they were able to resolve the enantiomers of one of the test compounds (3) with an R_s of 8.02, an $\alpha = 1.49$ and efficiency of 196 000 plates per metre for peak 1, as shown in Fig. 29.

The enantiomers of compound (7) were resolved with an R_s of 30.95, an $\alpha = 3.82$ and efficiency of 200 000 plates per metre for peak 1 using the (3*R*,4*S*)-Whelk-O stationary phase under the same conditions as for compound (3) on the naproxen phase. An example of this separation is shown in Fig. 30.

It was pointed out that normally the efficiency of the more retained enantiomer shows less efficiency, i.e., $N_1 > N_2$. However, it was observed that the (3*R*,4*S*)-Whelk-O stationary phase showed relatively little loss of efficiency between the enantiomers, and in the case of the test compound benzoin, $N_1 < N_2$.

The macrocyclic antibiotic phases such as vancomycin and teicoplanin are finding increasing use in chiral separations [25–27] due to their ability to show selectivity for a large number and range of chiral analytes including amino acids, labetolol, naproxen, metoprolol, ibuprofen, flurbiprofen, clenbuterol, atenolol and oxazepam. The wide applicability of these materials is attributed to the fact that they have multiple chiral centres. Vancomycin has 18 chiral centres surrounding three cavities, bridged by five aromatic rings. Teicoplanin has 20 chiral centres surrounding four pockets or cavities, along with seven aromatic groups in close proximity giving rise to hydrogen bond donor and acceptor sites. Separation is achieved as a result of the complex chiral environment which allow for π–π interactions, chiral hydrogen bonding, peptide binding, carbohydrate binding and inclusion complexation to name just some of the many mechanisms that these materials are capable of utilising. The manufacturers also claim that these materials have similar selectivities to the glycoprotein phases but are stable in 0–100% organic modifiers and have greater sample capacity. The two phases are known as Chirobiotic V (vancomycin) and Chirobiotic T (teicoplanin), respectively.

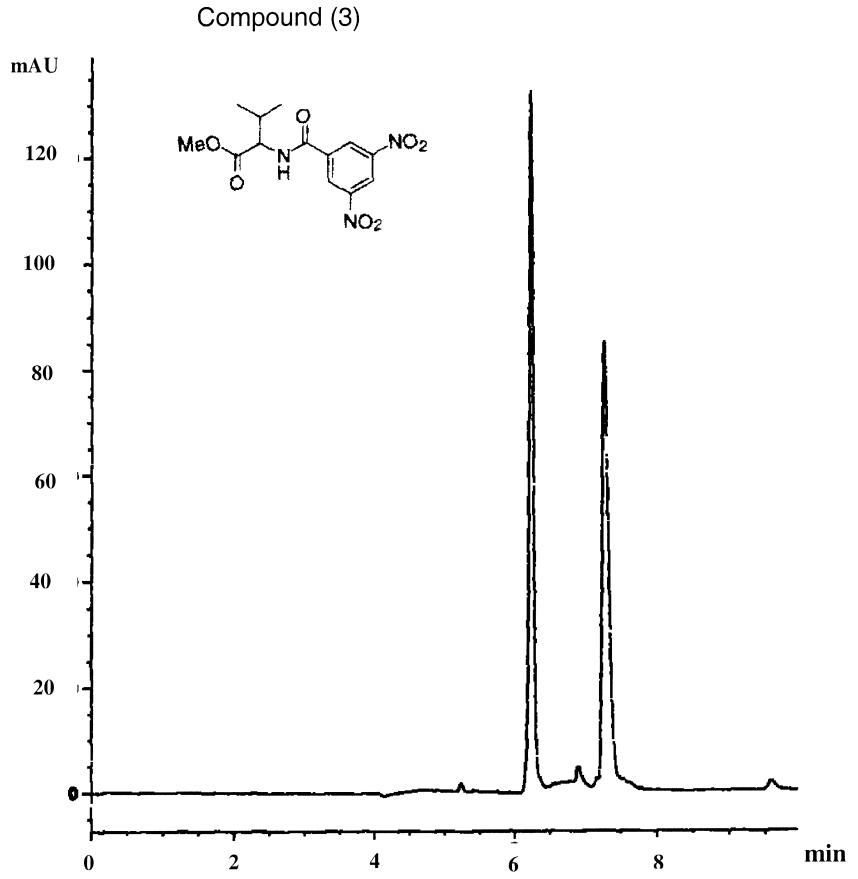


Fig. 29. CEC separation of the enantiomers of compound (3) on (*S*)-naproxen stationary phase. Capillary: 38 cm total length, 29.5 cm packed. Detection at 230 nm. Mobile phase: 25 m*M* MES, pH 6.0–acetonitrile (1:3.5, v/v). Applied voltage 25 kV. From Ref. [26] with permission ©.

Dermaux et al. [28] reported the separation of warfarin and hexobarbital using a 100 μm I.D. capillary packed to 40 cm with 5 μm vancomycin stationary phase. Optimum separation of the enantiomers of warfarin were achieved by modifying the HPLC method which consisted of 0.1% TEAA at pH 5.0–tetrahydrofuran (THF) (80:20, v/v). Because no resolution was obtained with this system, THF was replaced by acetonitrile and the separation illustrated in Fig. 31 was obtained.

The efficiency for the second enantiomer was 13 300 plates and resolution was 2.7. Using a mobile phase of 0.1% TEAA at pH 6.65–ACN (80:20, v/v) they were also able to resolve the enantiomers of hexobarbital with an efficiency of 15 500 plates with a resolution of 0.9. Baseline separation could be achieved by lowering the acetonitrile concentration to 10% but at the expense of an increase in analysis time to 20 min as shown in Fig. 32A and B.

Wikström et al. [29] demonstrated the separation of thalidomide enantiomers on a vancomycin stationary phase in both the reversed-phase mode and the polar organic mode. 5 μm LiChrospher 100 diol was slurry packed into 75 and 100 μm I.D. capillaries, followed by immobilisation with vancomycin. In the reversed-phase mode, they were able to separate the enantiomers using ACN–0.1% TEAA (pH 6.5) with an R_s of 2.6 and efficiency of 80 000 plates per metre. A typical separation is shown in Fig. 33A.

