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Review

Specific applications of capillary electrochromatography to biopolymers, including proteins, nucleic acids, peptide mapping, antibodies, and so forth

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Abstract

Separation of biopolymers is an obvious application of capillary electrochromatography (CEC) technology, since speed and resolution should increase significantly over high-performance liquid chromatography (HPLC). All too often, HPLC chromatograms of polymers show poorly resolved envelopes of overlapping peaks from oligomers. The practical limitation of column length and pressure drop has hindered development of high resolution separations of many polymers in HPLC. However, this generally applies only to packed beds of small particles, and not to continuous (or monolithic) beds, as introduced by Hjerten et al. [S. Hjerten, Ind. Eng. Chem. Res. 38 (1999) 1205; S. Hjerten, C. Ericson, Y.-M. Li, R. Zhang, Biomed. Chromatogr. 12 (1998) 120; C. Ericson, S. Hjerten, Anal. Chem. 71 (1999) 1621; J.-L. Liao, N. Chen, C. Ericson, S. Hjerten, Anal. Chem. 68 (1996) 3468; S. Hjerten, A. Vegvari, T. Srichaiyo, H.-X. Zhang, C. Ericson, D. Eaker, J. Capillary. Elec. 5 (1998) 13; C. Ericson, J.-L. Liao, K. Nakazato, S. Hjerten, J. Chromatogr. A 767 (1997) 33; S. Hjerten, D. Eaker, K. Elenbring, C. Ericson, K. Kubo, J.-L. Liao, C.-M. Zeng, P.-A. Lidstrom, C. Lindh, A. Palm, T. Srichiayo, L. Valtcheva, R. Zhang, Jpn. J. Electroph. 39 (1995) 1]. Throughout this review we will refer to such packings as monolithic or continuous beds, but they are identical type packings, formed by the in situ polymerization in the capillary or column. CEC capillaries can be much longer, and contain smaller particles than is practical for HPLC. This improves resolution significantly. CEC is able to capitalize on existing mobile phase technology developed over 30 years to improve separations. The requirement that the mobile phase simultaneously promote the separation and mobile phase mobility needs to be considered. In RPLC, this dual role is not much of a problem. It may be much more important in other modes, particularly ion-exchange (IEC). As the field develops, it is becoming clear that CEC is not just a simple extension of HPLC. Instruments, column technology and operating optima are clearly different than HPLC. CEC will develop into its own unique field. Open tubular HPLC is almost precluded by the high pressures required for forcing liquids through 10 μm or smaller capillaries. Electroosmotic pumping (EOF) avoids the pressure constraints and provides better flow profiles. Compared to HPCE, the ability to interact with the stationary phase may enable separations that would be difficult with electrophoresis alone. Since the mobile phase can be less complex than micellar electrokinetic chromatography (MEKC), CEC also avoids the problem of high background signals from the micelle forming compounds. Thus CEC–MS (mass spectrometry) is expected to be even more powerful than HPCE–MS. The fortuitous, simultaneous development of matrix assisted laser desorption–time of flight MS (MALDI–TOF-MS) technology will enable extension of the mass range to above 100 000 Da. Lack of familiarity is the perhaps the largest liability of CEC compared to other techniques. This paper critically compares the state-of-the-art of CEC with HPLC and HPCE, with a particular emphasis on separation of biopolymers. The goal is to help the reader overcome the fear of the unknown, in this case, CEC.

Keywords: Reviews; Electrochromatography; Proteins; Nucleic acids; Peptides; Carbohydrates; Oligonucleotides

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Contents

1. Introduction

1.1. Requirements for separations of biopolymers

Biopolymers are complex, with subtle differences, such as point mutations, being exceedingly important. Detection is another issue, since the amount of biopolymers is often limited, such as in the analysis of single cells or organelles. Capillary electrochromatography (CEC) is a new technique that potentially solves many of these problems, as summarized in Table 1. The table also reports the status as of the beginning of 1999.

Narrow peaks are probably the most outstanding attribute of CEC. Narrow peaks offer high peak capacity, meaning that it is easier to see details in the heterogeneity of the natural polymers. All too often HPLC of biopolymers produced ''lumpograms'', where the peaks of individual species excessively overlapped. This camouflaged the important details, thus obscuring structure–activity relationships.

CEC opens the door to separations employing hundreds of thousands of plates, perhaps even a million, per meter. With HPLC, the practical upper limit for plate count was about 25 000 plates. With a similar effort, it appears that CEC can deliver 400 000 plates. This provides a four fold improvement in chromatographic resolution. A fused peak with a resolution (R) of 0.3 will jump to a R of 1.2, which is almost baseline separation. Heterogeneity should be much more tractable with CEC.

For an equal number of plates, CEC offers similar linear flow-rates, (1 to 3 mm/s) as is common in HPLC. However, it is possible to use smaller particles than HPLC. This shortens columns for the same plate count. Since run time is proportional to column length, the run time of CEC is often less than half of HPLC.

Table 1
Summary of CEC goals for biopolymer separations

Goal	CEC results
1. Narrow, sharp eluting peaks with good peak symmetry (small asymmetry values)	Demonstrated Analyte dependent
2. High peak capacity per capillary length	Demonstrated
3. High speed of separation and analysis	Can demonstrate
4. Ease of quantitation	Not yet demonstrated
5. Ease of method development/optimization	Not yet demonstrated
6. Ease of interfacing with MS	Demonstrated
7. Trace level detection	Not demonstrated
8. Ability to separate variants of proteins and other biopolymers	Case dependent

1.2. Undeveloped areas for CEC

The development of CEC is following a predictable pattern, established by HPLC and later by HPCE. First, interesting separations are developed, but scientists are quick to ask, so what? What are they good for? Is the technique both qualitative and quantitative? What are detection limits? What detectors are most suitable? As expected, the UV and MS detectors were quickly investigated, but are they really optimized?

These are important questions, but they can only be answered after the separation is worked out and controlled. Since CEC technology has different operating optima than HPLC or HPCE, method development is not as well worked out. Operating constraints and guidelines need to be developed. These are beginning to appear for RPLC–CEC, but the other modes are much farther behind.

1.3. State-of-the-art

The development of CEC will depend upon fulfilling needs unmet by other techniques. Existing technology such as HPLC can separate biopolymers by exploiting differences in hydrophobicity (RPLC), isoelectric point (ion-exchange or chromatofocusing) and solubility (HIC) [1–10]. Another factor is the large number (perhaps 150 000) of HPLC instruments and corresponding larger number of experienced users that are active around the globe. Many will argue that there is only a marginal need (at best) for better resolution or faster separations. This is the conundrum faced by CEC today. What can CEC do that is impractical or impossible with other techniques? And how valuable is this?

Originally, CEC was presented as a blend of the best of HPLC and HPCE. This was true, but also misleading, since the optimum operating parameters occupies a unique space. The first investigations of column packings showed that it was important to have both charged groups to support EOF, as well as the traditional surface chemistry, such as C_{18} or C_8.

The HPLC model quickly failed since the pH dependence of the flow-rate with capillaries packed with silica based particles quickly showed that much of the separations of proteins and peptides at low pH, were just too slow. The slowness is due to the loss of EOF, due to the pH being near to the pK_a of the charge carriers ($=SiO^-$ and H^+). In the future, the ideal columns for CEC will have charge carrying groups that are compatible with EOF at pH values over the range of ca. 1 to 9.

A lack of convenient compatibility of HPCE with MS was a major problem hindering acceptance. Certainly, several creative groups have shown that HPCE–MS is already and potentially a very powerful technique, if one can but find the right buffer and running conditions [11–26]. The bonded phase of CEC avoids many of the problems with HPCE–MS, especially in trying to utilize various modes of MEKC–MS.

The ideal packing for the capillaries will have a surface chemistry with a combination of charge carrying groups (ion-exchange) and non-covalent and non-ionic chemical interactions with the analytes. First attempts have combined ion-exchange groups for charge carrying and interactive groups such as C_{18} [27–37]. A few references also point out that separations analogous to SEC in HPLC are also possible in CEC [38–41]. However, these are yet to be used with biopolymers, though they will prove most interesting in such applications.

The review below is based upon the CEC literature available as of early 1999. No work has been reported for certain major classes of biopolymers, such as modified DNA, including intact DNA adducts and lipids. There is work, as below, on individual nucleoside-adducts, but not intact DNA adducts, as yet. It will also touch on important instrumental techniques, including CEC or pressure assisted CEC (PEC). These will certainly be covered by other authors in this volume.

1.4. CEC of carbohydrates

Let us first start with the separation of carbohydrates, monosaccharides, polysaccharides, and related glycans. A more recent publication by Palm and Novotny has described the generation of a monolithic capillary bed formed by copolymerization of polyacrylamide and poly(ethylene glycol) with added ratios of acrylic or vinyl sulfonic acid (to produce the desired EOF) [42]. Hydrophobic ligands, including C_4, C_6, and C_{12}, were introduced via various acrylate esters, copolymerized with the

monomers leading to the basic backbone of a mixed polyacrylamide–poly(ethylene glycol) phase. Various ratios of these monomers were then polymerized in a capillary which had its walls already activated first by a bifunctional reagent (such as an unsaturated, trimethoxysilane) in order to coat the walls. The presence of free double bonds on the walls then permitted the covalent attachment of the monolithic polymer gel to the same walls. Various ratios of linear and crosslinked monomers (%T/%C) were then used to generate different gel matrices, with free sulfonic acid groups, and then utilized for the separations of peptides and carbohydrates, as well as smaller organics.

In order to visualize carbohydrates, which are usually UV transparent and lack natural fluorescence, the authors utilized a now-standard derivatization method involving 2-aminobenzamide, followed by reductive amination, for the laser-induced fluorescence (LIF) detection with a He–Cd laser (325 nm) as the excitation source [43]. These derivatization–detection approaches have been long-utilized in capillary LC (cLC) and HPCE modes. Precapillary tagging; however, usually leaves an excess of the reagent in the final injection solution, and often provides variable labeling. Excess reagent can interfere in the final chromatogram, and/or react with the stationary phase to alter the surface activity. This would almost certainly change the selectivity from one injection to the next, thereby adversely affecting the reproducibility of the separation. These studies involved isocratic CEC, without any external pressure driven flow, and without step-gradient conditions. Detection was off-gel, after the gel portion of the packed capillary, using a fiber-optic cable placed as near as possible to the interface between the gel and free solution. Under such separation–detection conditions, the efficiencies for the Glc1–Glc3 oligos were between 190 000 and 230 000 theoretical plates/meter.

Fig. 1 illustrates a typical isocratic chromatogram of a maltose oligosaccharide mixture, where the reagent peak appears between 14 and 16 min (Fig. 1B). Fig. 1A is an enlargement of Fig. 1B, which illustrates the elution of the excess tagging reagent after elution of the derivatized maltooligosaccharides (Glc7 to Glc1). These and other carbohydrate or protein separations with this particular packing/

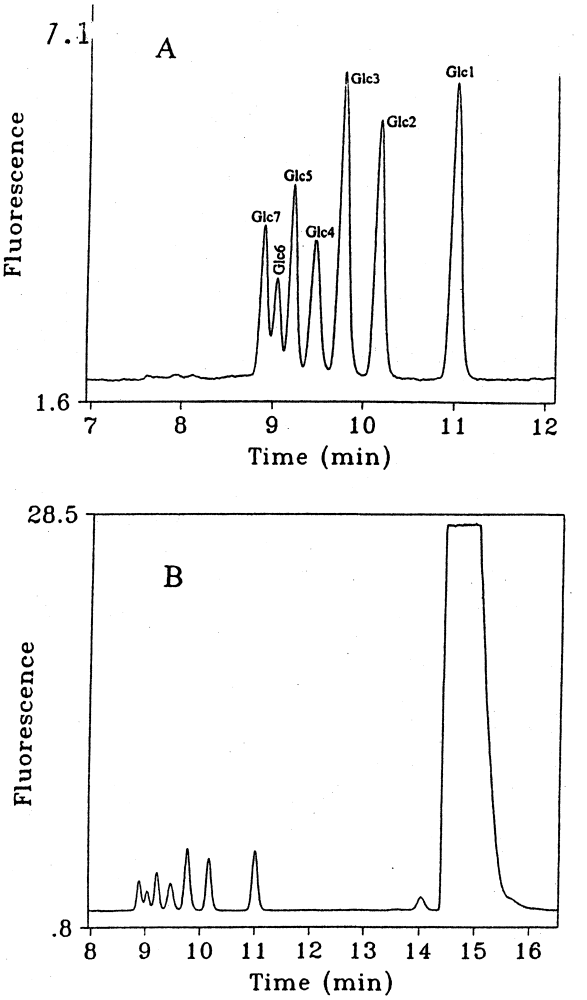

Fig. 1. (A) Isocratic electrochromatogram of maltooligosaccharides (glucose (Glc1)–maltohexaose (Glc6)) in a capillary filled with a macroporous polyacrylamide–poly(ethylene glycol) matrix, derivatized with a C_4 ligand (15%) and containing vinylsulfonic acid (10%). 2-Aminobenzamide was used to ''tag'' the oligosaccharides for the laser-induced fluorescence detection. (B) is the same analysis as in (A), including the peak of the derivatization agent, which appears at 14–16 min. Conditions: capillary, 32 cm (25 cm effective length); field strength 900 V/cm, 20 μA (current); sample concentration, 5–10 μ*M*; other conditions are described in the original publication [42]. (Reproduced with permission of the authors and copyright holder, American Chemical Society, Washington, DC).

