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Influence of temperature on the behaviour of small linear peptides in capillary electrochromatography ☆

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Abstract

The influence of temperature, *T*, on the retention times, peak widths, peak symmetry coefficients and theoretical plate numbers of two small linear peptides, [Met^5]enkephalin and [Leu^5]enkephalin, has been studied with capillary electrochromatography (CEC) capillary columns of 100 μm I.D. and 250 mm packed length with a total length of 335 mm, containing 3 μm Hypersil *n*-octadecyl bonded silica. With increasing column temperature from 15 to 60°C, the electroosmotic flow (EOF) and the column efficiencies increased, whereas the retention coefficients (κ_{cec}) of both peptides decreased. A linear relationship was found between the EOF value and the square root of the temperature over this temperature range, with a linear regression correlation of 0.998. Non linear Van 't Hoff plots (ln κ_{cec} versus $1/T$) were observed for these peptides between 15 and 60°C, suggesting that a phase-transition occurred with the *n*-octadecyl chains bonded on the silica surface, affecting the CEC retention behaviour of these peptides. In CEC systems, the κ_{cec} values of peptides incorporate contributions from both electrophoretic migration and chromatographic retention. Positive and negative κ_{cec} values can, in principle, thus arise with these charged analytes. However, the κ_{cec} values of the enkephalin peptides under all temperature conditions studied were positive with an eluent composed of water–50 m*M* NH_4OAc/AcOH, pH 5.2–acetonitrile (5:2:3, v/v) and therefore the chromatographic component dominates the retention process with these small peptides under these conditions.

Keywords: Temperature effects; Electrochromatography; Retention behaviour; Peak shape; Efficiency; Peptides

1. Introduction

Capillary electrochromatography (CEC) is a relatively under-explored separation technique, which combines the advantages of high-performance capillary electrophoresis (HPCE) and high-performance liquid chromatography (HPLC). CEC is a valuable complementary technique to micellar electrokinetic chromatography (MEKC) of neutral compounds. When *n*-alkylsilica sorbents are employed as the

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packing material, the separation of neutral solutes is based on their ability to partition between the *n*-alkyl chains bonded onto the silica surface and the eluent. With charged analytes, on the other hand, advantage can also be taken from the participation of an additional separation mechanism — electrophoretic migration. In this later case, it is well known that the electrophoretic migration of charged analytes is directly proportional to their charge densities and inversely proportional to their Stokes radii.

In CEC, the electroendosmotic flow (EOF) is used to drive the eluent through the packed capillary columns, with the magnitude of the EOF, when *n*-alkylsilica sorbents are used, controlled by the applied electric field strength, the composition of electrolyte solution, the temperature of the capillary column, and the charge density of the silanol groups present on the particles within the capillary column. The EOF originates at the solute–liquid interphase with the main contribution arising from the packing material per se in the capillary column with a lesser effect provided by the capillary walls [1,2]. When the applied electric field strength over the packed capillary is kept constant, the EOF increases with (i) increasing temperatures, since higher temperatures will decrease the viscosity of the eluent, increase the zeta-potential of the sorbent and capillary surfaces, as well as also increase the pH of the eluent; (ii) increasing the pH of the eluent, since higher pH values will favour a higher degree of ionisation of silanol groups; and (iii) decreasing the ionic strength of the electrolyte solution, since lower ion concentrations will cause a change in the thickness of the double layer and thereby affect the zeta-potential.

Significant fundamental CEC studies into the origin of the EOF effect have been performed by Jorgenson et al. [3,4], whilst other CEC studies have been concerned with the separation of neutral solutes, which involve only the chromatographic retention component with *n*-alkyl bonded silicas [5,6]. However, there is a growing interest in the separation of charged analytes by CEC methods as a complement to HPCE and HPLC [7], thereby combining the favourable features of both electrophoretic migration (HPCE) and chromatographic retention (HPLC). Work within our laboratories has focused on the CEC of peptides [8–10], particularly since these molecules have charge ratios that depend on their surroundings, e.g. the pH of the eluent. Moreover, small linear peptides typically have random coil, flexible structures in solution, whereas larger polypeptides can adopt different secondary structures such as α-helix or β-sheet motifs, depending on the amino acid sequence and the extent of stabilisation provided by the surrounding electrolyte, with respect to ionic strength and content of organic modifier in the eluent [11]. The retention coefficients, κ_{cec}, of neutral compounds in CEC are by definition the same as the retention factors, *k*, in HPLC [12,13], whereas the κ_{cec} values for peptides analysed by CEC will include contributions from both electrophoretic migration and chromatographic retention. The influence of capillary column temperature, *T*, on the retention behaviour of peptides in CEC has not been previously explored systematically. Accordingly, in the present study, the impact of changes in *T* on the retention behaviour of [Met^5]enkephalin and [Leu^5]enkephalin separated by CEC has been studied over the range of $T=15$°C to $T=60$°C with Hypersil *n*-octadecylsilica particles packed in capillaries with uracil as the EOF marker.

