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Determination of amino acids in *Sargassum fusiforme* by high performance capillary electrophoresis

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Abstract

High performance capillary electrophoresis (HPCE) was developed for quantitative determination of 18 phenylthiohydantoin (PTH)–amino acids. The influence of electrolyte concentration, pH, organic modifier and applied voltage on HPCE performance was investigated. The HPCE separation of a PTH–amino acids mixture was much improved by adding organic modifier and Tris–boric acid buffer to the run buffer. After optimization of the method, 17 PTH–amino acids in a solution containing 18 PTH–amino acids could be separated using 400 mmol l^{-1} Tris–boric acid, 1.0 mmol l^{-1} diethylamine at pH 9.5 adjusted with 0.1 mol l^{-1} NaOH as a run buffer, voltage of 25 kV was applied, temperature was maintained at 25 °C, detection wavelength was 254 nm. The precision ($n = 7$) of this method is less than 3.2% (peak area) and 1.1% (migration time) of relative standard deviation (R.S.D.). Linearity was established over the concentration range 50–1000 μM of each derivative, with correlation coefficients (r) ranging between 0.9904 and 0.9993. The detection limits (S/N = 3) range from 2 to 48 μmol l^{-1}. The method was applied to determine amino acids in *Sargassum fusiforme*, a marine algae collected from Tongtou County of Zhejiang Province in China with satisfactory results.

Keywords: *Sargassum fusiforme*; High performance capillary electrophoresis; PTH derivatives; Amino acids

1. Introduction

Sargassum fusiforme is marine algae classified as brown algae. In china and the Far East the marine algae is regularly utilized in human alimentation since ancient times [1,2]. The algal proteins contain all essential amino acids [3]. Amino acid analysis remains of great importance, not only in the food sciences but also in the characterization of natural products. Since its introduction by Per Edman as a sequencing reagent, phenylisothiocyanate (PITC) has been a popular derivatizing reagent for amino acids [4]. Under basic conditions, the reagent reacts with the alpha amine of the amino acids, forming the phenylthiocarbamyl (PTC) derivatives. Under acidic conditions, the phenylthiocarbamyl (PTC) derivatives rearrange to produce phenylthiohydantoin (PTH) derivatives.

Chromatography is a traditional method for the control of amino acid content: ion exchange chromatography (IEC), using amino acid analyzer [5] and reversed-phase (RP)-HPLC [6,7], using usually a post- or a pre-column (pre-capillary) derivatization with various kinds of chromophores [8] or fluorophores [9,10]; the non-modified amino acids are detected in rare cases [11]. HPLC has been the technique of choice for separation and analysis of amino acids [12–15]. Capillary electrophoresis (CE) is a powerful separation technique, which compared with HPLC, often provides better resolution, shorter analysis time, very low buffer consumption, and low consumption of sample [16–20]. Most often, derivatives of amino acids are exposed to separation and detection [21], widespread reagents being: fluorescein isothiocyanate (FITC) [22], dansyl chloride (Dns) [23], phenylisothiocyanate (PITC) [24] and *o*-phthaldialdehyde (OPA) [25]. In 1997 Smith reviewed the developments in amino acid analysis using capillary electrophoresis [26]. It is reported

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that micellar electrokinetic chromatography (MEKC) [23,27] and capillary electrochromatography (CEC) [28] have been adopted for the measurement of amino acids. The techniques of time-of-flight mass spectrometry (TOF-MS) [29] and electrospray ionization mass spectrometry (ESI-MS) [30–33] have been used for the detection of amino acids in HPCE. However, only a few CE protocols have been established for biological amino acid analysis to date. The methods reported need to be improved in resolution, running time, and number separated in a run.

The aim of the present paper is to establish a sensitive, simple and practical method for the determination of amino acids in *S. fusiforme*, which can be used for determining amino acids in other algae.