phase were, of course, pH and organic content dependent, both of which greatly affected resolutions and peak shapes, as well as total analysis times [42]. Fig. 2 (with conditions as indicated) further illus-

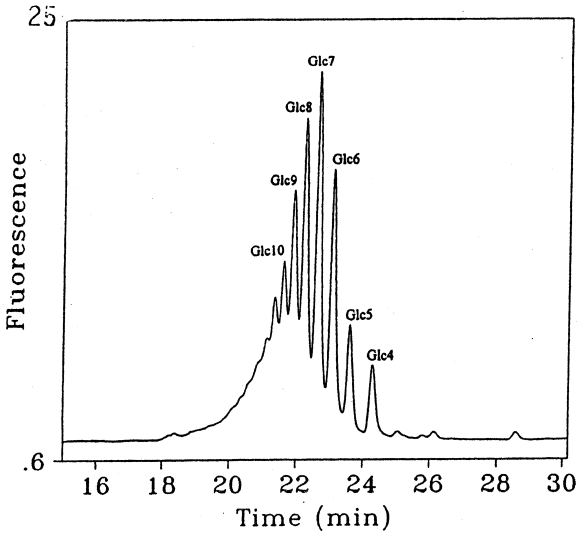

Fig. 2. Isocratic electrochromatography of the oligosaccharide ladder in a capillary filled with a macroporous polyacrylamide–poly(ethylene glycol) matrix, derivatized with a C_4 ligand (15%) and containing vinylsulfonic acid (10%). Conditions: capillary length, 50 cm (40 cm effective length); mobile phase, acetic acid 1:1000 containing 5% (v/v) acetonitrile; field strength, 600 V/cm, 14 μA; injection, 5 s (100 V/cm); sample concentration, 30 mg/ml in derivatization solvent and thereafter diluted 1:100 in the mobile phase; other conditions are described in the original publication [42]. (Reproduced with permission of the authors and copyright holder, American Chemical Society, Washington, DC).

trates the analysis of Glc4–Glc10 oligosaccharides, on a longer capillary and at a lower field strength. The larger oligomers (presumably, Glc11–Glc16) were also visible, though poorly resolved from one another. The peak shapes, resolutions, efficiencies, and total analysis times, were also a function of the nature of the hydrophobic moieties incorporated into the final, monolithic polymer gels (C_4, C_6, or C_{12} or others possible).

Advantages of the above type packing, a monolithic, gel polymerized in situ stationary phase, as in capillary gel electrophoresis (CGE) are several-fold. Good separations could be achieved, at times, in less than 10 min, often less than 5 min. The migration time reproducibility is better than 1% (RSD) from run-to-run and 2.5% from day-to-day. Finally, the gel is stable up to at least 50% acetonitrile used as a mobile phase, but not much higher than that [42].

Additional work in the area of carbohydrate analysis by CEC has been that of El Rassi and colleagues [44,45]. In their approach towards improved separations of carbohydrates, they designed a special octadecyl-silica (ODS) stationary phase for CEC that had a limited amount of hydrocarbon coverage, in order to leave 75% of the surface silanols unreacted. This yielded a relatively moderate EOF, and yet still exhibited RP behavior toward alkylbenzene homologs and a series of *p*-nitrophenylglycosides and *p*-nitrophenyl-maltooligosaccharides, all within a relatively short period of time [45]. Again, a prerequisite for achieving the separation and detection of carbohydrates by CEC (or CZE or cLC) with ODS capillary columns, is to derivatize the sugar analytes with fluorophores (as above) or chromophores to yield preferably neutral (though at times charged derivatives can be utilized) derivatives. In these studies, the *p*-nitrophenyl group was introduced at the terminal end of the carbohydrates, singly tagged. In this case, the rationale for carbohydrate derivatization then is really two-fold: (1) to increase the sensitivity of the detection; and (2) to confer the hydrophobicity necessary for RP CEC. Under these CEC conditions, even alpha- and beta-anomers of some *p*-nitrophenyl-monosaccharides were readily separated in the presence of a small amount of borate buffer in the hydroorganic eluent in CEC. Such conditions have also been utilized by these and other workers in the past in CZE/CE.

Fig. 3 illustrates the CEC electrochromatograms of *p*-nitrophenyl-alpha-D-glucopyranoside and *p*-nitrophenyl-alpha-D-maltooligosaccharides. The percentage of ACN (v/v) in the mobile phase was changed in order to determine the optimal mobile phase composition for rapid elution time and high separation efficiency. These studies were done in a true isocratic CEC mode, no pressure driven flow was used, and the capillaries were packed using a wet slurry packing approach, with wet bare silica of 5 μm average particle size being sintered at the ends by a Bunsen burner to form the end frits. Detection was on-column, through the packed bed, rather than off-bed, as above [44]. Satisfactory separation was obtained, Fig. 3, with a mobile phase of low acetonitrile content (20% v/v) and low electric field strength (370 V/cm).

The reproducibility of the CEC system, in terms of retention time, was quite good, with %RSD<0.55.

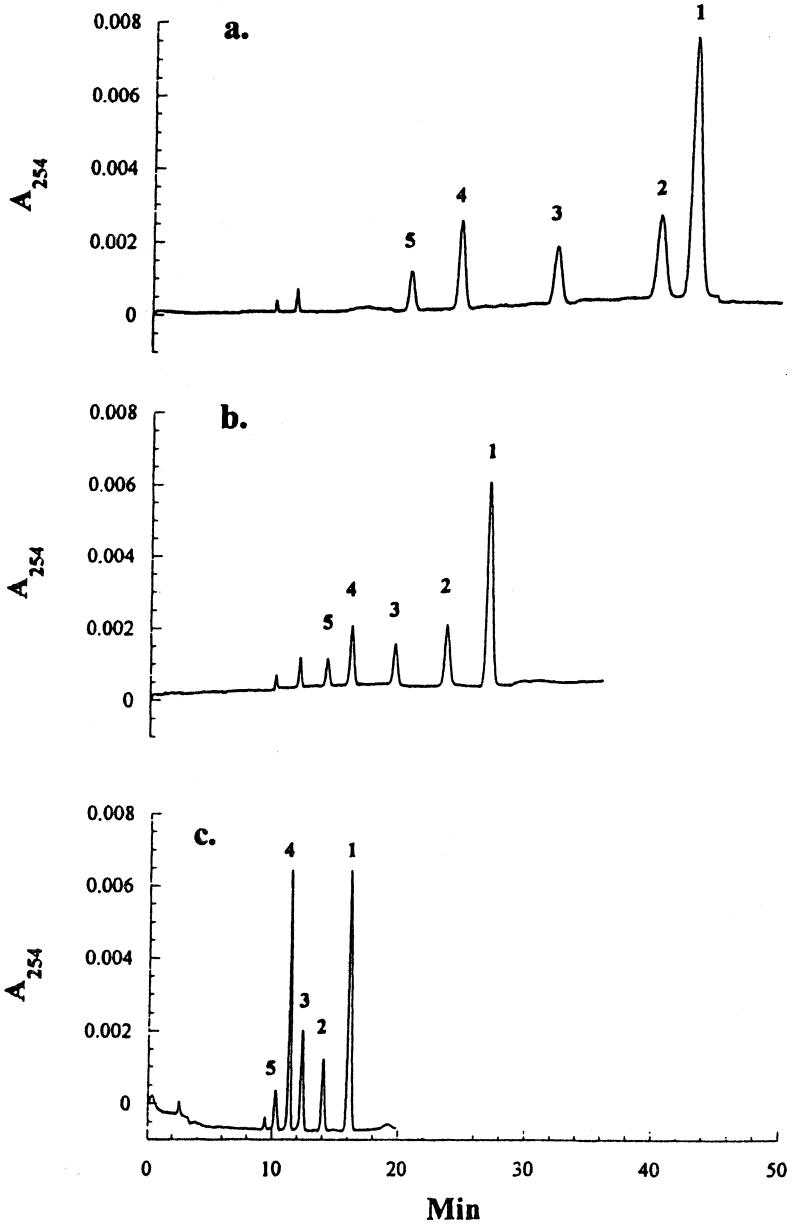

Fig. 3. Electrochromatograms of *p*-nitrophenyl-alpha-D-glucopyranosides and maltooligosaccharides. Mobile phase: (C), 40% v/v of 5 m*M* NaH_2PO_4 (pH 6.0), 40% v/v H_2O and 20% v/v acetonitrile; (B) 42.5% v/v of 5 m*M* NaH_2PO_4 (pH 6.0), 42.5% v/v H_2O and 15% v/v acetonitrile; (A) 45% v/v of 5 m*M* NaH_2PO_4 (pH 6.0), 45% v/v H_2O and 10% v/v acetonitrile; voltage, 10 kV, other conditions are described in the original publication [44]. Solutes: 1, *p*-nitrophenyl-alpha-D-glucopyranoside; 2, *p*-nitrophenyl-alpha-D-maltoside; 3, *p*-nitrophenyl-alpha-D-maltotrioside; 4, *p*-nitrophenyl-alpha-D-maltotetraoside; 5, *p*-nitrophenyl-alpha-D-maltopentaoside [44]. (Reproduced with permission of the authors and copyright holder, Wiley-VCH Verlag GmbH, Weinheim, Germany).

The column separation efficiency was high, varying between 72 000 and 152 000 plates/meter for the various derivatives. The elution order of typical *p*-nitrophenyl saccharides was the same as that observed in RPC, and is believed to be due to the hydrophobicity of the glycosides, as well as by organic induced conformational changes of the glycosides [46]. Changes in the elution order of the various *p*-nitrophenyl saccharides may be attributed to organic, solvent-induced conformational changes [47]. Several other examples of CEC separations of saccharides are offered in this study, including the separation of various alpha- and beta-anomers of glucopyranoside derivatives. Table 2 summarizes the realized column efficiencies in terms of plates/meter and retention time reproducibility (%RSD) for a number of these carbohydrate derivatives in this type of packed bed CEC [44].

1.5. CEC of peptides, proteins, and antibodies

CEC has shown utility in the separation of the enantiomers of amino acids [52–55]. Most of the separation methods utilized consisted of using molecularly imprinted polymers, with recognition sites as the stationary phase. Other groups have successfully utilized gradient elution to separate mixtures of dansylated amino acid mixtures on Zorbax ODS stationary phase [54].

Table 2
Column efficiency (plates/meter) and retention time reproducibility (%RSD) [44][a]

p-Nitrophenyl derivative of	Retention time (%RSD)	Column efficiency (plates/meter)
Galactose	0.54	95 000
Glucose	0.47	92 000
N-Acetylglucosamine	0.21	83 000
Mannose	0.31	84 000
Maltose	0.55	95 000
Maltotrioside	0.23	85 000
Maltotetraoside	0.49	152 000
Maltopentaoside	0.26	72 000

[a] Mobile phase: 20:80% v/v acetonitrile: 3.34 m*M* sodium phosphate, pH 6.0; capillary column: 20 cm (27 cm total length)× 100 μm ID packed with 5 μm ODS; 10 kV; $\lambda = 254$ nm; pressure injection at 20 p.s.i. for 10 s. (Reproduced with permission of the authors and copyright holder, Wiley-VCH Verlag GmbH, Weinheim, Germany).