Using a mobile phase in the polar organic mode consisting of MeOH–ACN–HOAc–triethylamine (TEA) (80:20:0.2:0.2, v/v) complete separation of

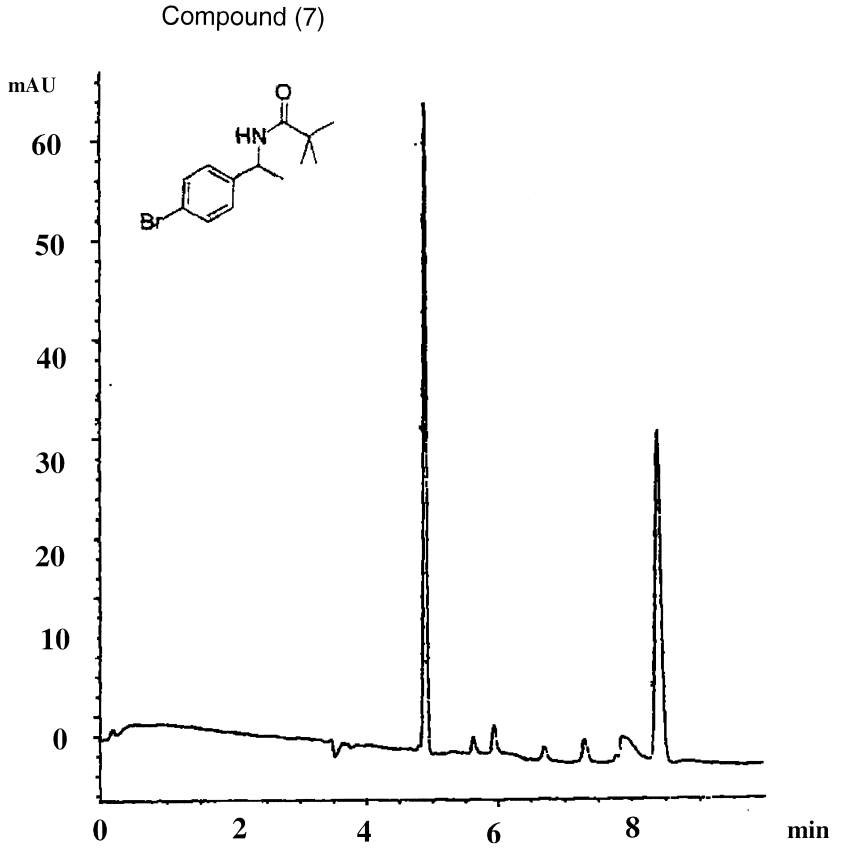


Fig. 30. CEC separation of the enantiomers of compound (7) on (3*R*,4*S*)-Whelk-O stationary phase. Capillary: 39.3 cm total length, 30.5 cm packed. Detection at 230 nm. Mobile phase: 25 m*M* MES, pH 6.0–acetonitrile (1:3.5, v/v). Applied voltage 25 kV. From Ref. [26] with permission ©.

the enantiomers was achieved in <15 min also with an R_s of 2.6 but with an efficiency of 120 000 plates per metre and this is illustrated in Fig. 33B.

Carter-Finch and Smith [30] described the suitability of the teicoplanin phase Chirobiotic T for chiral CEC. In their paper Armstrong et al. [26] showed via mobility measurements that teicoplanin was negatively charged above ~pH 3.8 and therefore in a CEC mode where the macrocyclic compound was bound to silica, any electrical double layer resulting from the stationary phase ligands would provide electroosmotic flow towards the cathode as would the residual silanols on the silica support itself. Company literature suggests that low levels of organic modifier (usually ethanol or methanol) were required to resolve enantiomers on this phase, often with the addition of up to 1% of TEAA at a pH of between 4.0 and 4.5. In the BAS Technicol Applications Note [31], tryptophan was resolved by HPLC in a mobile phase consisting of 30% ethanol in water, but attempts to reproduce this by CEC in our laboratory were unsuccessful. In fact the behaviour of the phase was very unpredictable giving extremely non-reproducible data making mobile phase selection very difficult. It was even difficult to obtain peaks of any description and it was only after equilibration over several days that some reasonable chromatography was achieved. Experiments showed that the neutral chiral compound benzoin could be eluted as a single symmetrical peak from the teicoplanin phase using acetonitrile–2 m*M* Na_2HPO_4, pH 7.0 (70:30, v/v) as the mobile phase.

Using these conditions tryptophan was injected to see if any resolution of the enantiomers was possible.

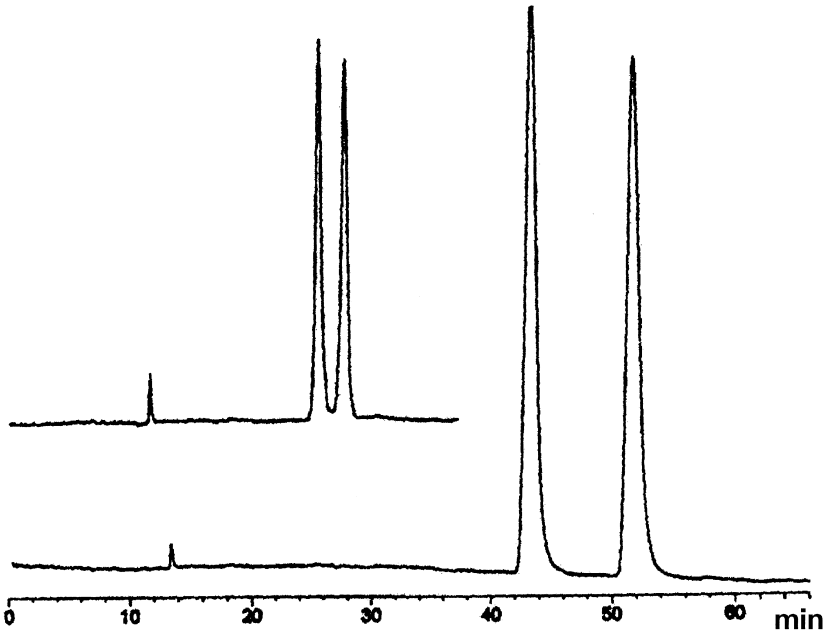


Fig. 31. Enantioseparation of warfarin by CEC. Column: 40 cm×0.1 mm I.D. FSOT. Injection: 10 kV/1 s. Detection at 200 nm. Temperature: 20°C. Voltage: 30 kV. Vancomycin CSP 5 μm. Mobile phase: 0.1% TEAA at pH 5–ACN (80:20, v/v). Inset: separation at 0.1% TEAA, pH 5–ACN (70:30, v/v). From Ref. [30] with permission ©.

Although a symmetrical peak eluted from the capillary, there was no evidence of chiral selectivity. After equilibration of ~30 min, the separation shown in Fig. 34 was obtained where the enantiomers of tryptophan are resolved in <4 min with an R_s of 1.7 an α of 1.22, and $N=35\,000$ plates per metre.

In the same paper, the separation of the enantiomers of dinitrobenzoyl leucine was described, but resolution was achieved only by the use of ACN–5 m*M* NaH_2PO_4, pH 2.3 (70:30, v/v). Almost baseline resolution is achieved in under 6 min with an R_s of 1.47 and α of 1.10, Fig. 35. Using the same mobile phase at a pH of 7.0 failed to resolve the enantiomers.

