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Review

Capillary electrochromatography of peptides and proteins

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Abstract

This review surveys the accomplishments in the separation of peptides and proteins by capillary electrochromatography (CEC) over the last decade. A significant number of research articles have been published on this topic since the last review. Peptide and proteins separations have been carried out in all three formats of CEC, i.e., packed bed, continuous bed and open-tubular (OT) format. In addition to electrophoresis, different chromatographic modes have been successfully exploited with the most prevalent being reversed-phase mode followed by ion-exchange. Although many researchers continue to use model proteins and peptides primarily to evaluate the performance of novel stationary phases some researchers have also applied CEC to the analysis of real-life samples. The potential of CEC to yield complementary information and sometimes a superior separation with respect to established techniques, i.e., microbore HPLC and capillary electrophoresis has been demonstrated. Instrumental modifications in order to facilitate coupling of CEC to mass spectrometry have further upgraded the value of CEC for proteomic analysis. Capillaries are still the separation vehicle of choice for most researchers yet the microfluidic platform is gaining momentum, propelled particularly by its potential for multitasking, e.g., performing different chromatographic modes in series.
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Contents

1. Introduction

Four years have passed since the last survey of peptide and protein separations by capillary electrochromatography (CEC) in this journal. Back then, the number of publications was so small (about a dozen, covering a time scale of approximately four years) that they were actually only a subchapter of a larger review dealing with CEC separations of biopolymers in general [1]. The fact that the number of publications has now increased to approximately a dozen per year permits the cautious assertion that the field of CEC of peptides and proteins has started to leave its infancy yet is still young enough to attract plenty of exciting research opportunities. This review article aims to provide a survey on all research papers and related reviews in the English language treating the separation of peptides and proteins by CEC both in the capillary and the microfluidic format. The authors utilized the SciFinder Scholar Chemical Abstracts Service Database using the key words "capillary electrochromatography proteins" and "capillary electrochromatography

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peptides". We would like to point out at this point that a complete coverage was attempted and apologize for any publication that did not find entry in this review. Additional to the actual separations of peptides or proteins by CEC, the search also yielded several hits regarding the use of immobilized proteins as stationary phases for the separation of drug enantiomers. Since this class of analytes does not fall into the category of either peptides or proteins they are not included in this paper (instead a recent review can be found in [2]).

After mapping the genomes of several species, including the human genome, the mapping of the proteome is most likely to be one of the most ambitious research projects mankind has ever undertaken. In this context, the growing importance of micro separation techniques, probably hyphenated with mass spectrometry (MS), is expected to provide a valuable alternative to conventional slab-gel electrophoresis or HPLC in terms of speed, reagent consumption and efficiency (for general reviews the reader might refer to [3,4]. Proteomics typically involves a digestion of the protein of interest into its constituent peptides followed by separating these peptides through one or more micro separation techniques and structural identification by MS. Additionally, the mapping of the complete set of peptides of an organism, i.e., peptidomics, will deliver further indispensable information on its functioning [5]). Yet apart from proteomics and peptidomics a large number of related disciplines in all areas of life sciences heavily rely on separations of peptides and proteins, for example, the profound importance of both protein and peptide separations in clinical chemistry. Moreover, since a growing number of synthetic peptides find applications as drugs, their separations also play an important role in quality and clinical assessment.

Proteins and peptides carry net charges at any pH different from their inherent p*I*-value, i.e., they are rarely found in their neutral state. Many peptides and most certainly all proteins, also contain hydrophobic residues. The combination of charged and hydrophobic moieties makes them an ideal class of analytes to fully exploit the intrinsic potential of CEC. Differences in their charge to volume ratios allow them to be separated electrokinetically and, if desired, to interact with ionic residues on the surface of the stationary phase for ion-exchange separations. On the other hand, differences in hydrophobicity permit separation based on a partitioning process with hydrophobic residues of the stationary phase. With this multitude of possible interactions, it comes as no surprise that many researchers are currently using model peptides, or model proteins, as probes to characterize novel stationary phases for the analysis of these biopolymers. All formats of CEC, i.e., packed beds, open-tubular (OT) and monolithic stationary phases (continuous beds) have found attention in this respect. The successful incorporation into the microfluidic platform with the intention to perform rapid and multidimensional separations has been demonstrated as well. Besides, a substantial amount of work has been carried out to interface CEC with MS (for a recent review on this particular aspect, the reader might refer to [6]). Despite the predominance of model systems, some groups have already proceeded a step further and started to utilize CEC methodology in the analysis of real-life samples.

Apart from the multitude of interactions with the stationary phase a large number of experimental variables enables the user to fine tune the separation of peptides or proteins, such as pH, temperature, the addition of ion-pairing reagents, assisting pressure, amount and type of organic modifier, salt content and gradient elution. Several articles have shown that through careful manipulation of these factors separations can be attained that cannot be achieved with either capillary zone electrophoresis (CZE) or with micro-HPLC thus making CEC a truly orthogonal technique to established micro separation techniques.

A variety of differences exists between peptides and proteins, for instance, proteins are synthesized in vivo exclusively via the transcription/translation pathway whereas peptides can also be synthesized via other pathways, or synthetically in vitro. Also, very small peptides typically possess flexible random coil structures in solution whereas larger peptides and proteins adopt secondary structures (α-helix, β-sheet, β-turn). These structures depend on the amino acid sequence but vary with the degree of stabilization that the surrounding solvent provides with regards to temperature, pH, ionic strength or percentage organic modifier. Naturally, the secondary structure also influences the degree of chromatographic interaction with the stationary phase [7]. Yet from a pure structural point of view, the major difference between proteins and peptides lies only in the underlying number of amino acid building blocks, with the cutoff often arbitrarily set at 10,000 g/mol, and in essence both belong to the same class of biopolymers. The authors of this review therefore do not differentiate between publications on peptide- or protein separations in the following. We rather grouped the publications into four categories with respect to the way the stationary phase is incorporated into the respective CEC format, i.e., packed beds using micro particles, open-tubular CEC and continuous beds (monoliths). An additional category describes work carried out in the microfluidic platform. Essentially all four categories are characterized by the simultaneous occurrence of three physicochemical parameters enabling the separation. In most cases, the stationary phase contains both hydrophobic moieties to permit partitioning (i.e., reversed-phase CEC), and acidic or basic moieties, e.g., silanols, sulfonic acids or quaternary amines. In their charged states, these latter moieties are essential for the generation of a zeta potential suitable to maintain a sufficiently stable and high electroosmotic flow (EOF). However, they also interact electrostatically with oppositely charged analytes and in some cases this is actually the desired type of interaction, i.e., ion-exchange CEC. In the case of reversed-phase CEC, though, this type of interaction is often found to lead to peak tailing and an overall

decrease in separation efficiency, convincing many researchers to select pH ranges where analytes carry charges to induce electrostatic repulsion. Both hydrophobic and electrostatic interactions are superimposed on the third physicochemical process, i.e., electrophoretic migration. Since a large number of operating parameters influence the significance of each of these three processes, the overall separation process is a rather complex interplay. The sheer abundance of operating parameters gives the user a wide range of parameters in order to optimize the separation of peptides and proteins in terms of speed, resolution and selectivity. To complicate things further, many operating parameters vary more than one of the three physicochemical processes at a time, and in the case of biopolymers might additionally induce changes in the secondary structure or the charged state of an analyte. For the case of charged peptides, Fig. 1 shows the dependence of the three physicochemical separation processes on two commonly adjustable experimental parameters, the content of organic modifier and the ionic strength in the mobile phase [8].

In terms of nomenclature we would like to point out at this point that we use the expressions capillary and column interchangeably, the same applies for the terms monoliths and continuous beds. Furthermore, to minimize potential confusion, if comparisons to liquid chromatography in the capillary format are made we always refer to them as μ-HPLC (instead of nano-HPLC, capillary chromatography or microbore-HPLC). Also, we utilize the expression pressurized CEC although some authors prefer to differentiate between pressurized CEC and electrically-assisted micro-HPLC depending on whether the dominating force for the flow of an eluent is the pressure induced by a pump or electroosmosis by an electric field [9]. The Appendix A will provide an overview of all separations in the capillary

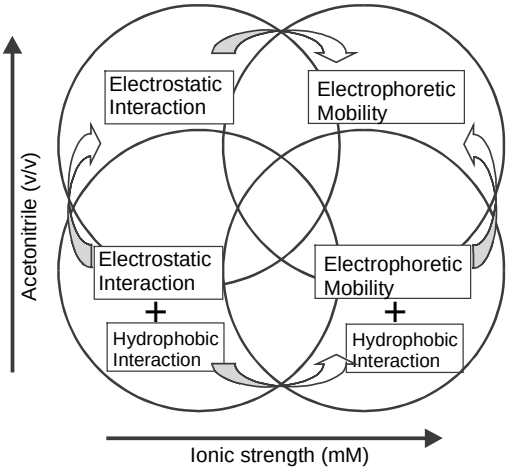

Fig. 1. Schematic illustration of the retention behaviour of a charged peptide in a CEC system related to the effect of variations in the content of the organic solvent, acetonitrile, or the ionic strength of the buffer, such as ammonium acetate. Other conditions are described in the original publication. From Ref. [8] with permission 2003 Blackwell Munksgaard).

platform in terms of format, type of packing, monolith or wall functionalization, respectively, capillary format, mode of detection and analytes separated.