2. Experimental

2.1. Instrumentations and reagents

Experiments were performed with a P/ACE^{TM} MDQ capillary electrophoresis system equipped with an on-column diode-array detector (DAD) system, operated under Gold system software for control, data acquisition, and analysis (Beckman Co., USA). The column used for the determination of 18 amino acids was an uncoated fused-silica capillary of 50 cm in length and 50 μm in internal diameter (Beckman Co., USA). A pHs-3C acidometer is made in Shanghai Leici Instrument Corporation (China). Eighteen kinds of amino acids were received from Sigma Corporation. Phenyl isothiocyanate (PITC; analytical grade), other chemicals and reagents used herein were of analytical grade. Ultrapure water (ρ = 18 MΩ) was used to prepare all of the solutions.

2.2. Synthesis of PTH–amino acids

Three hundred microliters of 1,4-dioxane–water (1:1, v/v), 5 μl of PITC and 50 μl of solution of 18 standard amino acids, containing 100 mg l^{-1} of each of amino acids, were added to 100 μl of 0.1 M solution of sodium carbonate. The components were vortex—mixed for 30 s and heated at 55 °C for 30 min. After the coupling reaction, the solvent was removed by evaporation and sublimation at 55 °C. 1 M HCl (1 ml) was added to the resultant residue, which was heated at 55 °C for 40 min or 80 °C for 5 min. The reaction products were extracted using ethyl acetate (3 × 1 ml). After drying under a stream of N_2, the PTH–amino acids obtained were washed with benzene (3 × 1 ml). After drying, the PTH–amino acids dissolved in 500 μl of aqueous methanol solution (50%, v/v). Concentration of every amino acid in the final solution (in the form of PTH-derivative) was 10 mg l^{-1}. These standards were stored at −20 °C, immediately before use; they were diluted with ultrapure water (methanol concentration less than 10%, v/v). It was filtered by 0.45 μm membrane filter. Finally this solution was degassed before to be used.

2.3. Preparing sample of S. fusiforme (acidic hydrolysis of proteins)

The sample of *S. fusiforme* collected from Tongtou County of Zhejiang Province in east China. It was washed with distilled water and dried under vacuum at 60 °C for 24 h. Then the dried sample was ground into powder. Powdered sample was transferred to plastic bottle and kept in the laboratory away from direct sunlight.

A weighed sample (100 mg) was put into a vial, and 10 ml of 6 mol l^{-1} HCl were added. The mixture was tightly capped and put into the air thermostat (T = 110 °C) for 10 h. The received hydrolysate was filtered through an ash-free filter, and after taking 500 μl aliquot, it was evaporated to dryness in the flow of warm air. The solid residual was dissolved in 500 μl of water.

For analysis of PTH–amino acids a 100 μl aliquot was taken, and then 100 μl of 0.1 mol l^{-1} sodium carbonate, 300 μl 1,4-dioxane–water (1:1, v/v), 5 μl of PITC were added, and next operations were followed according to Section 2.2.

If necessary, the final solutions were diluted by water in 5–10 times right before the analysis.

2.4. Conditions of electrophoresis

The buffers are prepared from 400 mol l^{-1} Tris–boric acid, and 1.0 mol l^{-1} diethylamine are adjusted with 0.1 mol l^{-1} NaOH to pH 9.5. Finally, buffer solutions are filtered through a 0.45 μm membrane filter and degassed in advance of being applied, and then operated at a condition of voltage at 25 kV; pressure injection at 5 psi s; the separation temperature at 25 °C and the wavelength at 254 nm. Prior to analysis, Fused-silica capillary is purged for 5 min with 0.1 mol l^{-1} NaOH under high pressure (138 kPa) with water and buffer, respectively. After the end of each day's analysis, the fused-silica capillary must be purged for 5 min with 0.1 mol l^{-1} NaOH and water.

3. Results and discussion

3.1. Effect of buffer concentration

According to literature [34], we know that the best buffer solution to separate PTH–amino acids is Tris–boric acid buffer. Separated PTH–amino acids, the key factor to influence the resolution are the concentration and pH of buffer solution.

