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Separation of peptides by pressurized capillary electrochromatography

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Abstract

A pressurized electrochromatography (pCEC) instrument with gradient capability was used in this work for separation of peptides. Three separation modes, namely, pCEC, high-performance liquid chromatography and capillary electrophoresis can be carried out with the instrument. In pCEC mode, the mobile phase is driven by both electroosmotic flow and pressurized flow, facilitating fine-tuning in selectivity of neutral and charged species. A continuous gradient elution can be carried out conveniently on this instrument, which demonstrates that it is more powerful than isocratic pCEC for separation of complicated samples. The effects of applied voltage, supplementary pressure and ion-pairing agents on separation of peptides in gradient pCEC were investigated. The effects of flow-rate of the pump and the volume of the mixer on resolution were also evaluated.

Keywords: Electrochromatography; Pressurized capillary electrochromatography; Gradient elution; Peptides

1. Introduction

Capillary electrochromatography (CEC) has become a powerful separation technique because it provides high column efficiency and selectivity by combining the advantages from both high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) [1–4]. Many articles and reviews of CEC have been published on the development of theory, application, instrumentation and column fabrications [5–10]. To further explore the potential of CEC, it is very important to develop the capability of gradient elution for successful separation of a wide variety of complex samples [11–13].

Step gradient elution has been realized in a conventional CE instrument with a packed capillary column, in which CEC separation was carried out with switching inlet vials containing different mobile phases [14]. Although the step gradient can be programmed with a conventional CE instrument, a considerable drawback is its inability to perform CEC with continuous solvent gradient elution. Continuous gradient elution can be formed through mixing two mobile phases, transferred by HPLC pumps or a syringe [15], by a magnetic stirrer [16], or through connecting a micro-LC system to the

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CEC column via a flow-injection analysis interface [17] or a mixer [18].

Another method for generating continuous gradient elution in CEC is to mix the two mobile phases by an electroosmotic pumping system, which consists of two capillaries and two independent high-voltage power supplies [13].

In our laboratory, a special CEC instrument, the Trisep 2000GV CEC system, has been developed in which the mobile phases are driven by electroosmotic flow (EOF), as well as pressurized flow. Bubble formation, which is often encountered in CEC, can be suppressed in the pressurized CEC system. Adjustment of the ratio between the pressure and voltage also facilitates the fine-tuning of separation selectivity of neutral and charged components. A continuous gradient elution can be carried out conveniently and quantitative injection though a rotary-type injection valve can be carried out on the instrument [19,20].

CEC separation of peptides has received considerable attention in recent years [21–25]. There are several reports that have shown potential in separating peptides by laboratory-made CEC instruments [11,12].

In this paper, we report the development of a pressurized CEC (pCEC) system. Separation of peptides was carried out by pCEC in both isocratic and gradient elution modes using capillary columns packed with C_{18} stationary phase. The performance of the gradient pCEC system was evaluated with different volumes of mixers under different flows. The effects of supplementary pressure, applied voltage and elution strength of mobile phase on the separation of the peptides were also investigated.

2. Experimental

2.1. Apparatus

In these studies, CEC was carried out on a Trisep 2000GV CEC system (Unimicro Technologies, Pleasanton, CA, USA) which comprised a solvent gradient delivery module, a high-voltage power supply (+30 kV and −30 kV), a variable-wavelength UV–Vis detector, a micro fluid manipulation module (including a 20-nl four-port injector) and a data acquisition module, as shown in Fig. 1. A continuous gradient is generated by merging two flows in a mixer. After splitting in a T-connector, the flow enters a capillary column through a rotary-type injector under constant pressure controlled with a backpressure regulator. An electrode is set to the tee in connection with the outlet end of the column. The samples were introduced through the rotary injector.