2. Capillary electrochromatography of peptides and proteins using packed beds

Packed beds using microporous particles, frequently made of silica, are still the packing material of choice for many researchers in CEC. Their use is spurred on by the large variety of commercially available packing materials for HPLC and μ-HPLC. Due to electroosmotic flow, they allow for higher velocities in CEC than in HPLC since backpressure is no limitation in CEC. One potential disadvantage associated with packed beds is the need for retaining frits. Apart from the degree of complexity added to the manufacturing process these frits often become a source for bubble formation which, at best, lead to noisy baselines but can also result in current breakdown. Many researchers therefore apply additional pressure to the capillary inlet to suppress bubble formation, especially for instruments interfaced to mass spectrometers. Unfortunately, a parabolic flow profile is superimposed on top of the flat electroosmotic one which can slightly compromise the efficiency of the system, and also adds complexity to the instrumentation. However, by applying pressure with a pump, gradient elution becomes practical. The capacity factor for charged analytes depends on both the applied field strength and on the assisting pressure, the latter parameter has been varied successfully to improve resolution of peptides as will be seen in the following.

To the best of our knowledge, the very first peptide separation by CEC was reported in 1995 by Schmeer et al. [10] using 1.5 μm octadecyl silica (ODS) particles for CEC in the reversed phase (RP) mode. Here, the authors' focus was less on the separation of the two enkephalin derivatives but rather on interfacing their CEC system to electrospray (ESI) MS. Although assisting pressure was required to stabilize the flow at high electric field strengths, their paper presented the first CEC–MS coupling that relied mainly on electroosmotically generated flow without sheath flow or transfer capillaries. The next example of a peptide separation using packed beds appeared two years later by Choudhary and Horváth [11]. Again, the focus of the paper did not lie in the separation of peptides but in evaluating the differences in electroosmotic flow and conductance between open and packed capillaries. However, it represented the first peptide separation in ion-exchange mode where three small peptides were separated within 15 min on a column packed with 8 μm strong cation exchanger. An early example for powerful resolution and rapid analysis by CEC in the RP mode was demonstrated with the separation of an *N*-methylated C- and N-protected tetrapeptide from its non-methylated analogue by Euerby et al. [12]. The separation could be accelerated significantly from 22 to 3.5 min by applying assisting pressure whereas

the fastest HPLC method would take 30 min. In the same year, Lubman and co-workers [13] showed baseline separation of a tryptic digest of bovine cytochrome *c* within 14 min on a 6 cm long capillary using pressurized gradient elution CEC (3 μm ODS particles) coupled via ESI to an ion trap storage/reflectron time-of-flight (TOF) MS. The tuning of pressure and applied field strength yielded peak resolutions not achievable by pure μ-HPLC. The authors furthermore presented the separation of a tryptic digest of chicken ovalbumin resulting in 20 peaks within 17 min. These first examples show how CEC might evolve as a valuable alternative to μ-HPLC in the separation of complex samples and establish itself as a complementary technique in proteomics and peptidomics. An optimization study for a tryptic digest of bovine β-lactoglobulin was presented by the same group a year later using the same instrumental setup [14]. Higher selectivity and speed was accomplished by tuning the separation voltage, minimizing diffusion by increasing the pH to obtain a higher EOF, and reducing the inner diameter (i.d.) of the capillary to minimize Joule heating. In another comparison to μ-HPLC, CEC using gradient elution achieved higher resolution for the separation of a cytochrome *c* tryptic digest, as demonstrated by Behnke and Metzger [15]. Using UV detection, the outlet end of the capillary was connected to a resistor column creating sufficient backpressure to suppress bubble formation and allowing currents of up to 100 μA. The importance of the applied electrical field as a variable to tune the separations of peptides was also emphasized by Apffel et al. [16] who obtained better resolution when comparing pressure-assisted CEC to μ-HPLC for the separation of a tryptic digest of recombinant human growth hormone and recombinant tissue plasminogen activator. In another publication from the Lubman and co-workers [17], mixed-mode stationary phase particles were investigated. In addition to the RP moiety (ODS), their stationary phase also contained dialkylamine residues for EOF generation. Since the dialkylamine groups are positively charged at low pH, this approach prevented electrostatic interactions between these functions and positively charged peptides. Another advantage of the dialkylamine is they generate a sufficiently high EOF at low pH unlike the silanol groups of conventional packing materials. A tryptic digest of horse heart myoglobin showed differences in the elution pattern between conventional ODS particles and mixed-mode particles at similar efficiencies. Another paper on the use of mixed-mode stationary phases, consisting of C_{18} residues and sulfonic acid residues appeared by Adam and Unger [18]. The authors measured higher EOF than with conventional ODS particles. Four peptides were baseline resolved within 6 min using isocratic conditions, by applying a gradient using pressure-assisted CEC a higher resolution was achieved for a tryptic digest of cytochrome *c* compared to μ-HPLC. Through dynamically coating bare silica particles with cetyltrimethylammonium bromide added to the mobile phase, Zou and co-workers [19] improved peak symmetry of three peptides at neutral pH. Adsorption of these basic analytes onto the silanol moieties was diminished since the positively charged quaternary ammonium chains adsorbed to the negatively charged silanol moieties. At the same time, the ammonium groups themselves can generate EOF and function as a moiety for RP interactions. In another publication a year later Zou and co-workers [20] investigated isocratic cation-exchange CEC for the separation of 10 small peptides. For 10 consecutive runs, they obtained remarkably high number of theoretical plates ranging up to 460 000 plates per meter and migration time reproducibilities of 0.3% R.S.D. Since the elution order differed from CZE it could be concluded that indeed an ion-exchange mechanism took place. Isocratic anion-exchange CEC of four proteins at physiological pH was also described by Horváth and co-workers [21] who derivatized the surface of silica spheres with a vinyl monomer containing quartenary ammonium groups thus obtaining “tentacular” strong anion-exchange functions. These separations could be significantly accelerated by increasing the salt content in the mobile phase due to attenuation of the electrostatic interactions between negatively charged proteins and positively charged ammonium groups. An additional advantage of this particular stationary phase lies in the fact that the same ammonium groups generate a sufficiently high zeta potential, and thus EOF, even at high salt concentrations (NaCl in this case). The methodology provided an example for the generation of a stationary phase specifically tailored for the needs of CEC. The authors furthermore calculated that higher peak capacities were achievable with isocratic CEC than with gradient HPLC provided the eluent strength in CEC was at a magnitude where chromatographic retention factors were fairly low. The same group applied a similar mechanism to the separation of peptides but this time exploiting the weak cation-exchanger 2-acrylamidoglycolic acid, again bound to silica beads [22]. This time, it was found that the EOF did decrease with increasing salt concentrations. Phase ratio was also shown to play an important role by comparing the selectivity to open-tubular CEC that employed the same anion-exchange function (but only attached to the capillary walls) and to CZE. At a given salt concentration, the elution orders in the open-tubular format were identical to CZE although the migration times were longer, thus the separation was dominated by electrophoretic migration. In the packed format, though, the elution order differed which emphasized the prevailing chromatographic mechanism. Fig. 2 shows the separation of four peptides at different salt concentrations. An experimental setup for detection of peptides by coupling CEC (containing 3 μm silica particles that had either a C_8 or C_{18} phase bonded to them) to an ion trap MS via nanoelectrospray interface at ambient pressure was presented by Guček et al. [23]. Since the nanoelectrospray matched the flow rates in CEC no sheath flow was required. Sensitivity in the attomole range was obtained as long as the applied electric field strengths for the CEC separation were low (presumably due to bubble formation at higher field strengths). The excellent sensitivity furthermore allowed for

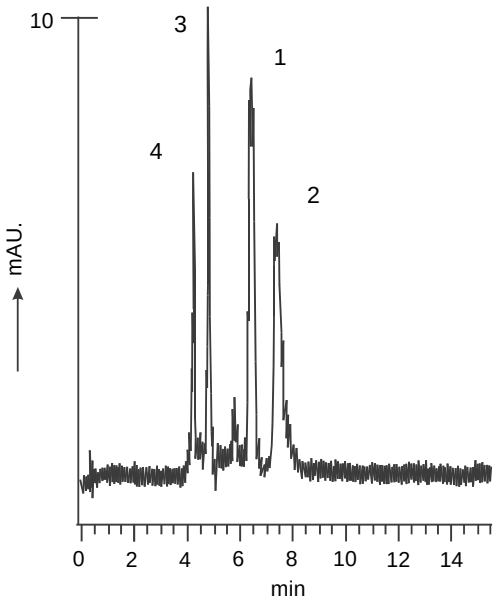

Fig. 2. Electrochromatogram of the four activin-related peptides separated with Hypersil *n*-octadecyl silica material: the background electrolyte was 60% (v/v) acetonitrile with 15 mmol/l NH_4OAc/AcOH pH 5.0. Other conditions are described in the original publication. From Ref. [8] with permission.