2. Experimental

2.1. Chemicals

HPLC ultra pure acetonitrile (gradient grade) was purchased from J.T. Baker (Deventer, Netherlands). Tris(hydroxymethyl)aminomethane, Tris, HCl 32%, and acetic acid (AcOH), all analytical-reagent grade were obtained from Merck (Darmstadt, Germany). Ammonium acetate (Sigma ultra) was purchased from Sigma (Deisenhofen, Germany). Deionised water was obtained with a Milli-Q water purification system (Millipore, Eschborn, Germany).

2.2. Samples

Uracil (U) (minimum 99%) was purchased from Sigma and used as the EOF marker. Ethylbenzene and *n*-butylbenzene were purchased from Aldrich (Deisenhofen, Germany) and *n*-pentylbenzene was purchased from Merck. [Met^5]Enkephalin and [Leu^5]enkephalin were purchased from Labkemi (Stockholm, Sweden). The peptides were dissolved

as stock-solutions in water (1 mg/ml) and then aliquots of the two peptides were mixed with the eluent before analysis.

2.3. Instrumentation

In these studies, a Hewlett-Packard Model HP^{3D}CE capillary electrophoresis system (Waldbronn, Germany) was used. The UV-detection was performed at 214 nm, whilst both the inlet and outlet capillary ends were pressurised at 10 bar during analysis. An applied voltage of 25 kV with a ramping time of 0.5 min was used for all runs. The CEC capillary column cassette was equilibrated for 30 min for each temperature step and the eluent in the vials was changed for each different temperature prior to the measurements.

2.4. CEC capillary columns

Hypersil *n*-octadecyl bonded silica CEC columns [250 (335) mm×100 μm I.D.], packed with 3 μm particles, were supplied by Agilent Technologies (Waldbronn, Germany). The CEC capillary columns were conditioned in 25 m*M* Tris·HCl, pH 8.0–acetonitrile (1:4, v/v) at 20°C, according to the standard procedure recommended by the manufacturer. Lifetime performance monitoring of the CEC capillary columns was carried out with the alkylbenzene test mixture before and after each temperature study with the peptides. The temperature investigations with the two peptides was performed with ammonium acetate buffer, pH 5.2, and the CEC capillary column was equilibrated for 2 h at 20°C with the eluent; water–50 m*M* NH_4OAc/AcOH, pH 5.2–acetonitrile (5:2:3, v/v) when the buffer was changed from pH 8.0 to pH 5.2. The neutral *n*-alkylbenzene samples were injected electrokinetically at 5 kV for 6 s as described elsewhere [14] for this test system, whilst the peptide samples were injected electrokinetically at 10 kV for 10 s.

2.5. Eluents

The eluents were prepared by adjusting the buffer to the desired pH and then mixing with an appropriate volume of acetonitrile. The ammonium acetate buffer was adjusted with acetic acid to pH 5.2. The eluents were then prepared by adding 3 ml of acetonitrile to 2 ml of 50 m*M* NH_4OAc/AcOH, pH 5.2, and finally 5 ml of water was added. The Tris buffer was adjusted to pH 8.0 with HCl, with the eluent for the test mixture prepared by adding 8 ml of acetonitrile to 2 ml of 25 m*M* Tris·HCl, pH 8.0. All eluents were filtered through 0.2 μm PTFE filters, Nalgene, Labotec (Wiesbaden, Germany).

2.6. Performance of the CEC capillary columns as monitored by a n-alkylbenzene test mixture

The performance of the Hypersil *n*-octadecyl bonded CEC capillary columns were independently monitored during each set of temperature studies with [Met^5]enkephalin and [Leu^5]enkephalin using a previously developed test system [14] containing several neutral analytes (ethyl-, *n*-butyl- and *n*-pentylbenzenes). These performance characteristics were determined with these *n*-alkylbenzenes at 20°C with an eluent of 25 m*M* Tris·HCl, pH 8.0–acetonitrile (1:4, v/v).