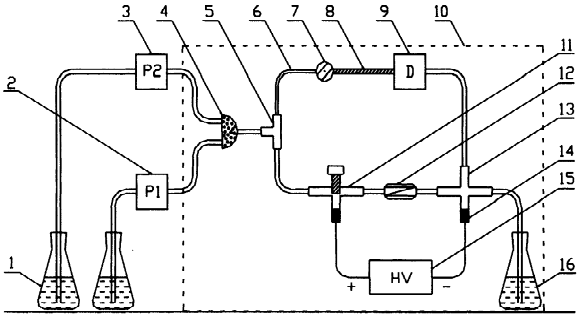

Fig. 1. Schematic diagram of the TriSep 2000GV CEC system. 1, Mobile phase reservoir; 2, pump one; 3, pump two; 4, micro-mixer; 5, tee; 6, low dead-volume connector; 7, injector; 8, column; 9, detector; 10, interlocked safety box; 11, micro-splitter valve; 12, back-pressure regulator; 13, cross; 14, electrode; 15, high-voltage power supply; 16, waste.

2.2. Materials

Capillary columns of 250 mm×100 μm I.D.×375 μm O.D. packed with 3 μm C_{18} particles were supplied from Unimicro Technologies. Trifluoroacetic acid (TFA) obtained from Aldrich (Milwaukee, WI, USA) and phosphate (H_3PO_4) obtained from Tianjin Chemical Reagents (Tianjin, China) were all of analytical grade. Micro-filters with 0.22-μm pores were obtained from Tianjin Chemical Reagents. Acetonitrile obtained from Xinke Chemical (Hebei, China) was chromatographic grade. Double deionized water was used. Backpressure regulator, mixer, cross, tee, filter and injector were all purchased from Upchurch Scientific (Oak Harbor, WA, USA). Peptide samples: Gly–Gly–Gly, Gly–Arg–Gly–Asp, Arg–Gly–Asp, Gly–Arg–Gly–Asp–Ser–Pro–Lys, Gly–Arg–Gly–Asp–Ser–Pro and Met–Met were obtained from Sigma (St Louis, MO, USA).

2.3. Procedures

Peptides were first dissolved in water to obtain a solution containing 1 mg/ml of each peptide and were stored at 4 °C. The sample solutions were further diluted with mobile phase to give the peptide an approximate concentration of 0.1 mg/ml. All these solutions were filtered with a 0.22-μm filter. Mobile phase solutions were prepared as follows: the appropriate volumes of acetonitrile and water were mixed, then the appropriate volume of TFA or phosphate was added to the mixture solution. Mobile phase solution was degassed in an ultrasonic bath for 10 min before use. A negative voltage was applied to the column outlet and the column inlet was grounded. Pressure was applied to the column inlet during the separation. Total flow-rate of the two pumps ranged from 10 to 100 μl/min. The wavelength of the UV–Vis detector was set at 214 nm. The injector has an internal loop of 20 nl.

3. Results and discussion

3.1. Separation of peptides by isocratic and gradient elution pCEC

The results of separation of six peptides by isocratic and gradient elution pCEC were obtained, as shown in Fig. 2. Six peptides were all positively charged thus the direction of EOF, pressure, and the migration of peptides were all the same. In the isocratic elution mode using mobile phase containing different concentrations of acetonitrile, separation of the pairs of peptides, 2 and 3, 4 and 5, were very difficult as shown in Fig. 2a,b. In the gradient elution mode, six peptides were baseline resolved as shown in Fig. 2c. It is evident that a gradient elution in pCEC is very effective in separating similar peptides, where an isocratic separation would be impractical.

