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Comparative study of capillary electroendosmotic chromatography and electrically assisted gradient nano-liquid chromatography for the separation of peptides

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Abstract

Capillary electroendoendosmotic chromatography (CEC), being a hybrid of high-performance liquid chromatography (HPLC) and capillary electrophoresis, offers considerable changes to enhance column efficiency, speed of analysis and additional selectivity as compared to the parent methods. The analytes are driven by the electroendosmotic flow (EOF) and separated by surface–solute interactions as well as by differences in electromigration. In this paper on the separation of peptides on C_{18} reversed-phase and mixed-mode (sulphonic acid–*n*-alkyl) packings in CEC and electrically assisted reversed-phase gradient nano-LC are investigated. It is shown that mixed mode packings generate a higher EOF than reversed-phase packings that is scarcely dependent on the pH of the eluent. Applying a potential in gradient elution reversed-phase nano-LC of peptides shortens the analysis time as compared to separations without a potential. Electrically assisted reversed-phase gradient elution nano-LC is a powerful separation tool for analysis of tryptic digests. Peptides can be successfully resolved in acidic organic mobile phase at pH 2–3 with and without trifluoroacid as ion pairing reagent under isocratic conditions. It is demonstrated that CEC with mixed mode packing and an eluent of pH 2.3 with varying acetonitrile content can be applied to monitor impurities in a synthetic peptide.

Keywords: Electrokinetic chromatography; Gradient elution; Electrically assisted gradient elution; Mixed-mode chromatography; Peptides

1. Introduction

CEC is a hybrid technique between micro-high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). It was pioneered by Pretorius et al. [1], Jorgenson and Lukacs [2], Tsuda [3] and Knox and Grant [4]. In CEC utilization of fused-silica capillaries of 50 to 100 μm inner diameter and 10 to 50 cm length packed with microparticulate reversed-phase silicas is widely spread. The columns are placed into a modified CE equipment that provides application of potentials between 0 and 30 kV. A buffered organic eluent of pH 8 is conveniently employed as mobile phase. As the capillary wall and the surface of the reversed-phase silica particles are negatively charged at pH>7 a diffuse layer is formed next to an electrical double layer. This causes an electroendosmotic flow (EOF)

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under the potential gradient conditions which is directed towards the cathode. Detection is accomplished by UV directly on the column or at the unpacked column end. The analytes are injected either by applying pressure or electrokinetically.

For neutral analytes the same retention behaviour in CEC as in HPLC is expected. In contrast to HPLC charged analytes are separated by both solute–surface interactions and by differences in electrophoretic mobility.

In HPLC a parabolic flow pattern is generated while in CE (open tube) a plug-like flow across the column cross-section is obtained [4–7]. Thus a higher efficiency in CEC than in HPLC is expected. Further, one can show that the eddy diffusion term in the plate height equation is much smaller in CEC than in HPLC [2].

Commonly used packings are 3 μm *n*-octadecyl bonded silicas, often specially designed to match suitability in CEC. Mixed-mode packings based on sulphonic acid–*n*-hexyl/*n*-octyl or /*n*-octadecyl silica packings are less frequently employed. It has been demonstrated that the EOF is predominantly generated by the charge of the silica particles in a CEC column rather than by the charged capillary wall [8]. The application of bonded silica particles as packings for CEC columns has two major effects on the magnitude of the EOF. With decreasing pH of the mobile phase from pH 8 to pH 3 the EOF decreases and becomes low at the pH of zero charge of the silica particles between pH 1.5 and 3.

The EOF is proportional to the concentration of silanol groups of a bonded silica.

Performance of CEC on columns with 3 μm reversed-phase silicas generates up to 200 000 theoretical plates per m column length. Separations have been performed with neutral analytes as well as with polar compounds, e.g. pharmaceuticals [9–15].

Very little has been reported on the resolution of peptidic mixtures by CEC [13,16,17]. In HPLC peptides are usually separated on reversed-phase silicas with acidic–organic mobile phases of pH 1 to 3 and ion pairing additives under gradient elution conditions [18]. Under such conditions the EOF of commonly used reversed-phase columns in CEC is very low and alternatives must be chosen to overcome this disadvantage. One solution is to apply CEC columns with mixed-mode packings [19]. These columns give rise to a sufficiently high EOF in an acidic pH range [8,19]. The addition of sodium dodecylsulphate (SDS) as detergent at a concentration of 5 m*M* as modifier to the mobile phase might be another solution [20].

A third alternative is to use micro-HPLC reversed-phase columns under gradient elution conditions and to additionally apply a potential gradient. This method is called electrically assisted reversed-phase HPLC and was used for the analysis of digest of glycoproteins coupled with matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) and electrospray ionization (ESI) MS [21].

In general the pressurized CEC mode introduced by Tsuda [3] can be applied under isocratic conditions. It was shown for nonpeptidic solutes that this technique is a powerful means to fine-tune the selectivity [22].

Recently, Wu et al. have demonstrated the usefulness of pressure driven CEC in the analysis of tryptic digests on a reversed-phase column coupled to MS under isocratic conditions [23].

