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Review

Capillary electroendoosmotic chromatography of peptides

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Abstract

This review focuses on the current state of peptide separation by capillary electroendoosmotic chromatography (CEC). When carried out under optimised conditions, peptide separation by CEC methods represents an orthogonal and complementary technique to micro-HPLC (μ-HPLC) and high-performance capillary zone electrophoresis (HPCZE). The origin of the selectivity differences that can be achieved with these three separation techniques (CEC, μ-HPLC and HPCZE), respectively are discussed, and the current limits of performance with CEC methods documented. Peptide separations by CEC methods with *n*-alkyl bonded silicas or mixed-mode phases are also illustrated. The development of different variants of CEC and pressurised CEC (also commonly referred to in the literature as electrically-assisted μ-HPLC) are examined. The potential of coupling CEC systems to mass spectrometers for real-time analyses of peptides or protein digests has been examined. Several future directions for the application of this technique in phenotype/proteomic and zeomic mapping of naturally occurring peptides and proteins are highlighted.

Keywords: Reviews; Electrochromatography; Optimisation; Peptides

Contents

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1. Introduction

In this article, recent progress in the high-resolution separation of peptides by capillary electroendoosmotic chromatography or, as more commonly known, capillary electrochromatography (CEC) has been reviewed. During the last several years, progress in the separation of neutral analytes as well as inorganic ions by CEC [1] has resulted in several aspects of the technique being considered for validation purposes by industry. The CEC of neutral compounds is an excellent complementary technique to micellar electrokinetic chromatography (MEKC), where the analyte partitions between the eluent and the micelles [2–4]. Charged compounds, including peptides, are more commonly separated by reversed-phase high-performance liquid chromatography (RP-HPLC) or by high-performance capillary zone electrophoresis (HPCZE). In RP-HPLC with bonded *n*-alkylsilicas, peptides are retained by solute–sorbent interactions mediated by hydrophobic and silanophilic effects [5–7]. In HPCZE, selectivity is mainly generated through the electrophoretic migration of peptide analytes according to their intrinsic charge characteristics and differences in Stokes' radii [8,9]. Compared to HPCZE, MEKC or CEC methods, the analytical separation technique most often applied today in peptide chemistry remains RP-HPLC. The facile ability to manipulate retention and selectivity of peptides in RP-HPLC by changing the eluent composition and/or the type of non-polar stationary phase are the main reasons for its continuous growth in popularity, since the technique was pioneered for application in peptide chemistry in the mid 1970s [10,11]. Because different physicochemical properties can be exploited, the various capillary electrophoresis methods are often considered as complementary or orthogonal analytical separation techniques to RP-HPLC procedures with peptides.

The application of CEC procedures to the analysis of polar compounds such as peptides is a relatively new development in the analytical separation sciences. Very little attention has yet been given to the selection of sorbent properties, field strength or buffer composition for the optimal separation of peptides by CEC procedures. The focus in CEC developments up to the present time has largely been placed on the separation of neutral pharmaceutical compounds or low-molecular-mass drug substances, and to a lesser extent on the analysis of inorganic ions. In addition, several groups of investigators have examined the development of suitable packing materials with optimum stationary phases and particle or pore sizes, the manufacture of frits, or the preparation stable columns [12–16]. These fundamental studies on the CEC performance are nevertheless mandatory if rugged procedures are to be established or the separation mechanisms with different classes of analytes are to be understood [17–23]. These investigations have shown that the retention behaviour of neutral compounds in CEC generally corresponds well to that observed in RP-HPLC. In comparison, the impact of the reversed-phase/multimodal chromatographic retention phenomena and electrophoretic migration effects with charged analytes, such as peptides, in CEC has not yet been studied in a comprehensive, systematic manner.

Peptides due to their charge, hydrophobicity, size and surface topology are ideal probes to elucidate the retention mechanism in CEC. The net charged of a peptide depends on the pH of the buffer. As will become apparent later in this article, most peptide separations by CEC can be performed on the walls of *n*-alkyl modified silica-based capillaries or with capillaries packed with reversed-phase material such as C_8- and C_{18}-bonded silicas, although mixed-mode phases can also be used. In order to achieve suitable mixed-mode phases, recourse can be made to the use of the commonly employed *n*-alkyl bonded silica packing materials with strong cationic groups attached, e.g., sulfonic acid groups. In addition, partially or non-end capped *n*-alkylsilica sorbents can be

employed, thus permitting the generation of an electroendoosmotic flow (EOF) effect. As a consequence, analytical CEC separation methods can either be performed under isocratic or gradient elution procedures. Detection is typically achieved using UV or diode array detection (DAD) although the number of publications where a mass spectrometer is used for detection of peptides separated by CEC is increasing. In this review, the current status of CEC for the analyses of peptides with packed and also surface modified etched open tubular fused-silica capillaries has been evaluated from the most relevant findings.

2. Fundamental issues

2.1. Peptide properties

Pharmacological studies with peptides usually require the synthesis not only naturally occurring bioactive peptides, but also of numerous analogues, thus permitting investigation into the relationship between structure and biological activity. A large number of homologous series of synthetic peptides have been studied by RP-HPLC, exploring their surface properties, solution conformations, thermodynamics behaviour on interaction with the sorbent and associated biophysical and functional features [6,7,11,24–26]. Since the side chain and N- and C-terminal charge moieties of peptide are most relevant in CEC separations, evaluation of the intrinsic charge features, Stoke's radius properties, solvational propensities and hydrophobic moment characteristics of peptides are essential requirements behind the evolution of successful separations. Peptides with free amino- and carboxyl termini are amphoteric molecules, and frequently contain both basic and acidic amino acid residues at their N-, C- and endo-positions. A particular peptide will thus exhibit a unique isoelectric point (p*I*) value whereby the net charge of the molecule reaches zero with a buffer of specific pH value. The p*I* value of a peptide is determined by the nature of the amino acid sequence, the type of the N- and C-terminal groups present and the extent of secondary structure/folding that has occurred under the particular buffer/solvent conditions. The intrinsic charge of a peptide is thus determined by the composition and pH of the buffer, the pK_a values of the N-terminal α-amino and C-terminal carboxyl groups, the pK_a values of the amino acid side chains, and the dipole moment and hydrogen bonding characteristics of the peptide backbone. Illustrative properties of the amino acid residues in terms of their occurrence within proteins and other biophysical features are listed in Table 1.

With many peptides, particularly those of synthetic origin, the N-terminal α-amino group can be acetylated, whilst the C-terminal group may be amidated. These modifications eliminate the possibility of protic equilibria occurring at the N- and C-terminal positions in buffers of high or low pH value. In these cases, the charge and p*I* characteristics of the peptide are totally determined by the nature of the amino acid side chains within the sequence. Thus, a peptide with the following structure Ac–Val–Phe–Leu–NH_2 has no charged moieties and will behave more like a hydrocarbon, compared to H–Val–Phe–Leu–OH, which has amphoteric properties due to the presence of both the basic N-terminal amino group and the acidic C-terminal carboxyl group.

With current solid-phase peptide synthesis technologies, essentially an infinite array of possibilities can be generated from the 20 naturally occurring L-α-amino acids, which form a genetically encoded subset, or the even larger sets of D-α-, or β-, γ-, δ-, ϵ-, etc., types of amino acids that can be prepared and incorporated into a peptide structure. Thus, for the tripeptide cited above, $20^3-1=7999$ additional variants can be generated using only the protein L-α-amino acid residues as the building blocks by exploiting either multiple split-and-combine or the ''selectide'' solid-phase peptide synthesis procedures [27,28], whilst for a naturally occurring hexapeptide, 20^6 ($\approx 64\cdot 10^6$) peptide isomers could in principle be generated from the common L-α-amino acid building blocks alone. This level of molecular diversity can be increased exponentially using D-α-amino acid residues, non-L-α-amino acids containing β-, γ-, δ-, ϵ-linkages, or by subsequent chemical modification in situ of the synthetic peptide construct at specific amino acid side chains. The opportunity to synthesise very large numbers of peptide analogues (as combinatorial libraries), as well as the practical realities of solid-phase peptide synthesis (SPPS) per

Table 1
Fundamental properties of amino acid residues in terms of occurrence in proteins, molecular mass, molecule volume, accessible surface area, partial specific volume, pK_a of ionising side chains and relative hydrophobicity [6]

	One-letter abbreviation	Occurrence in proteins [6,85,86] (%)	Molecular mass	Volume [87] (Å^3)	Accessible surface area [88] (Å)	Partial specific volume [87] (ml/g)	pK_a of ionising side chain [89]	Relative hydrophobicity [90]
Ala	A	9	71.08	88.6	115	0.748		0.06
Arg	R	4.7	156.2	173.4	225	0.666	~12	−0.85
Asn	N	4.4	114.11	117.7	160	0.619		0.25
Asp	D	5.5	115.09	111.1	150	0.579	4.5	−0.20
Cys	C	2.8	103.14	108.5	135	0.631	9.1 to 9.5	0.49
Gln	Q	3.9	128.14	143.9	180	0.674		0.31
Glu	E	6.2	129.12	138.4	190	0.643	4.6	−0.10
Gly	G	7.5	57.06	60.1	75	0.632		0.21
His	H	2.1	137.15	153.2	195	0.67	6.2	−2.24
Ile	I	4.6	113.17	166.7	175	0.884		3.48
Leu	L	7.5	113.17	166.7	170	0.884		3.50
Lys	K	7	128.18	168.6	200	0.789	10.4	−1.62
Met	M	1.7	131.21	162.9	185	0.745		0.21
Phe	F	3.5	147.18	189.9	210	0.774		4.80
Pro	P	4.6	97.12	122.7	145	0.758		0.71
Ser	S	7.1	87.08	89	115	0.613		−0.62
Thr	T	6	101.11	116.1	140	0.689		0.65
Trp	W	1.1	186.21	227.8	255	0.734		2.29
Tyr	Y	3.5	163.18	193.6	230	0.712	9.7	1.89
Val	V	6.9	99.14	140	155	0.847		1.59
			α-Amino 6.8 to 7.9			α-Carboxyl 3.5 to 4.3		

se, whereby the repetitive yield never reaches 100% and truncation, deletion and racemisation events arise, demand the availability of analytical screening methods with very high throughput and high resolution capabilities. Application of innovative CEC procedures, either alone or in conjunction with micro-HPLC (μ-HPLC) and HPCZE methods, could, in principle, achieve this outcome.