For the purposes of simplifying further discussions, we shall refer to this general class of biopolymers (peptides, proteins, and antibodies) as peptides, realizing that **proteins can also be small (tri-) peptides**, of higher molecular mass (M_r). And, that antibodies are really larger proteins of specific conformations, shape, size, and immunogenicity together with antigenic recognition properties [48–51]. To date, the majority of applications of CEC for biopolymers have dealt with peptides, of varying sizes and complexity, utilizing different modes of CEC (open tubular, (OT-CEC); conventional isocratic CEC, gradient CEC, PEC, and others). The following are not necessarily listed in their order of appearance or importance, but rather grouped according to specific authors.

For example, Palm and Novotny recently applied their above described polymeric gel beds (monoliths) for peptide resolutions in CEC [42]. This approach used the very same packing in isocratic CEC, but now with 29% of C_{12} as the ligand. Additional CEC conditions are indicated in Fig. 4, which illustrates the separation of a series of tyrosine (Tyr) containing peptides, providing detection at 270–280 nm. In this particular study, peptide elution patterns were very sensitive to changes in pH and acetonitrile (ACN) concentrations. A gradient elution technique, not employed here, would have been more appropriate for such samples of peptides having small differences in their constitution. Attempts to elute protein samples were unsuccessful with these particular gel matrices, perhaps due to the high hydrophobicity of the packings [42].

In some earlier work, Euerby et al., reported the separation of an *N*-methylated C- and N-protected tetrapeptide from its non-methylated analog (Fig. 5) [56]. These separations utilized a Spherisorb ODS-1, 3 μm packing material, without pressure driven flow (true CEC using a commercial, HP CE instrument), and an ACN–buffer composition, as indicated in Fig. 5. Other operating conditions are indicated in Fig. 5. Using non-optimized, non-pressurized CEC conditions, separation of the two tetrapeptides could be achieved in a run time of 21 min with efficiency values of 124 000 and 131 000 plates/meter.

In comparison, when a pressurized CE (buffer reservoirs and capillary were pressurized, without pressure driven flow of buffer) system was used,

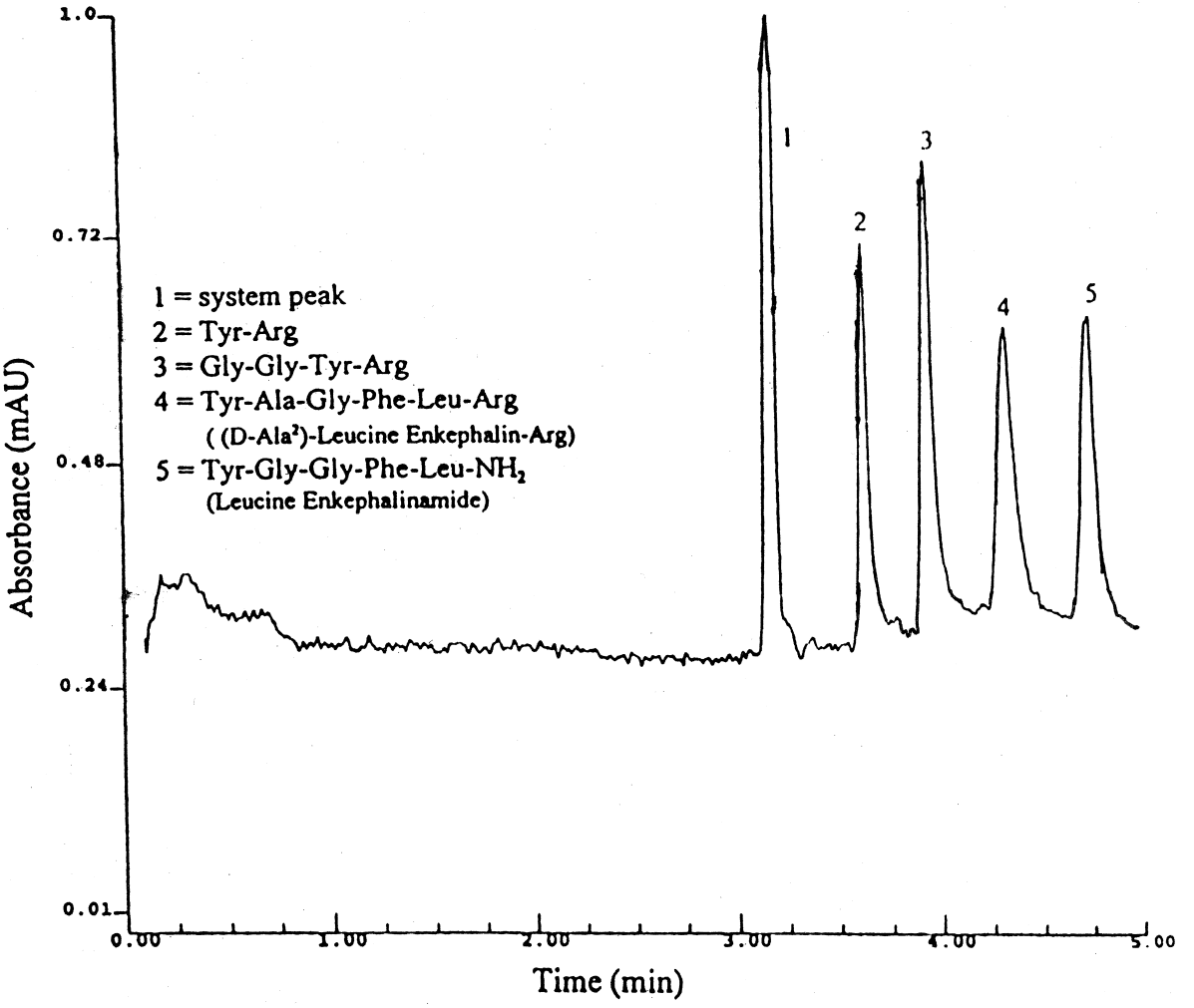

Fig. 4. Isocratic electrochromatography of peptides in a capillary filled with a macroporous polyacrylamide–poly(ethylene glycol) matrix, derivatized with a C_{12} ligand (29%) and containing acrylic acid. Conditions: mobile phase, 47% acetonitrile in a buffer; voltage, 22.5 kV (900 V/cm), 7 μA; sample concentration, 4–10 mg/ml; detection, UV absorbance at 270 nm; other conditions are described in the original publication [42]. (Reproduced with permission of the authors and copyright holder, American Chemical Society, Washington, DC).

separation of the components was achieved within 3.5 min. According to Euerby et al., the separation of these two peptides using a pressure driven HPLC gradient analysis took 30 min and gave comparable peak area results. Although this study illustrated the improved efficiency of both non-pressurized CEC and pressurized CEC over HPLC, no general conclusions should be drawn from this work. The scope of this peptide study was limited to two tetrapeptides of unspecified structures, differing only by a methylated nitrogen group. The low voltage used in the study produced a low EOF that allowed sufficient time (21 min) for chromatographic separation. The difference between the CEC elution time of 21 min and the HPLC elution time of 30 min is not remarkable. Furthermore, this study does not offer any insight into the capabilities of CEC with diastereomeric and enantiomeric peptides. In fact, based on the current literature discussing the failure to separate D,L amino acids, one concludes that the separation of enantiomeric peptides is problematic. Using recognition sites on molecularly imprinted polymers with pre-

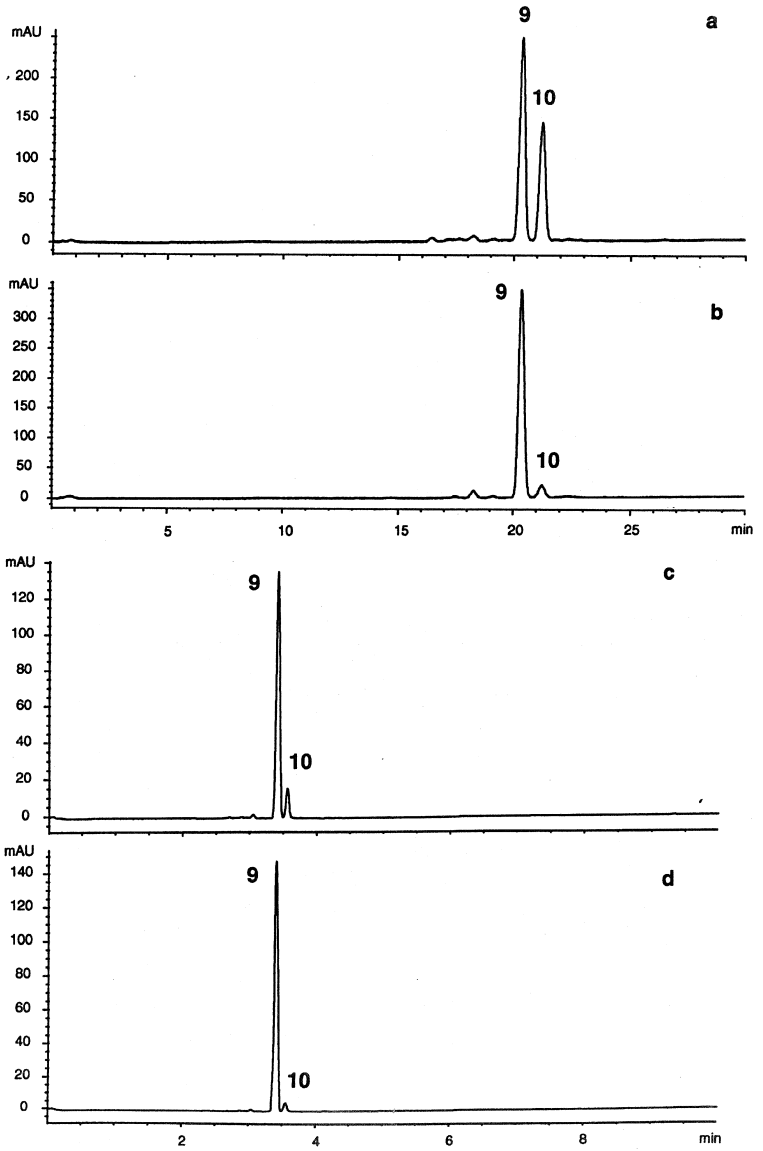

Fig. 5. Separation of synthetic, protected tetrapeptide intermediates. Peak assignments: [9] *N*-methyl C- and N-protected tetrapeptide; [10] non-*N*-methyl C- and N-protected tetrapeptide. The structures of these compounds is proprietary information and consequently cannot be disclosed. Detection wavelength 210 nm with a 10 nm bandwidth and a 1 s rise time. Electrochromatography was performed on a 250 mm×50 μm I.D. 3 μm Spherisorb ODS1 packed capillary using an acetonitrile–Tris (50 mmol/L, pH 7.8) buffer 80:20 v/v mobile phase, capillary temperature of 15°C, and an electrokinetic injection of 5 kV/15 s). (A) Synthetic mixture of protected tetrapeptides 9 and 10. Efficiency values of 124 000 and 131 000 plates per meter were obtained for analytes 9 and 10, respectively. Unpressurized HP3D CE system using an applied voltage of 5 kV. (B) Chromatogram of synthetically prepared 9, the presence of residual non-methylated tetrapeptide [10] can be clearly seen. Unpressurized HP3D CE system using an applied voltage of 5 kV. (C) Chromatogram of synthetically prepared 9, spiked with 10% of the non-methylated tetrapeptide [10]. Efficiency values of 83 000 and 101 000 plates per meter were obtained for analytes 9 and 10, respectively. Pressurized CE system using an applied voltage of 30 kV. (D) Chromatogram of synthetically prepared 9, the presence of residual non-methylated tetrapeptide [10] can be clearly seen at the 3% level. Pressurized CE system using an applied voltage of 30 kV [56]. Additional conditions are indicated in the original publication. (Reproduced with permission of the authors and copyright holder, John Wiley & Sons, Inc., New York, NY, USA).

determined selectivity, non-dansylated D,L-leucine could not be separated [57]. In addition, the study by Euerby et al. does not offer any insight into the simultaneous separation of numerous peptides, as in a complex peptide map from a large peptide or antibody.

The authors are disturbed about the apparent random selection of stationary phases and operating conditions. None of the literature to date shows chemometric optimization of the operating conditions in CEC. Such protocols have been used for years in HPLC and GC to optimize separations and shorten method development time [58–60]. In CEC, the fundamental parametrics are just being developed. Software aids (programs) to improve or speed-up method development and optimization are expected to be developed. This will probably make it easier to make better choices about stationary phases and operating conditions.