Another very popular series of chiral stationary phases are those from Daicel which are based on cellulose which is then derivatised in order to introduce certain functionalities into the structure. However, these phases tend only to be used in the normal-phase mode [32–37] although a reversed-phase variant of the popular OD phase is now available as OD-R. Chiralcel OD is a chiral stationary phase consisting of cellulose onto which is bonded 3,5-dimethylphenylcarbamate groups. Although it was designed to work in the normal-phase mode, it has been shown to work equally well in the reversed-phase mode. The structure of Chiralcel OD is illustrated in Fig. 36.

In order to test the versatility of the Chiralcel OD phase, 24 chiral analytes were run in our laboratory using the same conditions throughout but with mobile phase replenishment every six samples so that buffer depletion did not interfere with the results. Samples were dissolved in the mobile phase at a concentration of ~2 mg/ml. The 24 samples are listed in Table 1 along with the operating conditions

Of the 24 samples analysed, only benzoin was resolved. The efficiency for the two enantiomers was 38 805 plates per metre for peak 1 and 36 400 plates per metre for peak 2. The derivatised amino acids failed to elute presumably because their electrophoretic mobilities opposed and were greater than the electroosmotic flow. Many of the basic compounds produced broad peaks with severe fronting, although pindolol and ondansetron did show some separation.

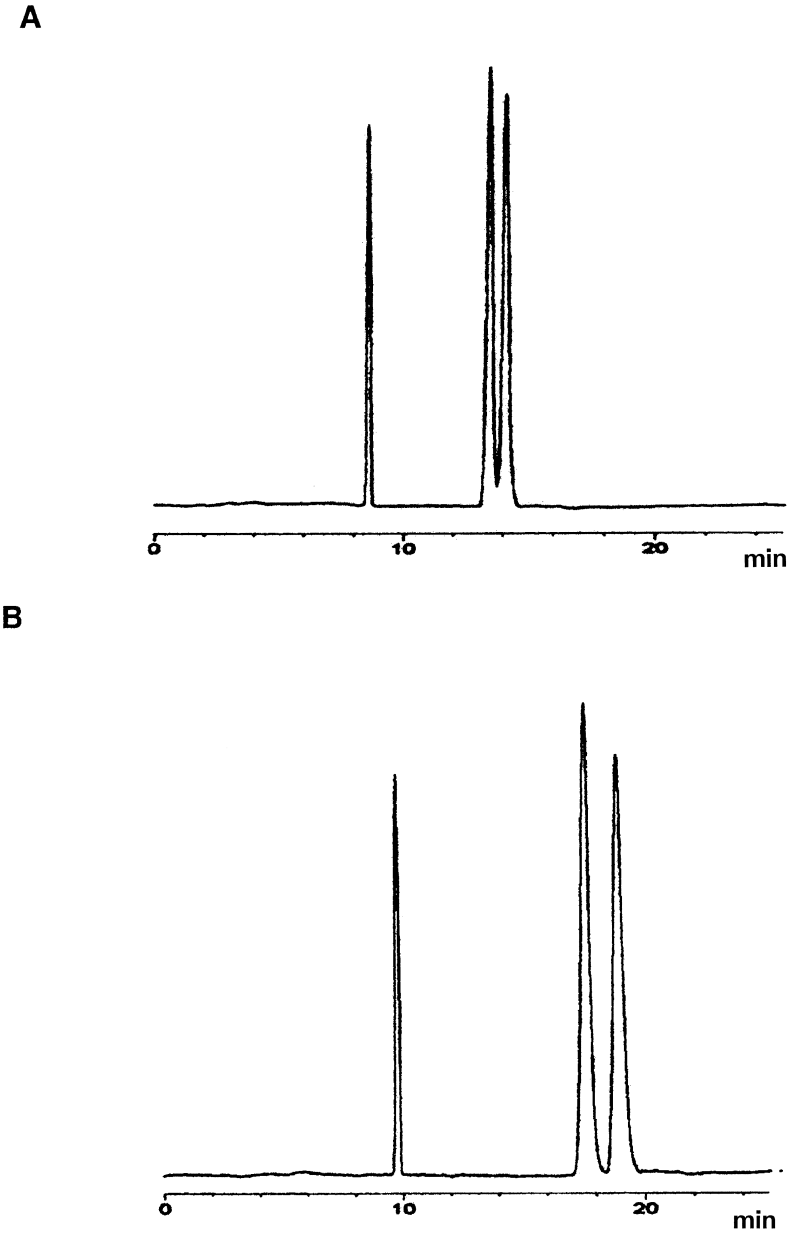


Fig. 32. Enantioseparation of hexobarbital by CEC. Column 40 cm×0.1 mm I.D. FSOT. Vancomycin CSP 5 μm. Mobile phase (A) 0.1% TEAA at pH 6.65–ACN (80:20, v/v). (B) 0.1% TEAA at pH 6.65–ACN (90:10, v/v). Injection: 10 kV/1 s. Detection at 200 nm. Temperature: 20°C. Voltage: 30 kV. From Ref. [30] with permission ©.

In order to try and reduce the analysis time and improve the peak shape, the acetonitrile concentration was increased to 90%, but because of solubility constraints, the Tris buffer concentration had to be reduced to 5 m*M* (pH 7.5). In fact the retention time increased and the plate numbers for the two enantiomers were now 21 322 and 39 073 per metre. The increase in retention time was somewhat surprising since the higher organic concentration would be expected to lead to reduced retention, as would the

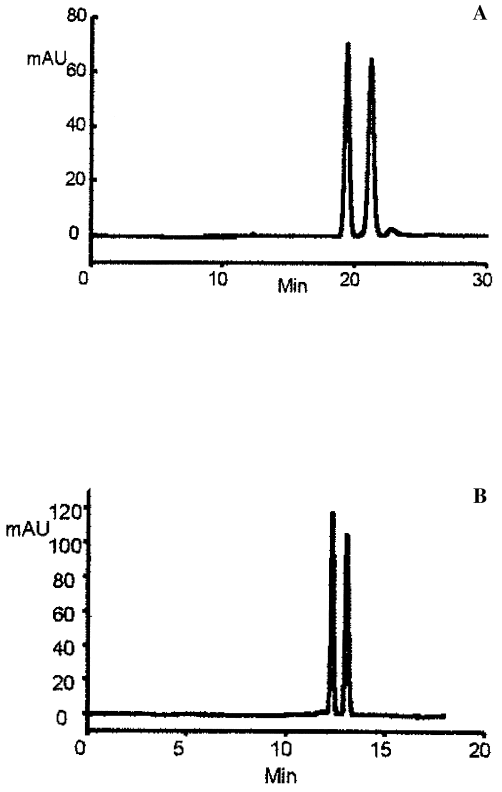


Fig. 33. (A) CEC reversed-phase mode; 26 cm×75 μm I.D. Vancomycin phase; mobile phase: ACN–0.1% TEAA, pH 6.5 (30:70, v/v); applied voltage: 15 kV; detection at 220 nm; temperature: 15°C; $R_s=2.6$, $\alpha=1.07$, N~80 000 plates/m. (B) Polar organic mode; 26 cm×75 μm I.D. Vancomycin phase; mobile phase: MeOH–ACN–HOAc–TEAA (80:20:0.2:0.2, v/v); applied voltage: 20 kV; detection at 220 nm; temperature: 15°C; $R_s=2.6$, $\alpha=1.06$, N~80 000 plates/m. From Ref. [31] with permission ©.

lower overall buffer concentration which should result in an increase in the EOF. This is illustrated in Fig. 37.