Simple peptide mixtures are preferably separated under isocratic conditions where significant changes in the retention are achieved by small changes of the

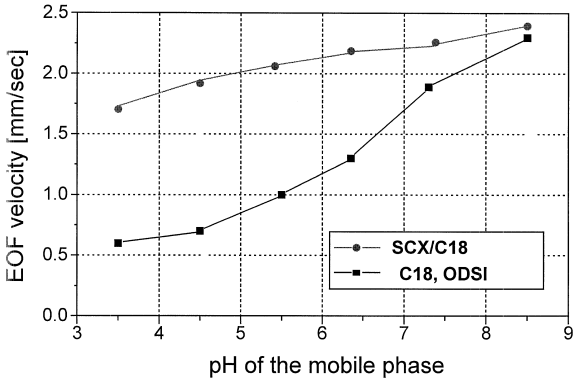

Fig. 1. Dependence of EOF on the pH of eluent for a *n*-octadecyl bonded silica and a mixed mode bonded silica (sulphonic acid–*n*-octadecyl). CEC conditions: 25 cm×100 μm packed capillaries with Waters Sperisorb SCX/C_{18}, ODS-Hypersil, 3 μm; mobile phase: 25 m*M* phosphate, pH see Figure–acetonitrile (20:80 v/v); injection: 5 kV, 6 s; UV detection: 254 nm; temperature: 20°C; sample: thiourea.

content of the organic solvent in the mobile phase. Complex mixtures such as digests require gradient elution conditions. In conclusion peptides are ideal analytes for CEC according to their hydrophobic properties and charge variation with pH.

Pressurized CEC and electrically assisted HPLC offer a number of distinct advantages with respect to selectivity as compared to MEKC (micellar electrokinetic chromatography) and to HPLC as orthogonal methods.

In this paper we report on the separation of peptides under CEC conditions and electrically assisted gradient nano-LC.

2. Experimental

2.1. Instrumentation

(1) A HP^{3D} CE system (Agilent Technologies, Waldbronn, Germany) was used throughout this work modified to allow the inlet and outlet buffer vials to be pressurized simultaneously or separately. A maximum pressure of approximately 1 MPa was applied.

(2) For the gradient system a laboratory-made instrument was used. The packed capillary columns were integrated into a Jasco Spectra-Physics vari-

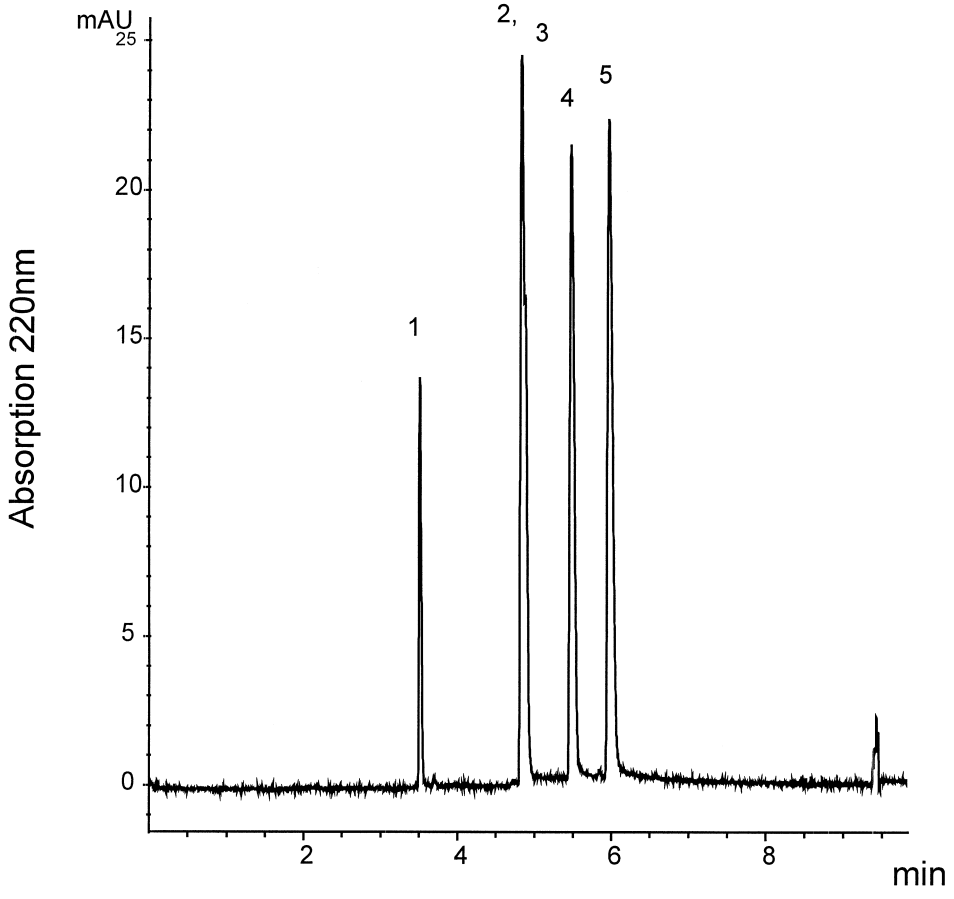

Fig. 2. CEC separation of the peptide mixture at pH 2.3. CEC conditions: 25 cm×100 μm packed capillaries with Waters Sperisorb SCX/C_6, 3 μm; mobile phase: 50 m*M* phosphate, pH 2.3–acetonitrile (50:50, v/v); injection: 5 kV, 6 s; UV detection: 220 nm; temperature: 20°C; voltage: 25 kV (746 V cm^{-1}); sample: 1=H–Gly–Val–NH_2, 2=H–Val–Tyr–Val– NH_2, 3=[Met^5]-enkephalin amide, 4=[Leu^5]-enkephalin amide, 5=angiotensin II amide.

