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Capillary electrochromatography with thermo-optical absorbance detection for the analysis of phenylthiohydantoin-amino acids

Ming Qi, Xing-Fang Li, Costas Stathakis, Norman J. Dovichi*

Department of Chemistry, University of Alberta, Edmonton, Alberta, T6G 2G2 Canada

Abstract

Capillary columns were packed with 3 μm C_{18} stationary phase, interfaced with an ultraviolet-laser based thermo-optical absorbance detector, and evaluated for separation of a mixture of phenylthiohydantoin–amino acids. These columns demonstrated consistent performance with a relative standard deviation (RSD) for migration time of less than 1.5% and a separation efficiency of 216 000 plates/m for the electroosmotic flow marker, thiourea. The thermo-optical absorbance detector was based on a 248 nm krypton–fluoride excimer laser. Detection limits (3σ) ranged from 1.6 to 4.8×10^{-7} *M* phenylthiohydantoin (PTH)–amino acid injected onto the column, which is a factor of three superior to those obtained in micellar electrokinetic chromatographic analysis of these compounds. A mixture of 17 PTH amino acids was injected onto the capillary; 13 components were nearly baseline resolved in 14 min.

Keywords: Thermo-optical absorbance detection; Detection; Electrophoresis; Amino acids; PTH derivatives

1. Introduction

Capillary electrochromatography (CEC) combines attributes of micro-high-performance liquid chromatography (μHPLC) and capillary zone electrophoresis (CZE). Like μHPLC, CEC uses a capillary column packed with conventional chromatographic packing materials. Like CZE, CEC uses electroosmotic flow to deliver the mobile phase through the column instead of using an HPLC pump. Neutral analytes are separated based on their differential partitioning between the stationary and mobile phases, whereas charged compounds are separated by differential partition and electrophoretic mobilities [1]. These two processes allow manipulation of the separation selectivity.

One of the major features of CEC is a higher separation efficiency than with μHPLC under the same conditions. High separation efficiency in CEC is attributed to the plug-like flow velocity of electroosmotic flow. Due to the high surface area of the packing beads, the electroosmotic flow in CEC is mainly generated by the electrical double layer on the liquid–solid interfaces of the reverse phase modified silica beads rather than that of the capillary wall.

During initial development of CEC, technical aspects related to column packing and frit fabrication have been explored [2–4] and its application has been demonstrated mainly for the separation of neutral compounds such as aromatic hydrocarbons [5]. Fundamental aspects concerning electroosmotic flow, band broadening, and separation mechanisms within a packed bed have been investigated and proposed [1,6,7]. To extend the application of this separation technique to biological substances and pharmaceuticals, different types of columns and instrument modifications have been developed in

*Corresponding author.

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recent years. For example, mixed-mode columns in which the packing material combined both a cation-exchanger and a reverse phase allowed the use of a low pH buffer to separate basic pharmaceuticals [8]. Huber and Horváth applied an HPLC gradient elution technique with CEC to separate twelve phenylthiohydantoin (PTH)–amino acids with a mobile phase containing only 30% acetonitrile [9]. Wu and Lubman demonstrated pressurized CEC to separate peptides in a protein digest [10].

CEC is still not widely used in laboratories today for several reasons. Column packing requires trained personnel and sophisticated equipment. Furthermore, the packing procedures are not well characterized and standardized. Instability or irreproducible performance and easy breakage of columns are common problems [4,5]. While CEC columns are commercially available, prices are high due to the extensive labor required for production. Last, the small injection volume results in mediocre concentration detection limit. Because of the short optical path length across the capillary, conventional transmission detection leads to limited sensitivity [11].

The objectives of this study are to address some practical problems in the preparation of CEC columns and to demonstrate the use of a photothermal absorbance detector for CEC separation. We report the characterization of laboratory-made CEC columns and demonstrate their performance using the reproducibility of migration time, theoretical plate numbers, and resolution. We also report the use of a photothermal ultraviolet absorbance detector for the analysis of phenylthiohydantoin amino acids. These amino acid derivatives are produced as the end product of the Edman degradation reaction in protein sequencing.