2.2. Micro-HPLC methods

The small sample volume and low abundance of proteins and peptides present in biological fluids and protein digest are often a limiting factor for analyses with conventional HPLC columns of 4.0–4.6 mm I.D. Downsizing of columns with respect to their inner diameter as well as length demands the utilisation of fully optimised equipment. The elution volume of peptide analytes with capillary columns (100–300 μm I.D.) with μ-HPLC systems when gradient elution conditions are employed are considerably smaller compared to conventional columns, an outcome directly reflected by the ratio of the internal diameters [as $(r_1)^2/(r_2)^2$)], and thereby permitting higher mass sensitivity in diode array UV (DAD) or laser-induced fluorescence (LIF) detection to be achieved with optimised μ-HPLC systems.

Mass spectrometer devices can also be easily coupled to a μ-HPLC systems for increased detection capabilities. Application of this hyphenating μ-HPLC–UV–DAD–LIF–MS detection approach increases the sensitivity further compared to a single channel (i.e., 215 nm) UV detector. The use of MS detection will also increase tremendously the amount of information related to the sample in terms of molecular mass, composition and sequence. In particular, with μ-HPLC–MS–MS systems, information on the fragmentation pattern of the separated peptides directly can be used to reveal the peptide sequence. Since in optimised circumstances, higher peak capacities (i.e., the number of eluting baseline resolved peaks per time unit) can be achieved for

μ-HPLC systems compared to conventional HPLC systems, this feature can save time and reagent costs for the analysis and can be important for high through-put peptide mapping.

In μ-HPLC, the peptide analytes are transported through the column by a hydraulic flow, with selectivity achieved through the interaction of the peptides with the stationary phase. In the case of RP-HPLC selectivities, separation of peptides on *n*-alkylsilica sorbents is mainly due to an adsorption/desorption mechanism and relies only to a limited extent on partition effects [6,7,29–32]. This on/off behaviour with peptides contributes dramatically to the subtle changes in the retention times that occur when as little as 1–2% change in the organic solvent modifier content is employed in the mobile phase. The fundamental term that describes this responsiveness to changes in chromatographic conditions is the retention time, t_R. A non-retained component will move with the velocity of the eluent and its elution time is commonly indicated by the void time, t_0. The extent of retardation of a peptide component is determined by the equilibrium distribution constant, K_a, and the phase ratio, Φ, of the chromatographic system. By convention, this extent of retention is expressed as a unitless capacity factor, k', thus permitting columns of different configuration to be compared, through the expressions:

$$k' = \frac{t_r - t_0}{t_0} = K_a \Phi = \Phi e^{-\Delta G_{assoc}/RT} \quad (1)$$

where ΔG_{assoc} is the Gibbs free energy change associated with the interaction of the peptide with the sorbent–mobile phase system and Φ is the phase ratio. In RP-HPLC, the extent of retention mediated by the dominant hydrophobic mechanism depends on the peptide sequence and conformation as well as the relative hydrophobicity of the sorbent and the extent that silanol groups are accessible. The hydrophilic/hydrophobic balance of peptides can vary considerably, due in part to their ability to undergo sequence dependent solvational and conformational processes as well as to carry different net positive/negative charges depending on the pH and composition of the mobile phase used for separation. This behaviour can result in selectivity reversals as well as pronounced dependencies on the mole fraction, ψ, of the organic solvent modifier used for the chromatographic elution. Since the concentration range of the organic solvent modifier needed to achieve an acceptable k' range (i.e., $1<k'<100$) is often extremely narrow for many peptides in the isocratic mode, gradient elution is usually necessary for the separation of peptide mixtures using conventional or μ-HPLC methods.

Due to contributions from wall effects and longitudinal mass transport processes within the column that arise between the particles and within the pores, parabolic flow profiles are usually generated in μ-HPLC separations. This behaviour results in band broadening, and can be contrasted to the plug flow properties evident in CEC, whereby the overall flow profile assumes a more uniform shape across the column cross-section. In order to achieve shorter analysis times, non-porous and porous silica-based reversed-phase sorbents of 1–2 μm particle diameter can be employed in short columns (i.e., ≤5 cm) thus avoiding the high column pressure drop. To reduce analysis times even further with μ-HPLC systems with peptides recourse to the use of elevated temperatures can be made, permitting lower eluent viscosities and higher flow-rates to be achieved. However, in μ-HPLC the reduced plate height, $h = H/d_p$, is predominantly controlled by Eddy diffusion and mass transfer effects, i.e., the *A* and the *C* terms of the van Deemter equation. At high linear velocities the *A* term remains a dominant contribution to band broadening, even with optimised monolithic sorbents, resulting in *h* never falling below a value of ca. 2.

2.3. High-performance capillary zone electrophoresis

In the high-performance capillary zone electrophoresis (HPCZE) of peptides, open fused capillaries of 25–75 μm I.D. and lengths of 25–100 cm are commonly used. When an electrical potential is applied along the axial direction of an open tubular capillary a net movement of solvated cations occurs towards the negative electrode (cathode). The velocity of this cation migration, which results in EOF, is given by Eq. (2). The EOF effect in HPCZE is generated by the electric double layer at the solid–liquid interfaces of the capillary wall, and gives rise

to a plug-like flow profile. The magnitude of the EOF can be expressed (Eqs. (2) and (3)) in terms of velocity (u_{eo}) or mobility (μ_{eo}). Charged analytes, such as peptides, are transported through the open fused capillary due to the combined effects of their electrophoretic migration and this EOF effect. The electrophoretic mobility of a peptide analyte is directly proportional to its intrinsic charge but inversely proportional to its Stoke's radius, i.e., the radius of the peptide as represented by the sphere swept out by the peptide in the solvent (Eq. (4)), and the viscosity of the buffer as illustrated in Fig. 1. The apparent electrophoretic mobility (μ_a) of the peptide is thus the sum of intrinsic electrophoretic mobility of the peptide analyte (μ_e) and the electrophoretic mobility of the EOF (μ_{eo}) (Eq. (5)):

$$u_{eo} = \frac{\epsilon_0 \epsilon_r \zeta E}{\eta} \quad (2)$$

$$\mu_{eo} = \frac{\epsilon_r \zeta}{\eta} \quad (3)$$

$$\mu_e = \frac{q}{6\pi\eta r} \quad (4)$$

$$\mu_a = \mu_{eo} + \mu_e \quad (5)$$

where ϵ_0 is the permittivity of in a vacuum, ϵ_r is the relative permittivity (dielectric constant) of the mobile phase, ζ is the zeta potential of the liquid–solid interface, η is the viscosity of the mobile phase, E is the electric field strength (kV/m), r_S is the ion radius or the Stoke's radius of the peptide analyte, and q is the intrinsic charge of the peptide, respectively. In electrophoresis, under ideal conditions, the peptide analyte is not retained by, or interacts with, the capillary walls and its transit time from the beginning of the capillary to the detector window can be denoted as the migration time, t_e. The dominant band broadening contribution in the HPCZE of peptides is associated with longitudinal diffusion, i.e., the B term of the van Deemter equation. The interactive properties of the fused-silica capillary wall can be modified by different kinds of coatings, e.g., with polyvinyl alcohol, polyacrylamide, polyethyleneglycol, etc., to diminish ionic or hydrophobic interactions with peptides, which cause adsorption and tailed peaks [2,34]. By minimising this peak distortion, efficiencies with coated capillaries can be

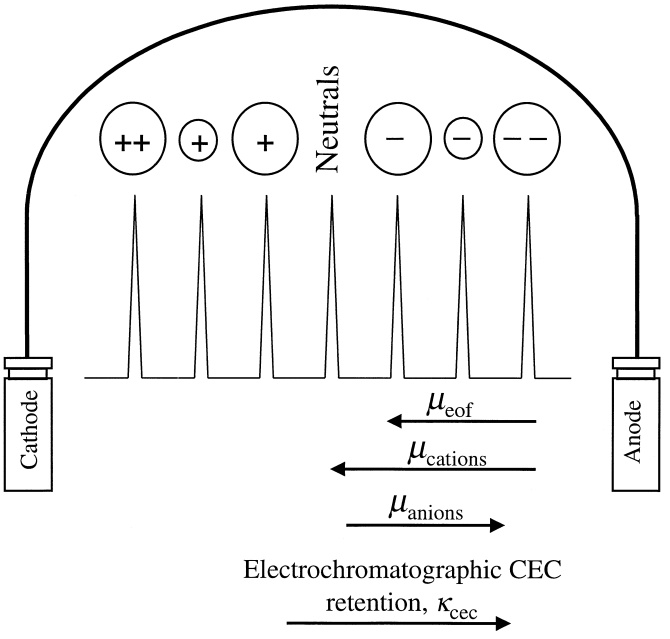

Fig. 1. The migration order of positively charged, negatively charged and uncharged (neutral) components in a separation by capillary electrophoresis electrochromatography are schematically illustrated.

increased significantly. Coated capillaries also have a very low or negligible EOF and therefore the separation is almost totally due to the intrinsic electrophoretic mobility of the peptide analyte. In HPCE, the peak shapes of peptides, separated at a low pH, can often be improved by increasing the buffer ionic strength. Joule heating in the capillary is however a limiting factor when buffers of very high molarity are employed.