Better results were obtained when the mobile phase was changed to acetonitrile–2 m*M* Na_2HPO_4, pH 7.0 (80:20, v/v) at a temperature of 40°C. Now baseline resolution was achieved for benzoin and pindolol, with near baseline resolution for ondansetron. Figs. 38 and 39 illustrate the separation of pindolol and ondansetron, respectively. The plate numbers for the individual isomers are shown in Table 2.

The plate counts for GR38032F appear to be excessive, but manual calculations on the expanded chromatogram confirm their validity. The reason for such high efficiencies is unclear.

Using these same conditions, a further four compounds were screened and these were aminoglutethimide, verapamil chlorpheniramine and chloroquine.

Of these, only aminoglutethimide was resolved, and the best separation was achieved by lowering the applied voltage to 25 kV. The resulting chromatogram is shown in Fig. 40.

The corresponding plate numbers for the two isomers was 15 110 per metre for peak 1 and 13 367 per metre for peak 2. Once again the efficiency for the second enantiomer was the lowest, i.e., $N_2<N_1$ so often the case with chiral separations.

3.5. Gradient elution

In isocratic CEC the flow of eluent across the column is generated and maintained by the EOF upon the application of a high electric field. In order to separate components of a mixture that have widely differing retention behaviour, gradient elution is employed to gradually increase the eluent strength during the chromatographic run. To realise the full potential of CEC, it is therefore necessary to employ instrumentation having gradient elution capabilities especially for the separation of complex mixtures. Presently there are no commercially available gradient CEC systems. However, research groups have designed appropriate instrumentation that either involves the use of a HPLC pump to deliver mobile phase of varying composition, or by electrically creating a gradient. If a HPLC pump is used the mobile phase is introduced to the inlet side of the capillary where the flow is split by the use of a T-piece. It is necessary to split the flow in order not to superimpose a parabolic flow profile onto the plug flow profile generated by EOF. From this point on the EOF is used to move the solvent through the capillary. An electrically created gradient is achieved by having the inlet end of the capillary coupled to a split capillary where each end is in a vial containing mobile phase of differing composition. By utilising two voltage supplies, one connected to each vial and then applying a voltage at various rates, the mobile phase ACN be mixed as desired. However, the

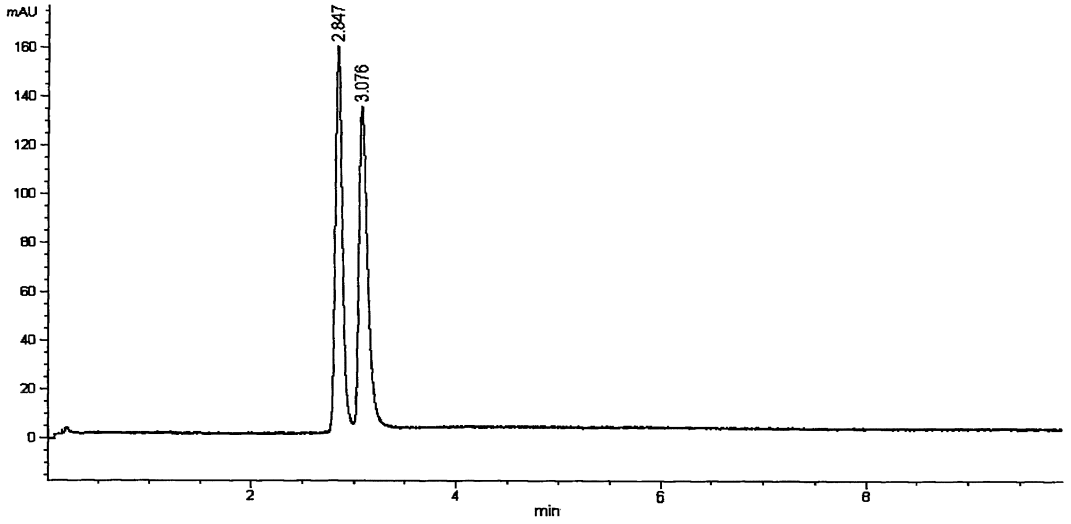


Fig. 34. CEC separation of tryptophan enantiomers on Chirobiotic T. Capillary: effective length 24.5 cm, total length 33 cm, 100 μm I.D. Conditions: acetonitrile–2 m*M* Na_2HPO_4, pH 7.0 (70:30, v/v); 30 kV; 25°C; injection: 10 kV/5 s; UV detection at 214 nm. Analyte concentration: 2 mg/ml in acetonitrile–water (50:50). From Ref. [32] with permission ©.

disadvantage is that the exact composition of the gradient is unknown.

Yan et al. [38] used instrumentation to deliver a purely electroosmotic gradient by merging two electroosmotic flows generated by separate power supplies. By varying the voltage from the two power supplies the solvent reaching the T-piece could be controlled. An improvement over isocratic elution

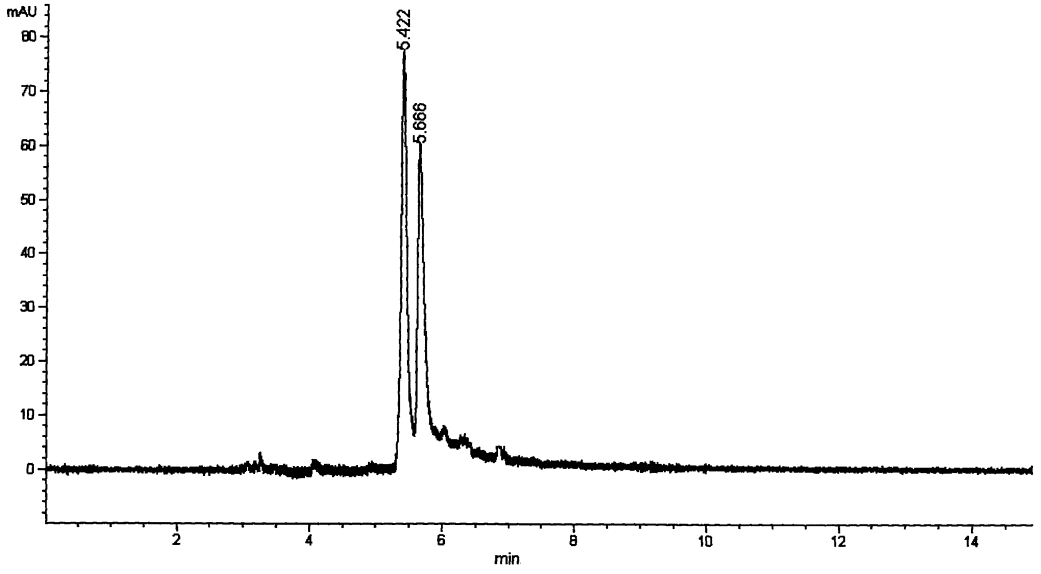


Fig. 35. CEC separation of dinitrobenzoyl leucine enantiomers on Chirobiotic T. Capillary: effective length 24.5 cm, total length 33 cm, 100 μm I.D. Conditions: acetonitrile–5 m*M* NaH_2PO_4, pH 2.3 (70:30, v/v); 30 kV; 25°C; injection: 10 kV/5 s; UV detection at 254 nm. Analyte concentration: 2 mg/ml in acetonitrile–water (50:50). From Ref. [32] with permission ©.