2. Experimental section

2.1. Material and reagents

Enzyme grade 4-morpholineethanesulphonic acid (MES) and sodium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ, USA). HPLC-grade acetonitrile (ACN) was purchased from EM Science (Gibbstown, NJ, USA). Thiourea was purchased from Sigma (Ontario, Canada). PTH–Amino acids were purchased from ABI (Foster City, CA, USA). The ODS1 3 μm packing material was a kind gift from Dr. Henry Chen of Waters (Franklin, MA, USA). Water used to prepare the buffer was filtered using a Milli-Q purification system (Millipore, Bedford MA, USA). The CEC mobile phase was sonicated for 5 min and filtered though a 0.2 μm Millex PTFE filter (Millipore) prior to use. The test mixture contains thiourea used as an electroosmotic flow marker and PTH–asparagine (N), PTH–leucine (L), PTH–tryptophan (W), PTH–glutamic acid (E); PTH–amino acids were prepared as reported earlier [12]. The stock solution of each compound was prepared in acetonitrile at a concentration of 10^{-3} *M*. The concentration of each compound in the test mixture was $5 \cdot 10^{-5}$ *M* in CEC mobile phase.

2.2. Column preparation

CEC columns used in this report were prepared using 50 μm I.D.×182 μm O.D. fused-silica capillaries (Polymicro Technology, Phoenix, AZ, USA). Our packing procedures were similar to those reported by Smith and Evans [13] with some modifications as follows. Before packing, a temporary frit was made by dipping one end of the capillary into a 5 μm silica gel pre-wetted with water, and this end was heated mildly on a micro-torch flame after drying at ambient temperature. This frit was used to retain the reverse phase particles during the packing process. The temporary frit was tested for mechanical stability and porosity by pumping acetonitrile at a pressure of 1000 pounds per square inch (p.s.i.) into the capillary (1 p.s.i.=6894.76 Pa). An HPLC pump (Waters 515) with a maximum pressure of 6000 p.s.i. was used for slurry packing instead of a special capillary column packer. The slurry of reverse phase material was made by mixing 10 mg ODS1 3 μm particles with 1 ml acetonitrile. After 5 min of sonication, the slurry was transferred into an empty HPLC column (70 mm×4.6 mm I.D.) serving as a slurry reservoir. Sonication of the slurry reservoir was omitted. The capillary was connected to the reservoir with a polyether ether ketone (PEEK) sleeve tubing (1.6 mm O.D.×180 μm I.D.) and column fittings. The C_{18} particles were pumped into the capillary at pressures up to 5000 p.s.i. The

flow-rate was below 0.010 ml/min without the use of a splitter. The packing process was monitored under a microscope (Unimicro Technology, CA, USA). Successful slurry packing usually took only 5 min for a 30 cm long column. Occasionally, the packing process stopped after only a few centimeter region was packed; packing could be resumed by turning the slurry reservoir upside-down and tapping it. This approach was more convenient than sonicating the slurry reservoir or the capillary because the sedimentation of particles in a small diameter tube under high pressure can not be easily avoided even by sonication. After the capillary was packed for the desired length, the pressure was slowly released to avoid disturbing the packing bed.

Frit fabrication is a crucial step in column preparation. We found that the use of an arc formed between two electrodes could sinter the packing material inside the capillary and produce good quality frits. A fiber optic splicer (Orionics FW-301, Scarborough, Canada) with a manually controlled heating power was utilized for this purpose. The formation of the frit was monitored under a microscope. During the heating, the polyimide coating was first burnt off, the mobile phase at the heating location was expelled in both directions and the packing turned white. Overheating must be avoided since it could affect the porosity. Inlet and outlet frits were generated in the same manner. It is important to point out that water was constantly pumped through the capillary at a pressure of about 2000 to 3000 p.s.i. during the heating process.

The region near the outlet frit became extremely fragile after the polyimide coating was burnt. To prevent breakage of the column in further experimental steps, the outlet frit was protected by gluing a 1 cm long PTFE sleeve (1.6 mm O.D.×280 μm I.D.) around it. A detection window was made behind the outlet using a heating coil. Finally, the excess packing behind the outlet frit was flushed by pumping acetonitrile though the column.

Columns used for the reproducibility studies were 22 cm of packed bed with a total length of 32.5 cm, with the on-column detection window 5.5 cm from the outlet end of capillary, unless otherwise specified. The evaluation of the column was carried out by separating the test mixture for 110 times within a period of nine days. The mobile phase used contained acetonitrile–20 m*M* MES with pH 7.0 (80:20, v/v).