very small sample volumes to be injected which diminished electrostatic interactions between silanol groups and positively charged peptides. The same group also investigated an experimental setup that did require sheath flow for peptide separations and conceded an average 30-fold loss in sensitivity. However, higher field strengths could be applied yielding shorter analysis times as compared to the sheathless interface [24]. An injection valve allowing pressurization of the inlet end was described by Walhagen et al. [25] with the intention to enhance sample throughput. Their device also permitted the use of short columns coupled to MS via electrospray but required a sheath liquid. Three peptides were employed as model analytes to test the performance of their setup and it was found that pressurization of the inlet end at 7 bar yielded more stable currents since bubble formation was suppressed. Hearn and co-workers [8,26–29] systematically investigated the retention behaviour of peptides in terms of contributions of the three underlying physicochemical separation mechanisms. The authors started with model peptides and applied the insights gained with the model analytes to "real-life samples", in this case the separation of structurally related synthetic peptides. Using 3 μm ODS particles they first inspected the retention behaviour of two small linear peptides as a function of the capillary temperature [26]. Their study suggested that a phase transition of the octadecyl chains occurred at elevated temperatures leading to a decrease in chromatographic retention factors. The influence that the temperature exerted on the zeta potential resulted in higher column efficiencies and shorter analysis times. The authors repeatedly verified the performance of the octadecyl stationary phase by separating a mixture of neutral test analytes after each set of temperature studies. In another study, the same group evaluated four different stationary phases for the retention behaviour of several linear and cyclic hormonal peptides [27]. The first two packings probed were 3 μm silica particles containing either octyl- or octadecyl groups, at low organic modifier content chromatographic retention governed the separation process whereas at a higher content, electrophoresis prevailed. This behaviour manifested itself in the chromatographic retention factors that were initially positive and became negative with higher organic content. By using low pH values and high buffer molarities electrostatic (silanophilic) interactions between charged peptides and silanol groups could be diminished thereby increasing the electrophoretic contributions even at low organic modifier content where chromatographic behaviour dominates. The other two packings also consisted of 3 μm silica particles but had mixed mode stationary phases, i.e., octadecyl or hexyl functionalities plus sulfonic acid groups as strong cation exchangers. Since the mixed mode packings were employed in order to provide a stable EOF rather than with the intention to deliver ion-exchange functions the undesired ion-exchange interactions were avoided by using high ionic strengths in the eluent. In their first analysis, 10 structurally related synthetic peptides ranging from 8 to 20 amino acid residues and covering isoelectric points (p*I*-values) from 3.7 to 10.1 were probed [28]. The longest peptide mimicked a human immunodeficiency virus epitope, while the other nine peptides were truncated analogues of the parent peptide. The study confirmed their previous findings with respect to the roles of temperature and organic modifier content. It also established structure–retention relationships by observing a linear relation between the increments in retention coefficient between two peptides at low organic modifier content to a product of both the sum of their intrinsic hydrophobicity and the ratio of their molecular masses. In a similar study the retention behaviour of another set of four synthetic peptides, this time derived from the loop 3 region of activin β_A–β_D, was examined [8]. An example electrochromatogram is given in Fig. 3. Due to the relatively broad range of p*I*-values, i.e., from 4.0 to 8.8, the set of synthetic peptides contained both positively and negatively charged peptides at pH 5.0. Interestingly, only the positively charged peptides showed a decrease in retention coefficient with increasing temperature whereas the opposite was observed for the negatively charged peptides, i.e., they experienced a slight increase in retention coefficient with increasing temperature.

Another common type of chromatography that has not been exploited very frequently in CEC is size-exclusion chromatography.

As part of a larger study with crude extracts of ergot fungus, Stahl et al. [30] compared pressurized CEC to μ-HPLC, both coupled to MS via electrospray interface. The authors obtained slightly higher resolutions and peak capacities in

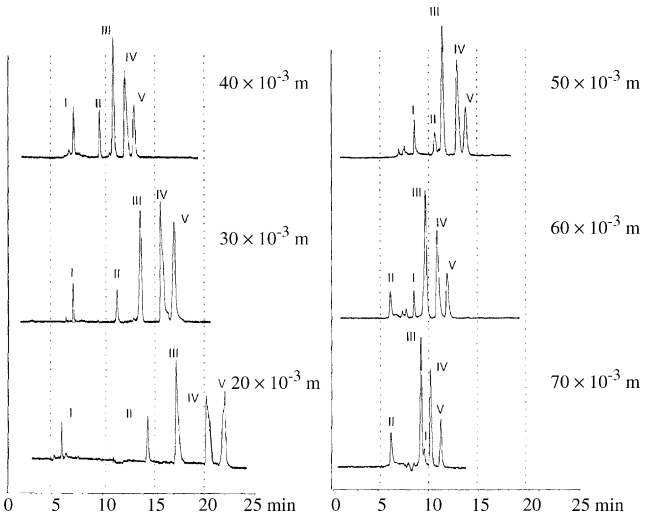

Fig. 3. Electrochromatograms of the peptides obtained at different salt concentrations. Column: 50 μm × 326/241 mm, packed with 5 μm WCX beads; mobile phase: 20 mM phosphate solution, pH 4.5 containing 50% (v/v) ACN and 20, 30, 40, 50, 60, 70 mM NaCl; applied voltage 20 kV; UV detection at 214 nm; Sample components: I = EOF marker, II = angiotensin II, III = [Phe^7]bradykinin, IV = [Sar^1,Ala^8]angiotensin II, V = angiotensin I. Other conditions are described in the original publication. From Ref. [22] with permission.

CEC with respect to the separation of three model proteins and two model peptides. Fig. 4 illustrates an example of an electrochromatogram and the resulting MS spectra.

Hydrophilic interaction CEC, which in essence is a type of normal-phase chromatography, was exploited for the separation of six dipeptides by Ye et al. [31]. The authors used 5 μm polymeric particles that provided a neutral and polar stationary phase while still being capable of generating sufficient EOF. Tuning of the selectivity became possible by varying the applied field strength or the applied pressure that was in excellent accordance with the theoretical model the authors developed to explain the influence of both applied field strength and applied pressure on the chromatographic retention coefficient. A separation of five different di- and tripeptides by mixed mode hydrophilic interaction and cation exchange CEC was described by Gao and co-workers [32]. A significant improvement in the separation in terms of decreasing analysis times became feasible since their pressurized CEC system also permitted gradient elution. In a related study, the same group described pressurized CEC and compared gradient elution to isocratic elution for the separation of peptides [33]. Using 3 μm ODS particles, only gradient elution had the capability of baseline-separating six structurally related small peptides within 20 min. By monitoring resolution as a function of mixer volume and pump flow rate, optimum operating conditions for the gradients were derived. Moreover, the authors evaluated the role of trifluoroacetic acid, a commonly used ion-pairing reagent, and observed enhanced retention of the peptides with the stationary phase due to a suspected increase in hydrophobicity of the peptides when forming ion-pairs with the trifluoroacetic acid.

3. Capillary electrochromatography of peptides and proteins employing monolithic columns (continuous beds)

Soon after the emergence of monolithic columns in HPLC, also referred to as continuous beds, they found entry as stationary phases for the separation of peptides and proteins in CEC. Several aspects fostered their development for applications in CEC. For one, they abolished the need for retaining frits since monoliths could easily be anchored to the capillary walls through functionalization of the walls. Consequently, many researchers claimed fewer problems with bubble formation using monoliths. Also, monoliths could be created in situ from a wide variety of monomers, crosslinkers and porogenic solvents. Apart from greatly facilitating the manufacturing process it gave researchers the opportunity to more easily control properties such as column length, porosity, chromatographic functionality and surface area. An additional advantage is in their high permeabilities, leading to greatly enhanced convective mass transfer rates. Therefore, unlike micro-particulate packing materials, monoliths allowed high electric field strengths to be applied without any significant increase in plate height. Such behaviour truly combines high efficiencies with rapid analyses (examples of experimentally recorded van Deemter plots can be found in [34–36]. Monoliths in CEC can essentially be divided into two categories: silica-based monoliths and organic polymer-based monoliths. The latter category includes acrylate, methacrylate, acrylamide and styrene-based monoliths. For more detailed reviews on monoliths in CEC the reader is directed to refs. [37,38]. As with any type of stationary phase employed in CEC, monoliths also have to fulfill the dual roles of generating EOF and providing chromatographic functionality. A wide variety of monomers makes monoliths a popular choice for the analysis of such complex analytes like peptides and proteins.

The first example of a peptide separation by monolithic CEC appeared in 1997 by Palm and Novotny [39]. Their monolith was based on polyacrylamide/poly(ethylene glycol) with a C_{12} functionality. Five small peptides could be baseline separated within 5 min using isocratic elution. Gusev et al. [34] described a monolithic column based on a styrenic monolith containing octyl groups to provide RP functionality plus quaternary ammonium groups to generate EOF. Insulin and three angiotensin-related polypeptides were baseline separated within 10 min. The complex interplay of electrophoresis and chromatography was illustrated by a plot of retention times versus content of organic modifier (in this case acetonitrile) in the mobile phase. With increasing acetonitrile content the migration times first reached a minimum and then ascended which defied results expected if only chromatography took place. A similar re-