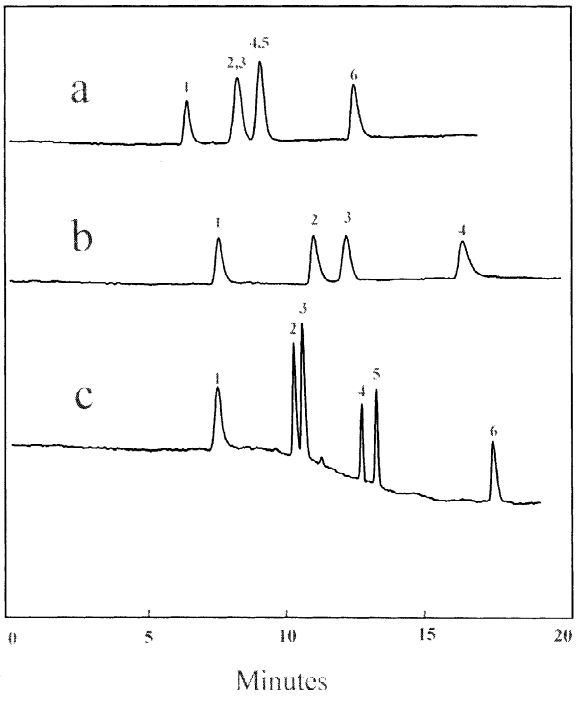

Fig. 2. Separation of six peptides on isocratic and gradient pCEC. Experiment conditions: column: 250 mm×100 μm I.D.×375 μm O.D. packed with 3 μm C_{18}; mobile phase: (a) isocratic 0.1% (v/v) TFA in 12% (v/v) CH_3CN; (b) isocratic 0.1% (v/v) TFA in 3% acetonitrile; (c) gradient (A) 0.1% (v/v) TFA, (B) 0.1% (v/v) TFA in 25% (v/v) acetonitrile; linear gradient: 10–20% B in 3 min, 20–60% B in 4 min; pressure added on-column: 10.335 MPa; voltage: 2 kV; UV–Vis detector: 214 nm; injection volume: 20 nl; Peaks: 1=Gly–Gly–Gly; 2=Gly–Arg–Gly–Asp; 3=Arg–Gly–Asp; 4=Gly–Arg–Gly–Asp–Ser–Pro–Lys; 5=Gly–Arg–Gly–Asp–Ser–Pro; 6=Met–Met.

3.2. The effect of applied voltage on resolution

Compared to micro-HPLC, pCEC can bring some changes: applied electrical field allows fine-tuning of the retention of charged solutes and improves selectivity. In gradient pCEC mode, the flow-rate on the column may be faster when driven by a combination of pressure and EOF than that produced by pressure only, and the shape of gradient may also be altered. Fig. 3 illustrates the relationship between resolution of two pairs of peptides and applied voltage. Resolution of peptides 4 and 5 increased with increase in applied voltage, while that of 2 and 3 decreased slightly. Differentiation between migration rate of 4 and 5 increased with increase in voltage, while that of 2 and 3 decreased. It is seen from Fig. 3 that the separation can be tuned by adjusting the electrical field on the column according to sample characteristics. The electrophoretic mobility of the peptides contributed to the selectivity.

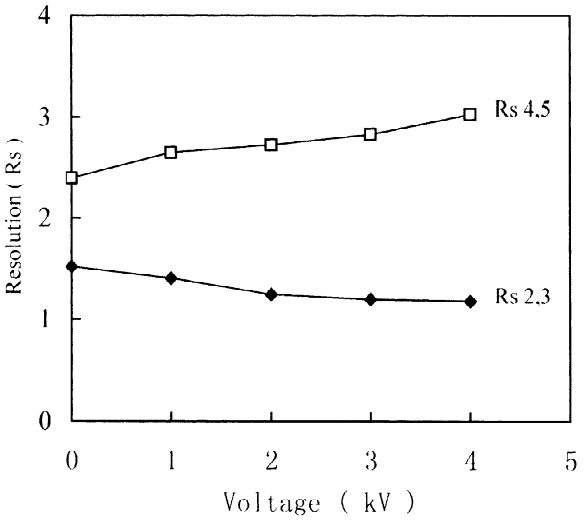

Fig. 3. Effect of voltage on resolution in gradient pCEC separation of peptides. Experiment conditions: column: 250 mm×100 μm I.D.×375 μm O.D. packed with 3 μm C_{18}; mobile phase: (A) 0.1% (v/v) TFA, (B) 0.1% (v/v) TFA in 25% (v/v) acetonitrile; linear gradient: 10–20% B in 3 min, 20–60% B in 4 min; pressure added on-column: 6.89 MPa; UV–Vis detector: 214 nm; injection volume: 20 nl; upper line: resolution between peaks 4 and 5; lower line: resolution between peaks 2 and 3. Peak order as in Fig. 2.