2.4. Capillary electroendoosmotic chromatography

In 1974 Pretorius et al. [35] demonstrated the possibility of using the EOF effect to transport the eluent through a packed column, whilst in 1981 Jorgenson and Lukacs described the use of CEC in pyrex glass capillaries [36,37]. Further, fundamentally studies of CEC and the role of the EOF profiles have been performed by Knox and Grant [38] and Smith and Evans [22,39], providing a theoretical and practical framework for the application of CEC with low-molecular-mass neutral compounds using reversed-phase and mixed-mode sorbents. As noted above, the origin of the EOF effect stems from the electrical double layer existing at all solid–liquid interfaces. In capillary zone electrophoresis, the walls of the fused-silica tubing contribute to the EOF, whereas in CEC the overall contribution to EOF is predominantly caused by the charged surface of the particles in the column bed [40].

Thus in CEC, the EOF velocity depends on surface charge of the sorbent particles, the temperature, and the composition of the eluent regarding its density, dielectric constant, viscosity, pH and ionic strength. As currently operated, in CEC a fused-silica capillary (typically of 50–100 μm I.D.) is packed with desired packing material, typically ≤3 μm in mean particle diameter, prior to the analysis. Today, several types of commercial CEC columns with different types of packing materials are available [41], based on fused-silica capillaries, packed with 3–5 μm particles with different types of hydrophobic or mixed-mode coatings. In the CEC mode with *n*-alkyl bonded silicas, neutral analytes separate because of their ability to partition into the immobilised *n*-alkyl chains, whilst in the case of charged analytes such as peptides, the separation is achieved through the interplay of adsorption effects and electrophoretic migration processes when an electric field is applied over the column. When silica-based sorbents are employed, basic compounds can cause tailing because of interactions with the free silanol groups due to the participation of ionic effects.

An expression for the velocity of EOF in a packed column (Eq. (8)) is obtained by combining Eqs. (2), (6) and (7). In CEC, a plug flow profile is generated in the channels between the sorbent particles in the packed column bed but the velocity is independent of the channel width. With current CEC instrumentation and columns, efficiencies approaching 250 000 plates/m can be achieved with 3 μm reversed-phase sorbents. The magnitude of EOF is virtually independent of the particle size since no additional column back pressure is generated when operating in the CEC mode. Plots based on the van Deemter equation of h versus ν for sorbents in the mean particle size range of 0.5–3.0 μm indicate that the B term is the dominant factor controlling overall efficiency, i.e., h is mainly controlled by longitudinal diffusion. If the solutes are charged they will undergo an acceleration or deceleration depending on their actual net charge. The separation of charge solutes, e.g., peptides, does, however, take on a more complex dimension since the apparent velocity is the summation of the EOF velocity, the electrophoretic migration of the peptide analyte (which may be in a positive or negative direction) and the retention of the analyte due to chromatographic interactions with the packing material in the column, as illustrated in Fig. 1:

$$\zeta = \frac{\sigma}{\epsilon_0 \epsilon_r \kappa} \tag{6}$$

$$\kappa = \left(\frac{2cF^2}{\epsilon_0 \epsilon_r RT} \right)^{1/2} \tag{7}$$

$$u_{eo} = \frac{\sigma \left(\frac{\epsilon_0 \epsilon_r RT}{2cF^2} \right)^{1/2}}{\eta} \cdot E \tag{8}$$

where ϵ_0 is the permittivity in vacuo, ϵ_r and η are the relative permittivity (dielectric constant) and viscosity of the mobile phase, respectively, ζ is the zeta potential across the diffuse liquid–solid interface, σ is the charge density of the surface of shear, κ is the structural packing parameter, E is the electric field

strength (kV/m), R is the gas constant, T is the temperature, c is the electrolyte concentration and F is the Faraday constant.

As evident from Eqs. (6–8), the u_{eo} term is inversely related to the viscosity of the eluent, but proportional to $\sqrt{T}$. These dependencies will result in non-linear retention behaviour in terms of the overall effects of the EOF effect and the chromatographic interaction process when unexpected or deliberately induced changes in temperature occur in the system. Complex non linear van't Hoff behaviour, expressed as a function of $\ln k'_{CEC}$ versus $1/T$, can thus be anticipated for the CEC of peptides. By analogy to the recently described homo- and heterothermic behaviour [6,7,26] of peptides in RP-HPLC or other interactive HPLC modes, where non-linear dependencies of the change in the enthalpy (ΔH_{assoc}), entropy (ΔS_{assoc}) and heat capacity (ΔC_p) of the system as a function of T have been discovered, the CEC of peptides under temperature programmed conditions can be anticipated to provide enhanced resolution and important additional biophysical data on the conformational integrity, peptide surface–solvent interaction characteristics and the mechanism of binding and desorption from the surface modified microparticulate support materials.

3. Characterisation of CEC systems

3.1. EOF properties

To obtain high EOF effects in CEC with reversed-phase packing materials, such as a C_8- or C_{18}-bonded silica, is necessary for these sorbents not to be end-capped [40,42,43]. A high surface density of ionised silanol groups on the sorbent particle increases the magnitude of the EOF, but on the other hand it also increases the silanophilic interaction with the peptide analyte. At pH$\geq$8, type I and type II silanols are fully ionised and therefore a high EOF will be obtained when the pH of the eluent/buffer is near this value. In contrast, at a low pH values, i.e., pH$<$3.0, the silanol groups are partially protonated and a significant decrease (or lack) of the EOF effect can be arise with the currently available reversed-phase packings. Alternatively, hydrolytically stabilised amino- and diol-phase coated silica materials [44,45] or macroporous, polyacrylamide/polyethylene glycol matrices [46] can be used for manipulation of the electroosmotic flow. Another way to establish a high EOF effect at a low pH is to use mixed-mode phases. For example, *n*-alkyl bonded silica materials containing sulfonic acid groups give a high EOF even at low pH as illustrated in Fig. 2.

3.2. Performance

Currently, most CEC analyses have been performed in the isocratic mode for the separation of uncharged, neutral compounds. Gradient elution profiles for the separation of pharmaceutical compounds as well as peptide maps has been advocated by several research groups. Although viable gradient systems have recently become available from two instrument manufacturers, wider usage of gradient elution protocols in CEC has yet to become popular. The combination of electrochromatography (EC) and liquid chromatography (LC) was first described by Tsuda [47], who used a pump at the inlet side of the column to suppress bubble formation. Smith and Evans [22,48] have also emphasised the need to pressurise the buffer vials to suppress the formation of gas bubbles in the eluent at high currents. Pseudo-electrochromatographic (pEC) separation methods based on both the chromatographic and electrophoretic behaviour of neutral compounds combined with continuous flow fast atom bombardment mass spectrometry (CF-FAB-MS) was demonstrated by Verheij et al. [49]. Subsequently, Hugener et al. [50] described the use of a similar pEC system with electrospray ionisation mass spectrometry (ESI-MS). The application of pressure-assistance on the inlet side of the CEC column through solvent delivery by a HPLC pump has been demonstrated by Eimer and Unger [51] as well as by Behnke and Bayer [52]. A schematic illustrating such systems is shown in Fig. 3. Irrespective of whether the technique is called pressurised- or pseudo-EC or electrically-assisted-LC, the separation mechanism is the same. In the beginning of the development of this new technique, the pressure on the inlet capillary end was used to suppress bubble formation, but later it was realised [51] that the selectivity could also be fine tuned either by changing the pressure or the electrical potential.

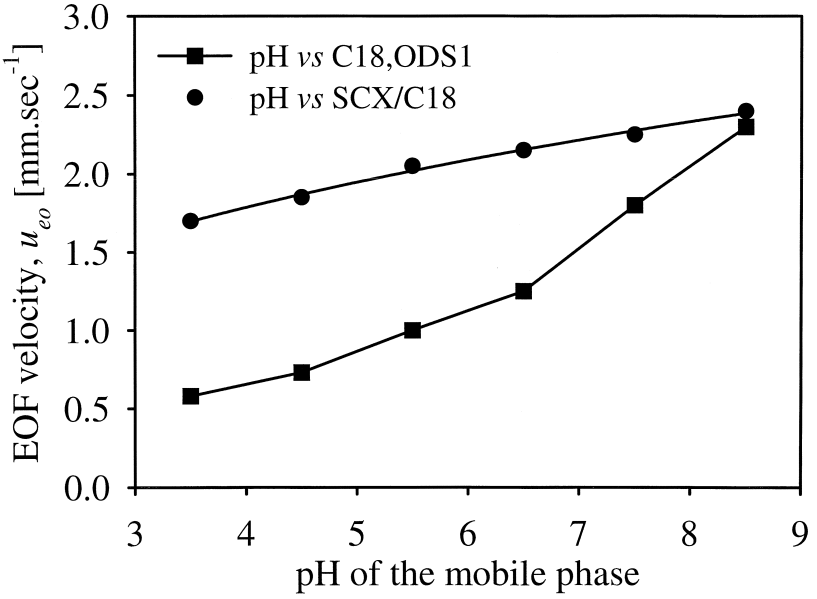

Fig. 2. Dependence of EOF on the pH of eluent for an *n*-octadecyl bonded silica (C_{18}, ODS1) and a mixed-mode bonded silica (sulfonic acid/*n*-octadecyl, SCX/C_{18}). Data derived from Ref. [74].