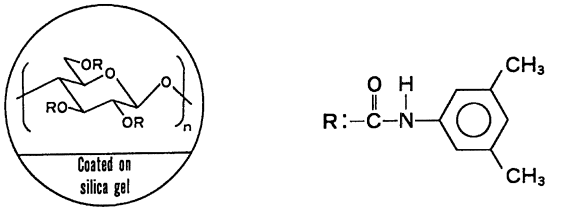


Fig. 36. Structure of Chiralcel OD. From Daicell Company literature ©.

Table 1
Compounds analysed on Chiralcel OD phase[a]

Pindolol	Bendroflumethiazide	Propranolol
Trimiprazine	Cyclophosphamide	Fenoprofen
Ketoprofen	3,5-Dnb-DL-leucine	Hexobarbital
Oxphencyclamine	3,5-Dnb-DL-phenylglycine	Pentobarbital
Metoprolol	2,4-Dnp-DL-norleucine	GR57888X
Chlorthalidone	Dns-DL-serine	GR50360A
Ibuprofen	Dns-DL-glutamic acid	Ondansetron
Terfenidine	Disopyramide	Carbovir

[a] Dnb = Dinitrobenzoyl; Dnp = dinitrophenyl; Dns = dansyl. Operating conditions: instrument: HP[3D] CEC; packed length: 24.5 cm; total length: 33 cm; mobile phase: acetonitrile–10 m*M* Tris, pH 7.5 (85:15, v/v); applied voltage: 30 kV; temperature: 30°C; injection: 5 kV for 5 s.

was demonstrated by the separation of 16 polycyclic aromatic hydrocarbons (PAHs). A disadvantage of this system was that in order to make an injection the column had to be disconnected from the system.

Behnke and Bayer [39] reported a pressurised gradient CEC system employing split flow from a conventional HPLC pump. Injections were performed using a rotary injection valve which was connected to a splitter device that delivered solvent to an inlet interface. In 1997 Taylor et al. [40] also developed a CEC system capable of delivering gradient flow and this was similar in design to that of Behnke and Bayer. They used this system to separate a mixture of corticosteroids in extracts of horse plasma and urine. An Apex 3 μm ODS-1 capillary was used with a packed length of 16 cm to the detector and a total length of 24 cm. This system applied a positive potential to the capillary inlet so that the outlet could be coupled with electrospray ionisation mass spectrometry (ESI-MS) if required. The gradient system employed by Huber et al. [41] was used to separate a number of amino acids and steroids. They were especially interested in decreasing the retention time of phenylthiohydantoin (PTH)-

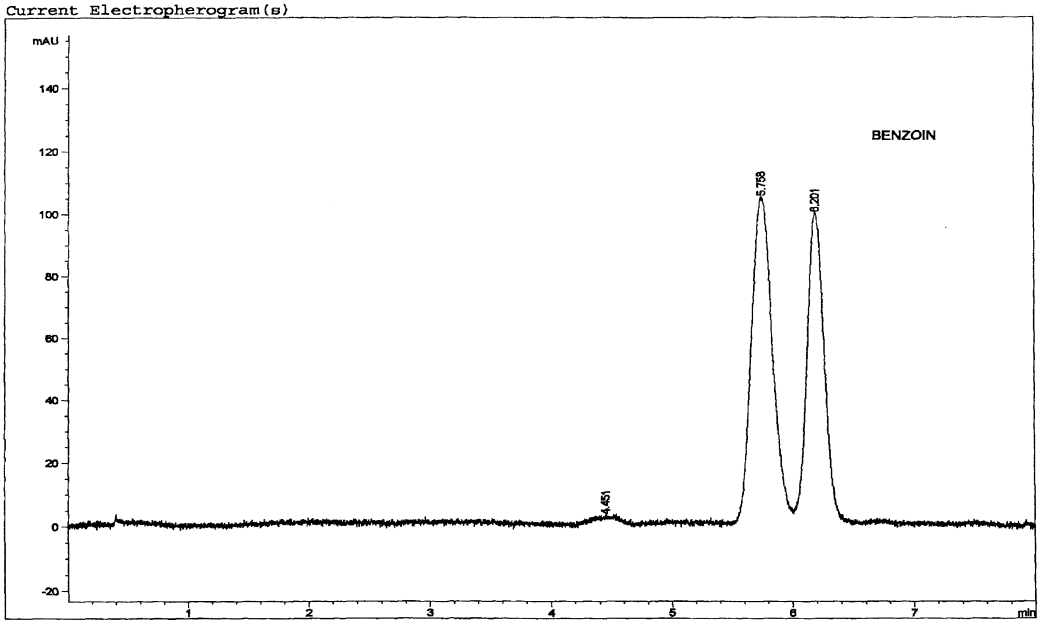


Fig. 37. Separation of benzoin on Chiralcel OD. Packed bed: 24.5 cm; total length: 33 cm. Mobile phase: ACN–5 m*M* Tris, pH 7.5 (90:10, v/v). Applied voltage: 30 kV; temperature: 40°C; injection: 5 kV/10 s.