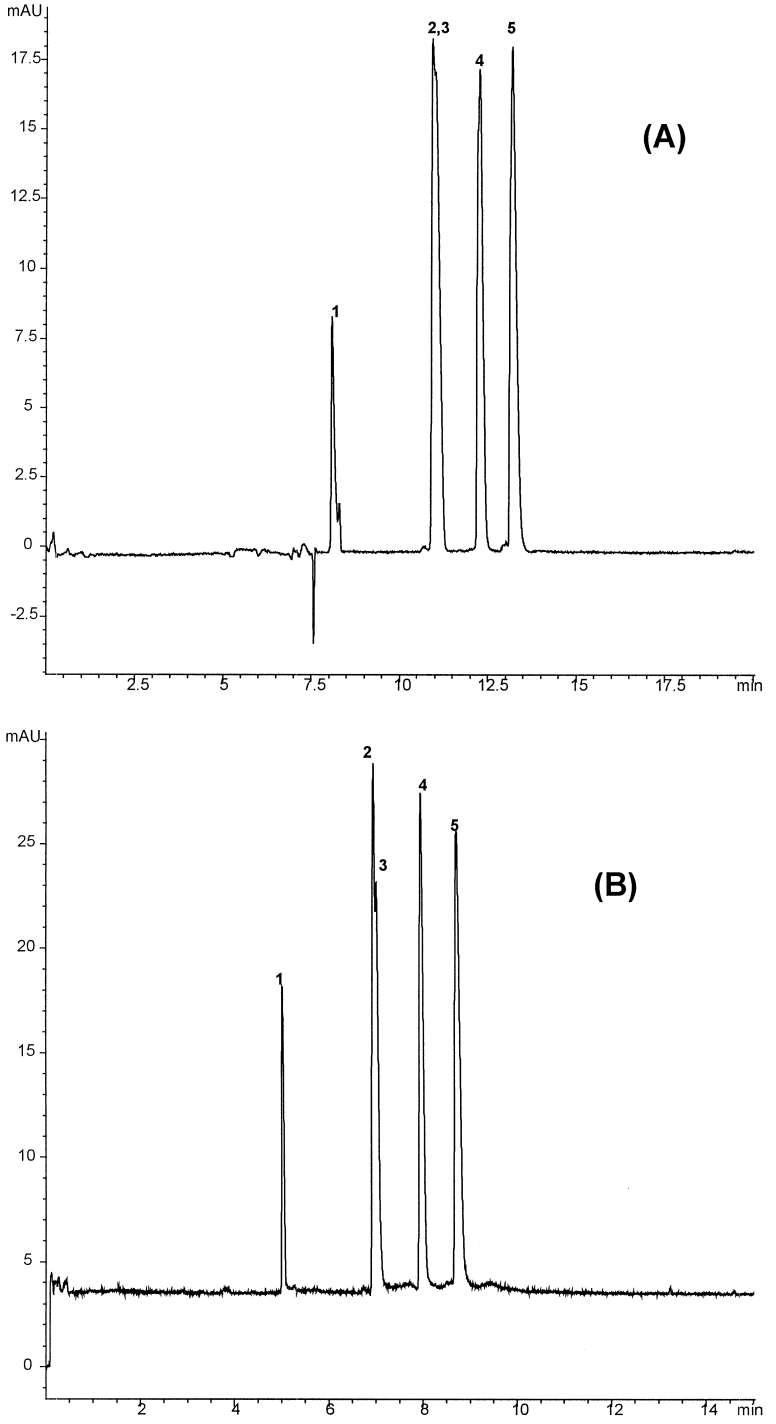

Fig. 3. Separation of peptides with and without TFA at pH 2.3 on a mixed mode stationary phase (Waters Sperisorb SCX/C_6). CEC conditions: 25 cm×100 μm packed capillaries with Waters Sperisorb SCX/C_6, 3 μm; mobile phase: 50 m*M* (A) TFA, (B) phosphate, pH 2.3–acetonitrile (50:50, v/v); injection: 5 kV, 6 s; UV detection: 220 nm; temperature: 20°C; voltage: 20 kV (597 V cm^{-1}); Sample: 1=H–Gly–Val–NH_2, 2=H–Val–Tyr–Val–NH_2, 3=[Met^5]-enkephalin amide, 4=[Leu^5]-enkephalin amide, 5=angiotensin II amide.

able-wavelength UV–Vis detector Spectra 100. The injection valve was a Swiss-made Valco injector with internal loop of 20 nl volume. The mobile phase was delivered at constant pressure by two Bischoff 2200 Micro-HPLC pumps (Bischoff Analysentechnik, Leonberg, Germany). To generate flow-rates of 200 nl/min a LC Packings splitter Acurate with a 40 cm×50 μm I.D. fused-silica capillary was used as a flow splitter. The split ratio ranged between 1/1000 and 1/2000. The split point was the capillary inlet before injection. A power supply with reversible polarity (Spellman Electronics, New York, NY, USA) was used.