The effect of the concentration of the Tris–boric acid run buffer at pH 9.5 on these separations was examined at concentrations of 100, 200, 300, 400, 500 mmol l^{-1}, with 1.0 mmol l^{-1} diethylamine. An applied voltage of 25 kV was used in all cases. As shown in Fig. 1, the migration order of each amino acid is changed slightly, and the separation was much improved on increasing the concentration of buffer. But

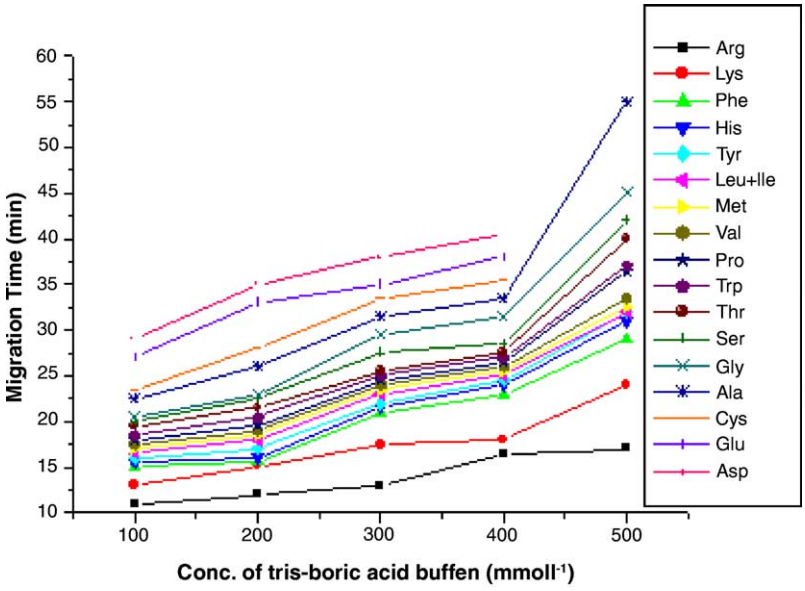

Fig. 1. Effect of the Tris–boric acid buffer concentration on migration time.

the migration time will become longer. As a result of this studies, a Tris–boric acid buffer concentration of 400 mmol l^{-1} was taken to be optimal.

3.2. Effect of the pH

The pH of running buffer was the most important parameter for changing the selectivity of capillary electrophoresis. Separation by electrophoresis depended upon differing mobilities of amino acid derivatives, which were directly related to their size and net charge. The effect of pH value of running buffer on the separation of PTH–amino acids was examined by varying the pH value from 8.0 to 10.0. The concentrations of buffer and diethylamine were fixed at 400 and 1.0 mmol l^{-1}, respectively. An applied voltage of 25 kV was used. Temperature was maintained at 25 °C. The experiment results were shown in Fig. 2. It was found that with the increase of pH value, the migration time of PTH–amino acids prolonged, but the resolution was improved. Under the pH value (pH 9.5) of running buffer, satisfactory separation efficiency was obtained.

3.3. Effect of organic modifier

Organic modifiers added to the running buffer altered the polarity and the viscosity of electrolyte, thus affecting both electroosmotic flow and the electrophoretic mobility of the analytes. Dang and Chen [34] reported that amine

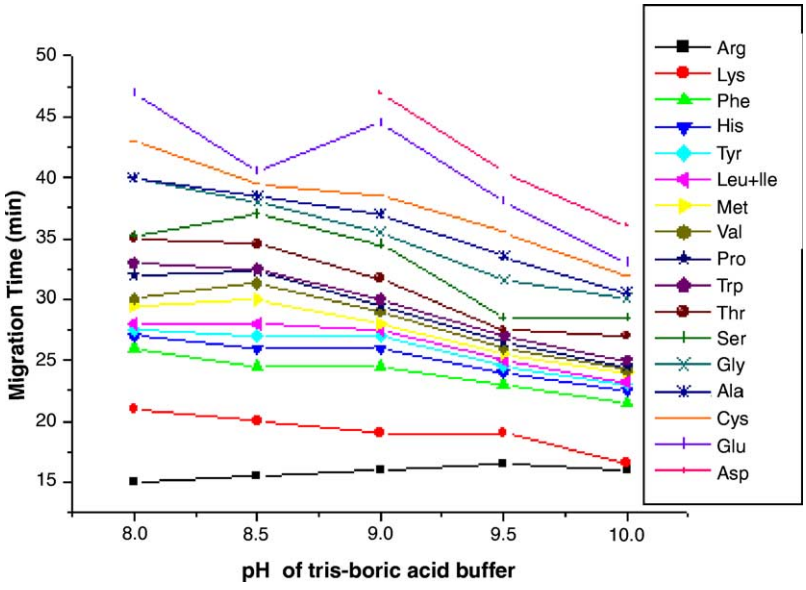

Fig. 2. Effect of the pH of Tris–boric acid buffer on migration time.