3.3. Selectivity

The use of pressurised CEC provides a special advantage for the separation of charged species, since the electrophoretic migration rate and the mobile phase flow-rate can be optimised independently. The retention factor, κ_{CEC}, in CEC is, in theory, independent of the applied electric field, whereas when an additional pressure is used the retention factor is dependent on both the electric field and the applied pressure [53] according to Eq. (9):

$$\kappa_{CEC} = \frac{(a - c)E + dk'_{LC}P}{cE + dP} \quad (9)$$

where a, c and d are constants using the same column and solvent system, E is the field strength (in

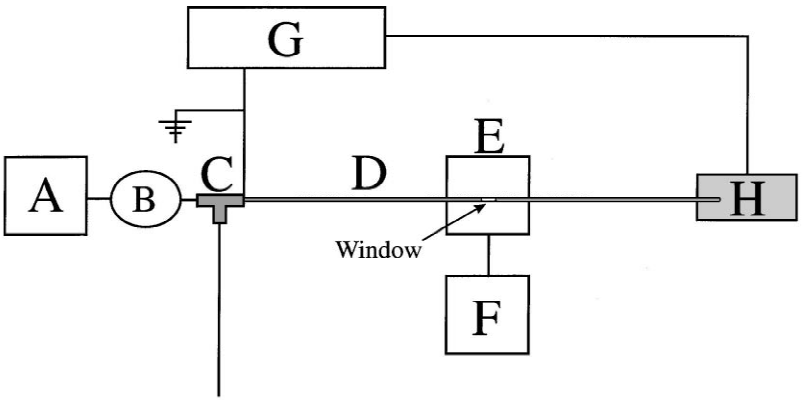

Fig. 3. Instrumentation design for pressurised flow electrochromatography. A: LC pump, B: injector, C: flow splitter, D: packed capillary column, E: UV detector, F: data acquisition computer, G: high-voltage power supply, H: electrode vessel.

kV/m), k'_{LC} is the capacity factor of the analyte in the HPLC mode, P is the supplementary pressure drop. The constant c is also dependent on the properties of the analyte. If no pressure is applied, then the retention factor, κ_{CEC}, of a peptide is, in theory, independent of the applied electric field. When an additional pressure is used, both the applied electric field and the pressure can be used to tune the κ_{CEC} of the analyte and thereby increase selectivity in the separation. In this context, CEC procedures provide attractive opportunities for instrument system miniaturisation, where pressure modulation can be readily factored into the equipment design to minimise bubble effects or effects due to frit incompatibilities [51] due to either inappropriate geometrical characteristics or surface chemistries.

4. Resolution of peptides by CEC

4.1. Peptide interactions

A common and well-known phenomenon of peptides is their propensity to interact and to be strongly (irreversibly) adsorbed to naked silica capillary walls or to packing materials of silica-based systems [4–7,54,55]. These interactions are caused via electrostatic effects mediated by charged silanol groups and the oppositely charged functionalities present within the peptide structure, mainly the free ε-amino group of lysine residues, the free N-terminal group and the guanidine moiety of the side chain of arginine. Silanophilic interactions between peptides and the silica surface also cause severe band broadening. This well known problem in the separation of basic peptides by RP-HPLC can be partially solved by end-capping the residual free silanol groups in the packing material. Hydrophobic interaction as well as hydrogen bonding effects also occur between the peptide and the sorbent surface. In the case of reversed-phase sorbents, where the silica surface has been chemically modified with *n*-alkyl groups, i.e., C_3- to C_{18}-chains, and extensively end-capped, these latter effects are popularly exploited to achieve high selectivity μ-HPLC separations with different types of solvent combinations. However, end-capped C_8- or C_{18}-bonded silica do not represent acceptable alternatives in CEC due to the significant decrease in the EOF effect. The tailing of basic compounds in RP-HPLC and CEC can be suppressed by adding a base to the eluent or alternatively through the use of an ion-pairing reagent [56–59], and similar approaches are relevant [42,60] to the separation of peptides by CEC methods. Euerby et al. [61] demonstrated the effect of adding a competing base such as triethylamine or triethanolamine in CEC to improve the peak symmetry of basic pharmaceutical compounds. Similar work has also been performed by Lurie et al. [62], who employed hexylamine in the eluent. As an alternative approach to overcome peak tailing and to enhance resolution, chemically modified etched capillaries have been proposed for the CEC separation of biopolymers [2,44,63,64]. This etching strategy with acid washed capillaries and treatment with ammonium hydrogen fluoride, followed by modification of the surface with triethoxysilane and subsequent hydrosilation with a suitable organic moiety (as the corresponding *n*-alkene) in the presence of Spiers catalysis (hexachloroplatinic acid), enables various reversed-phase (i.e., *n*-alkyl- and liquid crystal-type, i.e., cholesteryl-10-undecanoate) phases to be introduced onto the capillary surface. Importantly, the etching process permits an increase in surface area (by a factor of up a 1000-fold compared to the unmodified capillary) for interaction with the analytes as well as generation of the EOF.

Since peptides, depending on their amino acid sequence, can be acidic, basic or neutral, selection of the relevant buffer and pH condition to ensure that an appropriate charge state is achieved forms part of an essential optimisation protocol for successful CEC resolution. When low pH buffers are used the EOF decreases significantly [42,65,66] mainly because many of the residual free silanol groups on the silica-based sorbent become protonated. One way to retain high EOF with buffers of low pH is to use columns packed with ion-exchange material or mixed-mode phases, such as sulfonic acid modified *n*-alkyl bonded silicas [22,42,66]. Nevertheless, basic peptides can still exhibit severe tailing due to interaction with ionised functional groups of the packing material and the positively charged functional groups of the peptide.

4.2. Peptide separation by CEC

The high selectivity and separation power of electrochromatography has been documented by Schmeer et al. [67], who separated a mixture of enkephalin methyl ester and enkephalin amide with a pressurised CEC system combined with an ESI-MS detection system. A supplementary pressure system was used to obtain stable flow conditions. The eluent used for the separation was acetonitrile–water (80:20) containing 70 μl trifluoroacetic acid per litre of the eluent. These investigators also showed that with their instrumental system the contribution of pressure-driven and electro-driven flow effects were not additive. With increasing electroosmotic flow, the influence of the pressure decreased whilst at an electric field strength of 100 kV/m, the overall flow velocity was almost independent of the applied pressure. Pesek and Matyska [68] have separated a mixture of peptides on a C_{18}-modified etched capillary at pH 3.0. In this study, an open tubular capillary approach was used for the CEC investigations. Four different modified capillary surfaces were examined with bradykinin as a test-peptide: bare capillary, etched capillary, hydride-modified etched capillary and finally in a C_{18}-modified etched capillary. The peak shape of bradykinin was found to be fairly symmetrical and relatively narrow with the open tube system but became broader as the surface area of the capillary increased or the surfaces chemically modified. The C_{18}-modified, etched capillaries gave the largest peak width. In order to further characterise the behaviour of this new electrochromatography format, a test mixture of peptide analytes, namely lysozyme (turkey); angiotensin I and III; bradykinin; and ribonuclease A, was examined. With these hydride-modified open tubular capillary systems, an increase in migration time was observed for these bioanalytes compared to packed capillaries. The increase in elution time was probably a direct result of the lower EOF, and thereby the separation was more based on the differential electrophoretic mobilities of the analytes. The same analyte set was also examined with the C_{18}-modified etched capillary systems. In this case, the elution time of all analytes were increased significantly due to stronger interactions between the analytes and the bonded octadecyl moiety. In an associated study, Pesek et al. [44] demonstrated the separation of angiotensin I, II and III on bare, diol-modified and finally C_{18}-modified capillaries. All three peptides were baseline resolved on the diol- as well as the C_{18}-modified surfaces. It should be noted, however, that extremely long migration times arose for angiotensin II with the C_{18}-modified sorbent compared to the diol modified sorbent. With such open tubular, etched fused-silica capillaries with surfaces chemically modified with liquid crystal phases, good peak shape was observed [64] with several small basic proteins (cytochrome *c*, lysozyme and ribonuclease A), suggesting that mass transfer effects in these CEC systems was not compromised by the potential for phase transitions or ligand protein intercalation effects.