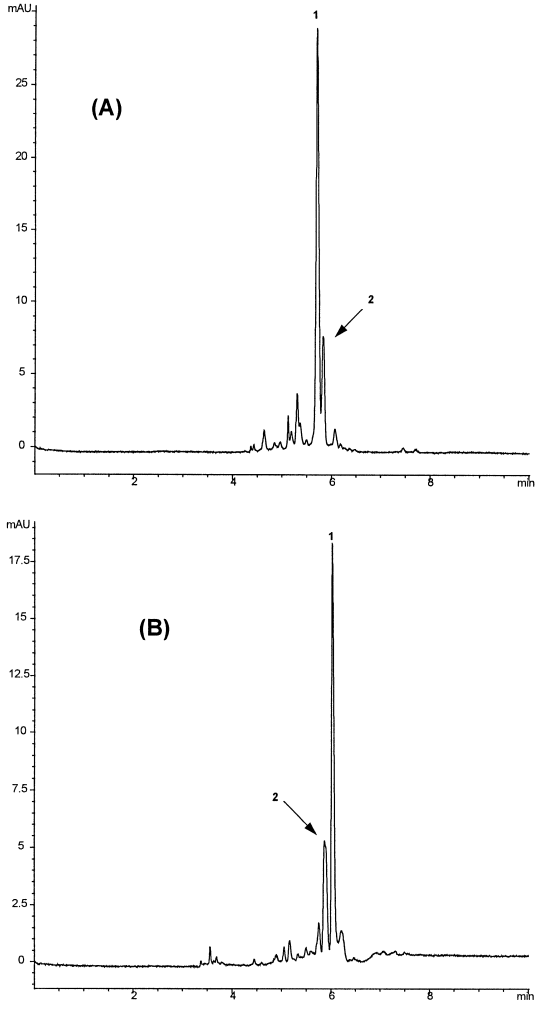

Fig. 4. Influence of the change of the mobile phase on the selectivity of the separation. CEC conditions: 25 cm×100 μm packed capillaries with Waters Sperisorb SCX/C_6, 3 μm; mobile phase: 50 m*M* phosphate, pH 2.3–acetonitrile (A) (50:50, v/v), (B) (30:70, v/v); injection: 5 kV, 6 s; UV detection: 220 nm; temperature: 20°C; voltage: 25 kV (746 V cm^{-1}); Sample: 1=peptide R126, 2=impurity.

2.2. Chemicals and stationary phases

The mixed-mode 3 μm SCX/C_6 and SCX/C_{18} were purchased from Waters Phase Separation, UK. The Hypersil ODS and Hypersil C_{18} 3 μm were obtained from Agilent Technologies. Buffer salts and analytes were purchased from Sigma–Aldrich, Deisendorf, Germany. Water was purified using a Milli-Q apparatus (Millipore, Bedford, MA, USA).

2.3. Preparation of capillary columns

Fused-silica capillaries (CS Chromatographie Service, Langerwehe, Germany) of 30 cm length were slurry packed with different stationary phases. When the progress of packing was completed, the column was equilibrated with the mobile phase to be used for CEC separations. Two frits were inserted using a

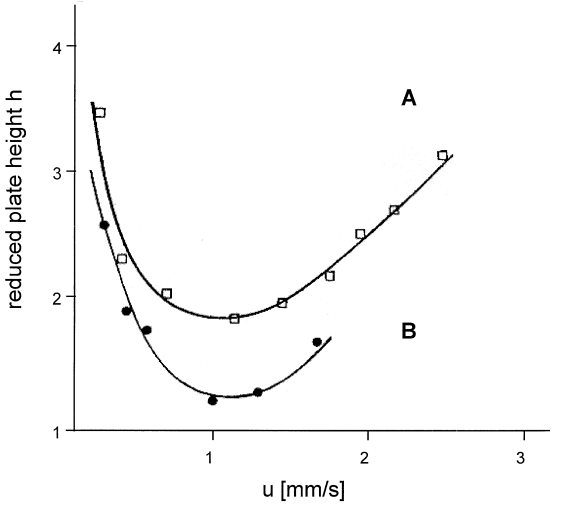

Fig. 5. Reduced plate height vs. the linear velocity in gradient nano-LC (A) and in electrically assisted gradient nano-LC (B). Conditions: 15 cm×100 μm packed capillaries with Hypersil ODS, 3 μm; mobile phase: 25 m*M* Tris–HCl, pH 8–acetonitrile (20:80, v/v); (A) without voltage, (B) with voltage: 15 kV; injection: 20 nl; UV detection: 254 nm; sample: 1=thiourea.

laboratory-made heater. The detection window was directly located behind the outlet frit of the capillary.

2.4. Peptide synthesis

Peptide synthesis was performed on a model 9050 peptide synthesizer (Milligen, Eschborn, Germany) using 9-fluorenylmethoxycarbonyl/1-hydroxybenzotriazole (Fmoc/HOBt) chemistry. Peptides were synthesized in the amide form using 9-fluoroenylmethoxycarbonyl-peptide amide linker-polyethylene glycole-polystyrene (Fmoc-PAL-PEG-PS) resin (Milligen). Synthetic peptides were purified after cleavage from the resin by reversed-phase HPLC. Identity was shown by mass analysis and co-elution with the native peptides.