The coupling of an electrospray (ESI) mass spectrometer (MS) with a pressurized CEC system (PEC) has been shown to separate peptides [61]. This particular study of Schmeer et al. utilized a commercial packing material, namely a reversed-phase silica gel, Gromsil ODS-2, dp=1.5 μm, already utilized in capillary HPLC for peptide separations. It was never made perfectly clear why this particular packing material was selected, or why PEC was selected for MS interfacing over conventional, isocratic CEC conditions. It is possible that the EOF alone with this packing was insufficient to elute all peptides in a reasonable time and thus pressurized flow was introduced? A knowledgeable reviewer has indicated that pressure was employed here to stabilize the EOF at high electrical field strength. No gradient elution PEC was demonstrated in this particular study. A mixture of enkephalin methyl ester and enkephalin amide were separated using the packed capillary column (Fig. 6). The coupling of these two methods showed enhanced sensitivity and detectability (limits of detection, LODs) at low concentrations (20 ppm (w/w) injected, 3 pmol detected at MS).

As in the above study, this too offers little insight into the true capabilities of CEC to separate peptides, as in a complex mixture of peptides. These two peptides contained significant physical differences, which influenced their elution times. As one would expect, the mass (electro)chromatogram reveals that the enkephalin amide eluted almost 2 min faster than the enkephalin methyl ester. Since the acetonitrile–water–trifluoroacetic acid buffer solution was at a low pH, one would expect a distinctive separation based on the electrophoretic separation of these two peptides. At a low pH, the amide should migrate faster to the cathode than the ester, since it carries an additional positive charge under acidic conditions. In addition, since these two compounds are structurally distinctive, a chromatographic separation was probably also taking place. In summary, the report by Schmeer et al. provides little insight into the CEC separation of peptides; however, the study does provide a nice example of a peptide separation based on chromatographic and electrophoretic separation mechanisms probably occurring simultaneously. This report also described, for perhaps the first time with peptides, the ability of easily interfacing CEC and PEC with ESI-MS, something that was and would also be reported by several other groups, usually for low M_r pharmaceuticals.

Along the lines of using open tubular (OT) or packed bed capillaries in CEC, at times with external pressure driven flow to generate PEC, several papers have recently appeared by Lubman's group [62–65]. In this work, both OTC-CEC as well as packed CEC approaches were interfaced with an on-line, ion trap storage/reflectron time-of-flight mass spectrometer (ITS–TOF-MS) [62]. Wu et al. here reported the separation of a six peptide mixture using an open tubular capillary (OTC) with a (C_8 surface chemistry) coupled to an on-line ITS–TOF-MS. The work described included an interesting experimental scheme to enhance the EOF under acidic conditions, without using pressure driven buffer flow. After preparing the CEC column with a commercial, C_8 stationary phase, the inner surface of the capillary wall was chemically coated with an amine, (3-aminopropyl)trimethoxysilane (APS). This coating served two purposes. First, it significantly enhanced the EOF in acidic buffer solutions, so that a large EOF flow-rate was obtained, without using a very high voltage, which in turn resulted in a stable flow. Second, the surface silanol groups were covered by the amine groups which carried positive charges in an acidic solution, so that non-specific adsorption between the peptide sample and the inner surface was greatly reduced. A six peptide mixture could be

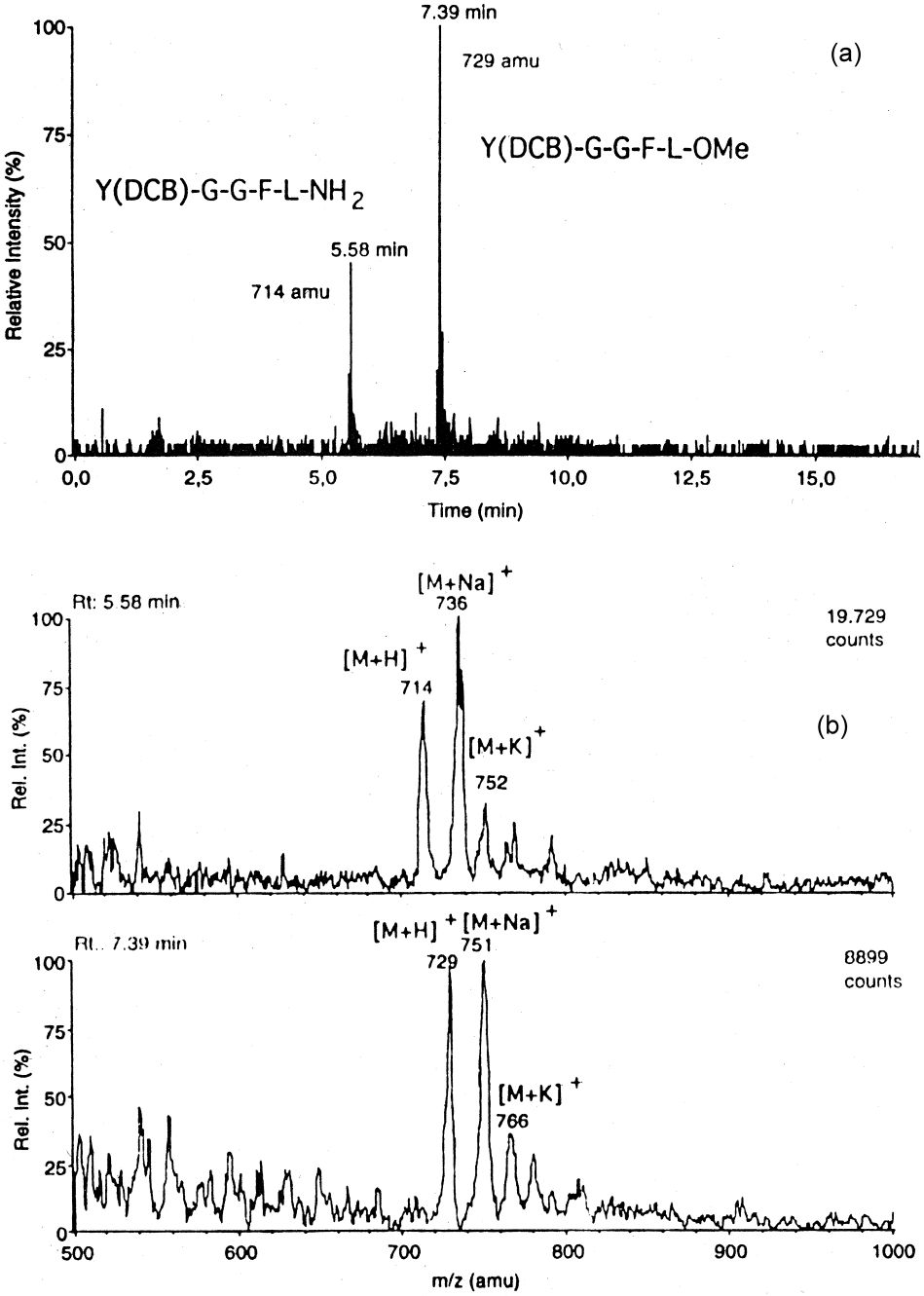

Fig. 6. Interfacing of pressure driven CEC (PEC) for the separation of two simple peptides, enkephalin methyl ester (5.58 min) and enkephalin amide (7.39 min). (A) Extracted mass chromatogram of m/z 714 and 729 for the on-line peptide separation. Specific operating conditions are indicated in the original publication [61]. (B) Mass spectra taken from the chromatographic peaks in (A) above, illustrating true Mr, and presence of M+H, M+Na, and M+K cations at appropriate m/z (amu) values. (Reproduced with permission of the authors and copyright holder, American Chemical Society, Washington, DC).

separated to baseline within 3 min on this system, with absolute identification of each peptide peak by the ITS–TOF-MS combination. Because of the high duty cycle of the MS and the column path length-independent concentration-sensitive feature of the ESI process, high quality total ion chromatograms could be obtained with injections from only 1–2 fmol of peptide samples. A concentration limit of detection of 1×10^{-6} *M* was also achieved due to the preconcentration ability of CEC. Fig. 7 shows the separation of the six peptide mixture on an APS-coated OT-CEC column. Because of the high flow velocity of the EOF, the separation was completed within 3 min. Of course, it was also possible to obtain complete ESI mass spectra (multiply charged M_r ions) for each of the peaks in Fig. 7.

In this same publication, the authors also demonstrated the ability of interfacing true gradient elution CEC with OTC to the same ITS–TOF-MS detector, again with ESI [62]. This too was performed without any external pressure driven mobile phase flow, but in true CEC fashion, now using a very simple gradient formation device with a single syringe pump and a anodic buffer vial that underwent constant,

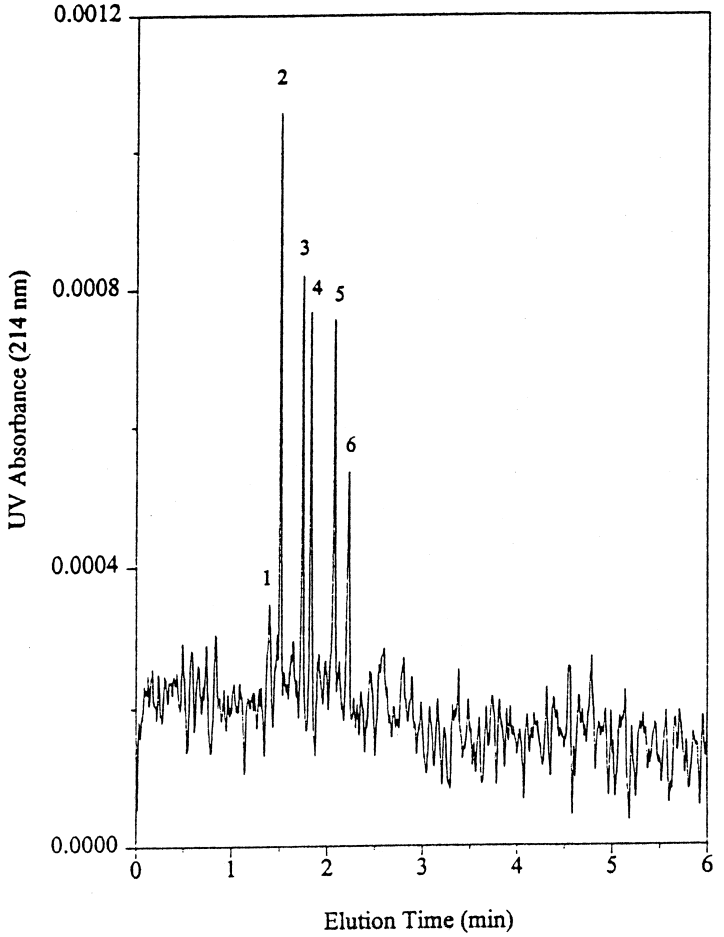

Fig. 7. Open tubular CEC (pressurized flow, PEC) separation of a six-peptide mixture using a column with APS coating. Separation conditions: column length 30 cm (25 cm to detector); separation voltage, −12 kV; injection, −2 kV×3 s; sample concentration, 1×10^{-5} M; UV detection at 214 nm. Six peptides are: (1) methionine enkephalin; (2) bradkykinin; (3) angiotensin III; (4) methionine enkephalin-Arg-Phe; (5), substance P; and (6) neurotensin. Additional conditions are indicated in the original publication [62]. (Reproduced with permission of the authors and copyright holder, American Chemical Society, Washington, DC).

reproducible changes in its composition. This, therefore, generated a true gradient buffer formation in the packed bed CEC–MS system. Fig. 8 here illustrates both the UV and TIC traces of a gradient CEC separation of the tryptic horse heart myoglobin digest, illustrating 10 peaks, almost fully baseline resolved within 6 min. Among the peaks shown in the TIC, 15 usable mass spectra (including coeluting components) could be obtained to cover about 90% of the amino acid residues in the protein. The calculated and measured masses were found to be in excellent agreement, Table 3. These mass spectra were all obtained with high-resolution conditions (ca. 1500 Da), even though they were all obtained at a