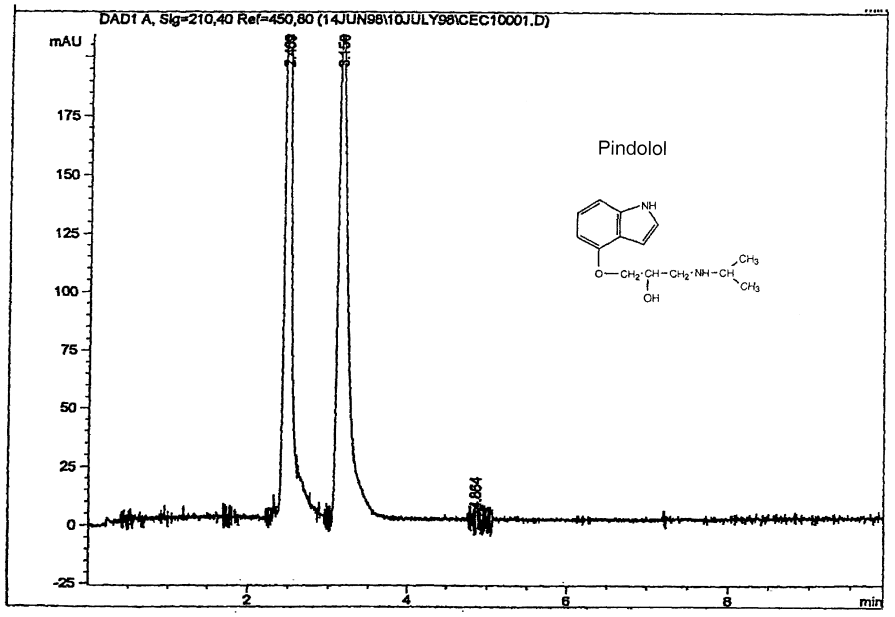


Fig. 38. Separation of pindolol on Chiralcel OD. Packed bed: 24.5 cm; total length: 33 cm. Mobile phase: ACN–2 m*M* Na_2HPO_4, pH 7.0 (80:20, v/v). Applied voltage: 30 kV; temperature: 40°C; injection: 5 kV/10 s.

methionine and PTH-proline amino acids, which had previously been separated isocratically. The instrument was similar in design to the ones previously mentioned however, an additional heating system was installed to provide uniform temperature control across the entire length of the column.

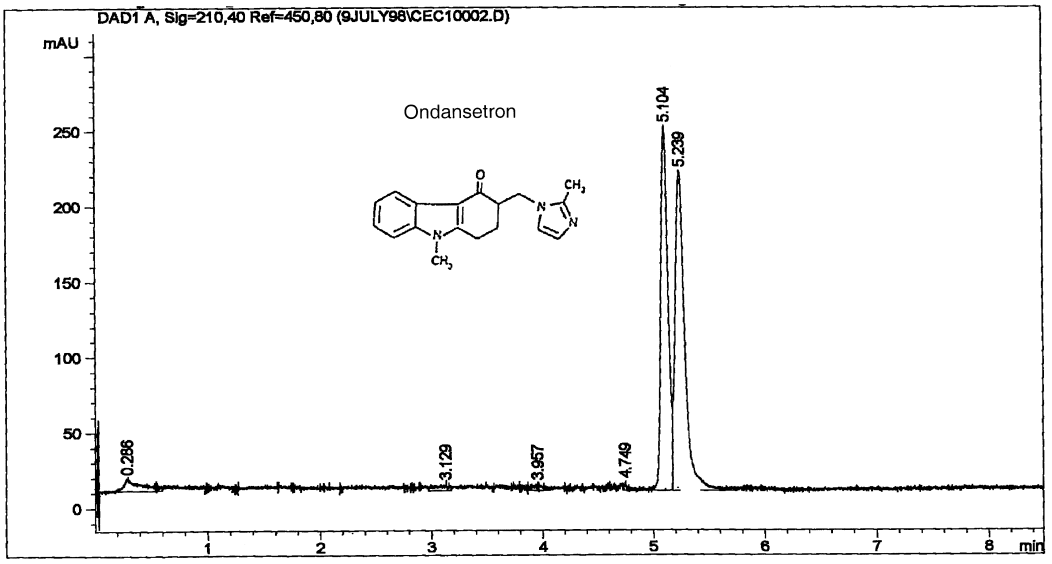


Fig. 39. Separation of ondansetron on Chiralcel OD. Packed bed: 24.5 cm; total length: 33 cm. Mobile phase: ACN–2 m*M* Na_2HPO_4, pH 7.0 (80:20, v/v). Applied voltage: 30 kV; temperature: 40°C; injection: 10 kV/10 s.

Table 2
Efficiencies of individual enantiomers of pindolol and ondansetron

Compound	Efficiency peak 1 N (m^{-1})	Efficiency peak 2 N (m^{-1})
Pindolol	30 257	14 551
Ondansetron	120 216	76 620

Ericson and Hjertén [42] designed a gradient system and applied it to the analysis of a mixture of proteins. By employing a gradient, cationic proteins were separated on a column derivatised with C_{18} groups and either stearyl methacrylate or dimethyldiallylammonium chloride. Two kinds of CEC columns were synthesised, one to achieve a moderate EOF the other to provide a high EOF. When the moderate EOF column was used the direction of the EOF was reversed so that it was counter to that of the separation. When they studied the separation profiles and resolution achieved by CEC and RP-HPLC on the same column with the same gradient propelled by an HPLC pump, they noted that the electrophoretic contribution to the separation was small. The design reported by Lister et al. [43] was of the type previously described, however in order to ground the inlet end of the set-up, the power supply was switched to reversed-polarity mode and a negative potential applied to the capillary outlet. Recently Banholczer and Pyell [44] illustrated the use of their gradient CEC system for the application of a separation of a number of benzoates. By using a gradient they were able to reduce the analysis time of this mixture from 25 min to 15 min and achieve baseline separation of all the components. We assembled a gradient CEC system based upon an ABI 270A instrument. Several modifications were made before the optimum set-up was found. Gradients were formed by using two Gilson HPLC pumps, Models 305 and 306. Samples were injected using a Rheodyne 7125 injection valve fitted with a 6-μl sample loop. A schematic of the gradient system is shown in Fig. 41.

The dimensions of connecting tubing were those which we found gave us most trouble free operation whilst maintaining good chromatography in terms of minimising band broadening and dead volumes. Despite many variations tried, we found the best inlet was constructed from a 1/16 in. (0.158 cm) stainless steel union, drilled out to take 1/16 in. PEEK (polyether ether ketone) tubing. What was crucial to the chromatography was the manner in

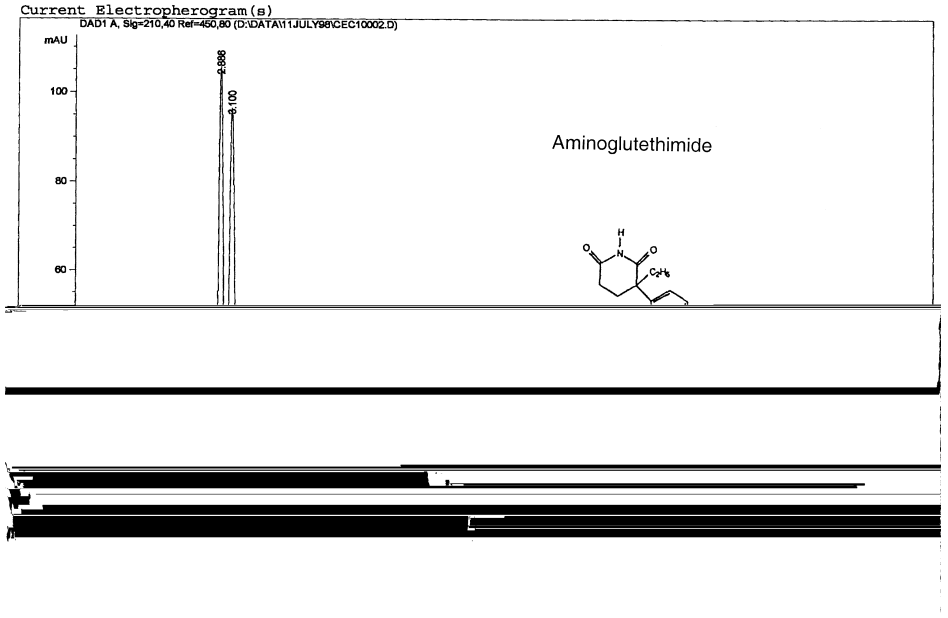


Fig. 40. Separation of aminoglutethimide on Chiralcel OD. Packed bed: 24.5 cm; total length: 33 cm. Mobile phase: ACN–2 m*M* Na_2HPO_4, pH 7.0 (80:20, v/v). Applied voltage: 25 kV; temperature: 40°C; injection: 10 kV/10 s.