2.5. Results and discussion

Peptides are potent pharmaceuticals. The intense research activities on peptides require new and highly sophisticated analytical methodologies to determine content, purity and stability of peptide drugs. CEC combines the advantages of separation techniques HPLC and CE. The high selectivity of HPLC and the high efficiency of CE result in an attractive analytical method, which can separate both neutral and charged compounds with a higher resolution and a faster analysis time than the individual one. Capillaries with appropriate packings can be applied. The mixed-mode phases consisting of a strong cation exchanger and *n*-alkyl chains on the silica surface deliver some advantages compared to conventional reversed-phase packing materials. Among these are accelerated EOF as compared to conventional RP phases and relative independency of the EOF over a wide pH range. Thus mixed-mode phases are ideal for further investigations on the separation of peptides. The measurements are carried out at acidic pH values. The peptides are positively charged and thus attracted by the negatively charged sulphonic acid groups of the mixed-mode phase.

In practice it is desirable to vary the pH of the mobile phase in terms of optimizing the separation.

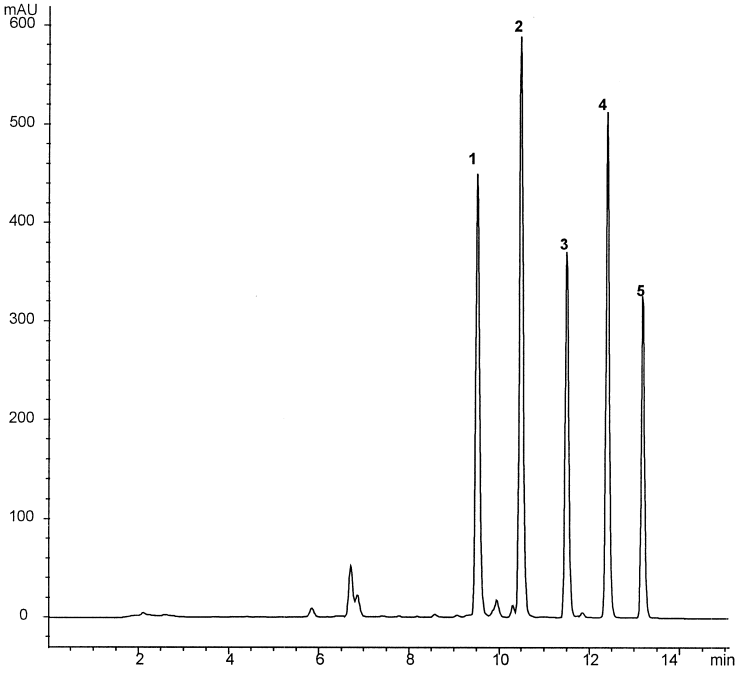

Fig. 6. Separation of *n*-alkylbenzenes with gradient-nano-LC. Conditions: 15 cm×100 μm packed capillaries with Hypersil ODS, 3 μm; mobile phase: (A) 25 m*M* Tris–HCl, pH 8, (B) acetonitrile; 20–80% B in 10 min; flow-rate: 800 nl/min; injection: 20 nl; detection: UV, 254 nm; temperature: 20°C; sample: 1=toluene, 2=ethylbenzene, 3=propylbenzene, 4=butylbenzene, 5=pentylbenzene.

In CEC the mobile phase velocity decreases when the pH of the mobile phase is lowered according to the decrease in EOF. For that reason, mixed-mode stationary phases that have both C_{18} alkyl chains and strong cation-exchange groups, propylsulfonic acid, attached to the surface have been prepared by column manufacturers. A strong cation exchanger carries sulphonic acid groups being permanently negatively charged over the whole pH range. Therefore such strong cation exchangers are expected to maintain a sufficiently high EOF over a broad pH range. In Fig. 1 the EOF is plotted versus the pH of the mobile phase for a C_{18}/SCX mixed mode-phase and a CEC-Hypersil C_{18} phase respectively.

As standard mixture the following peptides were employed: (1) H–Gly–Val–NH_2, (2) H–Val–Tyr–Val–NH_2, (3) [Met^5]-enkephalin amide, (4) [Leu^5]-enkephalin amide, (5) angiotensin II amide.

Fig. 2 shows the corresponding chromatogram. The peak-pair 2 and 3 was not baseline separated,