3. Results and discussion

3.1. Impact of temperature on various parameters in CEC

3.1.1. Effect of temperature on the performance of the CEC capillary columns

For these CEC studies with [Met^5]enkephalin and [Leu^5]enkephalin, a buffer composed of water–50 m*M* NH_4OAc/AcOH, pH 5.2–acetonitrile (5:2:3, v/v) was used over the temperature range of 15–60°C in increments of 5°C with the CEC capillary columns equilibrated for 30 min at each temperature. Fresh eluents were used every time the temperature condition was changed. In order to ensure that the performance of the CEC capillary columns containing Hypersil *n*-octadecylsilica remained consistently high and reproducible when used over extended periods of time as this operational condition was varied, our previously developed *n*-alkylbenzene (ethyl-, *n*-butyl- and pentyl-) neutral analyte test system [14] was employed before and after each set of temperature studies with [Met^5]enkephalin and [Leu^5]enkephalin. The theoretical plate number, *N*,

peak symmetry coefficients, λ_{sym}, and retention coefficients, κ_{cec} values, for the *n*-alkylbenzenes were recorded for each CEC capillary column concurrently during the peptide studies. The relative standard deviations, RSDs, of the κ_{cec} values were found to be <1%, and the RSD values of the peak efficiencies <6%, for sets of replicates ($n \geq 6$). The performance of the Hypersil *n*-octadecyl bonded CEC capillary columns with respect to the retention mechanism for *n*-alkylbenzenes was thus considered to be the same before and after the temperature studies with the selected peptides.

3.1.2. Effect of temperature on the EOF

As evident from Fig. 1, the EOF was found to be a linear function of the square root of the temperature, with the excellent correlation between the experimental data when the measurements were carried out over ascending or descending temperature values. The linear regression correlation for the ascending temperature plot was 0.9988 and 0.9979 for the descending temperature plot. The value of EOF was almost twice as high at 60°C (1.3 mm/s) compared to 15°C (0.7 mm/s) at pH 5.2. The temperature, *T*, affects the pH value and viscosity of the eluent, the

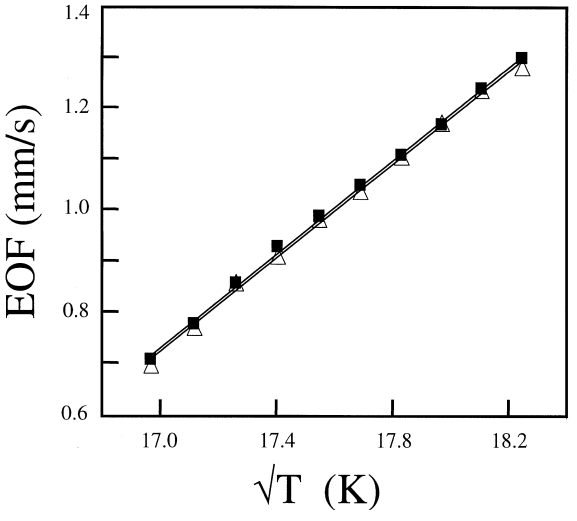

Fig. 1. Plot of the EOF versus $\sqrt{T}$ for the ascending (–■–■–) and descending (–△–△–) temperature measurements with a Hypersil *n*-octadecyl bonded silica capillary column. The eluent was water–50 m*M* NH_4OAc/AcOH, pH 5.2–acetonitrile (5:2:3, v/v) and uracil was used as the EOF marker. Other details are given in Section 2.

dielectric constant, the zeta-potential and the equilibrium constants of partitioning processes. The viscosity (η) is empirically related to the temperature and can be represented by the Arrhenius equation:

$$\eta = Ae^{\Im/RT} \tag{1}$$

where $\Im$ is an activation energy related to the heat of evaporation (mole cohesion) and *A* is a weakly temperature dependent pre-exponential factor, *T* is the absolute temperature and *R* is the universal gas constant [15]. The EOF is directly proportional to the zeta-potential (ζ) and inversely proportional to the viscosity (η) as shown in Eq. (2),

$$u_{eo} = \frac{\epsilon_o \epsilon_r \zeta E}{\eta} \tag{2}$$

where ϵ_o is the permittivity of the analyte in a vacuum, ϵ_r is the relative permittivity (dielectric constant) of the eluent, and *E* is the electric field strength (kV/m). In free zone electrophoresis, the product of the dielectric constant and the zeta potential has been found to be essentially independent of temperature, with the dielectric constant of water-based eluents decreasing as the temperature increases [16–19]. The zeta-potential (ζ) is a function of the thickness of the double layer (δ) according to the following equation:

$$\zeta = \frac{\delta\sigma}{\epsilon_r \epsilon_o} \tag{3}$$

where σ is the superficial excess charge density. The δ term, in turn, is related to *T* according to the following relationship:

$$\delta = \sqrt{\frac{\epsilon_r \epsilon_o RT}{2cF^2}} \tag{4}$$

where *c* is the molar concentration and *F* is the Faraday constant [5,18]. The contribution to the increase in EOF with increasing temperature is mainly dependent on the zeta-potential, which is thus predicted to be proportional to the square root of the temperature, i.e. $\sqrt{T}$. The decrease in the viscosity with increasing *T* will have little effect on the EOF compared to the impact of *T* on ζ, which results in an empirical linear relationship linking the EOF value with $\sqrt{T}$. These findings confirm and extend previous studies on the influence of temperature on the

EOF in CEC as reported by other investigators [20–26].