The CEC separation of a peptide mixture (Met-enkephalin, bradykinin, angiotensin III, Met-enkephalin–Arg–Phe, substance P and neurotensin) with a *n*-octyl coated open tubular capillary coupled to an on-line ion trap storage/reflectron time-of-flight mass spectrometry (CEC–ESI-IT/reTOF-MS) system has been described by Wu et al. [69]. With a capillary of internal diameter (I.D.) of about 100 μm, the peptides were baseline separated in about 2 min of analysis time. In this study, a C_8 stationary phase was loaded onto the inner capillary wall and then after a drying and flushing with nitrogen overnight, the capillary was finally modified with APS [(3-aminopropyl)trimethoxysilane] in toluene. Since the capillary inner wall was modified with APS, which replaced some of the residual charged silanol groups as amines, the separation potential was negative. The amine groups are fully protonated in an acidic solution and as a result a much higher EOF velocity ~3 mm/s was achieved with an electrical field as low as 350 V/cm. By replacing the negatively charged silanol groups with the positively charged amine groups, non-specific adsorption between the peptide and the column wall was somewhat minimised. However, it was found that optimisation of peptide separation with this modified capillary systems was difficult using an isocratic mode, especially for complex peptide mixtures such as protein digest. A gradient of 0–35% (v/v) acetonitrile–water in 6 min was attempted by placing a small buffer vial (inlet vial) on a mini-stirrer, and then adding the buffer with the higher acetonitrile content by a

syringe pump at a rate predetermined by the gradient conditions. In this way, a gradient CEC system could be created without using a pressure-driven flow connection to the CEC column.

In recent studies, we have documented [42] that the efficacy of isocratic and gradient separation methods for the resolution of cyclic peptides such as desmopressin, analogues of desmopressin, oxytocin and carbetocin using Hypersil C_8 and C_{18} materials and also mixed-mode phases. In the case of such CEC separations, the retention factor, κ_{CEC} (Eq. (10)), can be defined by analogy to the capacity factor in HPLC, although an alternative definition has been proposed by Rathore and Horváth [70]:

$$\kappa_{CEC} = \frac{(t_m - t_{eo})}{t_{eo}} \quad (10)$$

According to Eq. (10), the retention factor, κ_{CEC}, of a peptide includes contributions from the electrophoretic migration, the EOF effect as well as the chromatographic retention behaviour. Electrophoretic migration is the dominant effect for peptides when the value of the κ_{CEC} is negative, that is when the peptide elutes before the EOF marker. When changes from high organic solvent contents, i.e., from acetonitrile–water (60:40, v/v) are employed, chromatographic interaction effects can be used to tune the peptide separation. For example, in the case of cyclic peptides related to desmopressin [42], when the acetonitrile content was decreased, the κ_{CEC} value become positive with the dominant CEC effect with these peptides mediated by chromatographic interaction effects. Significant peak tailing can arise with non-end capped reversed-phase sorbents with peptides under these low pH CEC conditions, as illustrated for the desmopressin-related peptides when the organic solvent content was reduced to 20% (v/v) acetonitrile as shown in Fig. 4, which probably was due to silanophilic interactions. When the same eluent, triethylamine/phosphoric acid buffer at pH 3.0, was used in CZE, there was no resolution between desmopressin and oxytocin. The same buffer system was also used in an analytical RP-HPLC separation of desmopressin, oxytocin and carbetocin on a C_8 Hypersil column. The elution order of these peptides was different compared to their CEC and CZE behaviour, further documenting the orthogonal separation properties of the CEC, RP-HPLC and HPCZE methods with peptides.

The chromatographic and electrophoretic behaviour as CEC separations of two desmopressin-related peptides versus their electrophoretic migration (CZE) are illustrated in Fig. 5. As apparent from this plot, the chromatographic effect was small at higher contents of acetonitrile but increased as the content of acetonitrile decreased. These results can be contrasted to the findings of the same set of peptides when separated [42] on a Hypersil mixed-mode (C_{18}/SCX) CEC column and also on the Spherisorb C_{18}/SCX material. The elution order of these peptides on the mixed-mode CEC columns was different from the one obtained by Hypersil C_8 and C_{18} material although a common eluent was used. The main interaction on the mixed-mode phase were mediated through electrostatic effects with the side chain of Arg and the N-terminal amino group contributing strongly to the interaction. As also evident from these results, the selectivity and elution order of these peptides were different for these various mixed-mode sorbents compared to the non-end capped reversed-phase sorbents. For example, these desmopressin peptides eluted after the EOF marker even at high concentration of acetonitrile with these mixed-mode sorbents, emphasising that the main interaction with mixed-mode phases are based on coulombic interactions. In associated studies with families of closely related loop polypeptides derived from the activin $\beta_a \rightarrow \beta_a$ subunits [60], we have shown that pH modulation of the mobile phase provides a very effective avenue to optimise the resolution of large polypeptides that exhibit a significant percentage of β-sheet secondary structure due to their extensive hydrogen bonding framework. In these cases, resolution increased as the pH of the buffer was raised from pH 2.3 to pH 5.0, indicating that the EOF effect provided a more significant contribution to the retention mechanism. Moreover, such observations suggest application of ion-pairing reagents and other compounds that influence the surface charge or hydrogen bonding properties of peptides will have considerable benefit in circumstances where the structures of the peptides within a mixture are very similar.

4.3. Peptide separation by electrically-assisted LC

Separation of peptide mixtures on a pressurised CEC using an IT/reTOF-MS detection system has

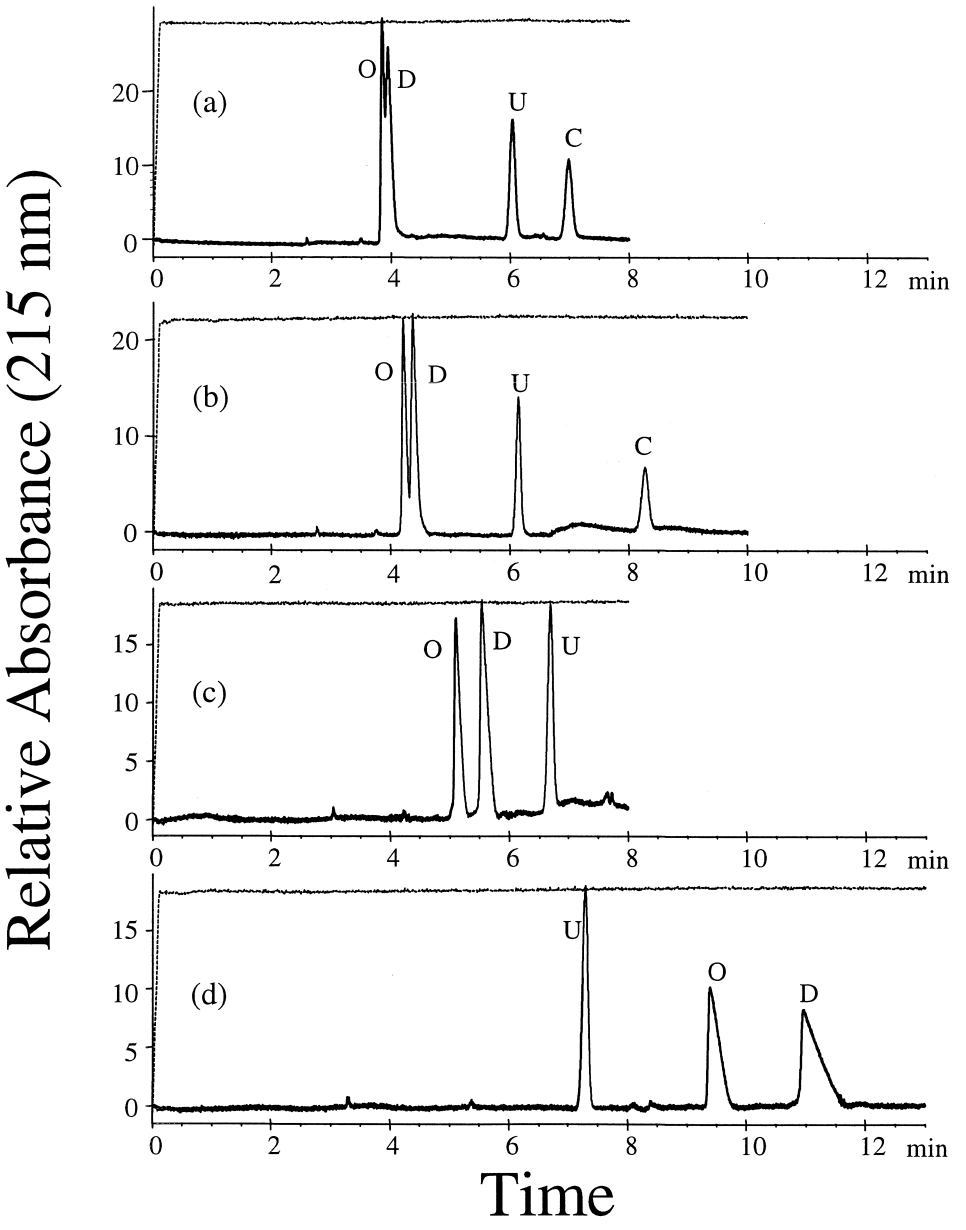

Fig. 4. Elution profile for oxytocin (O), carbetocin (C), and desmopressin (D) separated with a Hypersil C_8 CEC column at different concentrations of acetonitrile, EOF-marker Uracil (U), 25 kV, eluents: *X* ml acetonitrile added to 2 ml 38 m*M* TEAP at pH 3.0, and (10–2–*X*) ml water added to a final volume of 10 ml. (a) 50% acetonitrile; (b) 40% acetonitrile; (c) 30% acetonitrile; and (d) 20% acetonitrile. Data replotted from Ref. [42].