2.3. Apparatus

All the CEC separations were performed on an in-house designed instrument with a crossed beam thermo-optic UV absorbance detection system [12–18]. A UV excimer laser (248 nm) was used as a pump laser modulated at a frequency of 625 Hz and a He–Ne laser (632.8 nm) was used as a probe laser. The signal intensity of the diffraction fringe was monitored by a silicon photodiode and the signal was amplified by a lock-in amplifier, which sent the data to a computer for recording. The electrochromatograms for the separation efficiency study were processed using PeakFit v. 4.00 (Jandel Scientific, San Rafael, CA, USA).

3. Results and discussion

3.1. Separation efficiency and reproducibility of the CEC columns

CEC should provide higher separation efficiency than HPLC because of the plug-like flow profile. To test the quality of the capillary columns prepared using our simplified packing procedure, we separated a test mixture containing thiourea, PTH–N, PTH–L, PTH–W, and PTH–E under certain conditions. A typical capillary electrochromatographic separation of the test compounds was obtained within 4 min and is shown in Fig. 1. Theoretical plate numbers for these compounds were calculated using equation $N = 5.54(t_m/\Delta t_{1/2})^2$ and ranged from 216 000 plates/m for thiourea to 115 000 plates/m for PTH–E. Decreasing the amount of acetonitrile by about 10 to 20% in the sample, compared to the mobile phase, can further improve the separation efficiency because a lower strength of the solvent in the sample matrix than in the mobile phase induces analyte zone focusing on the stationary phase during the early stage of separation [19].

One of the major obstacles to CEC becoming a routine analytical tool is the need for reproducible performance of CEC columns. The results of the column performance in terms of capacity factor (k'),

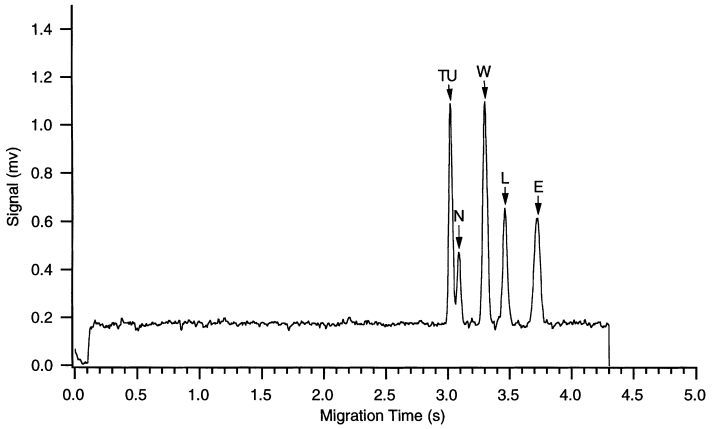

Fig. 1. CEC separation of mixture of PTH–amino acids. Packing material: phase separation 3 μm C_{18}. Column: 32.5 cm (effective length 22 cm). Mobile phase: acetonitrile–20 m*M* MES (80:20, v/v) pH 6.5. Electrokinetic injection at 5 kV for 10 s. TU is thiourea, E is glutamic acid, W is tryptophan, L is leucine, and N is arginine. Applied voltage was 25 kV.

theoretical plate numbers, and resolution within nine days were calculated and are summarized in Table 1. The percentage relative standard deviations (RSD%) of capacity factors representing the migration times of the analytes varied from 0.6% to 5.6% for over one hundred runs on the same column. The RSD of the capacity factors were less than 1.2% for all the well-resolved compounds except PTH–N, which is comparable to the reproducibility of capacity factors reported by others [20]. The middle columns in Table 1 present average values of theoretical plate numbers from 60 500 plates/m for the negatively charged compound PTH–E to 212 000 plates/m for the neutral electroosmotic flow marker, thiourea, along with the RSDs from 10% to 4%. The results show that reversed-phase CEC column has higher separation efficiency for neutral compounds than charged compounds. The reproducible separations obtained using this column were further demonstrated by the low RSD values (2 to 4%) of the resolution from the 110 runs. The low RSD values indicate the column gave a long lifetime and stable performance.

A stable electroosmotic flow determines the overall separation efficiency of a column. Therefore, electroosmotic flow is also a crucial parameter for examining the stability of a column. As described above, one column gave reproducible separations over 110 runs. The electroosmotic flow stability given by this column was demonstrated by measuring the migration time of thiourea over nine days, as shown in Fig. 2. The largest within-day RSD is less than 1%. Reproducible electroosmotic flow confirms the day-to-day stable performance of the column.