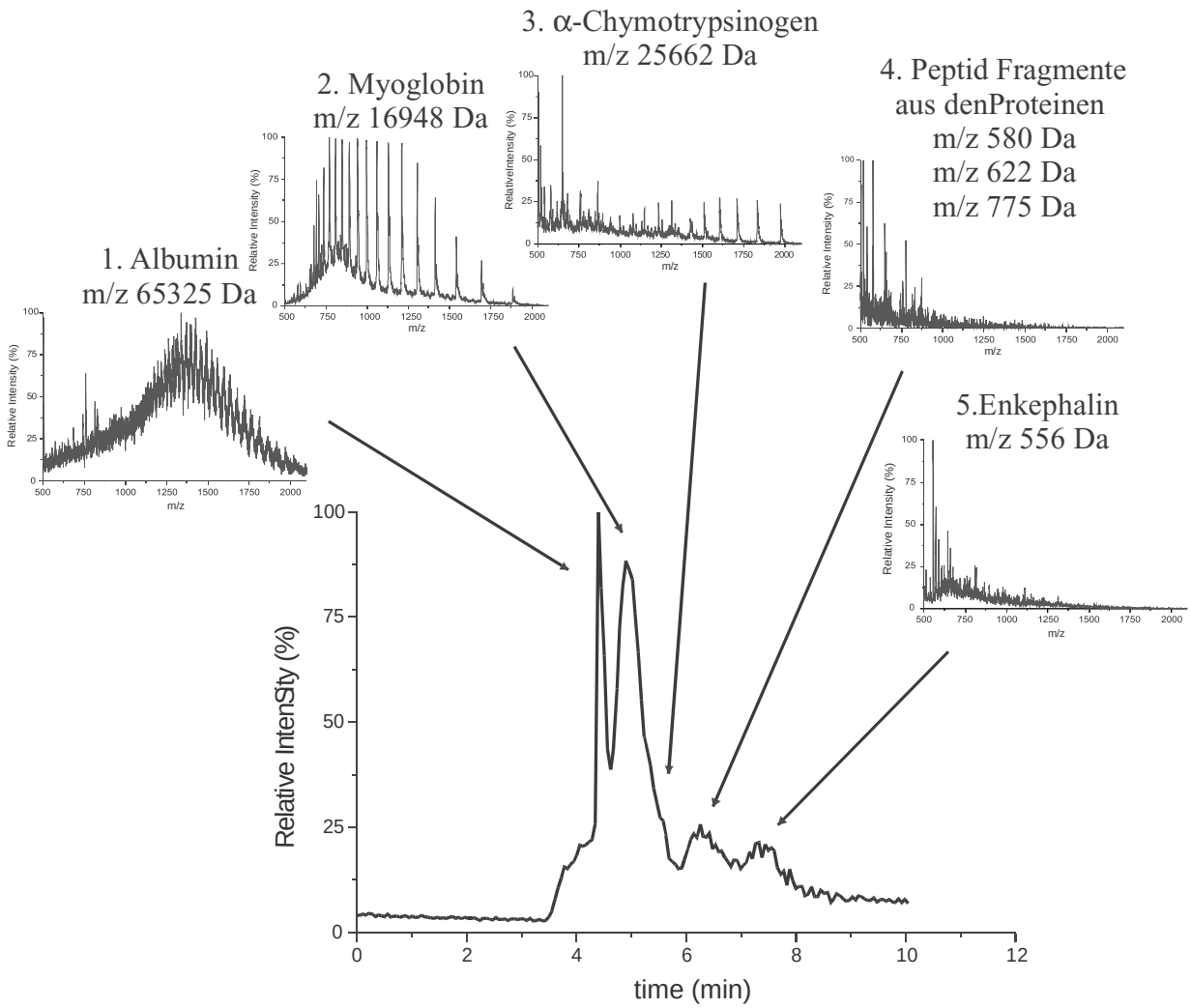

Fig. 4. +10 kV pCEC-CSEC-MS chromatogram and mass spectra. The isocratic separation of the protein mixture was performed using a 79.95% water, 20% acetonitrile and 0.05% formic acid mixture as mobile phase and a flow rate of 2 μL/min. ESI was performed with a voltage of 4500 V and an orifice voltage of 80 V. Mass spectra were recorded using dwell times of 1.0 ms per step of 0.5 u scanning the mass range from 200 to 2000 Da. Other conditions are described in the original publication. From Ref. [30] with permission.

lation was observed by varying the salt content in the mobile phase. The first separation of proteins by CEC using a monolithic column was published by Ericson and Hjertén where the authors employed a stearyl methacrylate-based polymer containing both C_{18} groups and quaternary ammonium functions provided by the crosslinker [40]. By varying the amount of crosslinker it became possible to tune the level of EOF in the manufactured column. Gradient elution was made possible through a nifty split-valve design connecting the inlet end of the capillary to an HPLC pump with solvent reservoirs. By allowing a small hydrodynamic flow, two different types of gradients were achieved: a counter-flow gradient using the moderate EOF column, where the net migration velocity was against the direction of the EOF (inlet electrode: anode, outlet electrode: cathode) and a conventional normal-flow gradient using the high EOF column where EOF and net migration velocity occurred in the same direction (inlet electrode: cathode, outlet electrode: anode), i.e., gradient and sample migrated in the same direction. The counter-flow gradient essentially extended the effective electrophoretic migration distance beyond the actual column length and should allow the use of shorter columns without sacrificing resolution. Carrying out the separation at a sufficiently low pH essentially eliminated any undesired electrostatic interactions between the then positively charged proteins and quaternary ammonium moieties and resulted in the baseline separation of four model proteins within 20 min. Comparisons with μ-HPLC using the same column and CZE showed that the separation was dominated by RP chromatography with little contribution from electrophoresis. Horváth and co-workers [41] tested a cationic acrylic monolith for the separation of both peptides and proteins. Tertiary amino groups with C_2 and C_4 chains provided the EOF in this case and established RP functionality. Un-

like quaternary ammonium groups, tertiary amines did not remain positively charged at elevated pH values and lead to diminished EOF. In order to obtain high EOF the separations should be carried out at low pH thereby adding the advantage of most proteins and peptides being positively charged as well and so minimizing electrostatic interactions with the tertiary amines. With isocratic elution, four model proteins were baseline separated within 8 min, the same was achieved with a mixture of four angiotensin type model peptides. By increasing the content of organic modifier in the mobile phase the authors observed an increase in migration factors for peptides yet experienced the opposite behaviour for proteins which was attributed to more pronounced chromatographic interactions of proteins with the stationary phase. In a follow-up publication, Zhang et al. [42] compared the previous monolith with a styrenic monolith now involving quaternary ammonium moieties. For the four model proteins the two columns showed almost identical behaviour in terms of selectivity and resolution. A comparison with CZE demonstrated CEC yielding higher selectivity and also resulting in a different order of elution. The authors furthermore evaluated the significance of temperature as a variable controlling the retention behaviour of both peptides and proteins. Without compromising the resolution, a two-fold increase in speed of analysis was attained by increasing the temperature from 25 to 55 °C. The analysis time could also be reduced through an increase in temperature for the separation of a tryptic digest of cytochrome *c*. This analysis is one of the first examples of employing monoliths in CEC for the analysis of more complex samples, especially in the context of proteomics.

A variety of hydrophobic moieties intended for reversed-phase CEC after surface derivatization of a sol–gel based monolith were investigated by Quirino and co-workers [43]. With a pentafluorylphenyl moiety six peptides could be eluted within 7 min. The same monolith was used to demonstrate the potential for on-line preconcentration due to its dual functionality as both solid-phase extractor and stationary phase [44,45]. By ensuring long injection plug lengths, a 20-fold preconcentration for a mixture of five model peptides was reported. Since sample matrix and mobile phase were identical, the authors excluded the possibility of electrophoretic stacking or chromatographic field enhancement as the underlying cause. Instead, they attributed the observed preconcentration to the fast mass transfer rates achievable in the porous monolith. When including electrophoretic stacking, i.e., dissolving the sample in a solvent with lower conductivity compared to the mobile phase, a 1000-fold enhancement in preconcentration took place.

Unlike all previously described publications, which have exploited thermally initiated polymerization reactions, Yu et al. [46] presented a photoinitiated porous polymer containing a butyl-, i.e., C_4 functions and sulfonic acid residues to generate EOF. Photopolymerization facilitates polymerization within restricted areas, e.g., selected parts of a capillary can be irradiated although the whole capillary is filled with the reaction mixture. Tailored pore sizes became possible by varying the amount of porogenic solvent in the reaction mixture yielding pore sizes ranging from 100 nm to 4 μm. Although the authors conceded strong electrostatic interactions between peptides and sulfonate moieties, four peptides could be baseline separated within 8 min when adding an ion-pairing reagent. Our own group also employed a photopolymerization procedure in UV-transparent, PTFE-coated capillaries to fabricate a porous monolith with C_4 functionality [36]. Contrarily to most other researchers, we employed a high pH (i.e., pH 10) to diminish electrostatic interactions between mostly negatively charged proteins and the sulfonate residues. Baseline separation of three model proteins was accomplished within 6 min.

Chirica and Remcho [47] developed templated porous monolith that included silica beads in the reaction mixture. The beads were dissolved after completing the polymerization resulting in monoliths with tailored pore sizes, thereby eliminating the need for porogenic solvents. With a step-wise gradient a crude separation of myoglobin and lysozyme became feasible but higher separation efficiencies should be possible in the future with their styrene based monolith, as results using μ-HPLC suggest. By developing a monolith with C_{12} functionality that only generated negligible EOF, Zou and co-workers [48] baseline separated 10 structurally related di- and tripeptides within 10 min as shown in Fig. 5. The separation was solely driven by the differences in electrophoretic mobilities and found different (and superior) in terms of both resolution and selectivity from a separation obtained by CZE thereby highlighting the role of chromatographic interaction for CEC. Depending on how strong the peptides interacted with the stationary phase different relations of retention factor versus content of organic modifier

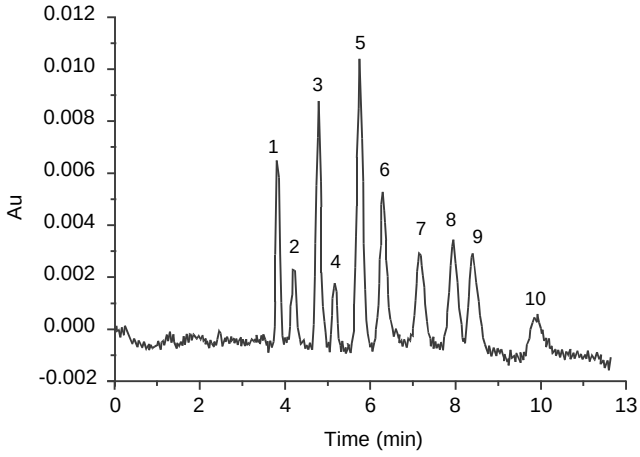

Fig. 5. Electrochromatogram of a peptide mixture on a monolithic column. Experimental conditions: column, effective length 10 cm (total length, 30 cm) with 75 μm i.d. and 365 μm o.d.; mobile phase, 40 mM phosphate buffer (pH 2.1); UV detection wavelength, 214 nm; electrokinetic injection, 5 kV for 5 s. Peaks: 1, Val–Lys; 2, Lys–Glu; 3, His–Phe; 4, Lys–Thr–Tyr; 5, Gly–Ser; 6, Gly–Ile; 7, Met–Val; 8, Met–Met; 9, Met–Leu; 10, Met–Tyr. Other conditions are described in the original publication. From Ref. [48] with permission.