been performed by Wu et al. [69]. These investigators used capillaries of 180 μm I.D., packed with 3 μm particles of C_{18}-silica. A mixture of six peptides (bradykinin, angiotensin I and II, Met-enkephalin–Arg–Phe, neurotensin and substance P) were separated by optimising both the pressure and the applied electrical voltage. The separation of a bovine cytochrome *c* digest was tuned by adjusting both the applied electric field and the pressure as shown in Fig. 6. The use of pressure-mediated effects in CEC may thus possible represent an additional parameter to fine tuning the separation of

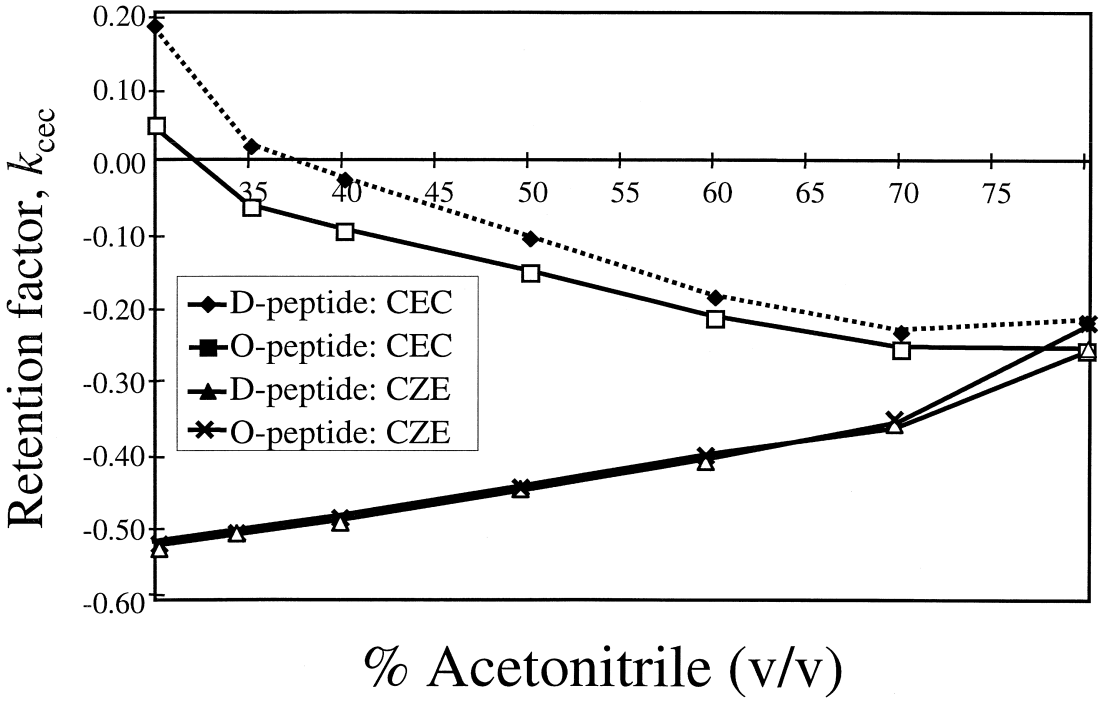

Fig. 5. Overlay of the plots of the retention factors of desmopressin (D) and oxytocin (O) versus the concentration of acetonitrile, using the CEC Hypersil C_8 and CZE-opened fused-silica capillary systems operated at 20 kV, with eluents containing *X* ml acetonitrile added to 2 ml 25 m*M* TEAP at pH 3.0, and (10–2–*X*) ml water added to a final volume of 10 ml. Data replotted from Ref. [42].

complex mixtures such as protein digests. Recently Apffel et al. [71] have also described the separation of proteolytic digest of proteins and glyco-proteins by electrically-assisted capillary LC (cLC). An electrospray ionisation interface was developed for the coupling of electrically-assisted cLC with an ESI-MS instrument. The electrically-assisted cLC was operated at pH 2.5 with a CEC column packed with Vydac 218TPB5 C_{18} material (5 μm, 300 Å) so that the EOF could be essentially negated. The selectivity of the peptides was then fine tuned by applying an electrical field continuously or selectively during the separation time. Under these conditions, the effect of changes in the electric field on chromatographic selectivity of charged molecules at low pH could be evaluated both in the isocratic as well as the gradient elution modes. Illustrative of this approach is the gradient electrically-assisted cLC separation of a peptide standard mixture at applied potentials of −10 kV, 0 kV and +10 kV shown in Fig. 7. Analogous types of mass spectrometry interfaces have been described by Choudhary et al. [72] for the on-line analysis by CEC coupled to ESI-MS or TOF-MS of phenylthiohydantoin (PTH) amino acids with electroosmotically driven solvent gradients.

The use of pressurised gradient electrochromatography, also known as electrically-assisted LC, for peptide mapping have recently been presented by Behnke and Metzger [73] using columns packed with C_{18} silica material, 3 μm particle, pore size of 100 Å, to a length of 6 cm bed. These investigators concluded that the resolution of a tryptic digest of cytochrome *c* obtained by gradient electrochromatography at 100 kV/m was superior compared to the analysis achieved by nano-LC. Several of the peaks unresolved by nano-LC were separated by pressurised gradient electrochromatography. Bubble formation was successfully suppressed by using a resistor capillary of 25 μm I.D. Connected to the outlet of the packed column. Similarly, Adam and Unger [74] have separated peptides by isocratic CEC

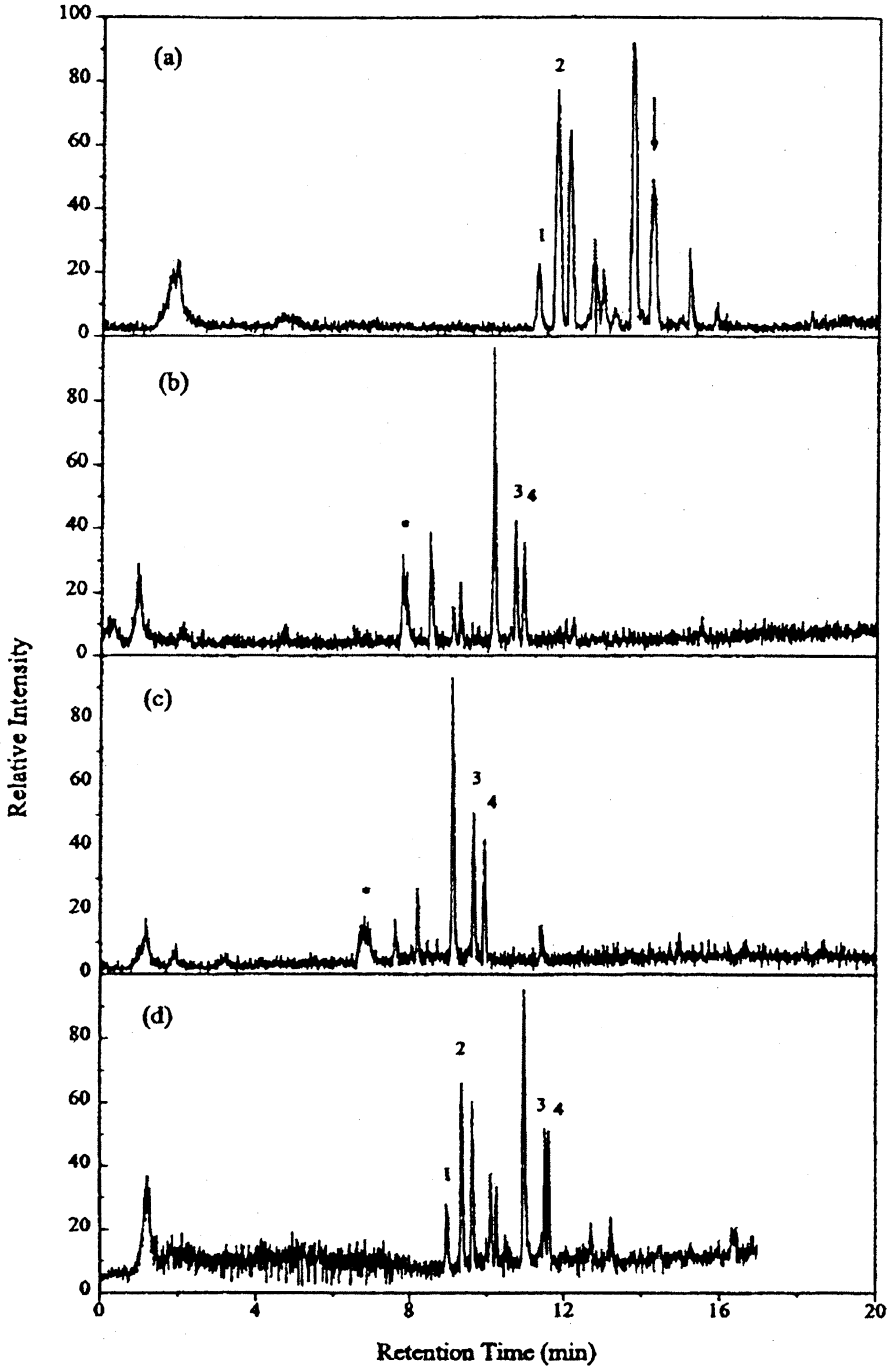

Fig. 6. Mass spectrometric total ion currents (TICs) following the separation of a bovine cytochrome *c* peptide digest using a 20-min, 0–50% acetonitrile gradient with sample injections of 8 pmol corresponding to the original protein. Column length, 6 cm. Column operation conditions: (a) HPLC mode with a back-pressure of 90 bar; (b) 1000 V applied voltage with 50 bar supplementary pressure; (c) 1400 V applied voltage with 50 bar supplementary pressure; and (d) 600 V applied voltage with 70 bar supplementary pressure. Data reproduced from Ref. [53].