We further evaluated the reproducibility of the packing procedure and demonstrated reproducible performance from column-to-column. Five columns were packed with the same slurry concentration and

Table 1
Average and RSD ($n=110$) of capacity factor (k'), efficiency (N) and resolution (R) on a single column

Solute	k'		N (plates/m)		R	
	AVG	RSD (%)	AVG	RSD (%)	AVG	RSD (%)
Thiourea	–	–	213 000	3.7	–	–
PTH–N	0.018	5.6	178 000	6.8	0.79	2.0
PTH–W	0.081	1.2	107 000	5.9	2.18	2.6
PTH–L	0.132	0.8	115 000	4.2	1.53	2.2
PTH–E	1.515	0.6	60 000	10	20.2	3.8

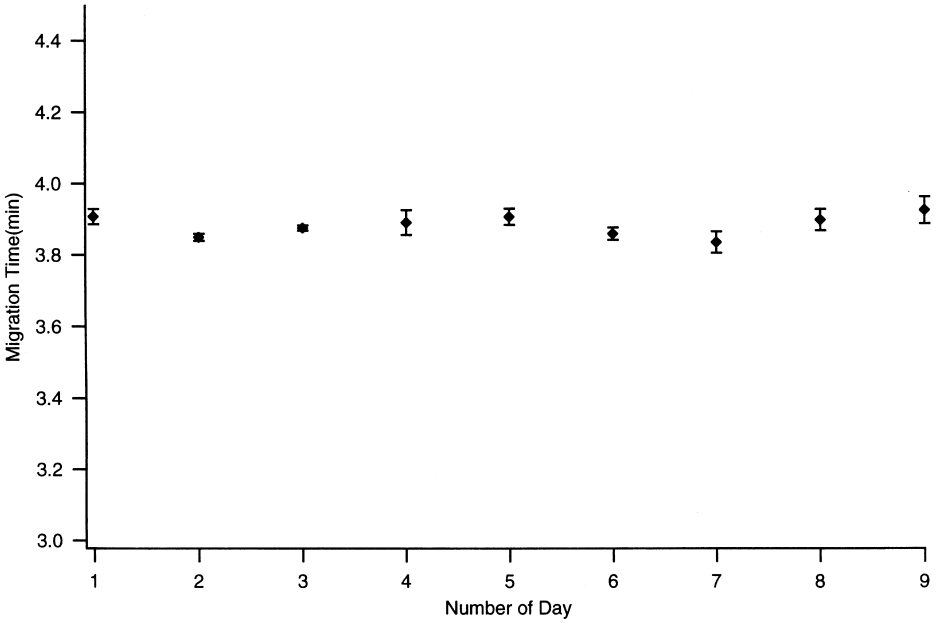

Fig. 2. Migration time of electroosmotic flow marker (thiourea) and its RSD over nine days. Electrokinetic injection at 5 kV for 10 s. Applied voltage 20 kV. Capillary dimension: 22 cm/29 cm.

procedure as described in the experiment, but were made at different times and in different batches. The performance of each column was evaluated by separating the same test mixture with a buffer of 20 m*M* MES (pH 7.0)–acetonitrile (20:80, v/v). Migration time, theoretical plate numbers, and resolutions were calculated over six runs for each column. Fig. 3 shows mean and RSD of migration time (A) and of theoretical plate numbers (B) for thiourea, PTH–L, and PTH–W obtained from the five columns. Fig. 3 demonstrated the reproducible separations of the test compounds using different columns and confirmed that our packing procedure was reproducible. The slightly lower efficiency obtained by columns two to five may be due to a long time storage of the columns in water while over one hundred runs were carried out on column one. Reproducible resolutions between peak and the adjacent early eluting peak were also obtained and are shown in Fig. 3C.

We observed that after many runs a gap of a few millimeters long was always generated near the inlet end. It may due to the influence of different characteristics of the mobile phases used in the preparation of the column and separation. A gap shorter than 2 mm did not noticeably affect the migration reproducibility, although the resolution degraded. Whereas the damage of the inlet frit often resulted in losing the packing from the inlet forced by electrophoretic mobility and therefore increasing the electroosmotic flow dramatically.