3.1.3. Temperature effects on the retention of peptides in CEC

In reversed-phase HPLC, the retention of peptides has been frequently been observed to be shorter at elevated temperature than at room temperature, with typically non-linear Van 't Hoff behaviour evident [27–31]. When analytes manifest only chromatographic retention behaviour, their retention coefficients can be rigorously defined in physicochemical terms for the system from the retention factors, k'. Under such conditions, the k' value can then be used to compare the chromatographic retention behaviour of analytes with one stationary phases to another one. The chromatographic retention of analytes can then be related to the temperature, T, according to the following equation:

$$k' = \frac{t_r - t_o}{t_o} = K_a \Phi = \Phi e^{-\Delta G_{assoc}/RT} \quad (5)$$

where t_r is the chromatographic retention time of a peptide and t_o is the time taken for a non-retained component to move through the capillary column with the velocity of the eluent. The extent of retention of the peptide is determined by the equilibrium distribution constant, K_a, and the phase ratio, Φ, of the chromatographic system, whilst the ΔG_{assoc} is the Gibbs free energy change associated with the solute–surface interactions of the analyte, R is the universal gas constant and T is the absolute temperature (K). If no change in the conformation of an analyte, such as a peptide, is induced by the temperature change, then in reversed-phase HPLC the Van 't Hoff plots (i.e. ln k' versus $1/T$) can be linear but more frequently follow curvilinear dependencies. In our associated studies, we have previously documented numerous examples of polypeptides and proteins separated by reversed-phase HPLC procedures where non-linear Van 't Hoff behaviour has been observed [27–31], indicative of thermodynamic processes that are characteristic of *homo*- and *hetero*-thermic behaviour where the change in heat capacity of the system is not equal to zero and temperature-dependent.

In contrast, in CEC the apparent migration of a peptide is the sum of the apparent chromatographic retention factor and the apparent electrophoretic mobility. From a physicochemical perspective the CEC migration factor, κ_{cec}, of a peptide can also be defined in terms of the overall distribution constant, K_{cec} and the phase ratio of the system, in a manner analogous to that employed to describe to link the k' value to K_a, and Φ for a solely chromatographic system [10,32]. As far as CEC experiments are concerned, a linear ln κ_{cec} versus $1/T$ relationship has previously been observed [23] for several phenylthiohydantoin (PTH) amino acid derivatives at pH 7.55. However, for PTH-amino acid derivatives as well as for higher-molecular mass charged polypeptides, the CEC retention coefficients, κ_{cec} values, with *n*-alkylsilica sorbents will also include contributions from both electrophoretic migration as well as chromatographic retention, i.e. from both silanophilic and hydrophobic effects. Accordingly, the sign of κ_{cec} values for polypeptides and proteins can be positive or negative, depending on which contribution dominates and the net charge of the analyte under a specific set of CEC conditions. As evident from Fig. 2a, the κ_{cec} values decreased for [Met^5]enkephalin and [Leu^5]enkephalin with increasing T, in part because the electrophoretic migration increased with increasing T due to the decrease in viscosity of the eluent. It is noteworthy from the above results that despite their inability to assume stabilised secondary structures in solution (and thus these peptides will exist predominantly in random coil conformations in the buffer used in the present studies), these enkephalin peptides exhibit non-linear dependencies of ln κ_{cec} versus $1/T$ in their Van 't Hoff plots. Over specific temperature ranges, pseudo-linear behaviour can be discerned, i.e. between 15 and 30°C or between 40 and 60°C, as shown in Fig. 3, with the linear regression coefficients over the narrower temperature interval from 15 to 30°C of 0.96 for the ascending and 0.98 for the descending temperature measurements and over the temperature interval from 40 to 60°C of 0.96 for the ascending and 0.99 for the descending temperature measurements. The discontinuity evident in these plots of ln κ_{cec} versus $1/T$ between 30 and 40°C could be caused by a phase transition of the bonded *n*-octadecyl chains at the surface of the Hypersil ODS packing. Associated studies with neutral peptides as well as with various *n*-alkylbenzenes have examined