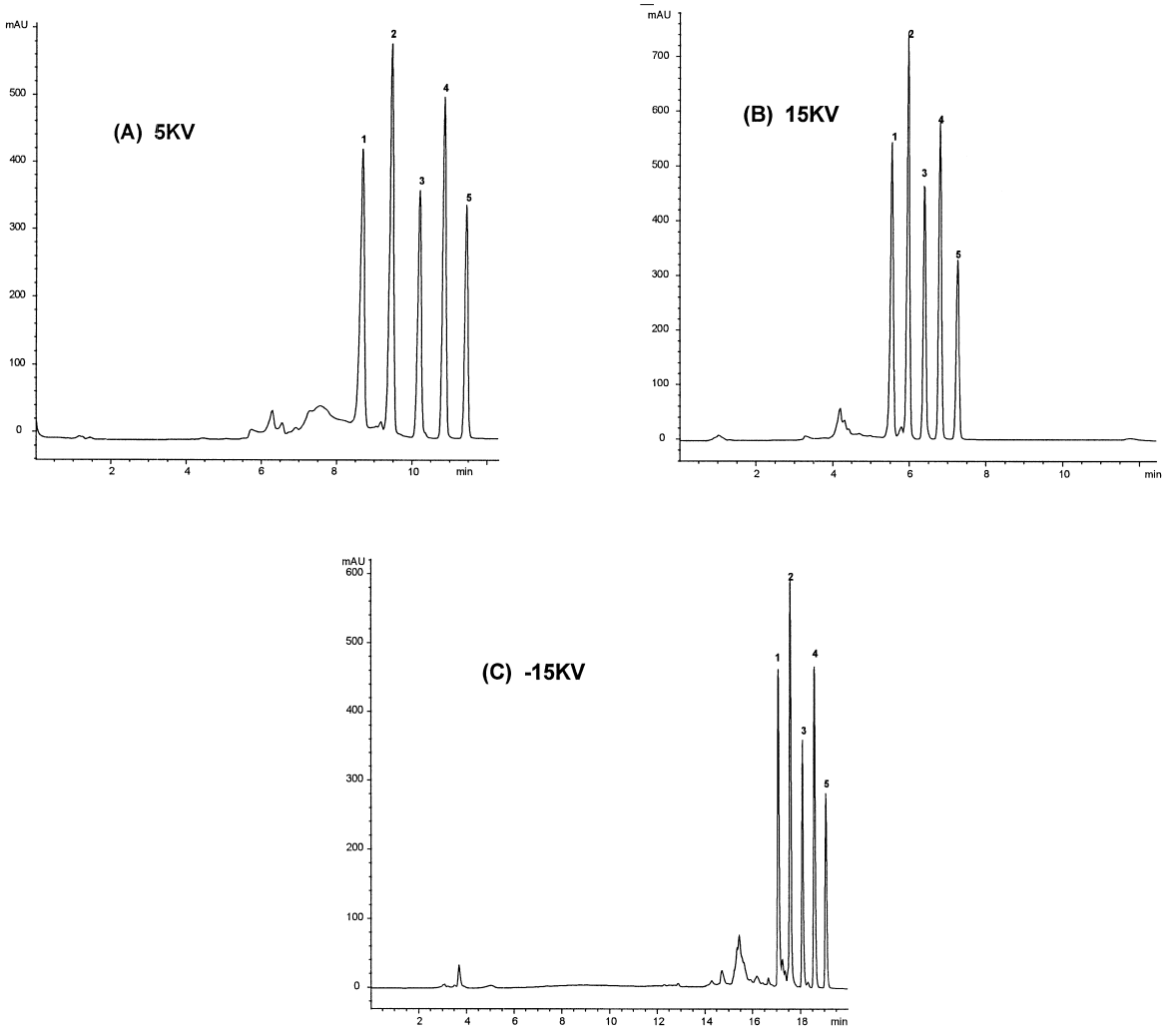

Fig. 7. Influence of the value and the polarity of the applied voltage on the retention of *n*-alkylbenzenes as analytes. Conditions: 15 cm×100 μm packed capillaries with Hypersil ODS, 3 μm; mobile phase: (A) 25 m*M* Tris–HCl, pH 8, (B) acetonitrile; 20–80% B in 10 min; flow-rate: 800 nl/min; injection: 20 nl; detection: UV, 254 nm; temperature: 20°C; voltage: (A) 5 kV, (B) 15 kV, (C) −15 kV; sample: 1=toluene, 2=ethylbenzene, 3=propylbenzene, 4=butylbenzene, 5=pentylbenzene.

because the selectivity of the C_6 chains of the stationary phase was not sufficiently hydrophobic. Moreover an ionic strength of 50 m*M* phosphate buffer (pH 2.3) was necessary supressing the interaction of peptides with the cation-exchange groups of the stationary phase. The disadvantage this system displayed is a very high current, which afforded additional thermostating of the capillary.

Changing the mobile phase to lower organic solvent content did not improve the resolution of the peak-pair 2 and 3. In the separation of peptides by gradient elution on reversed-phase silica at pH 2 trifluoroacetic acid (TFA) is often added as ion-pairing reagent. Fig. 3 shows the separation of a peptidic mixture with and without TFA in the mobile phase.

The resolution does not seem to be improving. However, the analysis time is much larger in the presence of TFA.

Fig. 4 shows a purity check of a peptide, which was purified by HPLC. Due to the higher efficiency of CEC a further impurity could be observed. The change of the mobile phase influences the selectivity such that the impurities were eluted before (A) and after (B) of the main compound.

2.6. Electrically assisted gradient nano-LC of peptides

A large number of examples is published showing the advantages of nano-LC systems as compared to conventional HPLC systems [3,21,22]. The consumption and disposal of mobile phase are minimized. Dilution of the sample caused by the smaller column-volume is lower resulting in higher detection sensitivity. Furthermore the injection volume is very small and thus the method is attractive for biochemical applications. The voltage additionally applied