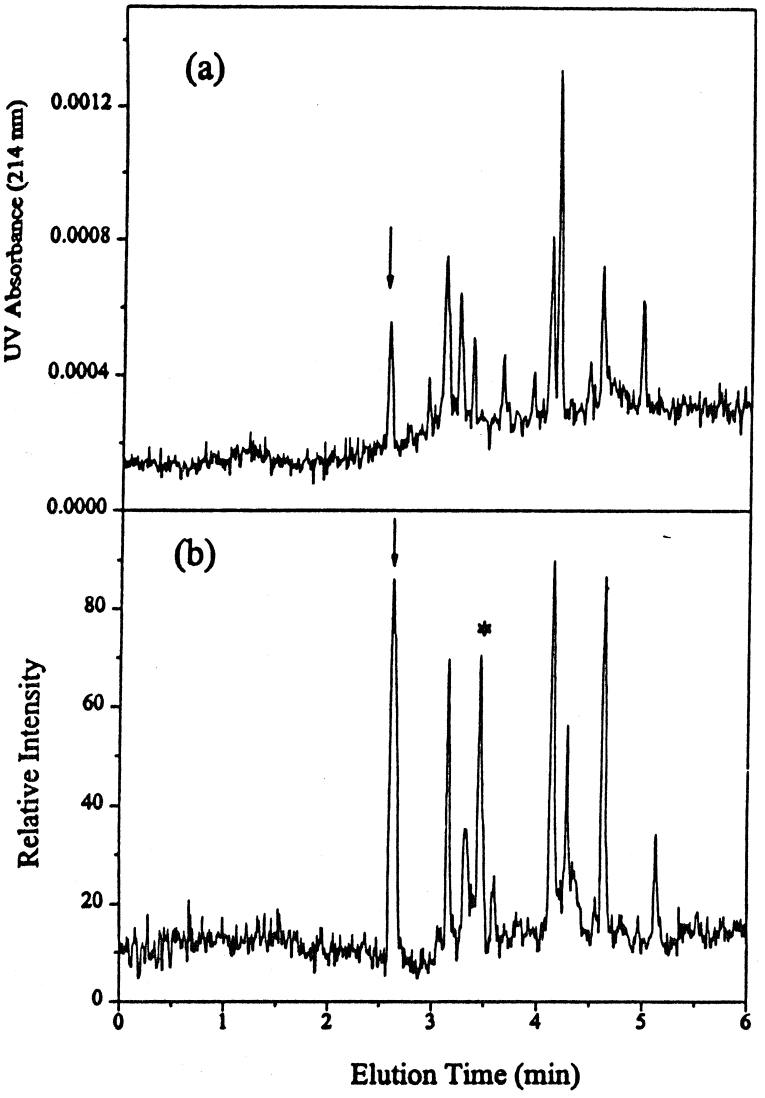

Fig. 8. Analysis of a protein digest using gradient elution CEC with on-line ITS–TOF-MS. UV trace (a) and TIC (b) of gradient CEC separation of a tryptic horse heart myoglobin digest. Conditions: 0–35% acetonitrile gradient in 6 min; column length, 40 cm (for UV detection, 35 cm to UV detector); separation voltage, −14 kV; injection, −2 kV×5 s; UV detection at 214 nm; MS detection speed, 8 Hz. Additional conditions are indicated in the original publication [62]. (Reproduced with permission of the authors and copyright holder, American Chemical Society, Washington, DC).

Table 3
Comparison of calculated and measured tryptic fragments of horse heart myoglobin from CEC–MS analysis

No.	Fragment	Calcd. mass (Da)	Measd. mass[a] (Da)	Sequence
1	1–16	1816.0	1816.4	GLSDGEWQQVLNVWGK
2	17–31	1606.8	1606	VEADIAGHGQEVLIR
3	32–42	12171.4	1271.4	LFTGHPETLEK
6	48–50	396.5	396.4	HIK
7	51–56	707.8	707.6	TEAEMK
8	57–62	661.7	661.8	ASEDIK
10	64–77	1378.7	1379.0	HGTVVLTALGGILK
13	80–96	1854.1	1853.6	GHHEAELKPLAQSHATK
14,15	97–102	752.9	752.7	HKIPIK
15	99–102	469.6	469.4	IPIK
16	103–118	1885.2	1885.5	YLEFISDAIIHVLHSK
17	119–133	1501.6	1501.2	HPGNFGADAQGAMTK
18	134–139	747.9	748.1	ALELFR
20	146–147	309.4	309.5	YK
21	148–153	649.7	649.6	ELGFQG

[a] Average mass of all charge states of the fragment observed. Reproduced with permission of the authors and copyright holder, American Chemical Society, Washington, DC.

full mass range sampling speed of 8 Hz. Clearly, another advantage of using the MS as an on-line detector for CEC, or for HPLC or CE for that matter, is that it can identify some partially resolved or unresolved peaks, thereby increasing the resolving power of the basic CEC system. This has been amply demonstrated in the past for HPLC–MS and CE–MS approaches, also with peptide maps and intact proteins.

There are several other recent papers by the Lubman group dealing with peptide analysis using various forms of CEC interfaced to ITS–TOF-MS, again using ESI as the interface [63–65]. Much of this work deals with the separation of tryptic digests of proteins using various buffers in pressurized CEC or PEC, again always coupled to ITS–TOF-MS [64,65]. This particular work utilized packed bed CEC, often a Vydac C_{18} silica gel phase (3 μm), as utilized in the past for similar peptide separations in HPLC and cLC. In this and related work, PEC was utilized for the analysis of peptide mixtures and protein digests. By using both EOF and pressure driven flow, the total time for analysis of peptide mixtures could be reduced over just EOF alone, even losing some of the efficiency and plate counts that would have accrued by isocratic or gradient CEC alone. With certain packings, such as this Vydac material, there are often very few residual, uncapped silanols and therefore EOF, hence the need for pressure driven flows. Gradient elution, packed bed PEC was now utilized with on-line IT–TOF-MS, in order to again demonstrate the advantages of combining these two separation schemes. The supplementary pressure driven flow suppressed bubble formation and also allowed for the tuning of the elution of peptides using the applied electrical field. In this way, a very fast separation of six peptides could be performed. Using very short, 6 cm columns, with PEC, a tryptic digest of bovine cytochrome c was fully separated in about 14 min by properly tuning the applied voltage and the external pressure from an on-line HPLC pump. Fairly complex protein digests, such as that from chicken ovalbumin, containing more than 20 peaks, could be resolved in the total ion chromatogram (TIC) in 17 min. Again, the use of an on-line ITS–TOF-MS detector increased the resolving power of the PEC system, by providing for absolute identification of coeluting components, as above with OTC–CEC. Sample concentrations were again in the range of 5×10^{-6} *M* with a volume of 1.5 μl injected. This then corresponded to a sample load injected in the low picomole range [63–65].

In a recent review by the Lubman group, they point out (quite correctly) that there are some significant advantages favoring use of OTC for CEC

as compared with packed beds. OTCs with inner diameters around 10 μm have been found to have a smaller plate height when compared to packed columns. This is due to the lack of band broadening effects associated with the presence of packing particles and end column frits. OTC capillaries do not require end frits. High concentration sensitivity is another advantage of OTCs, since columns with very small dimensions are used. The small diameters of the OTCs allow for the use of a higher voltage in CEC, without significant Joule heating. OTCs can often provide more rapid separations than packed columns, by eliminating intraparticle diffusion, which is the dominant limitation for ultrafast separations in packed columns. However, there are some grave difficulties involved in using OTCs, perhaps because of the real difficulties with sample injection (loadability, peak capacity, sensitivity) and detection. The injection volume of OTCs is in the low nanolitre or even picolitre range. The very small inner diameters of most OTCs makes optical detection difficult, at best, but they are clearly very compatible with a concentration sensitive detection method, such as ESI-ITS–TOF-MS. This approach is independent of the optical path length of the capillary, and thus the major disadvantages of OTCs may be overcome in a CEC–ESI-MS approach and configuration. Again, with peptide mixtures, gradient elution CEC with or without pressure driven flow, is almost required over isocratic or step-gradient methods, since small changes in the mobile phase composition results in large changes in peptide retention times. It is thus difficult, at best, to separate a complex peptide mixture, such as a large protein digest, using the isocratic mode in CEC or HPLC or cLC.

One final point that should be recognized from Lubman's studies. They almost always use pressurized flow together with applied voltages. For very complex peptide mixtures, it may prove difficult to optimize the selectivity of peptides using just the applied field in CEC without any supplementary pressure. If one attempts to improve selectivity by altering pH or ionic strength, then the EOF may be adversely affected and the total analysis times may be impractically long. On the other hand, by applying the external pressure driven flow, total analysis times can be controlled and reduced. The effect of applied voltage on peptide selectivity can be optimized independent of external flow-rates. Selectivity can be obtained without giving up total analysis times. Thus, the tuning of the selectivity of peptides becomes possible since the applied voltage and the supplementary pressure are available as two totally separate, tunable parameters that can be optimized for achieving a higher selectivity than using either the HPLC or CE modes alone.

We can illustrate this in Fig. 9, taken from a 1997 publication, which illustrates the advantages of using combined pressure driven flow with an external applied (variable) voltage and gradient elution RP packed bed CEC conditions [65]. Fig. 9 shows the TICs of the separation of a bovine cytochrome c digest, using a 6 cm long column with a gradient elution and a packed bed CEC capillary with a commercial C_{18} packing. In Fig. 9A, no separation voltage was applied, so this was just conventional gradient elution, RP cLC of a peptide mixture (90 bar). The use of such a short column in HPLC made it difficult to resolve all the components in the digest. The peaks marked by asterisks (*) contain two eluting components. In Fig. 9B, a 2000 V voltage was applied on the column, and the back pressure was reduced to 50 bar. It is often advantageous when using pressure driven CEC (PEC) to balance the EOF being controlled by the applied voltage with the applied pressure flow, in order to maintain a relatively constant, overall mobile phase flow-rate. The total flow-rate will affect the overall, observed separation process. In Fig. 9B, all of the peptides eluted faster than in Fig. 9A, due to the contribution from EOF and the EPF migrations. Due to the applied voltage and EOF vs. parabolic flow profiles, the peaks in Fig. 9B are all sharper, indicating an increase in separation efficiency. Certain unresolved peaks in Fig. 9A have now been resolved in Fig. 9B, mainly due to the EPF separation of these two components. However, the first two peaks in Fig. 9A have now coalesced in Fig. 9B, since for these two particular fragments, the EPF is in the opposite direction of the separation resulting from purely RP partition mechanisms. Fig. 9C illustrates the overall effect of a larger applied voltage, now 1400 V, but the peak marked by the asterisk in Fig. 9C is still unresolved. In Fig. 9D, it was now possible to vary the applied voltage to 600 V, with a supplementary pressure of 70 bar. At this particular pressure and

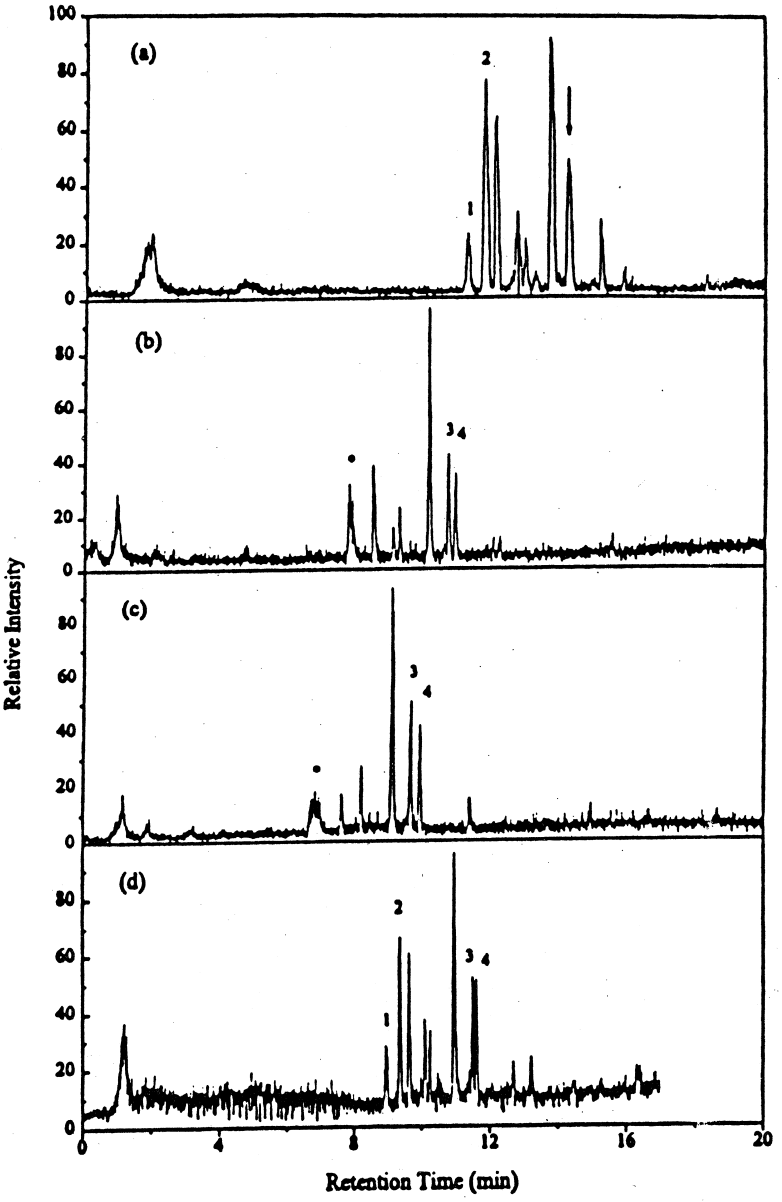

Fig. 9. TICs of the separation of a bovine cytochrome c digest using a 20 min, 0–50% acetonitrile gradient with a packed C_{18} bed, with sample injections of 8 pmol corresponding to the original protein. Column length, 6 cm; column operating conditions: (A) HPLC mode with a back pressure of 90 bar; (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. Additional conditions are indicated in the original publication [65]. (Reproduced with permission of the authors and copyright holder, American Chemical Society, Washington, DC).

voltage, all four of the involved peaks (unresolved before) were clearly resolved [65].