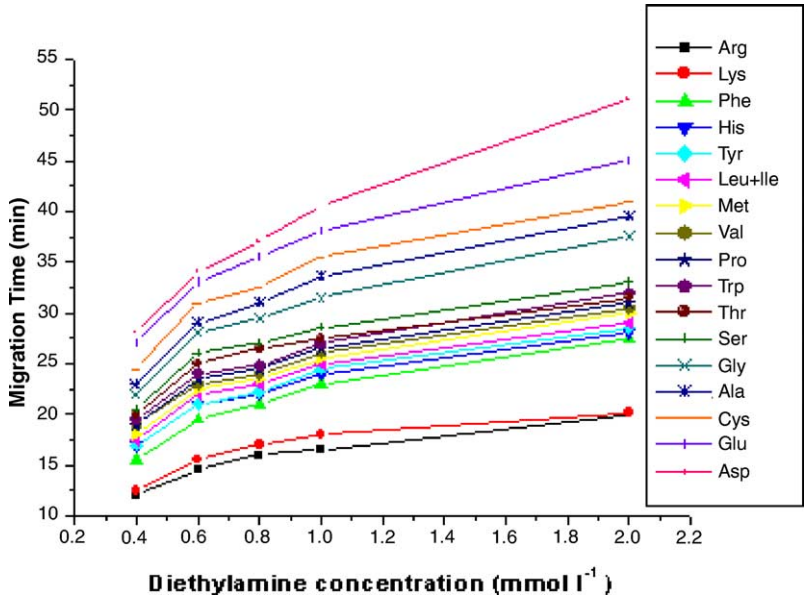

Fig. 3. Effect of the diethylamine concentration on migration time.

modifiers have a certain effect to the separation efficiency of PTH–amino acids. By the means of testing the ethylamine, ethylenediamine, phenethylamine and diethylamine, we found that diethylamine took important role to improve the separation efficiency of PTH–amino acids. Corresponding to the rising of the concentration of diethylamine, the resolution would increase, and the migration time would be prolonged. The effect of concentration of diethylamine on the migration time of each amino acid was investigated over a concentration range from 0.4 to 2.0 mmol l^{-1}, using a buffer concentration of 400 mmol l^{-1} Tris–boric acid at pH 9.5. The applied voltage used for these studies was 25 kV; temperature was maintained at 25 °C. The detection wavelength was 254 nm. The results are shown in Fig. 3, a diethylamine concentration of 1.0 mM was taken to be optimal.

3.4. Effect of applied voltage

The effect of the applied voltage on the separation of amino acids was examined by varying the applied voltage from 15 to 30 kV. The concentrations of buffer (pH 9.5) and diethylamine were fixed at 400 and 1.0 mmol l^{-1}, respectively. The applied voltage used for these studies was 25 kV; temperature was maintained at 25 °C. The detection wavelength was 254 nm. As shown in Fig. 4, the migration time of the analytes was significantly shortened and their corresponding electrophoretic peaks were sharpened when