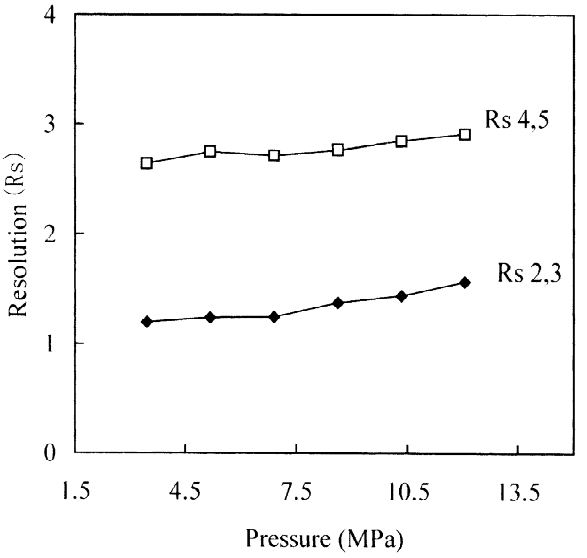

Fig. 4. Effect of pressure on resolution in gradient pCEC separation of peptides. Experiment conditions: column: 250 mm×100 μm I.D.×375 μm O.D. packed with 3 μm C_{18}; mobile phase: (A) 0.1% (v/v) TFA, (B) 0.1% (v/v) TFA in 25% (v/v) acetonitrile; linear gradient: 10–20% B in 3 min, 20–60% B in 4 min; voltage: 2 kV; UV–Vis detector: 214 nm; injection volume: 20 nl; upper line: resolution between peaks 4 and 5; lower line: resolution between peaks 2 and 3. Peak order as in Fig. 2.

3.3. Effect of pressure on gradient pCEC separation

Supplementary pressure prevents bubble formation and provides reliability and reproducibility of the electrochromatographic performance. In the separation system, a gradient elution was generated with two micro-HPLC pumps. The mobile phases were mixed in a mixer. After splitting, a fraction of gradient elution entered the separation column under controlled pressure. In the pCEC system, a high pressure over 6.89 MPa can be applied. The effect of pressure on gradient pCEC was studied using different backpressure regulators. It is seen from Fig. 4 that, under a constant voltage, a slight increase in resolution may be gained by changing the pressure. We believe that the increase in the resolution is due to the increase in the linear velocity of the mobile phase, which, under the experimental conditions, is lower than the optimum velocity in the Van Deemter plot. Therefore, the linear velocity is gradually approaching the optimum velocity when the pressure is increased. The closer the velocity of the mobile phase to the optimum velocity, the higher the column efficiency, and consequently, the higher the resolution.

3.4. Effect of ion-pairing agents on gradient pCEC

TFA is the most common ion-pairing agent in peptide separation by RP-HPLC. After forming ion-pairs with TFA, peptides become more hydrophobic, therefore, retention of peptides was increased on the C_{18} column with increase in TFA concentration. TFA is very effective in improving peak shape and resolution. However, the increase in TFA concentration in pCEC also increases the current resulting in Joule heating which can cause bubble formation in the column and unstable baseline. These phenomena may be improved using phosphate as the ion-pairing agent. The effects of TFA and phosphate as ion-pair agents in the same mobile phase on separation are illustrated in Fig. 5. TFA resulted in a longer elution time for these peptides than phosphate, while res-