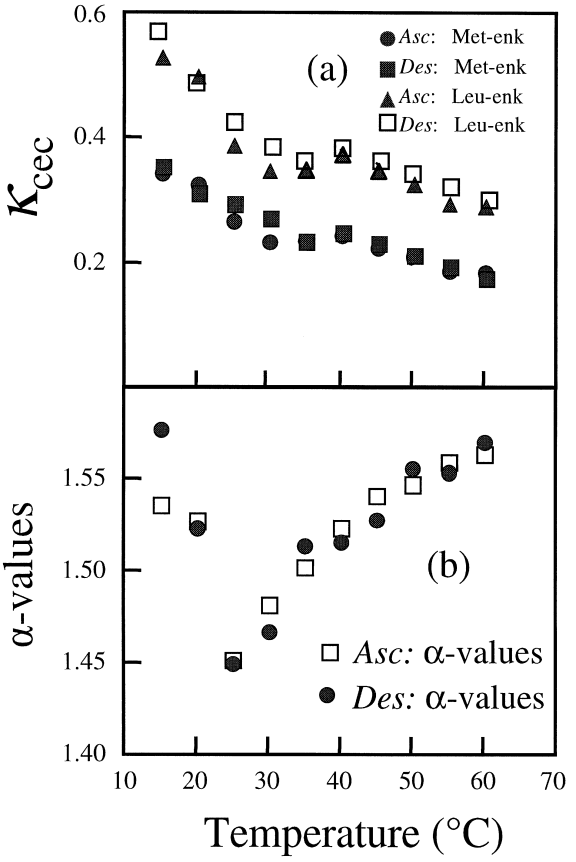

Fig. 2. (a) Effect of T on the κ_{cec} as plots of κ_{cec} versus T values for [Met^5]enkephalin (Met-enk) (–■–■– and –●–●–) and [Leu^5]enkephalin (Leu-enk) (–▲–▲– and –□–□–) separated under CEC conditions on a Hypersil *n*-octadecyl bonded silica capillary column. The CEC retention studies for the determination of the κ_{cec} values were performed with ascending (*Asc*: –■–■– and –▲–▲–) and descending (*Des*: –●–●– and –□–□–) temperature measurements under the same conditions as described in Fig. 1. (b) Effect of T on selectivity as determined from the plots of the α-values ($\kappa_{cec,2}/\kappa_{cec,1}$) versus T measured under ascending (*Asc*: –□–□–) and descending (*Des*: –●–●–) temperature conditions where $\kappa_{cec,2}$ relates to [Leu^5]enkephalin and $\kappa_{cec,1}$ relates to [Met^5]enkephalin.

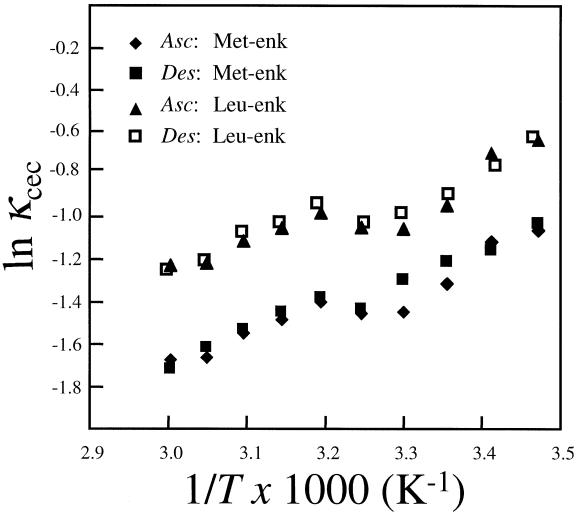

Fig. 3. Plots of ln κ_{cec} versus $1/T$ ($\times$1000) for [Met^5]enkephalin and [Leu^5]enkephalin separated under CEC conditions on a Hypersil *n*-octadecyl bonded silica capillary column between 15 and 60°C. The CEC data were acquired with ascending (*Asc*) and descending (*Des*) temperature measurements for [Met^5]enkephalin (Met-enk) (–♦–♦– and –■–■–) and [Leu^5]enkephalin (Leu-enk) (–▲–▲– and –□–□–) respectively under the same conditions as described in Fig. 1 in temperature intervals of 5°C.