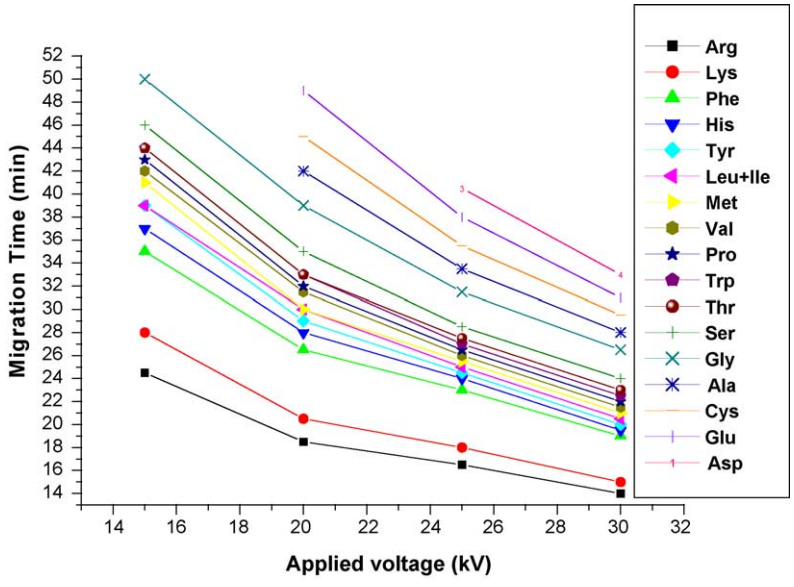

Fig. 4. Effect of the applied voltage on migration time.

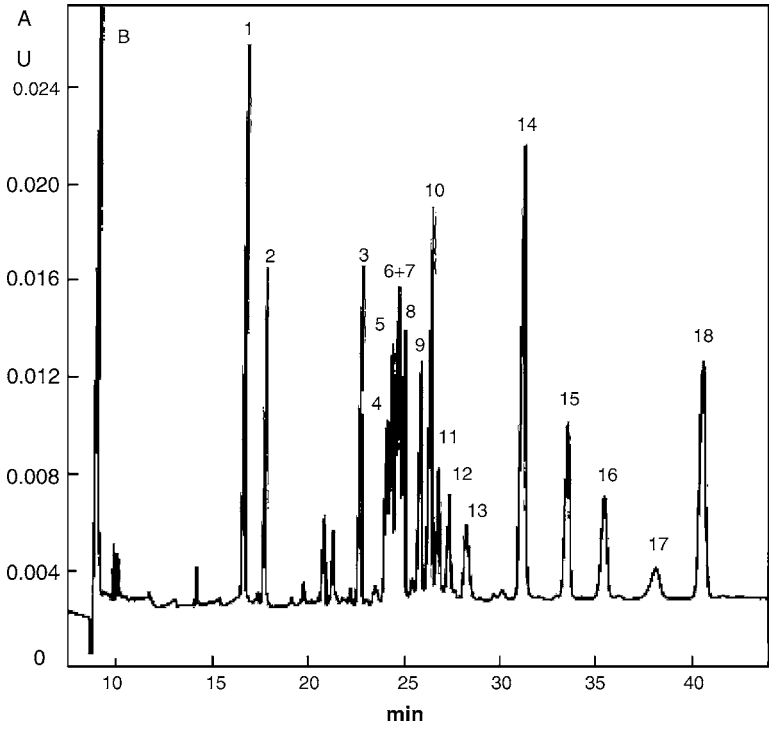

Fig. 5. Electropherogram of the separation of PTH–amino acids. R-EOF, 1-Arg, 2-Lys, 3-Phe, 4-His, 5-Tyr, 6-Leu, 7-Ile, 8-Met, 9-Val, 10-Pro, 11-Trp, 12-Thr, 13-Ser, 14-Gly, 15-Ala, 16-Cys, 17-Glu, 18-Asp, at the optimum conditions which are electrolyte buffer containing 400 mmol l^{-1} Tris–boric acid, 1.0 mmol l^{-1} diethylamine at pH 9.5 adjusted with 0.1 M NaOH, the applied voltage used for 25 kV, temperature was at 25 °C and with detection at 254 nm.

the applied voltage was increased. However, if the applied voltage was too large, more Joule heat was produced by the higher current inside of the capillary and separation efficiency was reduced. In these studies, 25 kV was chosen as the optimum applied voltage. Fig. 5 shows a typical electrophorogram of 18 PTH–amino acids standard solution; 17 PTH–amino acids were well separated from each other.