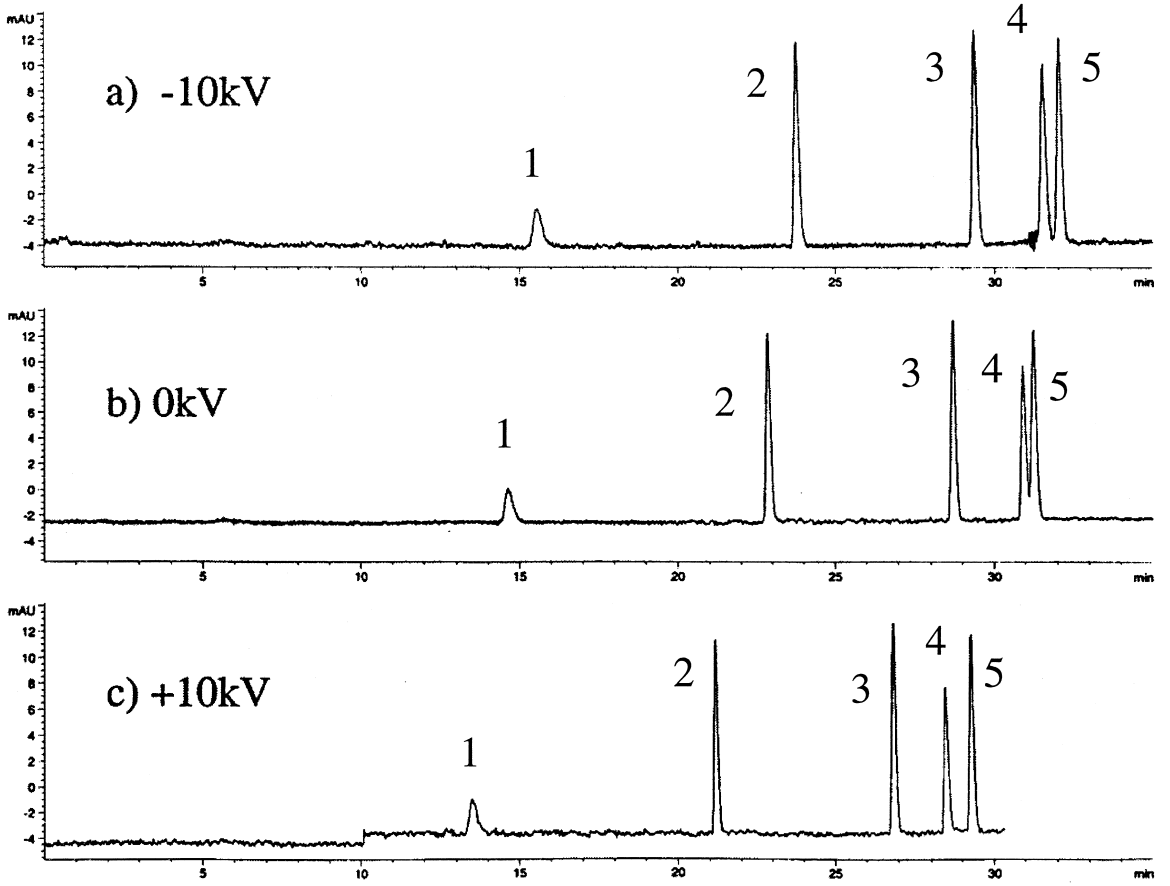

Fig. 7. Gradient electrically-assisted cLC. Separation of peptide mixture derived from recombinant human growth hormone. (a) −10 kV, (b) 0 kV, (c) 10 kV. Column: 25 cm×100 μm I.D., 5 μm, Vydac C_{18}. Mobile phase: A: 0.05% TFA–water, B: 0.045% TFA–acetonitrile. Gradient: initial 0% for 5 min; 0–35% B/30 min. Data reproduced from Ref. [71].

as well as with electrically-assisted nano-LC, using Hypersil C_{18} and the mixed-mode Spherisorb SCX/C_6 or SCX/C_{18} phases. As illustrated earlier in Fig. 2, mixed-mode phase with strong sulfonic groups can be chosen for their propensity to generate a high EOF even with low pH eluents. Moreover, high ionic strength buffers can be used to suppress the ionic interaction of the positively charged peptides with the CEC sorbent when buffers of low pH value are employed. According to the results reported by Adam and Unger [74], the isocratic CEC elution order and selectivity was the same for the set of enkephalin-related peptides used when the analyses were performed in TFA or phosphate buffer systems. Interestingly, when a TFA-based buffer was used the overall elution times were however much longer than for the corresponding phosphate buffer. In the isocratic analysis on a mixed-mode CEC column there was no resolution between H–Val–Tyr–Val–OH and Met [5] enkephalin amide. Instead these peptides were baseline resolved on a Hypersil C_{18} silica material packed column with a gradient electrically-assisted nano-LC system as illustrated in Fig. 8. As anticipated, these five peptides could be separated in a shorter time with a gradient system compared to the isocratic system, whilst the peak shape could be improved by tuning the separation with 15 kV compared to 10 kV.

A further advantage of employing voltage tuning with pressurised CEC based on mixed-mode sorbents and trifluoroacetic acid, or preferably acetic acid–ammonium acetate buffers, with acetonitrile–water

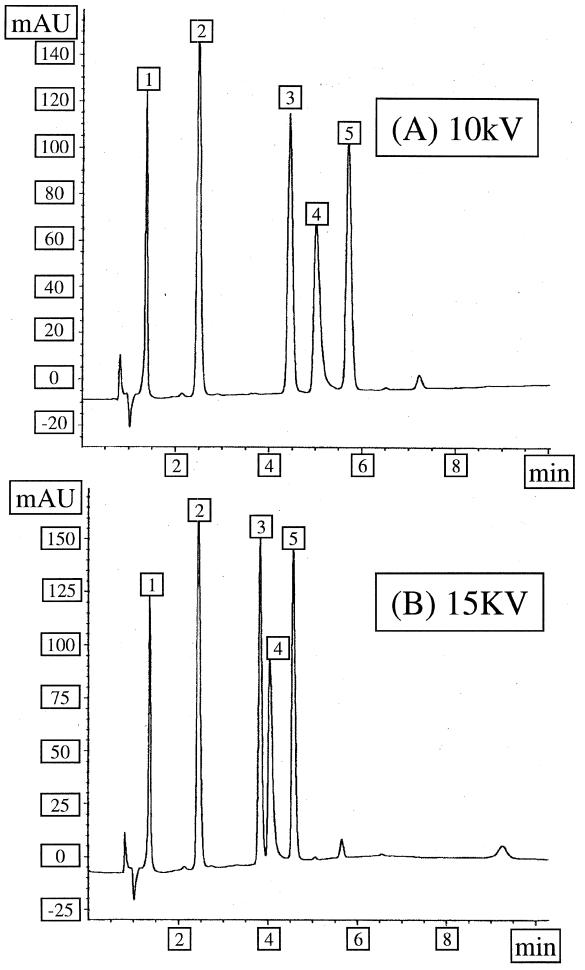

Fig. 8. Influence of the applied voltage of 10 kV and 15 kV on the gradient separation of several low-molecular-mass peptides. A CEC capillary of 15 cm×100 μm I.D., packed with 3 μm Hypersil ODS was employed with the elution conditions of mobile phase A: water–0.1% TFA/acetonitrile (95:5, v/v); and mobile phase B: water–0.1% TFA/acetonitrile (5:95, v/v), with a linear gradient of 20–40% B in 10 min, flow-rate: 800 nl/min; voltage: (A) 10 kV; (B) 15 kV; injection: 20 nl; UV detection at 220 nm. The peptide sample was: 1, Gly–Tyr; 2, Val–Tyr–Val; 3, Met-enkephalin; 4, Leu-enkephalin; 5, angiotensin II. Data reproduced from Ref. [74].

gradients is their potential for use with ion trap storage/reflectron time-of-flight mass spectrometry and other MS modes. Huang et al. [75] have used a commercially available 5 μm silica chemically modified with an *n*-octadecyl–dialkylamine (1:1) coating as a C_{18}/anion-exchange sorbent for the analysis of a horse heart myoglobin (HHMYO) tryptic digest. In common with our other experiences [42,66] comparing the resolution behaviour of peptides separated under RP-HPLC conditions or CEC conditions with *n*-alkylsilica and mixed-mode silica sorbents, distinctly different selectivities were also found with this HHMYO digest when these alternative procedures were employed. Moreover, as noted above, with mixed-mode sorbents a constant EOF effect can be achieved over the pH 2–5 range in the CEC mode, whilst with the more conventional *n*-alkylsilica sorbents, the EOF varies with pH. Clearly, other types of *n*-alkylamine-coated, as well as novel heterocycle-coated silicas can be contemplated to extend this range of selectivities without compromising the separation time. Alternatively, reversal of the voltage polarity with these ''tailored'' surfaces can be expected to result in totally different migration profiles with the same peptide mixture.