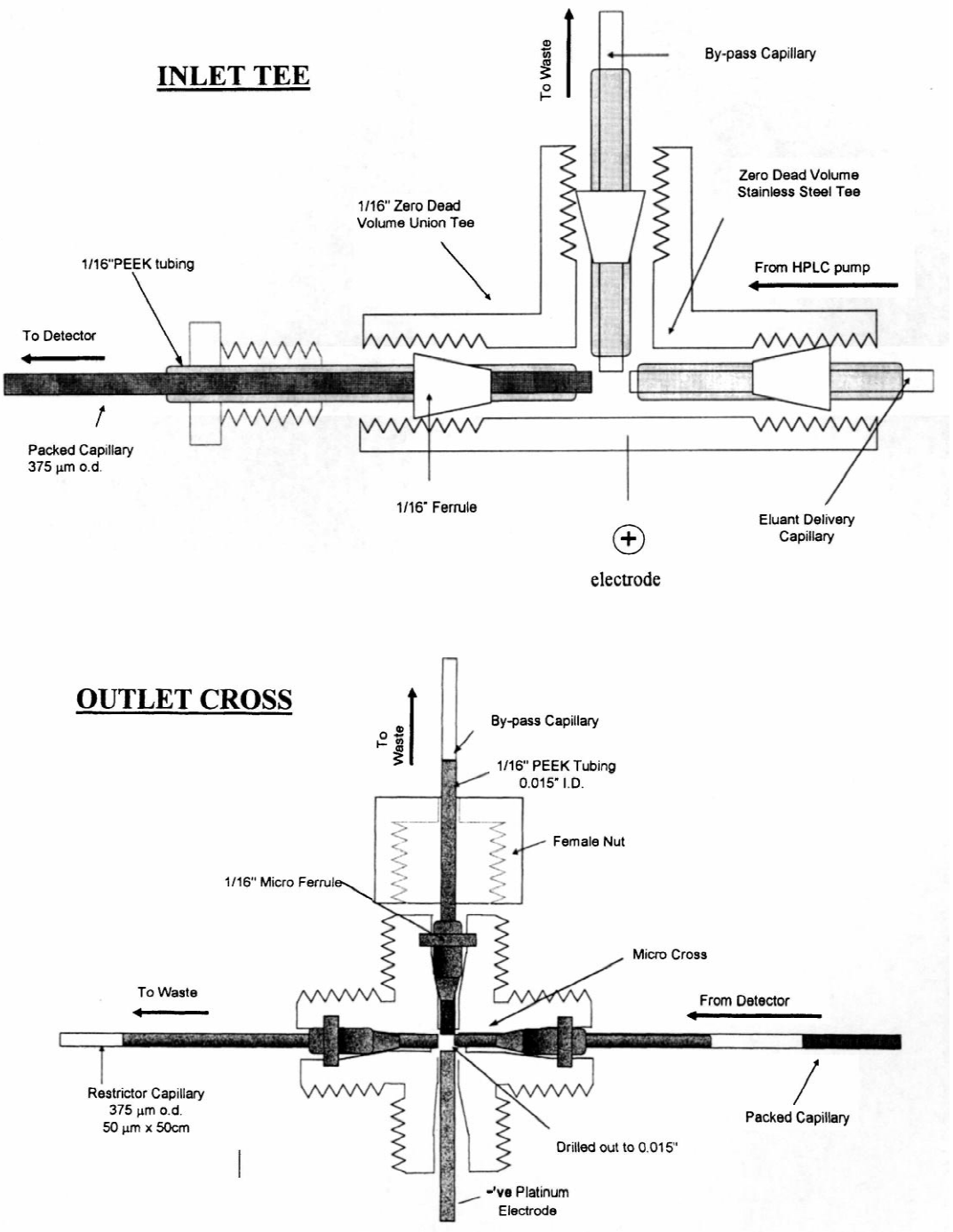


Fig. 42. Schematic of inlet and outlet devices. From A.S. Carter-Finch thesis.

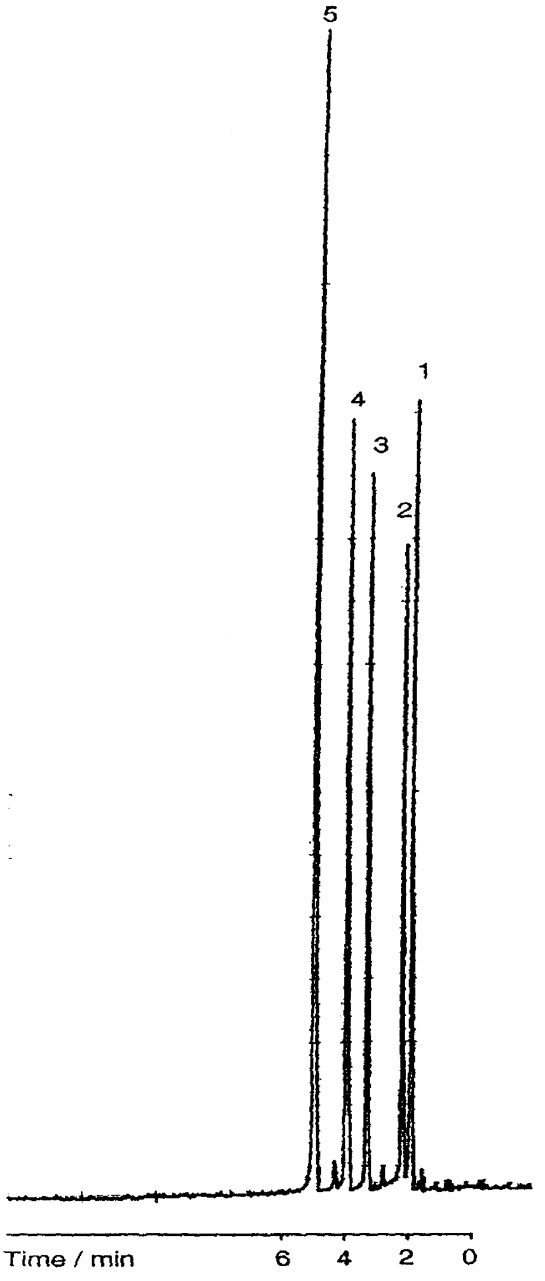
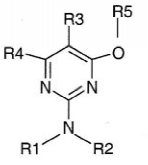


Fig. 43. Separation of a five-component test mixture. 3 μm Spherisorb ODS-1; packed bed: 25 cm; total length: 38 cm×100 μm. Mobile phase: ACN–10 m*M* Na_2HPO_4, pH 8.2 unadjusted (70:30, v/v). Applied voltage: 30 kV; temperature: ambient; detection at 214 nm; flow-rate: 0.1 ml/min; rise time: 0.5 s; range 0.05 AUFS. 1=Thiourea; 2=benzamide; 3=anisole; 4=benzophenone; 5=biphenyl. Analyte concentration=0.1 mg/ml in acetonitrile–water. From A.S. Carter-Finch thesis.