in the eluent were recorded. Later eluting peptides showed a decrease in retention factor as their interaction with the stationary phase became weaker at higher content of organic modifier whereas early eluting peptides most likely did not interact strongly with the stationary phase in the first place. Instead, an increase in organic modifier content decreased their electrophoretic mobilities, which explained the observed effect. Zou and co-workers [35] then developed a mixed-mode monolith, i.e., it contained both RP and strong cation-exchange functionality. This monolith was capable of generating EOF since it contained a sulfate group that fulfilled the dual function of both providing EOF and serving as a cation exchange moiety. At pH 3, based on an approximately logarithmic relation between retention factor and buffer concentration in the mobile phase, the authors concluded that an ion-exchange mechanism is occurring superimposed on the RP type hydrophobic interaction. As to be expected, the electrostatic interactions could be diminished when increasing the buffer concentration. An *N*,*N*-dimethylacrylamide-piperazine-based mixed-mode stationary phase was developed by Hoegger and Freitag [49]. In two separate experiments Gly–Phe and Phe–Gly–Phe–Gly as well as Gly–His and Gly–Gly–His could be baseline resolved. Allen and El Rassi presented the development of several silica-based monolithic columns with C_{18} chains for RP-CEC and applied them to protein separation [50,51]. At pH 2.5, four model proteins were baseline separated within 12 min using a monolith containing a secondary amine and C_{17} functionality. Under identical conditions (apart from the monolith), this separation was not possible using CZE which once more highlighted the role of chromatographic contributions to the overall separation. Using a ternary porogenic solvent, Bedair and El-Rassi [52–54] developed a variety of monoliths containing a C_{17} functionality. Unlike commonly employed binary porogenic solvents, the ternary porogenic solvent provided enough solubility for the C_{17} residues. This resulted in an increased retentive behaviour of the monoliths as compared to using shorter chain lengths. When a quaternary ammonium group for EOF generation was incorporated, six water-soluble model proteins were eluted within less than 3 min, although not all of them were baseline resolved. Migration times increased with elevated organic modifier content in the eluent. The authors explained this by accounting for several factors influenced by an increase in organic modifier content. For one, hydrophobic interactions between analyte and stationary phase decreased. However, both the electrophoretic mobility of proteins and the EOF decreased as well, therefore resulting in an overall increase in migration times. The monolith proved particularly successful for the separation of two crude extracts of membrane proteins that are normally a class of proteins particularly difficult to analyze due to their high hydrophobicity. Both the extracts of galactosyl transferase and cytochrome *c* reductase could be resolved into several single components, in each case within less than 5 min. Fig. 6 gives the example for cytochrome *c* reductase. An ultra fast separation of three

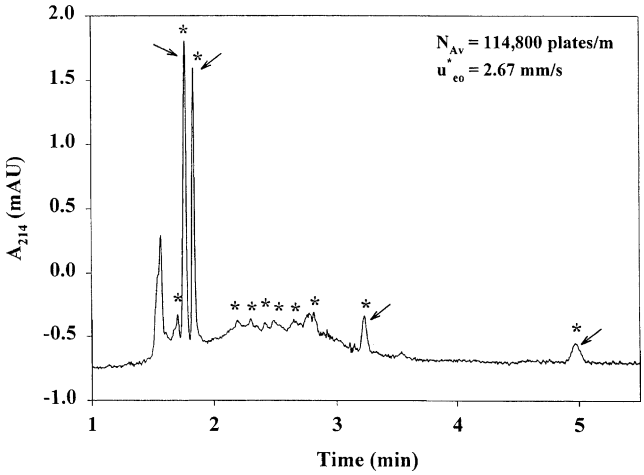

Fig. 6. Electrochromatogram of a crude extract of cytochrome *c* reductase obtained on column F. Column, 33.5 cm (25 cm effective length) × 100 μm I.D.; mobile phase, 10 mM sodium phosphate, pH 2.5 at 45% (v/v) acetonitrile; running voltage, −25 kV; electrokinetic injection, −10 kV for 2 s. Other conditions are described in the original publication. From Ref. [54] with permission.

model proteins within less than 1 min was also illustrated by the same authors.

Six years after the advent of monoliths in CEC, the first coupling of a pressure-assisted monolithic capillary to ESI-MS was recently published by Ivanov et al. [9]. As described earlier for packed beds, flow rates were compatible with the ESI which greatly facilitated interfacing to the MS. In their methacrylate based polymer a tertiary ethylbutylamine group both supplied EOF and provided the hydrophobic moiety. When increasing the ionic strength in the eluent at low pH a decrease in migration times for peptides was observed and was explained by the concurrent decrease in EOF and dominating role of electrophoretic migration over chromatographic retention. Under alkaline conditions chromatographic retention played a more prominent role. Using UV detection, separation efficiencies were not compromised by applying pressure thus successfully decreasing analysis times as demonstrated by the isocratic separation of a tryptic digest of cytochrome *c*. After separating a tryptic digest of bovine serum albumin on the monolithic column using gradient elution 73% of the total sequence could be identified by peptide mass fingerprinting.

A relatively novel method of controlling the surface properties of monoliths was applied towards the separation of peptides by Lander and co-workers [55,56]. The authors created chromatographic functionality on their silica-based monoliths through adsorption of polyelectrolyte multilayers, i.e., alternating layers of oppositely charged polymers that produced highly stable coatings that did not have to be added to the mobile phase, as confirmed by 100 consecutive EOF measurements. Depending on the charge of the exposed layer easy control of the direction of the EOF was possible.

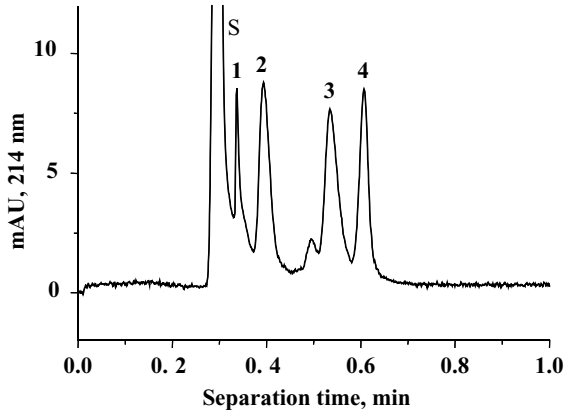

Fig. 7. Separation of peptides using monolithic capillary grafted with 2-acrylamido-2-methyl-1-propanesulfonic acid. Conditions: capillary column total length 34.5 cm, monolith 8.5 cm, 30 s grafting; mobile phase 100 mmol/L NaCl solution in 10 mmol/L phosphate buffer pH 6.0; voltage −15 kV; overpressure in both vials 0.8 MPa; temperature 60 °C; concentration of peptides 0.1 mg/mL; pressure driven injection at 0.8 MPa for 0.05 min. Peaks: system peak (S), Gly–Tyr (1),Val–Tyr–Val (2), methionine enkephalin (3), leucine enkephalin (4). Other conditions are described in the original publication. From Ref. [57] with permission.

Cation-exchange chromatography was carried out for eight peptides and the measured decrease in retention times with increasing ionic strength confirmed the presence of electrostatic interactions between the peptides and the negatively charged residues on the polymer. The presence of RP retentive behaviour was confirmed as well, but differed between the types of exposed polymer.

An ultra fast separation of four peptides within less than 1 min was demonstrated by Rohr et al. [57]. The authors employed photografting to attach 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) onto the monolith surface. The photografting process was carried out after manufacturing the monolith and therefore had less effect on the porous structure. Photografting permitted controlled surface functionalization by the incorporation of a very high number of AMPS residues onto the monolith surface which explained the unusually high EOF and the short analysis times as illustrated in Fig. 7.

4. Open-tubular capillary electrochromatography for the separation of peptides and proteins

Open-tubular CEC can be conceptualized as a transition region between packed CEC and CZE, in a similar way that pressure-assisted CEC is a transition between μ-HPLC and pure CEC (either using monoliths or packed beds) [58]. Like the monolith approach, the OT approach also alleviates the need for retaining frits and therefore diminishes problems associated with the “art of frit manufacture” and subsequent bubble formation. Consequently, it often simplifies the overall manufacturing process. Compared to packed beds using micro particles, OT-CEC can attain lower plate heights due the absence of intraparticular diffusion that is a primary source for band broadening in packed beds. However, OT-CEC possesses inherently lower capacity factors which make the use of small i.d. capillaries mandatory in order to obtain sufficiently high phase ratios. In addition, etching of the surface prior to derivatization is commonly carried out to enhance the surface area. The small i.d. capillaries also possess small pathlengths and so limit the sensitivity of most spectrophotometric detection methods. On the other side, the higher surface to volume areas of these capillaries make OT-CEC attractive in terms if analysis times since they allow higher electric fields to be applied without creating current instabilities caused by excess Joule heating.