5. CEC versus HPCZE and μ-HPLC

CEC of polar compounds is an orthogonal hybrid of CZE and HPLC. HPCZE separates highly charged peptide analytes on the basis of differences in charge and Stoke's radii, whereas MEKC procedures have been primarily developed to permit the separation of neutral or weakly charged peptide analytes where different properties must be exploited to permit an increase in selectivity. A major drawback of MEKC methods is the lack of MS compatibility due to the relatively high concentration of surfactants and other mobile phase additives present in the eluted fractions. To gain higher resolution, very high separation efficiencies are needed. In HPLC the efficiency increases with the use of smaller particle diameters at the cost of a higher back pressure and/or lower flow-rate of the mobile phase. In CEC, the EOF is virtually independent of the particle diameter and the column length. Instead the surface charge density is crucial for the EOF. An evident limitation of CEC is that the EOF changes with the mobile phase composition. Pressurised electrochromatography also known as electrically-assisted LC is one way to establish a stable flow in the capillary columns and also to achieving the desired possibility of tuning the selectivity of the charged peptides. Offsetting these

potential advantages of pressurised electrochromatography will be the larger peak dispersion inherent to this approach. Resolution and reproducibility will thus represent the usual compromise between system robustness, and maximal selectivity and peak efficiency.

One of the major differences between CEC and μ-HPLC is that peak dispersion of peptide analytes is larger in μ-HPLC than in CEC. As noted above, the dominating term controlling the total plate height based on the van Deemter equation in CEC is the longitudinal diffusion of the peptide analyte (B/ν), whereas in μ-HPLC the Eddy diffusion ($A\nu^{1/3}$) and the mass transfer ($C\nu$) terms dominate the peak dispersion of peptide analyte [76]. Since the B term is proportional to the diffusion coefficient of the peptide (i.e., $B=2\gamma D_m$) the molecular properties of the peptide analytes per se rather than the physical properties of the sorbent provide the dominant contribution to the peak dispersion. Some major difference between the three separation methods – HPCZE, μ-HPLC and CEC – are illustrated in Table 2. Since the separation mechanisms differ significantly between these three analytical methods, it is necessary to distinguish between the retention of analyte in terms of their definition, i.e., μ-HPLC: capacity factor k' (or chromatographic retention factor), CZE: electrophoretic velocity factor k'_e and CEC: electrochromatographic retention factor κ_{CEC} [33,42,77].

6. Concluding remarks and perspectives

If several remaining challenges can be solved, the CEC of peptides can in the future be anticipated to become a very high resolution, fast, separation method with high throughput complementary to those already existing as analytical methods in academia and industry. Its acceptance will partly reside as a hybrid technique of μ-HPLC and HPCZE. To generate in excess of 10^6 theoretical plates/m, however, CEC columns of 100 μm or less will be required, packed with particles with mean diameters in the range of 0.5–1.0 μm and operated with an EOF in the vicinity of 2000 V/cm. New concepts and procedures in capillary packing will be required, including procedures to immobilise the particles in the absence of frits. The synthesis of new types of tailored sorbents will have to be achieved in order to generate appropriate and controlled EOF effects.

From a theoretical perspective, a more comprehensive description of the fundamental principles and

Table 2
A comparison of HPCZE, μ-HPLC and CEC procedures

Properties	HPCZE	μ-HPLC	CEC
Velocity of eluent	Electro-driven	Pressure-driven	Electro-driven
Column I.D. (μm)	25–100	100–500	50–100
Separation by	Differential electrophoretic mobilities, charge state and Stoke's radii of analytes	Chromatographic interaction of analytes with the stationary phase	Both chromatographic interactions and electrophoretic mobility of the analytes
Capillary	Open tubular fused-silica capillary with/without wall modifications	Capillary packed with reversed-phase or mixed-mode sorbent materials	Capillary packed with reversed-phase or mixed-mode sorbent materials
Flow profile	Plug flow profile front is flat, peak dispersion dominated by the longitudinal diffusion term, $H=B/\nu$	Parabolic flow between particles in well packed capillaries; peak dispersion can be approximated to pseudo-plug flow shape under some optimised ν conditions but overall plate height, H, is given by van Deemter dependency, i.e., $H=A\nu^{1/3}+B/\nu+C\nu$, and dominated by the Eddy diffusion, $A\nu^{1/3}$, and the mass transfer, $C\nu$, terms as ν increases. Hence, H increases at high ν values	Plug flow between particles in well packed capillaries; overall profile front is also plug flow at high ν; peak dispersion, H, has very negligible contributions from the Eddy diffusion and mass transfer terms of the van Deemter dependency, i.e., both $A\nu^{1/3}$ and $C\nu\approx 0$, with H dominated by the longitudinal diffusion, B/ν, term. Hence, H decreases as ν increases.
Dispersion of zones relatively to each other	Small	Large	Medium

concepts of CEC will be required if reliable prediction of the changes in EOF as well as the variations in the retention, selectivity, band-broadening and overall resolution of the peptide analytes are to be achieved and interpreted in terms of the mobile phase composition and other operating parameters. In this regard, detailed investigations [77] into the effects of temperature, buffer composition and type of organic solvent with reversed-phase and mixed-mode CEC sorbents, can be expected to provide key data relevant to the potential application of CEC in proteomic screening as well as in the emerging field of zeomics [78]. For these approaches to gain wide acceptance, however, system reliability checks and assessment methods to document the robustness of a particular separation will be required. To this end, the recent studies [79] on the use of *n*-alkylbenzenes to capillary performance and stability in the temperature studies with peptides is indicative of the strategy that can be followed. Moreover, CEC as a separation technique operated in the isocratic or gradient elution mode, will undoubtedly gain in popularity when it is possible to very facilely achieve on-line connection to more sensitive detection units, such as ESI-MS instead of UV detection. This development will require special attention to the choice of the mobile phase buffer. Several studies with pharmaceutical compounds have shown [69–72,80–83] that coupling CEC to ESI-MS or nuclear magnetic resonance (NMR) spectrometers is technically feasible in some cases, and analogous procedures should be adaptable to peptide analysis. To proceed further, extensive development of new packing materials, instrumentation formats and rugged CEC columns remain however to be achieved before CEC methods with peptides assumes a deserved important place in the arsenal of applied generic procedures.

As the analytical field of CEC separation develops further, subsets of different approaches can be contemplated based around the already split facets of the technique, namely the ability to resolve complex mixtures of peptidic analytes by exploiting the EOF effect per se or by using electrically-assisted LC methods. These different attributes will be particularly relevant to the rapid, high throughput screen of peptides derived from combinatorial libraries or from proteome investigations associate with phenotype analysis. The types of columns and sorbents needed for these applications will be completely different. To obtain a sufficiently high EOF in CEC with low pH buffers, new reversed-phase and mixed-mode silica materials must be developed for peptide separations, whereas with electrically-assisted μ-HPLC, suitable packing materials suitable for peptide separations are already on the market. Conventionally end-capped or base-deactivated reversed-phase material with low or no EOF properties should be superior for use in these latter cases, since only the electrophoretic mobilities of the peptide analytes are used to fine tune the separation, which for electrically-assisted μ-HPLC is primarily based on the dominant contribution from the chromatographic interactions. Once solutions to these remaining challenges are found, then the application of CEC procedures as micro-machined nanoscale systems should follow, permitting high field strengths to be employed with small porous or non-porous particles packed into very short path lengths without seriously compromising selectivity or efficiency. Attainment of these objectives will undoubtedly lead to the emergence of CEC procedures as pivotal technologies underpinning the next revolution in the analytical life sciences, namely the field of *zeomics* [78,84], where the identification of how different supramolecular structures within a single cell are organised, interact and elicit their function can be described in comprehensive molecular terms.

7. Nomenclature

A	Eddy diffusion term ($=2\lambda d_p$)
B	Longitudinal diffusion term ($=2\gamma D_m$)
C	Mass transfer term $[=k'/(1+k')^2]d_p^2/D_s]$
c	Electrolyte concentration
D_m	Bulk diffusion coefficient in mobile phase
d_p	Average particle diameter of sorbent material
D_s	Overall diffusion coefficient of analyte in sorbent microenvironment
E	Electric field strength
F	Faraday constant
ΔG_{assoc}	Change in Gibbs free energy
K_a	Equilibrium distribution constant

k'	Unitless capacity factor in the μ-HPLC mode
κ_{CEC}	Unitless capacity factor in CEC mode
L	Column length
ΔP	Pressure drop across the column
q	Ion charge
r	Ion radius
r_S	Stoke's radius
R	Gas constant
T	Temperature
ϵ	Molar absorptivity
ϵ_0	Permittivity in vacuo
ϵ_r	Relative permittivity (dielectric constant) of mobile phase
γ	Packing tortuosity parameter
Φ	Phase ratio of the chromatographic system
ϕ	Column resistance factor
η	Mobile phase viscosity
κ	Structural packing parameter
λ	Column packing term
μ_a	Apparent electrophoretic mobility of analyte ($=\mu_{eo}+\mu_e$)
μ_{eo}	Electrophoretic mobility of EOF
μ_e	Electrophoretic mobility of analyte
σ	Charge density of the surface of shear
ζ	Zeta potential of the liquid–solid interface

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