3.2. Linear relationship between velocity of an analyte and the applied electric field

CEC separation involves the processes of partitioning and electrophoretic mobility. The apparent migration velocity of a charged analyte, u_a, is described by Rathore and Horváth as the sum of its velocities due to electroosmotic flow and electrophoretic migration in the mobile phase multiplied by the retardation factor $1/(1+k')$ as follows [1]

$$u_a = \frac{1}{1+k'} \cdot (u_{eo} + u_{ep}) \quad (1)$$

where k' is the chromatographic retention factor defined as

$$k' = \frac{t_r - t_0}{t_0} \quad (2)$$

Eq. (2) can only be used to obtain k' for neutral

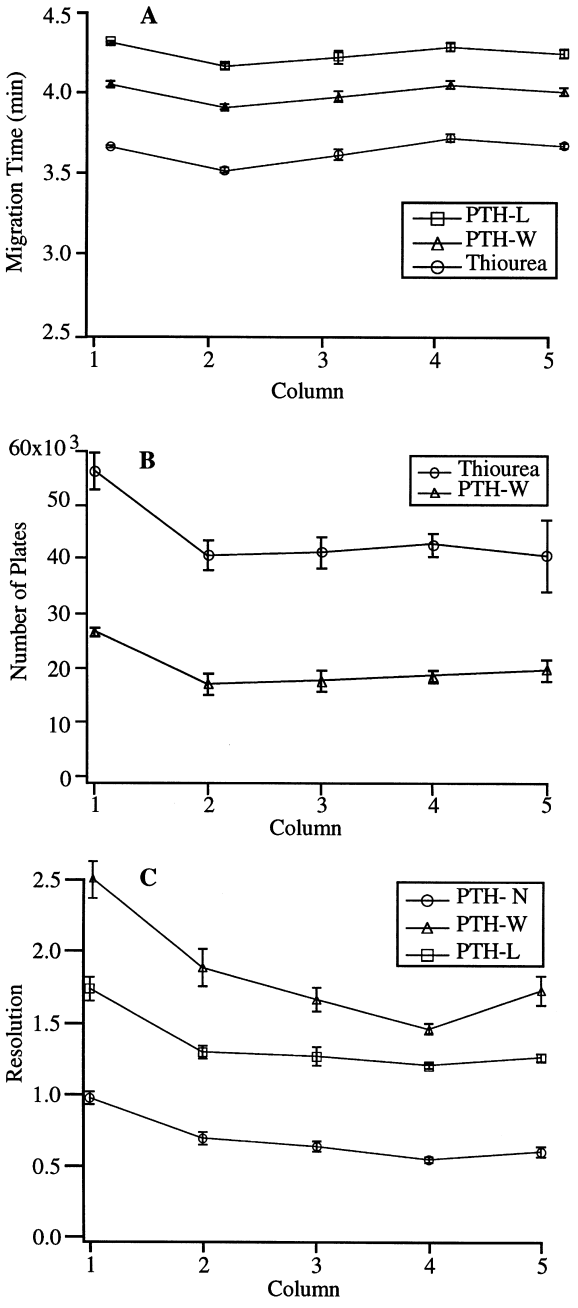

Fig. 3. Reproducibility of migration time (A), plate height (B), resolution (C) from 5 columns. Each data point is calculated from 6 runs on each column.

compounds because only partition is responsible for the separation. For charged compounds, one cannot simply use the apparent migration time and void time to calculate the k'.

Electroosmotic flow and electrophoretic mobility in CZE is described by Knox as [21]:

$$u_{eo} = \frac{\epsilon_r \epsilon_0 \zeta E}{\eta} = \mu_{eo} E \tag{3}$$

where ϵ_r is the dielectric constant of the mobile phase, ϵ_0 is the permittivity of vacuum, ζ is the zeta potential, E is the electric field strength, and η is the viscosity of the mobile phase.

The electrophoretic rate of the charged solute is described as [22]:

$$u_{ep} = \frac{qE}{6\pi\eta r} = \mu_{ep} E \tag{4}$$

where q is the charge of ionized solute, r is the solute radius. Substituting μ_{eo}, μ_{ep} in Eqs. (3) and (4) into Eq. (1), one can obtain

$$u_a = \frac{1}{1+k'} \cdot (\mu_{eo} + \mu_{ep}) E \tag{5}$$

Eq. (5) reveals that the migration velocity of an analyte in CEC is linearly related to the applied electric field, a fact that can be used to examine the quality of CEC columns. We conducted a number of CEC separations for the test mixture using different electric fields while other conditions were kept constant. The results are summarized in Fig. 4. It illustrates a linear relationship ($r > 0.999$) between the velocity of each analyte and the electric field. The linearity indicated that there was no significant Joule heating under the experimental conditions. We found that such a linear relationship was useful to determine if a column was ready for reproducible runs when the buffer was changed.