Using 50 μm i.d. capillaries Pesek and co-workers [59–62] first reported first use of OT-CEC for the separation of peptides or proteins. After enhancing the surface area through etching with concentrated HCl or H_2F_2, they covalently attached C_{18} chains or diol-residues onto the capillary walls through a silanization/hydrosilation process delivering a stable Si–C linkage. With the C_{18}-modified capillary, the authors separated turkey from chicken lysozyme which was not possible using CZE. Four model proteins were baseline-resolved using the diol capillary within 4 min. Separation of three angiotensin peptides became much faster employing the diol capillary than with the C_{18} capillary but the retentive behaviour of the C_{18} capillary was found to be stronger than with the diol. Furthermore, the authors observed an increase in separation factors at pH 2.1 when increasing the applied electric field whereas no increase was observed at pH 3.0 suggesting that only at the lower pH true chromatographic interaction took place between analyte and diol-residues. Pesek et al. [63] also investigated smaller i.d. capillaries, i.e., 20 μm, for use in OT-CEC and, after etching the surface, attached a C_{18} functionality onto the surface using the above mentioned procedure. Reproducible retention times (1.7% R.S.D.) were measured for more than 300 injections. The importance of the etching process became obvious by comparing the separation of a mixture of cytochrome *c*’s between a non-etched C_{18} capillary and an etched C_{18} one since only the latter provided baseline separation. For a polybrene-coated capillary just the etching itself improves resolution as was exemplified by an endoproteinase Lys-C digest of transferrin [64]. In another study, Matiska [65] evaluated OT-CEC using two liquid crystals as stationary phases, i.e., a cyanopentoxy-modified and a cholesteryl-modified capillary. Unlike a bare capillary in CZE, which yielded only one peak, both liquid-crystal modified capillaries separated four model proteins albeit they were not baseline-resolved. Both liquid crystals were found to show a similar elution pattern of for the proteins and furthermore possessed high reproducibility in terms of retention times up to 300 runs. However, since both crystals gave a similar elution pattern their high selectivity for planar molecules was not distinctively different from one another. In a related study, Matyska et al. [66] compared the performance characteristics of cholesteryl-modified

capillaries and C_{18} capillaries using six structurally related synthetic thrombin receptor antagonist heptapeptides as test probes. By varying experimental parameters like solvent composition, electric field strength and temperature the authors found different changes in the retentive behaviour of the two capillaries. At low pH, temperature variations had little effect on the retention of two peptides using the C_{18} column unlike at neutral pH where temperature increases lowered retention times. This suggested electrophoretic migration to be the dominating mechanism at low pH with all variations in temperature being compensated by changes in electrophoretic mobility or EOF. Chromatography appeared to be the dominating force at neutral pH. The results using the cholesteryl modified capillary contrasted this and the authors proposed temperature dependent changes of the surface morphology of the cholesteryl residues to be responsible. Also, for the analysis of the same peptides and impurities at different organic modifier content a decrease in resolution was found when increasing the methanol content using the C_{18} sample but the opposite was observed with the cholesteryl-capillary. In a comparison with RP gradient HPLC, an analysis of crude synthetic peptide yielded several peaks using the C_{18}-modified capillary but only one peak with HPLC. The authors excluded the presence of contaminating compounds based on MS data and suggested the OT-CEC method of being capable of resolving different charge states or different conformational states of the same peptide, as shown in Fig. 8 [67]. Additionally, for this specific separation, a way of increasing the detection sensitivity by an order of magnitude in the 20 μm i.d. capillaries was found by the use of the bubble cell. With respect

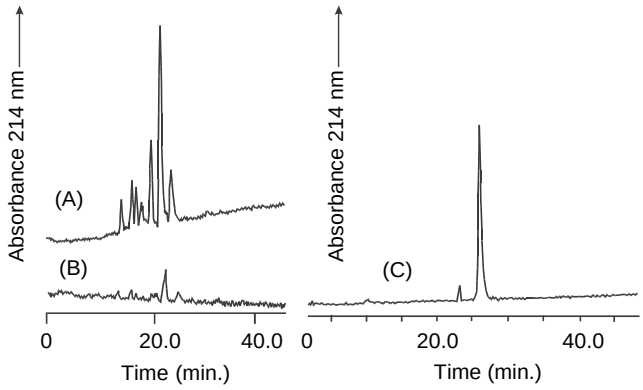

Fig. 8. Analyses of the TRAP-3 synthetic peptide sample. (A) By OT-CEC with an etched C_{18}-modified capillary with bubble cell for detection. Conditions: capillary i.d., 20 μm; total length 70 cm; length to detector 63 cm; voltage 25 kV; current 3.1 mA; electrolyte, buffer 1 (60 mM phosphoric acid and 38 mM Tris), pH 2.14 + 20% (v/v) methanol. (B) By OT-CEC with etched C_{18}-modified capillary and no bubble cell. Other conditions same as (A). (C) By RP-HPLC. Column, TSK-ODS 120T column (150 mm × 4.6 mm i.d.); mobile phase, linear gradient of 0–100% B over 60 min. Solvents: A, 0.1% TFA in water; B, 0.09% TFA in 60:40 (v/v) acetonitrile/water. Flow rate, 1 mL/min. Detection for OT-CEC and for HPLC at 214 nm. Other conditions are described in the original publication. From Ref. [67] with permission.

to column stability, no statistically measurable changes in reproducibility were detected by monitoring the separation factor between chicken and turkey lysozyme over 400 injections employing the cholesteryl-modified capillary. Pesek et al. [68] also presented a butylphenyl-modified capillary and compared it to the previously mentioned C_{18}- and cholesteryl-modified capillary for the separation of synthetic peptide samples. The butylphenyl-modified capillary revealed a higher number of peaks than the cholesteryl-one therefore once more proving the impact of chromatographic interactions since the mere occurrence of electrophoretic migration could not explain the different number of peaks. The number of peaks resolved on the C_{18} column could be enhanced by using ethanol as organic modifier.

Lubman's group presented the first coupling of an OT-CEC system to ESI-MS for the separation of peptides. Since the concentration-sensitive response of a MS is independent of the optical path length it may be the ideal type of detection for the small i.d. capillaries used for OT-CEC (9 μm in this case), apart from its ability to provide information on analyte structure and molecular mass. The authors employed a C_8-modified capillary that also contained an amine function for EOF generation. Both amine and peptides were positively charged at low pH so electrostatic interactions were successfully prevented and six model proteins were baseline resolved within 3 min. By applying a gradient, a tryptic digest of horse heart myoglobin could be eluted within 5 min.

The usability of a variety of different polymers as stationary phases in OT-CEC was investigated by Shao et al. [69]. Cationic, anionic and neutral surfaces were manufactured. The neutral surface was found to generate a negligible EOF due to the absence of any charged residues apart from some remaining, non-derivatized silanols on the walls. Three peptides could be baseline resolved on the capillary derivatized with the neutral polymer but not with the capillary containing the anionic polymer most probably due to strong electrostatic interactions. Huang et al. prepared a chromatographic surface in 20 μm i.d. capillaries by first polymerizing a porous styrenic support onto the capillary walls. This support was functionalized afterwards by attaching dodecyltrimethylammonium moieties in order to generate both permanent positive charges (for EOF and electrostatic repulsion of analytes at low pH) and hydrophobic residues (to achieve RP-chromatography) [70]. Compared with raw fused silica capillaries the column exhibited a greatly enhanced EOF. Unlike CZE, where variations of the acetonitrile content in the eluent did not bring any improvements in terms of resolution for the separation of four proteins, OT-CEC did show improved resolution. Both peptides and proteins, though, experienced increases in retention times when augmenting the acetonitrile content. Peak shapes were greatly improved by substituting parts of the acetonitrile with methanol.

Novel sol–gel technology consisting of hydrolysis and polycondensation of glycidoxypropyltrimethoxysilane fol-

lowed by an octadecyl silylation reaction yielded a C_{18} ester-bonded capillary shown by Zhao et al. [71]. Through the increase in surface area by the sol–gel process, a higher number of bonded C_{18} residues could be incorporated and thus higher mass loadabilities achieved. For the separation of a mixture of seven pentapeptides, higher resolution was obtained with the C_{18} column compared to a bare column (i.e., CZE) or a column that had undergone the sol–gel process but did not contain any ester-bonded C_{18} columns.

In an attempt to propel the development of OT-CEC towards the analysis of complex biofluids, Rehder-Silinski and McGown investigated G-quartet stationary phases in OT-CEC for the separation of bovine milk proteins [72]. The authors explained the potential of G-quartet forming aptamers for peptide and protein separations to lie in their natural tendency to undergo weak and non-denaturing interactions with amino-based structures. Stationary phases consisting of aptamers incapable of forming G-quartets, as well as CZE, were not able to separate two β-lactoglobulin variants (A and B) differing only in two amino acids. Contrarily, the 4-plane, G-quartet aptamer stationary phase achieved baseline resolution, with Tris buffer providing better resolution than phosphate buffer. These insights were then applied to the separation of both purified milk proteins (both casein and whey proteins) as well as bovine skim milk [73]. Seven baseline-resolved protein peaks were obtained (the separation is demonstrated in Fig. 9) although reproducibilities in terms of peak areas showed significant variations which the authors explained by inconsistent suspensions of untreated milk samples. In comparison, CZE yielded inferior resolution.

Utilizing poly(aryl ether)monodendrons to form dendrimers as bonded stationary phases was suggested by Chao and Hanson [74] and applied to the successful separation of three model proteins yet the authors conceded problems in controlling the experimental conditions. Porphyrin derivatives as stationary phases for OT-CEC of low-molecular weight peptides were investigated by Charvátová et al. [75,76]. Chromatographic interactions appeared to depend on the presence of aromatic amino acid residues in the peptide and on the type of central atom in the porphyrin. Although a separation of five tripeptides was dominated by electrophoretic migration, complementary chromatographic interactions were observed as demonstrated by comparison with CZE. Interestingly, CEC yielded much shorter analysis times at low pH, caused by more effective electrostatic repulsion between the positively charged porphyrin and positively charged peptides. Three types of non-covalent interactions were identified for the chromatographic contribution, i.e., p-p stacking, coordination bonds with other ligands and electrostatic interactions. A study of four structurally related tetrapeptides differing only in the presence or absence of a protecting group revealed the resolution to be strongly dependent on the type of central atom in the porphyrin with Ni and Cu yielding the best results [77].