This is perhaps an ideal illustration of utilizing all possible variable parameters in PEC, the nature of the gradient, the nature of the packing, the dimensions of the packed capillary, the applied voltage, the nature of the mobile phase (pH, ionic strength, organic/aqueous), and the applied pressure driven

flow component. By varying both applied pressure (bars) and applied voltage (V), so that these are truly optimized, even without varying the gradient conditions or the packed bed, it has been possible to resolve all of the peptides in Fig. 9. Again, the use of external applied pressure together with varying voltages, appears to provide additional analyte selectivity not possible in simple isocratic/gradient CEC. This is not possible in isocratic/gradient cLC, and surely not possible in CE, without any packing present. This approach also provides selectivity on the basis of partitioning mechanisms, as well as electrophoretic differences. Of course, in conventional cLC or HPLC, there is no applied voltage to introduce EPF migration differences or patterns. Thus, PEC with gradient elution, especially when combined with on-line MS, offers the greatest advantage for high resolution and speed. The only thing that may be lost is the maximum plate counts and column efficiency generated in pure CEC [65].

In contrast to the mainly packed column methods described above, Pesek et al. utilized the OT approach to CEC separation of peptides and proteins [66–69]. As mentioned above, OTC has several real advantages over packed bed capillaries in CEC or cLC. They have been shown to have smaller plate heights, due to the lack of band broadening effects associated with the existence of packing materials and end column frits. High concentration sensitivity is another advantage of OTCs, since columns with extremely small dimensions are used. Also, the small diameters of the OTCs allow for the use of a higher voltage in CEC without significant Joule heating or temperature effects. Furthermore, OTCs provide for much more rapid separations than packed columns, by eliminating intraparticulate diffusion, which is the dominant limitation for ultrafast separations in packed columns [66].

Pesek has engineered certain novel approaches to bonding of organic ligands to silica surfaces, and has demonstrated the increased stability of such an approach to covalent silicon bonding in HPLC, HPCE, and now CEC studies, with a wide variety of bonded phases and analyte applications. Whereas conventional attachment of organic ligands to silica has usually involved a siloxane (Si–O–Si) bond or variations thereof, Pesek's approach realizes a much more pH and aqueous stable Si–C bond. In his approach, the fused-silica inner surface was etched with ammonium hydrogen difluoride to increase the surface area. This then produced radial extensions of the ligand from the surface to facilitate solute-bonded phase interactions. In theory, the etching process increased the surface area of the inner walls sufficiently to induce solute interactions with the capillaries modified with an organic moiety. The chemistry used to modify the etched capillary was based on a silation–hydrosilation reaction scheme, which leads to a direct silicon–carbon bond on the surface, that is:

$$-\mathrm{Si}-\mathrm{OH} + (\mathrm{OEt})3\mathrm{Si}-\mathrm{H} \rightarrow -\mathrm{Si}-\mathrm{O}-\mathrm{Si}-\mathrm{H} + 3\ \mathrm{EtOH}$$

$$-\mathrm{Si}-\mathrm{H} + \mathrm{R}-\mathrm{CH}{=}\mathrm{CH}_2 \rightarrow -\mathrm{Si}-\mathrm{CH}_2-\mathrm{CH}_2-\mathrm{R}$$

In this process, the etched surface of the capillary was first reacted with triethoxysilane (TES) to produce a hydride surface. An organic moiety was then attached to the hydride intermediate by passing a solution containing a terminal olefin and a suitable catalyst, such as hexachloroplatinic acid, through the capillary. In order to characterize the behavior of this new CEC format, Pesek et al. ran a test mixture of five polypeptides and proteins on four types of capillaries, Table 4 [66,68].

As one would expect, in the bare capillary there was a relatively small difference between the migration times of the five components. The etched capillary also had a relatively small range of migration times, but each component had a much lower linear velocity than in the bare capillary. Even though the effective length of the etched capillary was twice as long as the bare capillary, the migration time was more than twice as long. This was likely due to the fact that the etching process reduced the number of free silanols on the surface, and hence lowered the EOF. Some improvements in separation as well as an increase of migration time over the bare capillary were seen for the hydride modified column. The increase in elution time was probably due to a decrease of free silanols, which led to a lower EOF. In any case, the relatively close elution of the solutes in the hydride capillary indicated that their separation was based mainly on EPF mobility and not on interactions with the capillary surface. When the same solutes were evaluated using the C_{18} modified,

Table 4
Migration times of various peptides and proteins [68][a]

Compound	Capillary and buffer				
	Bare: pH 3.7	Etched (300°C, 3 h); pH 3.7	Si–H modified; pH 3.7	C_{18} modified; pH 3.0	C_{18} modified; pH 3.0 with 10% MeOH
a=Lysozyme (turkey)	1.83	Wide peak	2.23	4.06	4.67
b=Angiotensin III	2.05	6.82	3.13	4.68	5.37
c=Bradykinin	2.09	6.98	3.00	6.01	5.68
d=Ribonuclease A	2.02	6.89	2.89	6.95	6.47
e=Angiotensin I	2.24	7.55	3.66	8.01	7.00

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etched capillary, some solutes had long retention times and/or poor peak shapes.

Since the result of the hydrosilation reaction was to replace hydrides with octadecyl (C_{18}) moieties, there should have been no increase or a decrease in the number of silanols which were on the surface. Therefore, the change in times reported in Table 4 must have been due to increased interaction with the newly modified surface. The increase in peak width also supports this conclusion, since there should be some decrease in efficiency due to mass transfer effects. Pesek et al. attributed the long retention times and poor peak shapes to the unfolding of the proteins and polypeptides, which was a result of the strong interactions between the solutes and the bonded octadecyl moiety. To alleviate this problem of long retention times and poor peak shapes, Pesek et al. lowered the pH from 3.7 to 3.0. As expected, lowering the pH increased the charge on the species and caused each solute to elute faster with better peak symmetry (Fig. 10) [68]. In any case, the increased peak widths and the larger range of elution times confirmed that chromatographic interactions between the solutes and the bonded moiety occurred as opposed to pure electrophoresis. This is the essential difference between CEC and CE. Further support to this conclusion was obtained when methanol was added to the mobile phase, thus causing a decrease in elution times for some solutes and a slight improvement in efficiency, indicated by a decrease in capacity factor, k'. Interestingly, the first two components in the mixture, Fig. 10, had an increase in elution time when methanol was added to the electrolyte. Adding organic solvents to the mobile phase can change both the EPF mobility as well as bonded phase interactions contributing to the retention. The effect is to sometimes increase retention time, and at other times, it produces a decrease. The effect of adding organic solvents on general elution does not appear to be predictable at this time.

A study performed by Pesek et al. showed outstanding stability and reproducibility of their capillaries. Thirty one consecutive injections of lysozyme, followed by an identical series of 31 injections of ribonuclease A were performed. No discernible increase or decrease in retention times for either protein was observed. The reproducibility of each result was $\pm 1.5\%$ [68]. The imprecision is still larger than HPLC, but it is probably sufficient to be a base for finding the other contributing sources for variance in retention behavior.

In a later study, Pesek et al. reported the separation of other proteins using a diol stationary phase. The use of a diol stationary phase should result in a surface that is more hydrophilic than a typical alkyl bonded moiety, like C_{18} or $C_{8.}$ Fig. 11 illustrates the separation of basic proteins in a buffer with a pH value of 4.41. The peaks were relatively symmetrical, indicating that little adsorption of the solutes on the etched and modified surface took place. Although no mention was made of the small, broader peaks near the baseline, they could have been due to partial unfolding of some of these proteins. A lowering of pH could alleviate this problem by affecting the charge of the protein species and their conformations.

A comparison of the separation characteristics for

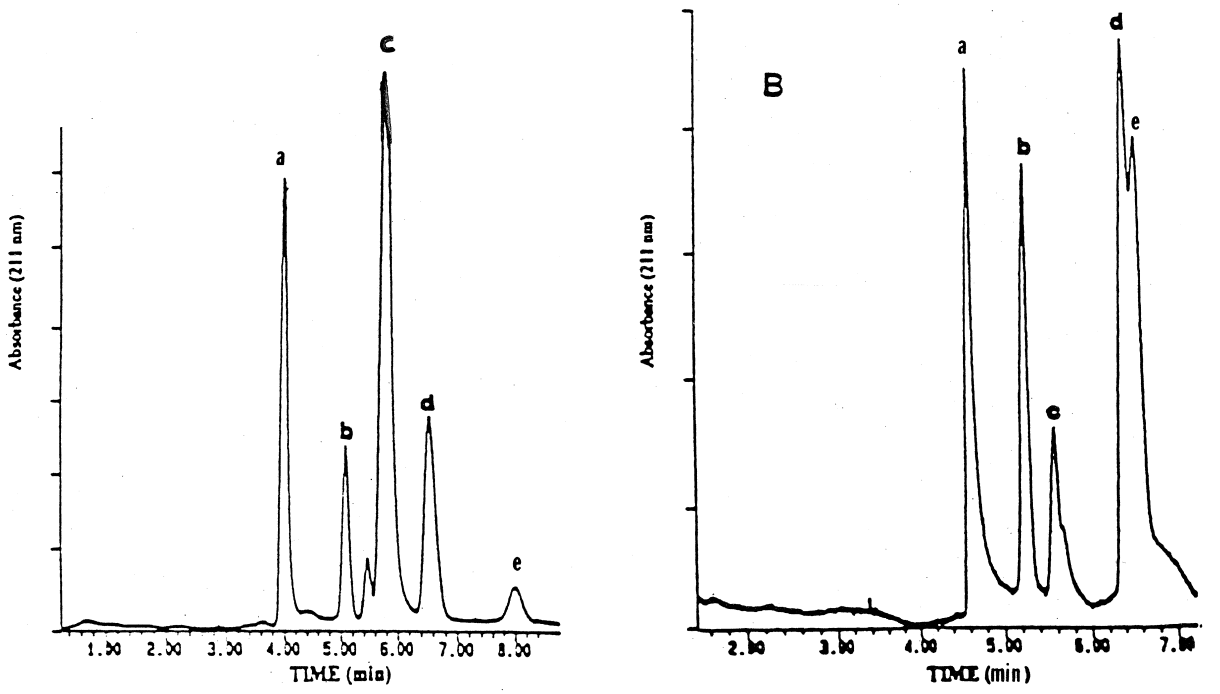

Fig. 10. Separation of a mixture of peptides and proteins on a C_{18}-modified etched capillary at pH 3.0 with (A) 0% methanol ($I=18$ μA) and (B) 10% methanol ($I=14$ μA) in the electrolyte. Conditions: L (total capillary length)=45 cm, l (effective length)=25 cm, $V=25$ kV, solutes: a=lysozyme (turkey); b=angiotensin III; c=bradykinin; d=ribonuclease A; and d=angiotensin I [68]. (Reproduced with permission of the authors and copyright holder, Elsevier Science Publishers, Ltd., Amsterdam, Holland).

a series of angiotensins, on bare unetched, diol-modified etched and C_{18} modified, etched capillaries is shown in Fig. 12. For each column, the elution order was the same, indicating that while solute-bonded phase interactions may have been significant, the differences in electrophoretic mobility were primarily responsible for the separations observed with the angiotensins. The longer migration times on the C_{18} column could have been due to more efficient bonding, i.e. a greater number of bonded moieties per unit area, or to stronger solute-bonded phase interactions. Pesek et al. attributed this greater retention time to the greater hydrophobic interactions between the bonded C_{18} and the solutes. In any case, it was clear that the separation for the two modified, etched capillary columns was a combination of differences in EPF mobility, as well as solute-bonded phase interactions. Again, these are the major differences between CEC vs. CE vs. HPLC.