further this possibility. Morel and Serpinet have identified [33] similar behaviour in their studies with bulk C_{18}- and C_{20}-bonded silicas by thermogravimetry, differential thermal analysis and differential scanning calorimetry. The studies by Schunk and Burke [34], as well as McGuffin et al. [35], with *n*-alkyl bonded silica columns have also provided evidence of a transition temperature between 298 and 308 K, which results in a step in the plot of ln k' versus $1/T$. This transition temperature of the stationary phase is dependent on the pore size of the parent silica and the ligand density, occurring on long-chain *n*-alkyl silica with $n>16$. As the dependency of κ_{cec} on column temperature for [Met^5]enkephalin and [Leu^5]enkephalin was examined under conditions whereby the chromatographic retention mechanism was dominant over electrophoretic migration, with these small peptides existing in solution without any preferred secondary structure(s), it seems highly probably that the effects observed in the present study are due to a phase change of the *n*-octadecyl chains. Overall, these non-linear dependencies of ln k' versus $1/T$, containing a plateau-like region between 30 and 40°C, suggest that a temperature-dependent phase-transition may have occurred with these CEC systems. This effect is probably caused by structural reorganisation of the *n*-octadecyl chains bonded to the silica surface, since these small linear peptides of only five amino acid-residues have very flexible, random coil conformations in the eluent.

In HPCE, the heights equivalent to a theoretical plate (HETP values) are only dependent on the longitudinal diffusion term (B) and therefore the efficiency of the capillary column decreases with increasing T. Our experience from the temperature studies with [Met^5]enkephalin and [Leu^5]enkephalin in CEC is that the capillary column efficiency increases with increasing T with the ammonium acetate buffer, pH 5.2. Thus, the contribution to HETP in our separation system will be dependent on all three terms in the van Deemter equation, namely the eddy diffusion A term, longitudinal diffusion B term, and the mass transfer C term. As shown in Fig. 2b, the α values for the separation of [Met^5]enkephalin and [Leu^5]enkephalin, i.e. $\kappa_{\text{cec[Leu5]enkephalin}}/\kappa_{\text{cec[Met5]enkephalin}}$ decreased rapidly between 15 and 25°C, followed by an increase in the α values with increasing T. Again, the experimental data obtained for ascending or descending temperature measurements highly correlated. These enkephalin peptides were baseline resolved at 20°C with broadened peak widths but with a high α value, whilst at 60°C the peak shape of the peptides was sharper as shown in chromatogram in Fig. 4.

3.2. Temperature effects on the capillary column efficiency

The effect of the temperature on the CEC capillary column efficiency of benzodiazepines has previously been studied by Cahours et al. [22] using Tris·HCl, pH 8.0–acetonitrile (2:3, v/v). These investigators reported that decrease in capillary column efficiency occurred with increasing T from 20 to 35°C with phenyl bonded silica packed capillaries, and concluded that the influence of T in CEC can be solely interpreted in terms of the capillary electrophoretic behaviour of different analytes and separation conditions. In contrast, our results indicate that the effect of T on peak efficiency depends very much on whether the dominating parameter is electrophoretic migration or chromatographic retention of the solute. The kinetic properties, as revealed from peak width and theoretical plate measurements, of [Met^5]enkephalin and [Leu^5]enkephalin are illustrated in Fig. 5. The capillary column efficiency, as N values, increased (Fig. 5a) as the peak width decreased (Fig. 5b) with elevated temperature for

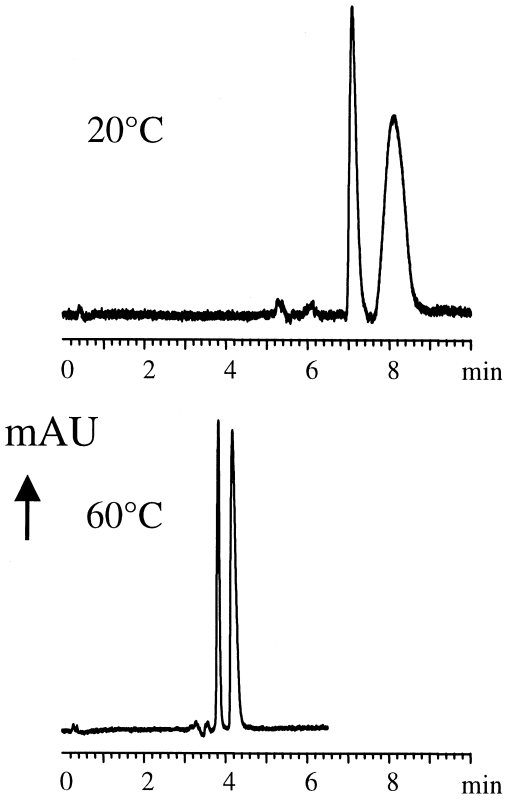

Fig. 4. CEC elution profiles for [Met^5]enkephalin (first eluting peak) and [Leu^5]enkephalin (second eluting peak) separated under CEC conditions on a Hypersil n-octadecyl bonded silica capillary column at 20 and 60°C. The eluent was water–50 mM NH_4OAc/AcOH, pH 5.2–acetonitrile (5:2:3, v/v).