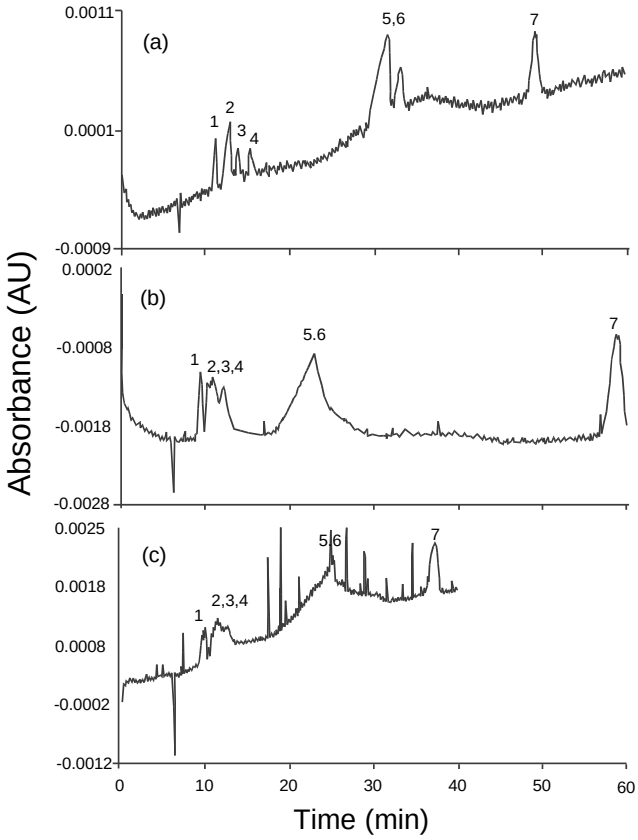

Fig. 9. Results for bovine milk obtained using (a) a 2-coated capillary (EOF = 7.0 min); (b) a 3-coated capillary (EOF = 6.3 min); and (c) a bare capillary (EOF = 6.4 min). Sample: untreated bovine skim milk, 25%, in mobile phase. Peaks: 1, a-lactalbumin; 2, b-casein; 3, β-lactoglobulin B; 4, β-lactoglobulin A; 5, 6, a-casein; 7, k-casein. Mobile phase: 10 mM phosphate buffer, pH 7.3; capillaries, 47 cm, 75 mm i.d.; 5-s low-pressure sample injection; 25 8C; separation voltage 15 kV. Negative peaks at 7.0, 6.3, and 6.4 min in (a), (b), and (c), respectively, are due to a change in refractive index when the sample plug migrates past the detector, and serve as EOF markers. Other conditions are described in the original publication. From Ref. [73] with permission.

5. Capillary electrochromatographic separations of peptides and proteins in microfluidic devices

Naturally, researchers in the field of CEC were attracted by a number of promising aspects that microfluidic devices had shown for conventional CZE. Notably, extremely short analysis times and tiny liquid volumes could be manipulated easily. The essentially unlimited availability of potential designs make integrated analysis systems possible that, for instance, are capable of executing multiple separations (and other analytical functions) in series or in parallel [78]. Moreover, the simplicity of applying potentials in the microfluidic platform to generate gradients and solvent mixtures provides a valuable alternative to μ-HPLC where the difficult task of building micropumps has yet to be satisfactorily resolved. One of the inherent problems of many materials, e.g., glass, used for building microfluidic devices is their limited UV transparency unless, for instance, the fairly expensive quartz

is employed. Early in the advent of microfluidic devices, laser-induced fluorescence detection became the detection method of choice for many authors. However, apart from the obvious necessity to perform a derivatization reaction in order to label analytes with a fluorescent tag (with reaction rates often being very slow, especially at low analyte concentrations), the presence of a fluorescent tag greatly influences the analyte's chromatographic interactions with the stationary phase. All three formats of CEC have been evaluated in the microfluidic platform, i.e., packed beds, monoliths and OT-CEC. Since the overall number of publications discussing CEC separations of peptides and proteins in the microfluidic format is fairly low so far, we decided to summarize all these approaches in one chapter.

An innovative alternative to the packed bed approach is the preparation of a microfluidic device containing an array of support particles (termed collocated monolithic support structures) etched into a quartz substrate as described by Regnier and co-workers [79]. Apart from abandoning the need to actually pack the manufactured device with particulate materials, these support particles were extremely homogeneous, could be placed at any position in the device and also manufactured at any size. Furthermore, they were totally immobilized, i.e., they did not show any movement during the separation. However, the authors conceded a lower phase ratio than conventional particulate material. By attaching a C_{18} function onto the support a fluorescein-5-isothiocyanate tryptic digest of ovalbumin under isocratic conditions digest exhibited the same peak capacities within 6 min as the comparison using RP-HPLC under gradient conditions which required 30 min. This study delivered one of the first examples demonstrating the potential of CEC in microfluidic devices to compete with HPLC in terms of peak capacities. Sufficient peak capacity is of great importance when attempting to establish CEC as a valuable complementary technique in proteomics. In a related study, Regnier and co-workers proposed the use of poly(dimethyl siloxane) as an inexpensive alternative substrate with respect to quartz [80,81]. Both C_8 and C_{18} moieties could be grafted onto the support particles as well as different sulfonic acid residues for EOF generation. A fluorescently labeled bovine serum albumin tryptic digest experienced the highest resolution on the C_{18}-modified substrate. A true three-dimensional analysis of a tryptic digest within one single microfluidic device was presented by the same group a year later [78]. The three dimensions consisted of a trypsin digestion, i.e., proteolysis, followed by affinity selection of histidine-containing peptides and RP-CEC. The first two steps were performed using particulate materials containing immobilized trypsin and immobilized metal affinity columns, respectively, both kept in place by retaining frits. The third step utilized the above mentioned collocated monolithic support structures. Fig. 10 displays an optical microscopy image of parts of the microfluidic device and an electrochromatogram of the separation of a fluorescently-labeled tryptic digest of bovine serum albumin. Singh and co-workers [82] demonstrated the

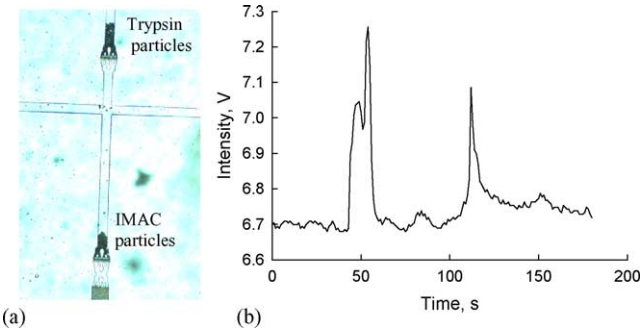

Fig. 10. Optical microscopy image taken with a CCD camera of microfabricated frit A with trapped immobilized trypsin particles and frit B with trapped Cu(II)-IMAC particles (a). A reversed-phase CEC separation of fluorescein-5-isothiocyanate-labeled bovine serum albumin after on chip trypsin digestion and Cu(II)-IMAC selection (b); separation conditions: 1 mM phosphate buffer (pH 7.0), 500 V/cm. Other conditions are described in the original publication. From Ref. [78] with permission.

feasibility to exploit porous photopolymer-based monoliths as stationary phases in microfluidic devices for the separation of fluorescently-labeled peptides. Fig. 11 illustrates the separation of six naphthalene-2-3-dialdehyde labeled peptides using monoliths possessing C_{12} functionality within less than 50 s by the same group [83]. Photopolymerization was found to be particularly attractive in the microfluidic platform since it allowed localized placement of the polymer. Typical retention time reproducibilities were in the order of 2% R.S.D.

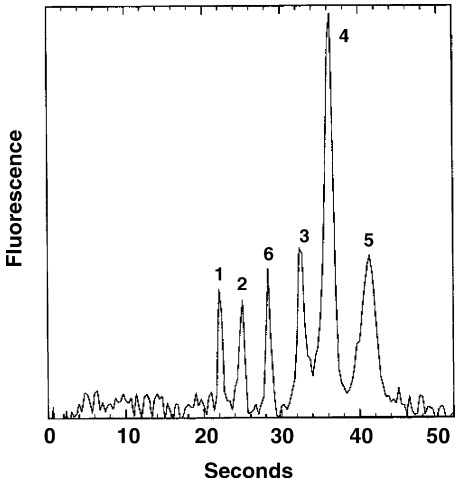

Fig. 11. Reversed-phase electrochromatography of peptides in a microchip. The polymer was negatively charged lauryl acrylate monolith, peptides were labeled with naphthalene-2,3-dialdehyde, and laser-induced fluorescence detection was performed using the 413-nm line of a Kr ion laser. Field strength 770 V/cm (5 kV). Mobile phase: 30:70, acetonitrile/25 mM borate, pH 8.2, containing 10 mM octane sulfonate. Offset-T microchip dimensions: 40 μm deep, 120 μm wide. The peptides are (1) papain inhibitor, (2) proctolin, (3) opioid peptide (a-casein fragment 90–95), (4) Ile-angiotensin III, (5) angiotensin III, and (6) GGG. Other conditions are described in the original publication. From Ref. [83]. with permission.

6. Concluding remarks

The review hopefully exposed the reader to the profound and exciting progress made over the last decade in the separation of peptides and proteins by CEC. Although the overall number of publications is still fairly limited, CEC has definitively been shown to possess the potential of becoming a truly complementary technique to other, more established separation techniques. A large number of stationary phases have now been developed for all formats of CEC and tested for the analysis of these biopolymers furnishing the user with a wide variety of options.

Also, significant advances have been made in gaining a deeper understanding of how the underlying operating variables and physicochemical separation processes influence the actual separation. An issue that has not yet gained much attention, and whose absence was already noticed in the last review in this journal, is the field of chemometrics in order to rationalize the large number of variables available and to determine the optimum operating conditions [1]. The future will hopefully see a rising number of papers further developing and optimizing CEC methodology and applying its full potential towards an ever higher number of 'real-life' applications.