The interesting results reported by Pesek et al. confirm the conclusion that proteins and peptides can be separated with chemically modified, etched fused-silica capillaries. The results showed that distinctive chemical modification (e.g. diol, C_{18}) showed significant variations in retention times due to differences in solute-bonded phase interactions. Other factors, such as pH could also influence this interaction, due to its influence on charge and protein conformations. Combining all these factors in the separation of peptides and proteins provides an experimentalist with many important decisions to be made in the optimized experimental conditions to be used. Other chemical modifications of etched fused capillaries need to be studied, in order to provide a better understanding of their interactions with proteins and peptides, as well as other biopolymer classes.

1.6. CEC of nucleic acids and oligonucleotides

There has perhaps been much less work reported in CEC for this particular class of biopolymers than for others. Though there are several publications on

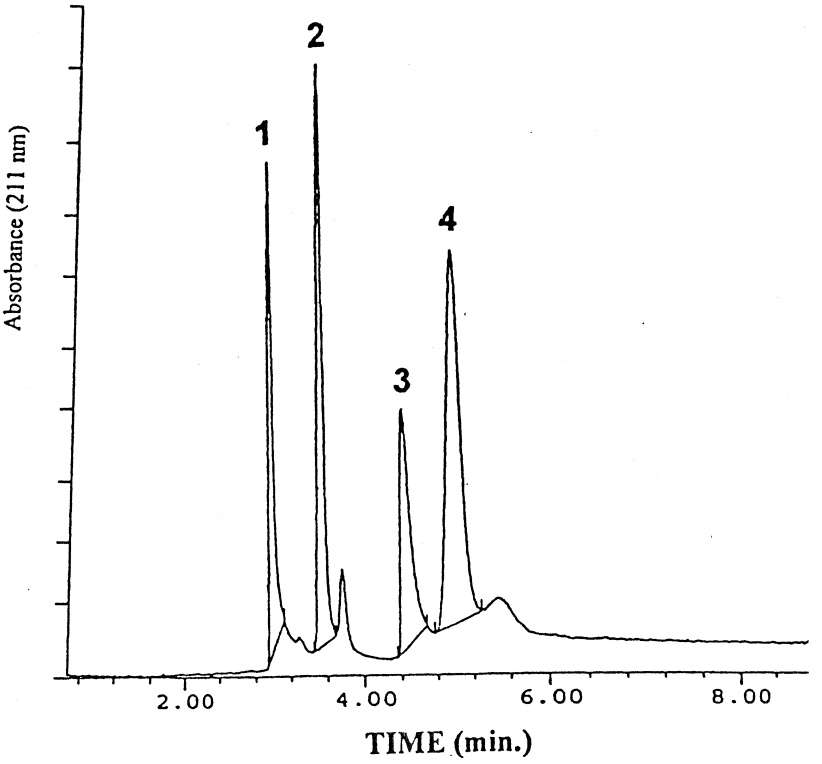

Fig. 11. Electrochromatograms of protein separation on a diol capillary. L (total capillary length)=45 cm, l (effective length)=25 cm, V=22 kV, I=7 μA, pH=4.41, solutes: 1=cytochrome c; 2=lysozyme; 3=myoglobin; and 4=ribonuclease A [67]. (Reproduced with permission of the authors and copyright holder, Elsevier Science Publishers, Ltd., Amsterdam, Holland).

the separation of monomeric nucleosides and nucleotides, we do not include those in this section, simply because they are not biopolymers [70–73]. One of the very few reports on the separation of oligonucleotides is that of Behnke and Bayer, using a pressurized, gradient elution CEC apparatus [74]. In their study, they used the term pressurized gradient electro high-performance liquid chromatography (electro-HPLC), which is a variation on PEC. It utilizes an HPLC system with a voltage applied across the capillary length. The authors demonstrated improved separations (RP) of charged analytes (anionic) on a 5 μm C_{18} reversed-phase silica gel packing. They studied the influence of applied voltage gradients up to 400 V/cm on the separation in both isocratic and gradient elution modes. The authors then made a direct comparison of microbore HPLC and MECC (aka MEKC). As in some of the PEC applications with Lubman et al., applying a voltage to a micro-HPLC column results in EOF. Its contribution to the overall velocity of the eluent increases with the electric field strength (V/L). Using this technique, analysis times are shortened and efficiency increases dramatically over just micro-HPLC. Electric field strength, direction of the voltage, voltage gradient, pH, applied external pressure (bar), eluent composition, and overall mobile phase gradient, are the most important, among other, parameters that can be varied to optimize the performance in PEC.

Fig. 13 then compares the influence of applied voltage on the chromatographic separation of a series of oligonucleotides (dC3–dC11) and a comparison with MECC [74]. Higher order oligos have increasing capacity factors in RP-HPLC and also increasing electrophoretic mobility. The overall direction of the electric field can be used to retard (or advance) the elution of the higher homologues in order to opti-

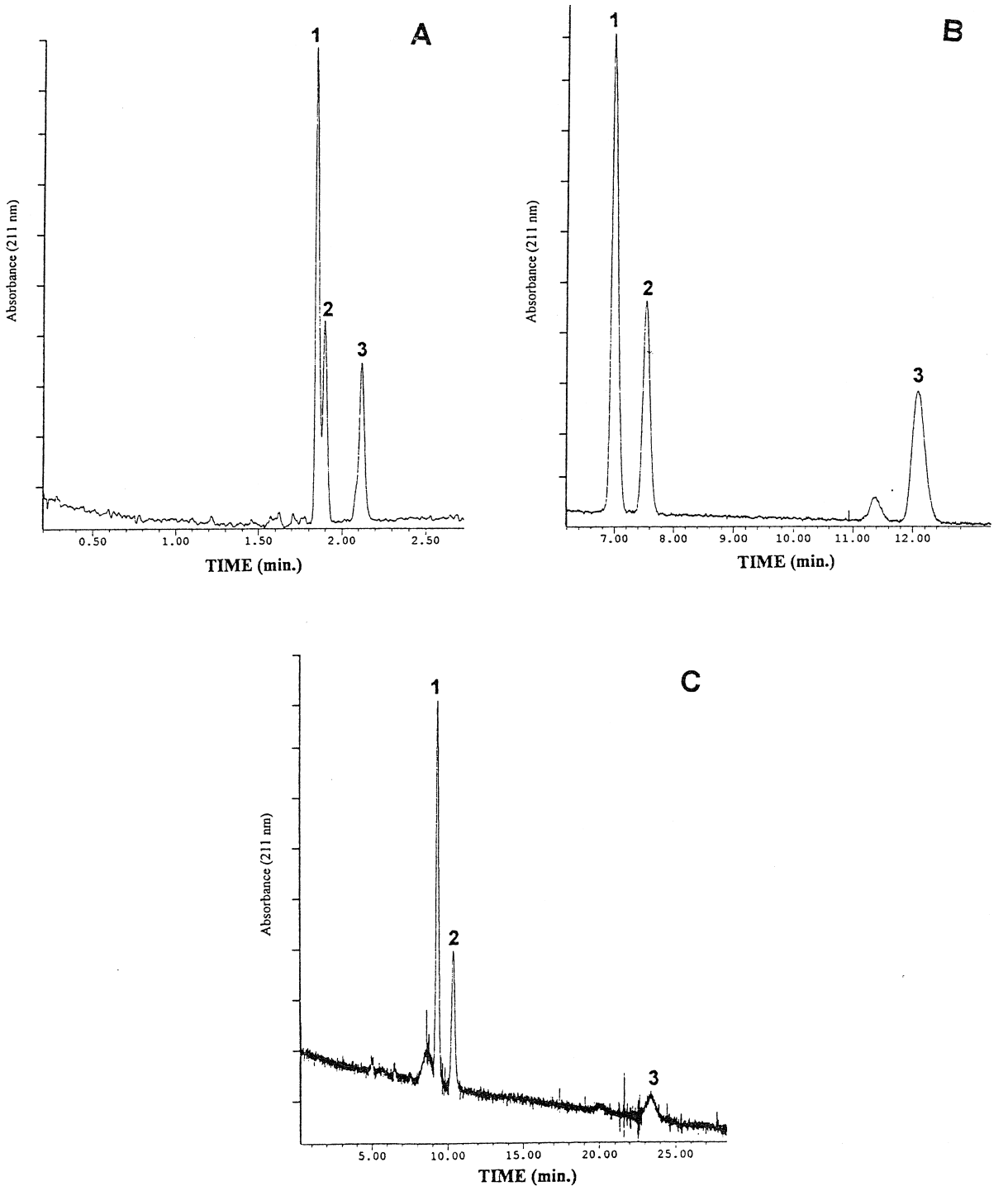

Fig. 12. Separation of angiotensins on (A) a bare capillary; (B) a diol capillary; and (C) a C_{18} capillary, $L=45$ cm, $l=25$ cm, $V=30$ kV, $I=29$ μA, pH = 2.14, solutes: 1 = angiotensin III; 2 = angiotensin I, and 3 = angiotensin II [67]. (Reproduced with permission of the authors and copyright holder, Elsevier Science Publishers, Ltd., Amsterdam, Holland).

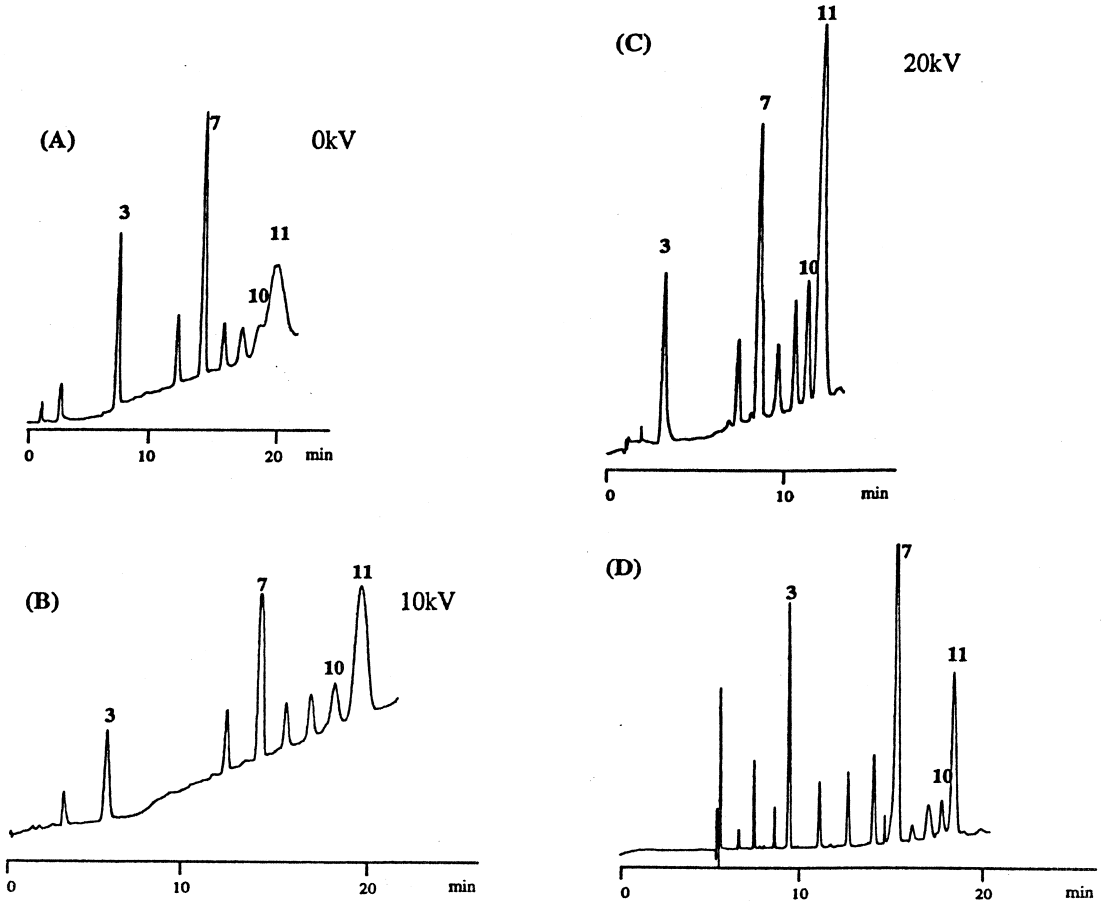

Fig. 13. Influence of applied voltage on the chromatographic separation of oligonucleotides (dC3–dC11) and a comparison with MECC. (A) gradient micro-HPLC; (B) electro-HPLC, applied voltage 10 kV; (C) electro-HPLC, applied voltage 20 kV; (D) MECC. Peaks 3, 7, 10 and 11 correspond to dC3, dC7, dC10, and dC11 oligonucleotides. Additional conditions are given in the original publication [74]. (Reproduced with permission of the authors and copyright holder, Elsevier Science Publishers, Ltd., Amsterdam, Holland).