these peptides with an eluent composed of water–50 mM NH_4OAc/AcOH, pH 5.2–acetonitrile (5:2:3, v/v). The peak widths and theoretical plate numbers of [Met^5]enkephalin and [Leu^5]enkephalin were calculated by the software within the HP Chem-Station according to the 5-sigma method, since the peptide peaks did not have a Gaussian shape. In contrast to the peak width or N values, the symmetry factors, λ_{sym}'s, of the enkephalin peptide peaks were not significantly affected by temperature changes >30°C. Although good peak symmetry was observed for [Leu^5]enkephalin at low temperature, since the peak width was large a low peak efficiency was obtained. The average of the peak areas of [Met^5]enkephalin and [Leu^5]enkephalin for three replicates at each temperature was calculated and the RSD values of the peak areas differed by less than

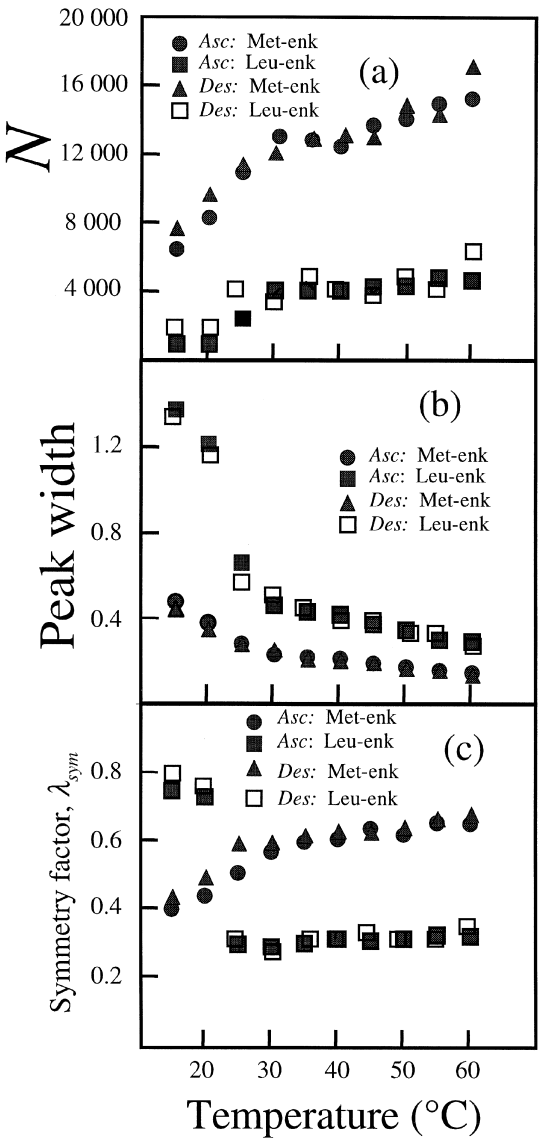

Fig. 5. Influence of temperature on the column efficiency for [Met^5]enkephalin (Met-enk) (ascending temperature, *Asc*: –●–●– and descending temperature, *Des*:–▲–▲– respectively) and [Leu^5]enkephalin (Leu-enk) (ascending temperature, *Asc*: –■–■– and descending temperature, *Des*: –□–□– respectively) separated under CEC conditions on a Hypersil *n*-octadecyl bonded silica capillary column, as determined from the change in theoretical plate numbers per column, peak width and peak symmetry coefficients as a function of *T*. (a) Number of plates per column, *N*, versus *T*; (b) Peak width versus *T*; (c) Symmetry coefficient, λ_{sym}'s, of the peak versus *T*. The number of plates and the peak widths were determined according to the 5-sigma method described in the extended performance report of the HP ChemStation.

2% when the same amount of peptide was electrokinetically injected. With this plug flow injection approach bandbroadening variations due to extracapillary effects could be minimised. Moreover, this injection strategy provided a practical approach in terms of reproducibility since the inlet of the capillary column was always kept at ambient temperature due to the construction of the instrument.

4. Conclusions

Based on the experimental data described in this paper, it can be concluded that it is advantageous to perform CEC separations with peptides at elevated temperatures, since the peak shape and capillary column efficiencies improve, whilst shorter analysis times can be achieved. Moreover, the findings of our temperature study with [Met^5]enkephalin and [Leu^5]enkephalin performed with *n*-octadecylsilica CEC capillary columns with an ammonium acetate, pH 5.2, buffer confirm that the EOF and peak efficiency increase and the retention times decrease as the *T* was increased. In addition, linearity between the EOF and $\sqrt{T}$ was found for temperatures between 15 and 60°C, which is in agreement with the theoretical predictions. Arising from these investigations, the opportunity now exists to further elucidate the thermodynamic basis of polypeptide and protein behaviour in CEC systems. Results of these studies will be reported in subsequent papers [10,36].