All the above compounds were neutral except PTH–E under the separation conditions. PTH–E migrated slowest, as expected, because it was negatively charged and had a reversed electrophoretic mobility to the electroosmotic flow at the pH of the buffer.

At a field strength of 1100 V/cm, the corresponding electroosmotic flow was about 1.5 mm/s in the CEC column, whereas the electroosmotic flow in

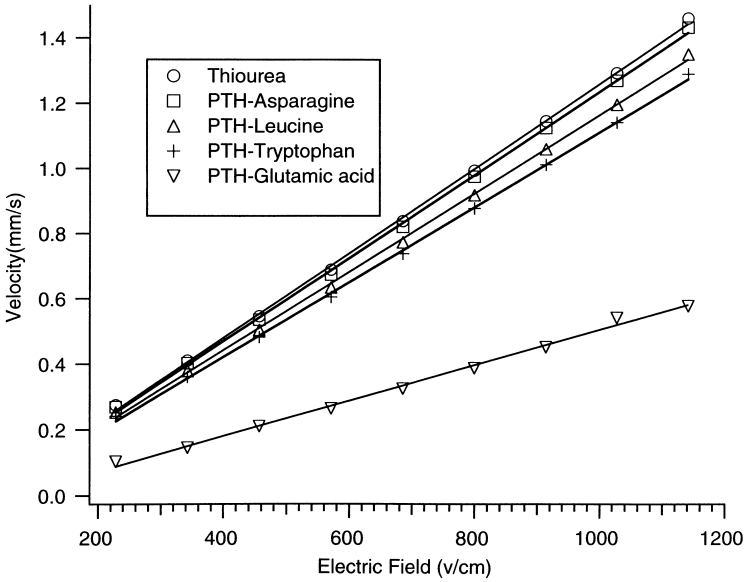

Fig. 4. Plot of velocities of test compounds under different electric fields. Separation conditions: Electrokinetic injection at 5 kV for 10 s. The mobile phase contains acetonitrile–20 m*M* MES, pH 7.0 (80:20 v/v).

an open tubular capillary was 1.8 mm/s at field of 200 V/cm with the same buffer. This indicates that CEC requires a much higher electric field strength than CZE in order to obtain the same electroosmotic flow.

3.3. Effect of acetonitrile content in the buffer

It has been reported that increasing the content of acetonitrile in the mobile phase increases electroosmotic flow [6]. We carried out a series of CEC separations for the test mixture using various volume ratios of acetonitrile to buffer as the mobile phases. When the volume ratio of acetonitrile to buffer (20 m*M* MES, pH 7.0) was increased from 50% to 90% without controlling the ionic strength, electroosmotic flow increased by 74%. This enhancement in electroosmotic flow is attributed to several factors: a higher ratio of dielectric constant to viscosity (ϵ/η) of the mobile phase at a higher concentration of acetonitrile as Eq. (3) illustrates and a lower concentration of electrolyte, which also leads to a higher electroosmotic flow. We further investigated the variation of electroosmotic flow with changes of the acetonitrile content in the mobile phase while the ionic strength was kept constant. In order to obtain a constant ionic strength in the mobile phase, a MES buffer was used and kept at 10 m*M*. The result is showed in Fig. 5. When the percentage of acetonitrile was increased from 50% to 90%, electroosmotic flow increased by 41%. Some difficulties were experienced when we tried to lower the organic content below 50%. The current was unstable and the relationship between the current and the voltage applied was nonlinear. This phenomenon was also noticed by others in non-pressurized CEC mode [6], which was explained as insufficient wetting of the C_{18} surface on the silica with higher aqueous content buffers.

Solvent strength affects the separation selectivity by changing the capacity factor k' of analyte. Fig. 6 shows the separation profiles of the test mixture under various percentages of acetonitrile. As the solvent strength decreases, the resolution of the separation improves, whereas the total separation time lasts longer. The mobile phase composed of 70% acetonitrile gives an elution window of about ten minutes from electroosmotic flow marker and PTH–glutamic acid. These data provide us some hint to further optimize separation conditions for a mixture of all PTH–amino acids. The peak of a charged