mize the selectivity of the separation. Thus, in PEC or CEC, one can vary in a true gradient or step-gradient isocratic mode, the effect of the stationary phase (C_1 to C_{18}), as well as the effect of the direction and total applied voltage on the overall separations of anionic oligos, as here. Fig. 13 then compares the three main capillary separation modes popular today for oligonucleotides. Fig. 13A is the chromatogram under gradient micro-HPLC conditions, with no applied voltage, while Fig. 13B is PEC, with an applied voltage of 10 kV. Obviously, it is possible to vary the applied voltage or pressure driven flow to any mixture of operational parameters and thus vary pressurized (parabolic) flow vs. EOF. The gradient PEC separations (Fig. 13B and C) for this oligonucleotide mixture of dC3–dC11 demonstrated the optimization of efficiency and speed with increasing participation of EOF. That is, Fig. 13B used an applied voltage of 10 kV, while Fig. 13C used a voltage of 20 kV, and Fig. 13A had no applied voltage. Applying a voltage gradient drastically improved the efficiency of the gradient microbore separation of the oligos, as well as peak shapes, efficiency, and overall resolution.

These separations were then compared with MECC, without any packing material or external, pressurized flow, under isocratic conditions, as is usual in HPCE. MECC discriminates between ana-

lytes by a combination of both chromatographic and electrophoretic mechanisms [74]. A comparison of Fig. 13C (PEC) and D (MECC) shows that PEC offers everything that MECC can provide in terms of analyte selectivity and resolution, as well as excellent peak shape and efficiency, but in much less time. However, the run time in Fig. 13D could easily be reduced by shortening the capillary, but with some loss of resolution.

The only text that has yet appeared devoted to CEC appears to have been that edited by Tsuda [30]. There are relatively few references in this text to true CEC, in any form, or PEC for that matter, devoted to biopolymer separations. Those examples of biopolymer separations using some form of EOF driven flow in capillaries, usually used OTC or gel-like media. There is also some confusion in the terms applied in this text, since the editor uses CEC to describe CGE and other gel-like media in CZE. We do not believe that CGE is really a form of CEC, but rather it belongs to CE. That is, wherein the solid support in the capillary (or coated on the capillary surface) does not adsorb or partition the analytes, but rather just acts as a sieving medium, that is not a true form of chromatography. It is rather like SEC, a form of HPLC. Thus, we believe that CGE belongs to CE, rather than to HPLC or CEC. In Tsuda's book, which is an excellent introduction and overview of electric field applications in chromatography, we believe that there is some confusion regarding what is CEC and what is CGE/CE. There do not appear to be any illustrations in this text of modern-day CEC, in any format, being applied to biopolymer separations. Why was that true as recently as 1994 and 1995? In Tsuda's own words: ''Despite its great potential, electroosmotically driven EC has undergone limited experimentation in the biosciences. This is due in part to the rapid developed and wide success of CZE and its related techniques. Since EC introduces a chromatographic component into the system, however, it is quite possible that electroosmotically driven EC techniques will some day outperform strictly electrophoretic procedures in a variety of biochemical applications.'' It would seem that within just the past 5 to 6 years, since the publication of Tsuda's text, most (if not all) descriptions of the application of CEC or PEC to biopolymer separations have appeared. We have not been able to find any such applications prior to 1994, such as Bayer's work, devoted to biopolymer separations using some variation of CEC/PEC [74]. Though there are examples of earlier CGE reports, these are not truly CEC applications. The reader is, nevertheless, strongly encouraged to review Tsuda's text for complete descriptions of the background, theory, principles, and applications of electric field applications in chromatography. These may not all be true examples of CEC or PEC.

1.7. General biopolymer separation comments

A lingering problem in all CEC of biopolymers, especially for charged analytes, is the unwanted (at times) irreversible adsorption or interactions with charged surface functionalities. In using conventional HPLC packings, such as Spherisorb and others, there is a certain degree of untagged (non-endcapped) silanol groups remaining. Other HPLC packings, such as Vydac, have very low residual silanol groups for peptide mapping in HPLC, leaving the preferred, pure reversed-phase interactions. However, if one (e.g. Lubman et al.) utilizes the Vydac packings in CEC, because there are so few free silanol groups present, residual EOF is very low [62–65]. Workers using packings such as Vydac must then apply external pressure (HPLC pump driven) to elute the proteins/biopolymers from such packings, and within a reasonable period of time. These types of packings do prevent unwanted biopolymer-packing interactions, namely ionic, but at the expense of greatly reduced EOF. In this sense, much of Lubmans work was really PEC, with questionable levels of EOF actually present and actually approaching cLC in scope and pressure driven flow. However, if one utilizes other packings with residual silanol groups to generate reasonable EOF, then one is faced with the (at times) unwanted, reversible protein adsorption (interaction) with the packings, especially if RP is the desired mode of separation in CEC. How can this seeming contradiction and anomaly be overcome?

In the past, in order to prevent ionic adsorption or interaction in IEC modes (HPLC), one commonly accepted approach is to introduce a salt (organic or inorganic) to compete with the analyte. However, the use of ionic salts in CE or CEC leads to current

buildup, heat generation, and eventually, system shutdown at high voltages (e.g. >15 kV). Therefore, one must use low concentrations of ionic species, just to enable reasonable voltages and the derived EOF, but low concentrations are usually insufficient to prevent unwanted protein–silanol surface interactions. If one really wishes to perform true CEC, with no or very little pressurized flow, then one simply must have a reasonable EOF generation. That means having residual, free silanols or ion-exchange sites (e.g. sulfate or sulfonate groups), which cause their own problems in irreversible interactions with the proteins or other biopolymers.

A possible solution to the above method requirements might reside in the use of zwitterionic buffers, species such as Z-1 Methyl reagent (Waters Corporation) or others. In the early days of CZE of proteins, before permanently coated capillaries were available from commercial vendors, analysts would add zwitterionic additives/reagents to the capillary buffer. These were really used to provide ionic conducting solutions (ionic strength), but also to prevent unwanted capillary wall (silanol) interactions with the free proteins (irreversible interactions with charged surface functionalities). The zwitterions would interact with the capillary walls and the proteins, keep one from the other by ionic repulsion, and peak shapes and efficiencies were more than adequate for simple, CZE separations. And, the use of zwitterionic additives did not lead to heat buildup, current generation, or system shutdown in CZE. It also allowed for application of high voltages and concurrent EOF, useful for protein analyses in CE. Thus, such approaches may well suffice in isocratic, step gradient, or true gradient CEC in the future, without the need for added, pressurized flow or PEC variants.

2. Conclusions

It is clear that CEC and PEC are both directly applicable to a wide variety of biopolymers, and that (at times) significant improvements in peak shape, plate counts, resolutions, efficiency, and time of analysis can be realized. However, what is also clear is that final optimization of these separations, in general, with a variety of packing materials, has not been always possible or realized, thus far. At times, workers have been forced to utilize pressurized flow together with EOF in PEC, just to realize reasonable analysis times, at the expense of additional band broadening and loss of plate counts and efficiency. It does not appear generally true that fully optimized CEC conditions for these different biopolymer classes have yet been demonstrated on a wide variety of packing materials. The often contradicting requirements of reasonable EOF but no silanol–analyte interactions (band broadening causes) do not appear to have been resolved or met. The solution to this general problem in biopolymer separations in CEC has been to introduce pressurized flow, which is not really solving the problem, but just forcing the analytes to elute and approaches electro-HPLC, rather than true CEC. That is, if there is no residual EOF then there is no CEC component, and one simply has forced the system to perform cLC with an applied voltage, so as to improve resolutions by the EPF factor, now added. That is not CEC, it is electro-HPLC, for PEC must have some EOF component remaining. That is perhaps a point to again emphasize, there are real differences between electro-HPLC, PEC, and pure CEC, and these should be recognized and respected, as well as admitted. Electro-HPLC has no EOF, PEC has some residual EOF combined with pressurized driven flows and EPF, and CEC has no pressurized component to the flow, but just EOF flow with EPF superimposed. This is perhaps the best way to summarize these three different variations on a theme of Haydn. Since biopolymer classes are usually charged, application of electro-HPLC, PEC, and/or CEC should find more widespread applications and positive results in the immediate future.

At the present time, though there are several applications of CEC/PEC to biopolymer classes, these are to be considered somewhat preliminary and not necessarily fully optimized in all possible parameters. There does not, in general, seem to have been any serious attempt, as yet, to utilize any software chemometric approaches in CEC/PEC for biopolymer separation optimizations or rationale for so doing. At times, perhaps too often, packings are simply used because they were on the shelf in a laboratory or commercially available, not necessarily

because, in theory, they were really the best for protein/peptide separations in CEC/PEC. Thus, there remains a need for research oriented column choices from commercial vendors or we will always remain packing our own capillaries with commercial HPLC supports. Or, we will finally start to prepare special packings just for CEC, pack our own capillaries, and then demonstrate that yes, these were really the right designs of packings for this newer type of separation. Perhaps that is what the future holds, as it did thirty or more years ago in HPLC and GC before that.

3. Notes added in proofs

One of the reviewers of this manuscript, Professor Hjerten, has quite correctly indicated that perhaps monolithic columns should more properly be termed continuous beds. These are the same packings just different descriptors. He has also pointed out that such capillaries provide, as above, virtually no limitations of back pressure in CEC or HPLC, since they are not pressure packed, as with small particle packings (packed beds). Several very recent papers have described true gradient separations of proteins in CEC and (at times) microchip electrochromatography [75–81]. The reader should see p. 1205 of one particular review paper [75]. As Professor Hjerten correctly indicated, In practice, it is difficult to use long CEC columns packed with small beads, since the back pressure is so high that one cannot wash out a bubble, once formed, and so the expensive column must be discarded [82]. There is little question but that continuous beds in CEC offer serious advantages over packed capillaries, but for the fact that very few of these are now commercially available. They may become more routinely available in the near future, but their overall market success is unclear at the moment [83]. Other workers have also reported, as above, on the use of continuous beds in CEC for biopolymers, such as Novotny's work [42]. Additional results have been recently presented elsewhere by the groups of Horvath, Novotny, and Hjerten, mainly using variously designed continuous beds in CEC, isocratic or gradient [84].

4. Nomenclature

ACN	acetonitrile
CGE	capillary gel electrophoresis
cLC	capillary liquid chromatography
CEC	capillary electrochromatography
IEC	ion-exchange chromatography
EOF	electroosmotic flow
EPF	electrophoretic flow
ESI	electrospray
HP	Hewlett-Packard Corporation
HPCE	high-performance capillary electrophoresis
HPLC	high-performance liquid chromatography
ITS	ion trap storage
LIF	laser-induced fluorescence
LOD	limits of detection
MEKC	micellar electrokinetic chromatography
MALDI–TOF	matrix assisted laser desorption–time of flight
M_r	molecular mass
OT	open tubular
ODS	octadecyl-silica
PEC	pressure driven (in part)
ppm	parts-per-million (w/w)
R	resolution
%RSD	percent relative standard deviation
RPLC	reversed-phase liquid chromatography
RPC	reversed-phase chromatography
TIC	total ion chromatogram
TES	triethoxysilane
v/v	volume to volume

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