3.5. Analytical reproducibility, linearity and detection limit

Analytical reproducibility was investigated by performing seven identical analyses on 17 PTH–amino acids of the 18 PTH–amino acids standard solution. Table 1 shows the results in terms of reproducibility (relative

Table 1
Regression equaton, limit of detection and reproducibility

Peak number	Amino acid	Regression equation[a]	r[b]	LOD (μmol l^{-1}) (S/N = 3)	Reproducibility (R.S.D., %)	
					Area	Time
1	Arg	$y = 2.107x + 0.0456$	0.9972	21	2.1	0.5
2	Lys	$y = 2.307x + 0.0210$	0.9981	30	1.8	0.5
3	Phe	$y = 4.168x - 0.0712$	0.9993	39	1.5	0.6
4	His	$y = 3.929x - 0.0168$	0.9962	27	2.4	0.5
5	Tyr	$y = 3.892x + 0.0634$	0.9945	2	1.7	0.6
6 + 7	Leu + Ile	$y = 2.481x + 0.0867$	0.9927	7	1.8	0.6
8	Met	$y = 3.066x + 0.0569$	0.9967	11	2.2	0.6
9	Val	$y = 3.568x - 0.0113$	0.9956	28	2.7	0.7
10	Pro	$y = 2.529x + 0.0112$	0.9966	31	2.3	0.7
11	Trp	$y = 1.841x + 0.0311$	0.9965	32	2.0	0.7
12	Thr	$y = 1.822x + 0.0199$	0.9971	38	2.8	0.7
13	Ser	$y = 1.586x + 0.0254$	0.9972	41	3.0	0.7
14	Gly	$y = 2.105x + 0.0280$	0.9975	29	2.3	0.8
15	Ala	$y = 1.680x + 0.0386$	0.9975	30	1.9	0.8
16	Cys	$y = 2.076x - 0.0196$	0.9918	48	3.1	0.9
17	Glu	$y = 2.842x - 0.0254$	0.9904	41	3.2	1.1
18	Asp	$y = 2.544x + 0.0427$	0.9977	18	2.1	1.1

Each value is the mean of seven independent assays.
[a] y (peak area count) = $A + Bx$ (amino acid concentration, 0.05–1.0 mmol l^{-1}).
[b] r = correlation coefficient.

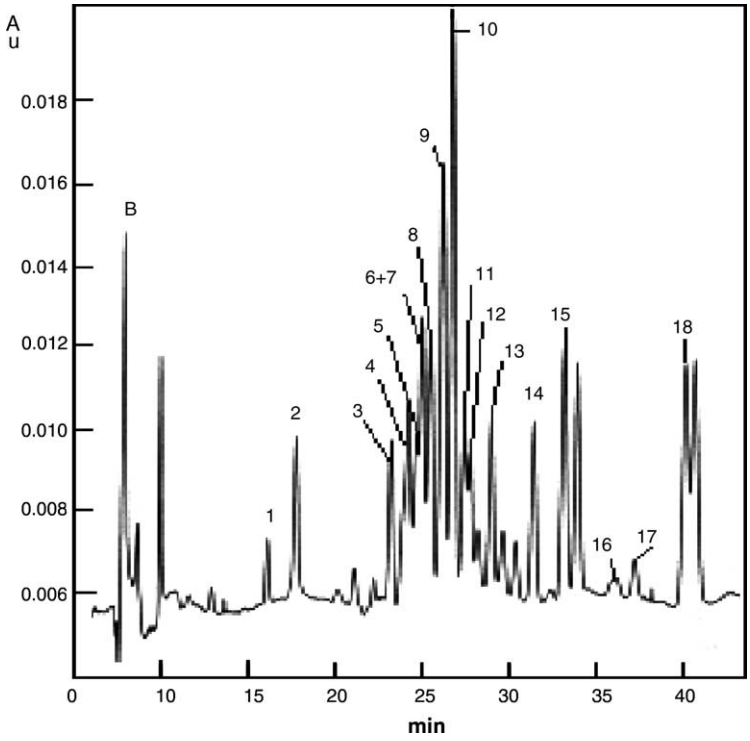

Fig. 6. Electropherogram of the separation of PTH–amino acids in *S. fusiforme* (condition as in Fig. 1).

standard deviation, R.S.D.) corresponding to peak area response and migration time, respectively. The R.S.D. values (%) for peak area and migration time were less than 3.2 and 1.1%, respectively, for each amino acid. Therefore, the reproducibility of our method is relatively good.