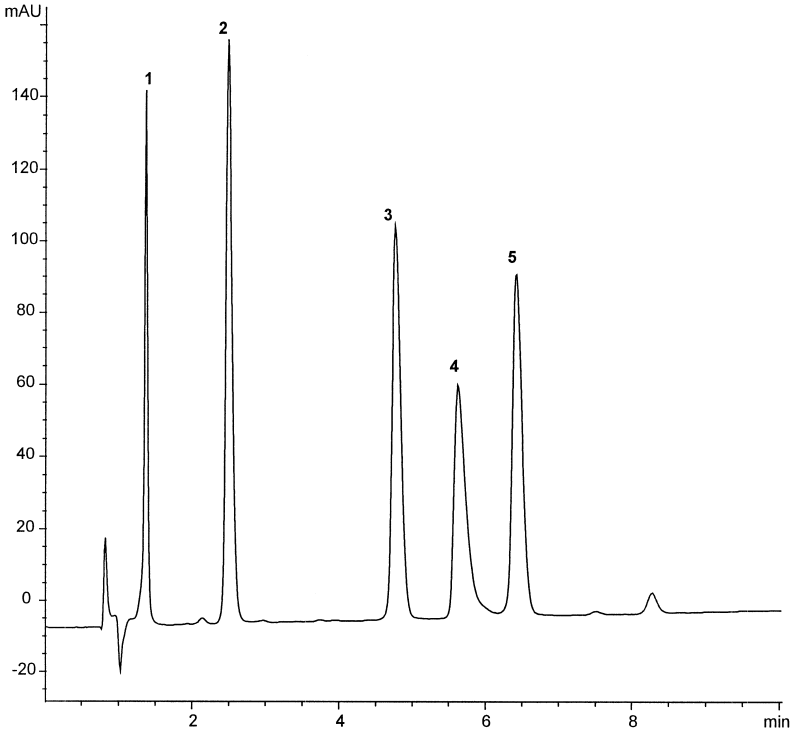

Fig. 8. Separation of peptides with gradient-nano-LC without applied voltage (EOF). CEC conditions: 15 cm×100 μm packed capillaries with Hypersil ODS, 3 μm; Mobile phase: (A) Water, 0.1% TFA–acetonitrile (95:5, v/v), (B) water, 0.1% TFA–acetonitrile (5:95, v/v), 20–40% B in 10 min; flow rate: 800 nl/min; injection: 20 nl; UV detection: 220 nm; sample: 1=H–Gly–Val–NH_2, 2=H–Val–Tyr–Val–NH_2, 3=[Met^5]-enkephalin amide, 4=[Leu^5]-enkephalin amide, 5=angiotensin II amide.

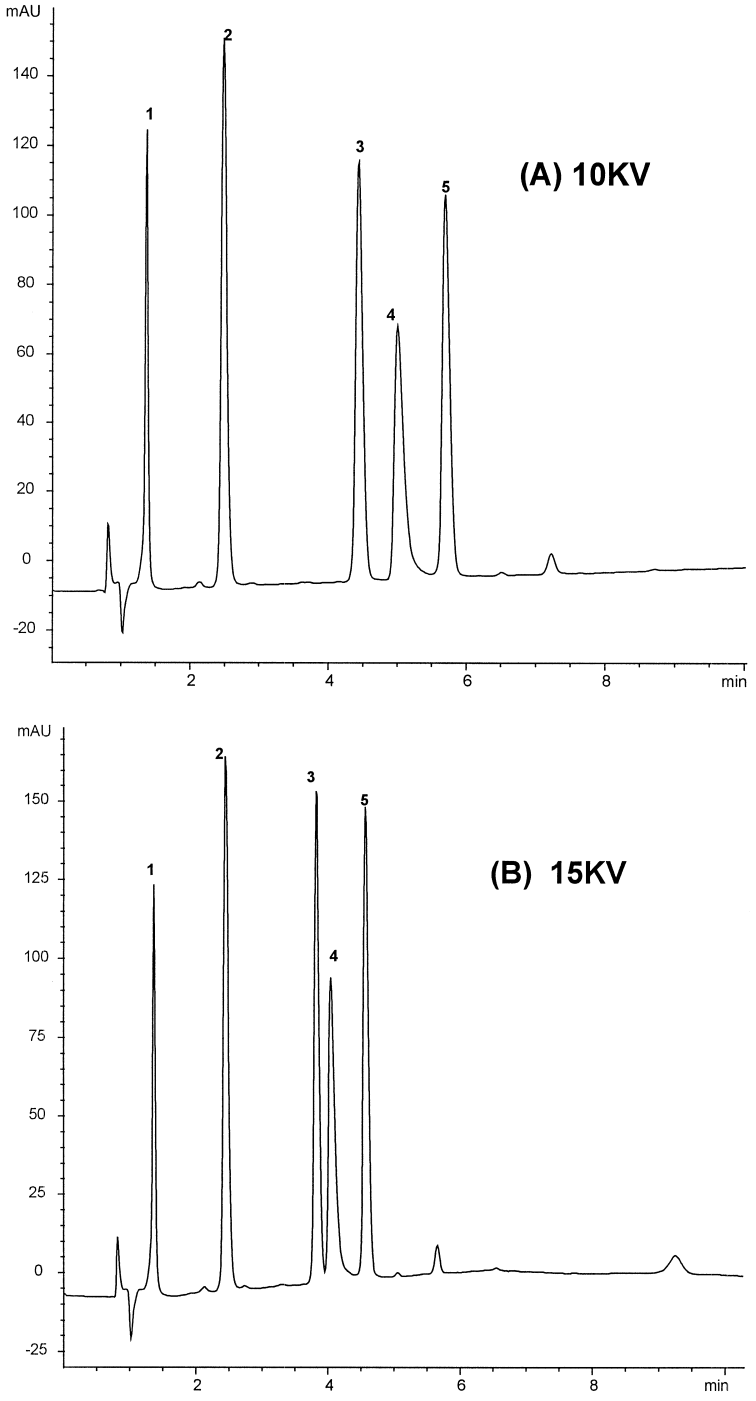

Fig. 9. Influence of the applied voltage on the separation of the peptides. CEC conditions: 15 cm×100 μm packed capillaries with Hypersil ODS, 3 μm; mobile phase: (A) water, 0.1% TFA–acetonitrile (95:5, v/v), (B) water, 0.1% TFA–acetonitrile (5:95, v/v), 20–40% B in 10 min; flow rate: 800 nl/min; voltage: (A) 10 kV, (B) 15 kV; injection: 20 nl; UV detection: 220 nm; sample: 1=H–Gly–Val–NH_2, 2=H–Val–Tyr–Val–NH_2, 3=[Met^5]-enkephalin amide, 4=[Leu^5]-enkephalin amide, 5=angiotensin II amide.

provides a powerful tool to influence selectivity, efficiency and analysis time.