Appendix A.

A.1. Packed beds

Mode	Capillary dimensions	Stationary phase particles	Detection	Analytes	Ref.
RPC	100 μm i.d., 40 cm total, 23 cm packed	Gromsil ODS-2, 1.5 μm	MS	2 model peptides	[10]
IEC (cation)	180 μm i.d., 37 cm total, 28 cm packed	8 μm, gigaporous, PLSCX	UV	2 model peptides	[11]
RPC	50 μm i.d., 25 cm total	3 μm Spherisorb ODS1	UV	4 synthetic protected peptide intermediates	[12]
RPC	180 μm i.d., 6 or 12 cm total	3 μm C_{18}, silica gel (Vydac)	MS	6 model peptides Bovine cytochrome *c* digest Chicken ovalbumin digest	[13]
RPC	180 or 130 μm i.d.	3 μm C_{18}, silica gel (Vydac)	MS	Bovine β-lactoglobulin A digest Human hemoglobin digest	[14]
RPC	100 μm i.d., 20 cm total, 6 cm packed	Gromsil ODS-2, 1.5 μm	UV	Bovine heart cytochrome *c* digest	[15]
RPC	100 μm i.d., 25 cm total	5 μm Vydac C_{18}	UV	Recombinant human growth hormone digest Recombinant tissue plasminogen activator	[16]
RPC/IEC (anion)	150 μm i.d., 8.5 cm total	Mixed-mode C_{18}/dialkylamine 5 μm (Alltech)	MS	5 model peptides Horse heart myoglobin digest	[70]
RPC/IEC (anion)	100 μm i.d., 25 cm packed	Mixed-mode, 5 μm Spherisorb SCX/C_{18}	UV	4 model peptides Cytochrome *c* digest	[18]
RPC	100 μm i.d., 31 cm total, 10 cm packed	5 μm Spherisorb ODS1	UV	3 model peptides	[19]
IEC (cation)	50 μm i.d., 31 cm total, 10 cm packed	Spherisorb SCX	UV	10 model peptides	[20]
IEC (anion)	50 μm i.d., 34 cm total, 26 cm packed	Derivatized 5 μm, Spherisorb S5-W, bare silica beads	UV	4 model proteins Bovine met- and oxy-hemoglobin	[21]
IEC (cation)	50 μm i.d., 33 cm total, 26 cm packed	Derivatized 5 μm, Spherisorb S5-W, bare silica beads	UV	5 model proteins	[22]
RPC	100 μm i.d., 33 cm total, 25 cm packed	3 μm Hypersil C_8 or C_{18}	MS	4 model peptides	[23]
RPC	100 μm i.d., 25 cm total	3 μm Hypersil C_{18}	MS	7 model peptides	[24]
RPC	100 μm i.d., 15 or 25 cm total	3 μm Hypersil C_{18}	MS	3 model peptides	[25]
RPC	100 μm i.d., 25 cm packed, 33 cm total	3 μm Hypersil C_{18}	UV	2 model peptides	[26]
RPC/IEC	100 μm i.d., 25 cm packed, 33 cm total	−3 μm Hypersil C_8 or C_{18}, mixed mode, Spherisorb C6/SCX or C_{18}/SCX	UV	6 linear model peptides 6 cyclic model peptides	[27]
RPC	100 μm i.d., 25 cm packed, 33 cm total	3 μm Hypersil C_{18}	UV	10 synthetic linear immunogenic peptides mimicking HIV-1 gp 120 epitope	[28]
RPC	100 μm i.d., 25 cm packed, 33 cm total	3 μm Hypersil C_{18}	UV	4 synthetic peptides related to activin β_A–β_D subunits	[8]
SEC	250 μm i.d., 25 cm total	5 μm Grom-Sil 120 SEC	MS	5 model proteins and peptides	[30]
HIC	75 μm i.d., 31 cm total, 10 cm packed	5 μm poly-hydroxylethyl A	UV	6 model peptides	[31]
IEC/RPC	100 μm i.d., 25 cm total	−5 μm poly(2-sulfoethylaspartamide)-silica for IEC −3 μm Unimicro C_{18} for RP	UV	6 model peptides	[32]

A.2. Monolithic columns (continuous beds)

Mode	Capillary dimensions	Monolith	Detection	Analytes	Ref.
RPC	100 μm i.d., 25 cm total, 21 cm monolith	Poly(acrylamide)/polyethyleneglycol	UV	5 model peptides	[39]
RPC	50 μm i.d., 8 cm total, 6 cm monolith	Stearylmethacrylate	UV	4 model proteins	[40]
RPC	75 μm i.d., 15 cm monolith	Methacryloxytrimethoxysilane pentafluorophenyltrimethoxysilane	UV	5 model peptides	[43]
RPC	75 μm i.d., 26 cm total, 19 cm monolith	(Methacryloxypropyl) trimethoxysilane (pentafluorophenylpropyl)trichlorosilane	UV	5 model peptides	[45]
RPC	75 μm i.d., 38 cm total, 27 cm monolith	Styrene–divinylbenzene	UV	4 model proteins 4 model peptides	[34]
RPC	100 μm i.d., 28 cm monolith	Butyl methacrylate	UV	4 model peptides	[46]
IEC (cation)	50 μm i.d., 39 cm total, 29 cm monolith	Glycidyl methacrylate ethylene glycol	UV	4 model proteins 4 model peptides	[41]
RPC	100 μm i.d., 33 cm total, 21 cm monolith	Butylacrylate	UV	3 model proteins	[36]
RPC	250 μm i.d., 15 cm total	Butylacrylate	UV	2 model proteins	[47]
RPC	75 μm i.d., 30 cm total, 10 cm monolith	Lauryl methacrylate	UV	10 model peptides	[48]
RPC/IEC	100 μm i.d., 30 cm total, 10 cm packed	2-(Sulfoxyethyl) methacrylate	UV	10 model peptides	[35]
RPC/IEC	75 μm i.d., 40 cm total, 30 cm monolith	Vinylbenzyl chloride ethylene glycol dimethacrylate	UV	5 model peptides 5 model proteins Cytochrome *c* digest	[42]
RPC/IEC	75 μm i.d., 36 cm total, 27 cm monolith	*N*,*N*-Dimethylacrylamide-piperazine diacrylamide	UV	4 model peptides	[49]
RPC	100 μm i.d., 27 cm total, 20 cm monolith	Tetramethyl orthosilicate	UV	4 model proteins	[51]
RPC	50 μm i.d., 19 cm total	Different methacrylates	MS	Cytochrome *c* digest Bovine serum albumin digest	[9]
RPC/IEC	75 μm i.d., 34 cm total, 27 monolith	Tetramethyl orthosilicate	UV	8 model peptides	[56]
RPC	100 μm i.d., 34 cm total, 25 cm monolith	Stearyl acrylate	UV	Galactosyl transferase Cytochrome *c* reductase	[54]

A.3. Open-tubular CEC

Mode	Capillary dimensions	Bonded phase to capillary walls	Detection	Analytes	Ref.
RPC	50 μm i.d., 45 cm total	1-Octadecene	UV	5 model proteins and peptides	[59]
RPC	50 μm i.d., 45 cm total	(a) 1-Octadecene (b) 7-Octene-1,2-diol	UV	10 model proteins and peptides	[61]
RPC	20 μm i.d., 50 cm total	1-Octadecene	UV	7 model proteins	[63]
RPC	50 μm i.d., 34 cm total	Polybrene	UV	4 model proteins Lys-C digest	[64]
RPC	50 μm i.d.	(a) Cholesterol-10-undecenoate (b) 4-Cyano-4′-*n*-pentoxybiphenyl	UV	4 model proteins 4 different cytochrome *c*	[65]
RPC	20 μm i.d., “bubble” cell, 69 cm total	(a) 1-Octadecene (b) Cholesterol-10-undecenoate	UV	6 thrombin receptor antagonist peptides 2 different lysozymes	[66,67,58]
RPC	50 μm i.d., 34 cm total	(a) 1-Octadecene (b) Cholesterol-10-undecenoate (c) Butylphenyl	UV	17 model peptides	[68]
(a) RPC (b) IEC (cation)	50 μm i.d., 45 cm total	(a) Ucon 75-H-90000 (b) 2-Acrylamido-2-methylpropanesulfonic acid	UV	3 model peptides	[69]
RPC/IEC	20 μm i.d., 47 cm total	*N*,*N*-Dimethyldodecylamine	UV	4 model proteins 3 model peptides	[70]
RPC	25 μm i.d. 48 cm total	Stearic acid	UV	7 model peptides	[71]
NPC	75 μm i.d., 47 cm total	4-Plane G-quartet-forming oligonucleotide	UV	Bovine β-lactoglobulin variants A and B	[72]
NPC	75 μm i.d. 47 cm total	(a) 4-Plane G-quartet-forming oligonucleotide (b) 2-Plane G-quartet-forming oligonucleotide	UV	7 bovine milk proteins Skimmed milk (bovine)	[73]
RPC	50 μm i.d., 54 cm total	Poly(aryl ether)monodendrons	UV	3 model proteins	[74]
NPC	50 μm i.d., 30 cm total	(Metallo)porphyrins	UV	5 model peptides	[75,76]
NPC	50 μm i.d., 30 cm total	(Metallo)porphyrins	UV	4 peptide derivatives of B23-B30 fragment of B-chain of human insulin	[77]

Abbreviations used in Appendix: RPC, reversed-phase chromatography; IEC, ion-exchange chromatography; HIC, hydrophilic interaction chromatography; NPC, normal-phase chromatography; SEC, size-exclusion chromatography.

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