The linearity of each peak area, corresponding to its amino acid response at concentrations of between 50 and 1000 μmol l^{-1}, was calculated by the least squares regression method for $y = A + Bx$, where y, x, A and B are peak area counts, concentration of amino acid (mmol l^{-1}), intercept at y-axis and slope, respectively. The results obtained

Table 2
The data of the amino acids determination in *S. fusiforme* using HPLC and ICE (amino acid analyzer)

Peak number	Amino acid	Percentage of the amino acids in *S. fusiforme*[a] (%)			
		HPCE[b]	R.S.D. (%)	ICE[c]	R.S.D. (%)
1	Arg	0.64	2.0	0.63	UD
2	Lys	0.66	1.7	0.68	UD
3	Phe	0.82	1.3	0.77	UD
4	His	0.27	3.3	0.23	UD
5	Tyr	0.42	2.4	0.41	UD
6 + 7	Leu + Ile	1.77	2.0	1.80	UD
8	Met	0.40	2.5	0.39	UD
9	Val	0.88	2.6	0.90	UD
10	Pro	0.81	2.0	0.76	UD
11	Trp	0.15	3.8	0.19	UD
12	Thr	0.47	1.8	0.63	UD
13	Ser	0.64	1.4	0.70	UD
14	Gly	1.83	1.9	1.81	UD
15	Ala	0.89	1.5	0.94	UD
16	Cys	0.22	2.4	0.19	UD
17	Glu	0.67	2.2	0.70	UD
18	Asp	1.45	1.8	1.43	UD

Each value is the mean of seven independent assays. UD: undetermined.
[a] Percentage (%) means amount of amino acid per 100 mg of *S. fusiforme*.
[b] The data are obtained using the present method.
[c] The data obtained using IEC (amino acid analyzer) are taken from Ref. [3].

were good, with correlation coefficients (r) ranging between 0.9904 and 0.9993. Limits of detection (LOD) were obtained using a signal to noise ratio (S/N) of 3. Their parameters are also listed in Table 1 based on these data, the detection limits range from 2 μmol l^{-1} for tyrosine to 48 μmol l^{-1} for cystine.

3.6. Determination of amino acids in S. fusiforme

The offered scheme of separation of PTH–amino acids allows analyzing practically the whole list of protein amino acids. For leucine and isoleucine the total content is measured.

Under the optimum condition described above, we had separated PTH–amino acids from *S. fusiforme*, and a typical electropherogram is shown in Fig. 6. To determine the accuracy of the present method, these data were compared with IEC (amino acid analyzer) data that were cited in a previous literature [3]. The results of data are listed in Table 2.

4. Conclusions

In order to obtain optimal separation conditions four experimental parameters, i.e. electrolyte concentration, pH, organic modifier and applied voltage were optimized. Increasing the electrolyte concentration could improve efficiency, resolution and sensitivity of the separation, because amino acid derivatives spent more time in the capillary column. The pH of running buffer was the most important parameter for changing the selectivity of capillary electrophoresis. Separation by electrophoresis depended upon differing mobilities of amino acid derivatives, which were directly related to their size and net charge. Increase in salt concentration increased the polarity of the running buffer, and should therefore increase both electroosmotic flow and the electrophoretic mobility. Organic modifiers added to the running buffer altered the polarity and the viscosity of electrolyte, thus affecting both electroosmotic flow and the electrophoretic mobility of the analytes. Applied voltage also affected the separation resolution. Because the migration time of the analytes was significantly shortened and their corresponding current peaks were sharpened when the applied voltage was increased. However, if the applied voltage was too large, more Joule heat was produced by the higher current inside the capillary and separation efficiency was reduced. In the optimum condition: 400 mmol l^{-1} Tris–boric acid (1:1) with 1.0 mmol l^{-1} ethylenedimine buffer (pH 9.5), the applied voltage used for 25 kV, temperature was maintained at 25 °C. The detection wavelength was 254 nm, and therefore, 17 PTH–amino acids were well separated from each other in a mixture of 18 PTH–amino acids. We determine the amino acids in *S. fusiforme* by this method, and find the content of the amino acids range from 0.15 to 1.83%. The relative standard deviation of the amino acids range from 1.3 to 3.8%. Above results demonstrated that this method was a sensitive, simple and practical method and could be used for determining amino acids in *S. fusiforme*.

Acknowledgements

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