Compound	R1	R2	R3	R4	R5
Carbamates					
Pirimicarb -1	Me	Me	Me	Me	$CO.N(Me)_2$
2	CHO	Me	Me	Me	$CO.N(Me)_2$
3	H	Me	Me	Me	$CO.N(Me)_2$
4	Me	Me	Me	CH_2OH	$CO.N(Me)_2$
5	H	H	Me	Me	$CO.N(Me)_2$
Hydroxypyrimidines					
6	H	Me	Me	Me	H
7	Me	CHO	Me	Me	H
8	Me	Me	Me	CH_2OH	H
9	H	H	Me	Me	H
10	H	Me	Me	CH_2OH	H
11	H	H	CH_2OH	Me	H

Fig. 44. Pirimicarb and related compounds. From A.S. Carter-Finch thesis.

possible to perform step-gradients on commercial instruments and this involves changing mobile phase vials during the run from a vial containing a low concentration of organic solvent to a vial with a higher organic concentration. After much method development, the best gradient conditions in terms of acceptable resolution and analysis time were as follows: 10% B $\overset{5\ min}{\rightarrow}$ 40% B where B=acetonitrile–5 m*M* Tris, pH 8.6 (80:20, v/v) and A=acetonitrile–5 m*M* Tris, pH 8.6 (12:88, v/v).

In all the separations including those performed isocratically, compounds 6 and 7 were not resolved. An example of the gradient CEC analysis is shown in Fig. 45.

Resolution of components 6 and 7 could possibly be achieved by the use of ternary solvent systems and/or a different stationary phase. The design of the instrument is such that shorter capillaries ACN be used thus facilitating the use of higher field strengths resulting in faster analysis times.

3.6. Limitations of CEC

In order for a new analytical technique to be

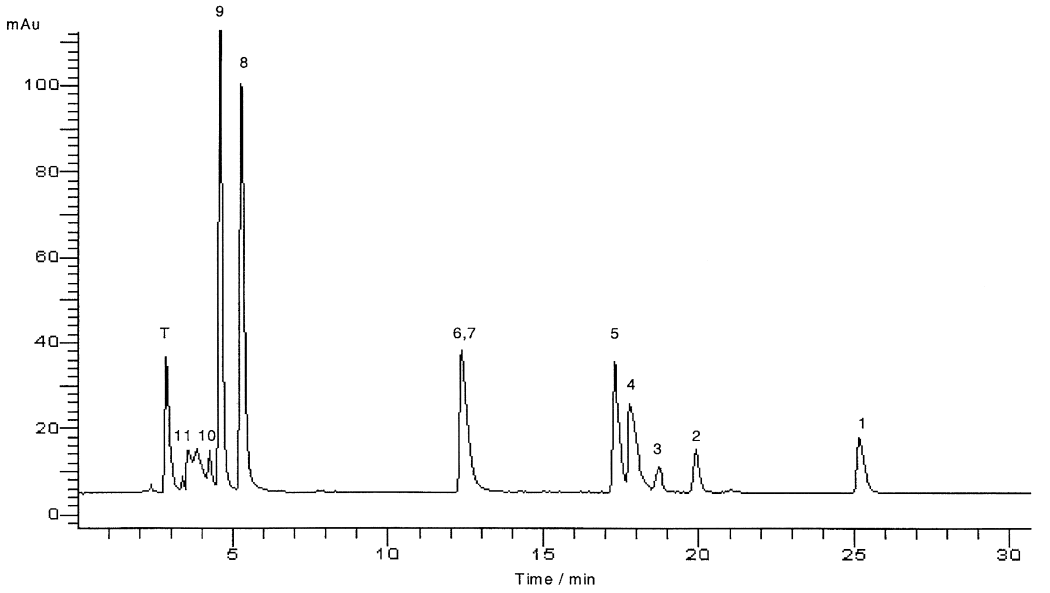


Fig. 45. Gradient CEC analysis of pirimicarb and related compounds. Separation of pirimicarb and related compounds 1–11 using a solvent composition gradient 10% B $\overset{5\ min}{\rightarrow}$ 40% B. Capillary: 3 μm Spherisorb ODS-1, packed length 24 cm, total length 36 cm, 100 μm I.D. Conditions: 10% B $\overset{5\ min}{\rightarrow}$ 40% B, where: A = acetonitrile–5 m*M* Tris, pH 8.6 (12:88, v/v) and B = acetonitrile–5 m*M* Tris, pH 8.6 (80:20, v/v); 30 kV; ambient temperature; injection: 6 μl; UV detection at 214 nm. Samples as in Fig. 44. From A.S. Carter-Finch thesis.

accepted, it is of tremendous importance to interest the pharmaceutical industry. Although CEC has shown tremendous promise with neutral and acidic compounds, it fails quite dramatically when it comes to highly basic compounds. Since the majority of pharmaceutical compounds contain basic amine functions, this presents a big hindrance to the progression of this technique. Although some progress has been made using mobile phase additives, there is a pressing need for a phase capable of analysing highly basic compounds.

The vast majority of CEC capillaries are fabricated from fused-silica and once a detection window is burnt, become extremely fragile. Since these capillaries are then easily broken, this puts a serious constraint on the use of the technique, especially in view of the high cost. There is then a need for CEC capillaries to be produced from materials other than fused-silica. There is also considerable concern over the fragility and behaviour of the retaining frits in CEC, and future developments should concentrate on ''fritless capillaries''.

Finally, CEC instrumentation is very limited. Although Hewlett-Packard and Beckman both offer instruments for CEC, neither are purpose built instruments but have been modified to work under pressure thus allowing their use for CEC. There is considerable concern that CEC is potentially unreliable because it is difficult to control the EOF. It is therefore considered that the only way to overcome this problem is to superimpose a hydraulic flow onto an electrically generated flow, thereby introducing reproducibility into the technique. This is only achievable by having an integrated μ-LC–CEC system but at this moment in time, manufacturers seem extremely reluctant to produce such an instrument.

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