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References

[1] M.M. Dittmann, G.P. Rozing, J. Chromatogr. A. 744 (1996) 63.

[2] M.M. Dittmann, G.P. Rozing, G. Ross, T. Adam, K.K. Unger, J. Cap. Electrophoresis 4 (1997) 201.
[3] J.W. Jorgenson, K.D. Lukacs, J. Chromatogr. 218 (1981) 209.
[4] J.W. Jorgenson, K.D. Lukacs, Anal. Chem. 53 (1981) 1298.
[5] J.H. Knox, I.H. Grant, Chromatographia 32 (1991) 317.
[6] N.W. Smith, M.B. Evans, Chromatographia 38 (1994) 649.
[7] I.S. Lurie, T.S. Conver, V.L. Ford, Anal. Chem. 70 (1998) 4563.
[8] K. Walhagen, K.K. Unger, A.M. Olsson, M.T.W. Hearn, J. Chromatogr. A 853 (1999) 263.
[9] T. Adam, K.K. Unger, (2000) in preparation.
[10] K. Walhagen, K.K. Unger, M.T.W. Hearn, J. Chromatogr. A 887 (2000) 165.
[11] M.T.W. Hearn, in: J.C. Janson, L. Ryden (Eds.), Protein Purification, Wiley-VCH Publ. Inc, New York, N.Y, 1998, p. 239.
[12] A.S. Rathore, Cs. Horváth, J. Chromatogr. A. 743 (1996) 231.
[13] F. Lelièvre, C. Yan, R.N. Zare, P. Gareil, J. Chromatogr. A 723 (1996) 145.
[14] K. Walhagen, K.K. Unger, M.T.W. Hearn, J. Chromatogr. A, (2000) in press.
[15] R.T. Lerner, G.L. Trigg, in: Encyclopedia of Physics, 2nd ed, VCH, New York, 1991, p. 1072.
[16] I. Watanabe, N. Ui, M. Nakamura, J. Phys. Colloid Chem. 54 (1950) 1366.
[17] J.H. Knox, K.A. McCormack, Chromatographia 39 (1994) 207.
[18] S. Hjertén, Chromatogr. Rev. 9 (1967) 174.
[19] J.C. Reijenga, Chromatographia 38 (1994) 658.
[20] M.M. Dittmann, G. P Rozing, J. Microcol. Sep. 5 (1997) 399.
[21] N.M. Djordjevic, P.W.J. Fowler, F. Houdiere, G. Lerch, J. Liq. Chromatogr. Rel. Technol. 21 (1998) 2219.
[22] X. Cahours, Ph. Morin, M. Dreux, J. Chromatogr. A 845 (1999) 203.
[23] C.G. Huber, G. Choudhary, Cs. Horváth, Anal. Chem. 69 (1997) 4429.
[24] I.S. Lurie, R.P. Meyers, T.S. Conver, Anal. Chem. 70 (1998) 3255.
[25] M.R. Euerby, C.M. Johnson, S.F. Smyth, N. Gillot, D.A. Barret, P.N. Shaw, J. Microcol. Sep. 11 (1999) 305.
[26] P.D.A. Angus, E. Victorino, K.M. Payne, C.W. Demarest, T. Catalano, J.F. Stobaugh, Electrophoresis 19 (1998) 2073.
[27] R.I. Boysen, Y. Wang, H.H. Keah, M.T. W Hearn, Biophys. Chem. 77 (1999) 79.
[28] M.T.W. Hearn, in: S. Ahuja (Ed.), Handbook of Bioseparation, Academic Press, 2000, p. 75.
[29] M.T.W. Hearn, G.L. Zhao, Anal. Chem. 71 (1999) 4874.
[30] M.T.W. Hearn, in: K.M. Gooding, F.E. Regnier (Eds.), HPLC of Biopolymers, Marcel Dekker, New York, 2000, in press.
[31] R.I. Boysen, A. Jong, Y. Wang, M.T.W. Hearn, (2000) in preparation.
[32] M.G. Khaledi, S.C. Smith, J.K. Stasters, Anal. Chem. 63 (1991) 1820.
[33] D. Morel, J. Serpinet, J. Chromatogr. 200 (1980) 95.
[34] T.C. Schunk, M.F. Burke, Int. J. Environ. Anal. 25 (1986) 81.
[35] V.L. McGuffin, C.E. Evans, S.H. Chen, J. Microcol. Sep. 5 (1993) 3.
[36] K. Walhagen, K.K. Unger, M.T.W. Hearn, (2000) in preparation.