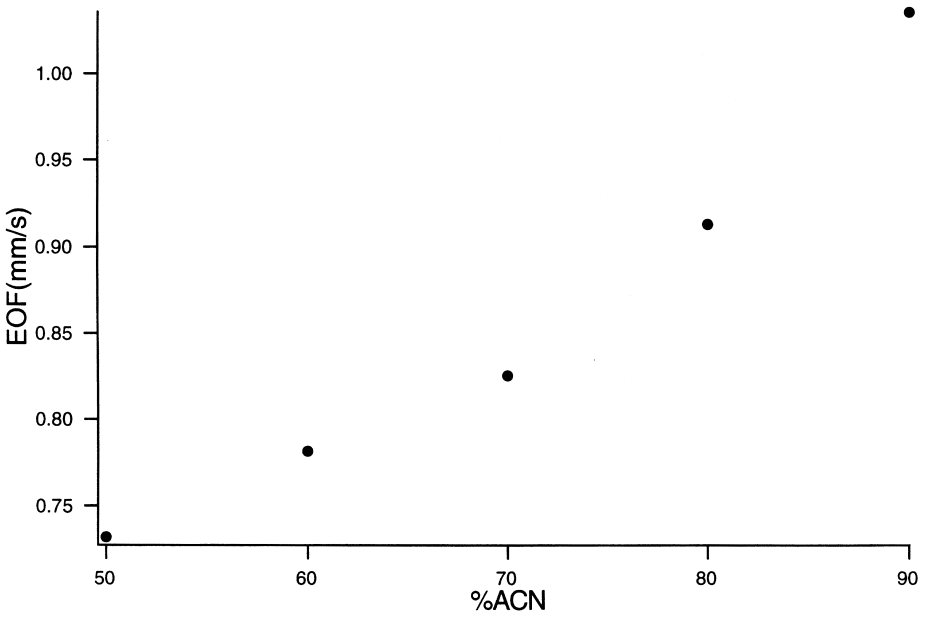

Fig. 5. Dependency of electroosmotic flow (EOF) velocity on the percentage of acetonitrile in buffer at constant ionic strength 10 m*M* MES at pH of 6.5. Column = 32.5 cm (effective length 22 cm). Marker: thiourea.

compound, PTH–E did not show up due to the extensive band broadening when the percentage of acetonitrile was below 60%.

3.4. Separation of PTH–amino acids

PTH–amino acids are important products of the

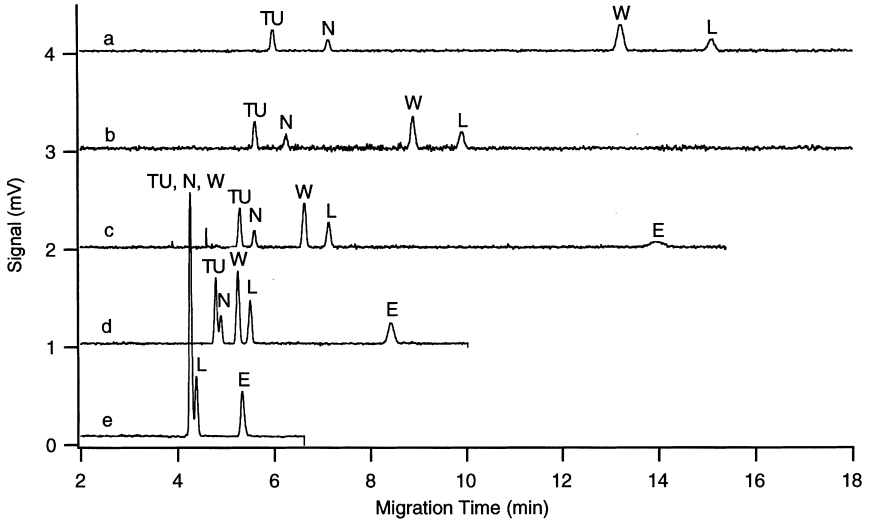

Fig. 6. Electrochromatograms of test mixture at different percentage of acetonitrile (ACN). a = 50% ACN, b = 60% ACN, c = 70% ACN, d = 80% ACN, e = 90% ACN. The ionic strength of the mobile phase was kept at 10 m*M* MES at pH of 6.5. Electrokinetic injection at 5 kV for 10 s, applied voltage 20 kV, Column = 32.5 cm (effective length 22 cm). TU: thiourea, N, W, L, E refer to correspondent PTH–amino acids.

Edman degradation reaction that is used in protein sequencing. These analytes are conventionally analyzed by liquid chromatography and UV-absorbance detection. Terabe introduced the use of micellar electrokinetic chromatography for the analysis of the PTH amino acids [23].