Eimer et al. [21] have shown that the combination of pressure and voltage enhances both resolution and efficiency. Their investigations were restricted to the isocratic mode. Another concept which is used in CEC is a solvent gradient. This implies application of both a pressure gradient and an electrical field across the packed capillary column.

The influence of the voltage applied on reduced plate height versus linear velocity is shown in Fig. 5. The voltage caused a lower reduced plate height and a higher efficiency as compared to the pure nano-LC variant.

Fig. 6 displays the separation of five alkylbenzenes in nano-LC with gradient elution on a Hypersil ODS 3 μm column. Fig. 7 shows the influence of the voltage on efficiency and analysis time. The efficiency could be drastically improved by applying voltage during the separation. Changing the polarity of the voltage accelerated the analysis of the five alkylbenzenzes.

The results for *n*-alkylbenzenes are transferred to peptide separation.

The peptide mixture was first separated with a very shallow gradient in less than 10 min (Fig. 8). Applying a positive voltage reduced analysis time for all peptides (Fig. 9). The addition of TFA formed ion-pairs with the positively charged peptides. The separation was carried out on a Hypersil ODS, 3 μm column.

A tryptical digest of cytochrome *c* was also investigated. Fig. 10 shows the degradation products after 1 h of digestion. The voltage applied led to higher resolution and efficiency of the compounds as compared to the separation without a potential.

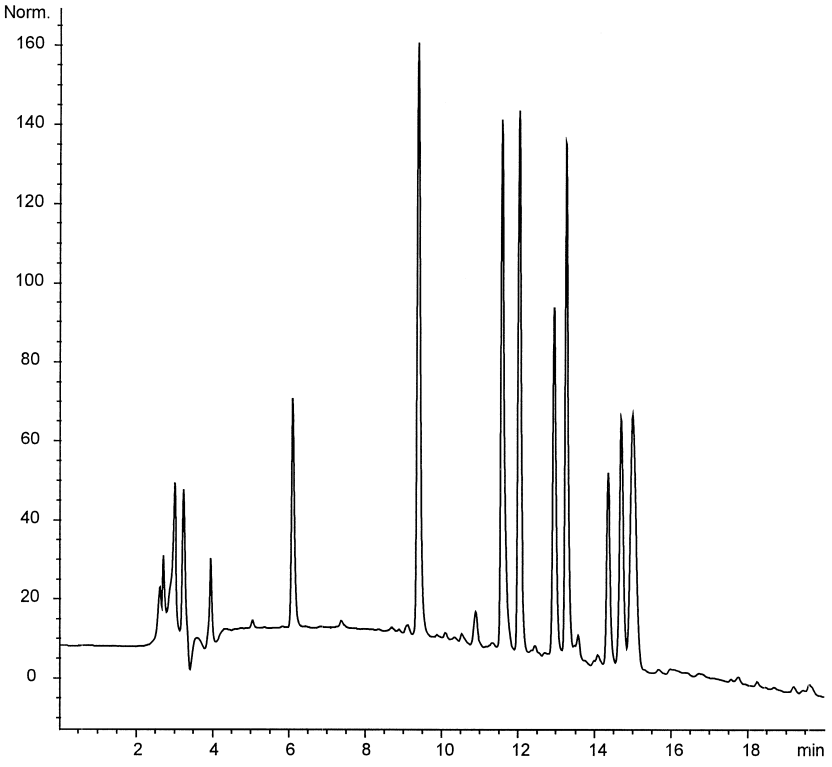

Fig. 10. Separation of tryptic digest of cytochrome *c* by electrically assisted reversed-phase gradient nano-LC. CEC conditions: 15 cm×100 μm packed capillaries with Hypersil ODS, 3 μm; mobile phase: (A) water, 0.1% TFA–acetonitrile (95:5, v/v), (B) water, 0.1% TFA–acetonitrile (5:95, v/v), 20–40% B in 10 min; flow rate: 800 nl/min; voltage: 10 kV; injection: 20 nl; UV detection: 220 nm; sample: tryptic digest of cytochrome *c*.

3. Conclusions

It was demonstrated that CEC is a promising miniaturised tool for the resolution of peptides. The mixed-mode columns in addition to the reversed-phase columns are most suitable for the separation of peptides. The advantages are displaced accelerated EOF as compared to the reversed-phase columns and relative independency of the EOF over a wide pH range. The use of gradient nano-LC causes additional selectivity as compared to the isocratic mode of CEC. Applying a potential yields shorter retention times of the peptides. By changing the polarity of the voltage applied and introducing a gradient condition the electrically assisted gradient nano-LC delivers optimum selectivity and analysis time for the peptide separations.

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