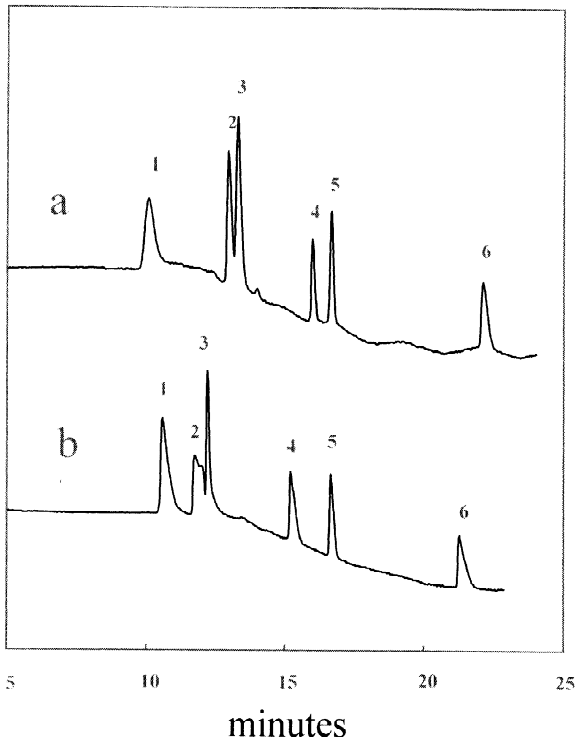

Fig. 5. Effect of ion-pairing agents on pCEC separation of peptides. Experiment conditions: column: 250 mm×100 μm I.D.×375 μm O.D. packed with 3 μm C_{18}; mobile phase: a: (A) 0.1% (v/v) TFA, (B) 0.1% (v/v) TFA in 25% acetonitrile; b: (A) 0.1% (v/v) H_3PO_4, (B) 0.1% (v/v) H_3PO_4 in 25% (v/v) acetonitrile; linear gradient: 10–60% B in 7 min; pressure added on-column: 6.89 MPa; voltage: 2.5 kV; UV–Vis detector: 214 nm; injection volume: 20 nl; Peaks: 1=Gly–Gly–Gly; 2=Gly–Arg–Gly–Asp; 3=Arg–Gly–Asp; 4=Gly–Arg–Gly–Asp–Ser–Pro–Lys; 5=Gly–Arg–Gly–Asp–Ser–Pro; 6=Met–Met.

olution of 4 and 5 was improved using phosphate. However, it is evident that peak shape is much better when TFA was used as ion-pairing agent.

3.5. Effect of flow-rate and mixer volume on gradient pCEC separation

With the limitation of precision on flow-rate of the pump, the gradient elution generated with two pumps was not quite stable at a total flow-rate below 30 μl/min. The effect of flow-rate on gradient pCEC separation was evaluated at flow-rates from 30 to 100 μl/min using three different volume mixers, 2.2, 20 and 50 μl. As shown in Table 1, the resolution of the peaks may be altered by different flow-rates and mixers. According to Table 1, the flow-rate of 30 μl/min and 50-μl volume mixer are preferred. Table 2 lists the RSD of the retention time of six peptides obtained from five runs in gradient pCEC. A reproducibility of less than 2% of RSD in retention times was achieved. The good reproducibility is obtainable because the rotary type of HPLC injection valve was used in this pCEC system.

Table 1
Effect of volume of mixer and total flow-rate of pumps on resolution in gradient pCEC

Volume of mixer (μl)	Total flow rate of pumps (μl/min)	$Resolution_{4,5}$
2.2	30	1.40
2.2	100	1.12
20	30	2.78
20	100	2.42
50	30	3.03
50	50	2.84
50	100	2.75

Other experimental conditions are the same as Fig. 3.

4. Conclusion

A gradient pCEC was developed and used for the separation of peptides. The experiment demonstrated that pCEC with gradient elution capability is more powerful in separating similar peptides than isocratic elution. In pCEC mode, the separation of the peptides can be influenced not only by adjusting the applied voltage and the supplementary pressure on the column, but also by changing the flow-rate and mixer volume.

Table 2
Reproducibility (five runs) of gradient pCEC

Peptide	Mean t_R (min)	RSD (%)
1	7.61	1.35
2	10.42	0.78
3	10.81	1.05
4	12.78	0.83
5	13.71	1.23
6	17.69	1.97

Other experimental conditions are the same as Fig. 3.

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