Fig. 7 presents a separation of 17 PTH–amino acids; aspartic and glutamic acid were not determined. Thirteen of the components are nearly baseline resolved. Arginine and histidine co-elute, as to phenylalanine and proline. Threonine generates two peaks, as was observed in micellar electrokinetic chromatography. Less than 14 min were required for this separation, which is comparable to micellar electrokinetic chromatography.

3.5. Photothermal absorbance detection

Photothermal absorbance techniques are well-established methods for the determination of small absorbance [11,24–26]. In these methods, non-radiative relaxation following absorbance of a laser beam produces a temperature rise within a sample. This temperature rise is proportional to both laser power and absorbance and induces a refractive index change within the sample. If a chopped or pulsed excitation laser beam is used, then the periodic change in refractive index can be detected with high precision by use of a phase-sensitive detector.

This group has developed a thermo-optical absorbance detector for micrometer capillaries [12,25]. A modulated pump laser beam periodically illuminates the sample at a point near the exit of the capillary. Complicated deflection and diffraction effects occur at the capillary-solution interface. Perturbation of the refractive index at the interface changes the intensity of the probe beam, measured after the capillary with a small photodiode. There are several examples of ultraviolet laser pumped thermo-optical detection for capillary separations.

This group introduced the use of photothermal absorbance detection in the analysis of PTH–amino acids [12]. We reported detection limits (3σ) for most amino acids ranged from 1 to 10 μM. The sensitivity of the photothermal signal depends on the thermo-optical constants for the solvent. Aqueous solvents have poor sensitivity because of the low change in refractive index with temperature for water. Addition of organic solvents often improves detection limits in photothermal absorbance measurements.

The detection limits for the 13 resolved PTH–amino acids are listed in Table 2. Detection limits ranged from 1.6 to $4.8 \cdot 10^{-7}$ M, which is a factor of

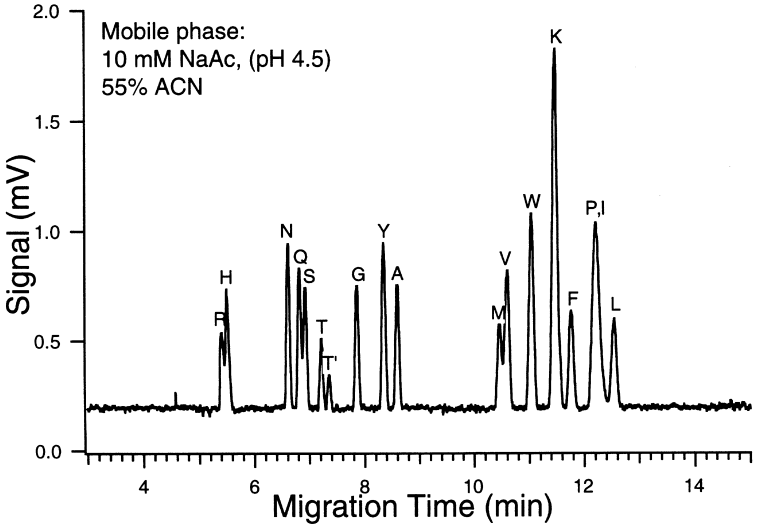

Fig. 7. Electrochromatogram of 17 PTH–amino acids. The standard single-letter abbreviation for the amino acids is listed above each peak. The separation buffer was a 10 mM acetate buffer at pH 4.5, 55% acetonitrile. Injection was at 20 kV for 10 s. Separation was at 25 kV. The column was 34 cm (effective length 25 cm).

Table 2
Detection limits (3σ) for PTH–amino acids

Amino acid	Detection limit, μM
Alanine (A)	0.34
Asparagine (N)	0.27
Serine (S)	0.29
Glutamine (Q)	0.30
Glycine (G)	0.34
Isoleucine (I)	0.33
PTH α, ϵ-PTC lysine (K)	0.16
Leucine (L)	0.44
Methionine (M)	0.41
Threonine (T)	0.48
Tyrosine (Y)	0.25
Tryptophan (W)	0.19
Valine (V)	0.29

ten superior to the detection limits reported for micellar electrokinetic chromatography [12]. Most of this improvement in detection limit is due to the higher thermo-optical enhancement produced by the high acetonitrile content of the buffer compared to the aqueous buffer used in the micellar electrokinetic separation. This enhanced sensitivity should be valuable in sequencing trace amounts of proteins [14–16].

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