

Simultaneous determination of six main nucleosides and bases in natural and cultured *Cordyceps* by capillary electrophoresis

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Abstract

A simple method is described for simultaneous determination of six main nucleosides and bases including adenine, uracil, adenosine, guanosine, uridine and inosine in *Cordyceps* by capillary electrophoresis (CE). Chemometric optimization based on central composite design was employed to find the optimum resolution. The optimum factor space was defined by three parameters: buffer concentration, pH and concentration of acetonitrile as organic modifier. Resolution (R_s) was employed to evaluate the response function. A running buffer composed of 500 mM boric acid, adjusted pH to 8.6 with sodium hydroxide and 12.2% acetonitrile as modifier was found to be the most appropriate for the separation. The contents of the six components were determined by using adenosine monophosphate as an internal standard. Furthermore, hierarchical clustering analysis based on characteristics of 32 peaks in CE profiles from the tested 12 samples showed that natural and cultured *Cordyceps* were in different clusters. Adenosine and inosine were extracted as markers for discrimination of natural *Cordyceps*. The result of clustering based on the two peaks characteristics was in excellent agreement with that based on 32 peaks'. Thus, adenosine and inosine could be used as markers for quality control of natural and cultured *Cordyceps*.

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Keywords: Central composite design; *Cordyceps*; Quality control; Determination

1. Introduction

Cordyceps, one of the well-known traditional Chinese medicines, consists of the dried fungus *Cordyceps sinensis* (Berk) Sacc. growing on the larva of the caterpillar. The parasitic complex of the fungus and the caterpillar is found in the soil of a prairie at an elevation of 3500–5000 m. It is commonly used in China to replenish the kidney and soothe the lung for the treatment of fatigue, night sweating, hyposexuality, hyperglycemia, hyperlipidemia, asthemia after severe illness, respiratory disease, renal dysfunction and renal failure, arrhythmias and other heart disease, and liver disease [1,2]. Recent studies have demonstrated its multiple pharmacological actions such as anti-oxidation [3–6], potentiating

the immune system [7–9] and hypoglycemic activities [10–12].

The natural *Cordyceps* (wild *C. sinensis*) is rare and expensive in the market; workers at China have extensively examined its lifecycle and isolated fermentable strains of *Cordyceps*. Several mycelial strains, indeed, have been manufactured in large quantity by fermentation technology in China [13]; these mycelial fermentation products have been sold as health food products. The market price of natural *C. sinensis* is ~300 times more expensive than that of cultured *Cordyceps* mycelia, though studies showed that the chemical constituents, or even some pharmacological activities, of the former are similar to those of the latter [4,14–16]. Nucleoside is commonly considered as a chemical marker for quality control of *Cordyceps*, and is believed to be one of the active components in *Cordyceps*. Several methods, including high-performance liquid chromatography (HPLC) [17,18], capillary electrophoresis (CE) [19] and thin layer chromatog-

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raphy (TLC) [20], have been reported to quantify the level of nucleosides in *Cordyceps*. However, the markers for quality control of natural and cultured *Cordyceps* are still controversial [21]. In this report, six main nucleosides and bases including adenine, uracil, adenosine, guanosine, uridine and inosine in natural and cultured *Cordyceps* were simultaneously determined by CE. Chemometric optimization based on central composite design was employed to find the optimum resolution. Hierarchical clustering analysis showed that adenosine and inosine could be used as markers for quality control of natural and cultured *Cordyceps*.

2. Materials and methods

2.1. Chemicals, reagents and materials

Boric acid, adenine, uracil, adenosine, guanosine, uridine, inosine and adenosine monophosphate were purchased from Sigma (St. Louis, MO, USA). Acetonitrile for liquid chromatography and sodium hydroxide was purchased from Merck (Darmstadt, Germany). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA). Reagents not mentioned here were from standard sources. Natural *C. sinensis* was obtained from different provinces of China: one from Qinghai and two from Tibet. The identities of these natural *Cordyceps* were confirmed by Professor Ping Li, China Pharmaceutical University, Nanjing, China. Cultured *C. sinensis* mycelia were obtained from Chinese Medicine Factory of Jiangxi (Jianxi), East China Pharmaceutical Factory (Huadong), Wanfeng Pharmaceutical Factory (Wanfeng), Boding Pharmaceutical Factory (Boding), Hiyeah Health Products Co. Ltd., Shenzhen (Hiyeah), Huade Institute of Bioengineering, Chengdu (Huade), Liangji Pharmaceutical Co. Ltd., Guizhou (Liangji), Yuxi Pharmaceutical Co. Ltd., Yunnan (Yuxi), Wanji Pharmaceutical Co. Ltd., Shenzhen (Wanji). All corresponding voucher specimens were deposited at the Institute of Chinese Medical Sciences, Macau University, Macau, China. Dried samples were ground into powder (0.13–0.15 mm, i.d.).

2.2. Instrumentation

All CE separations were performed on a Agilent CE instrument, equipped with a UV diode-array detector. A fused-silica capillary (56 cm × 75 μm i.d., 48 cm effective length) was used throughout this study. A running buffer composed of 500 mM boric acid with 12.2% acetonitrile as modifier and pH adjusted to 8.6 with sodium hydroxide was used for the separation. All solutions were filtered through a 0.25 μm filter (Gelman Science, Ann Arbor, MI, USA). Before sample injection, the capillary was rinsed with 1 M sodium hydroxide and running buffer for 10 min, respectively. Twenty kV applied over the capillary. No pair of running vials (inlet and outlet) was used for more than a total of 2 h running time. Pressure injection was 50 mbar for 6 s, and the detection

was performed at 254 nm. The running time was 25 min at 20 °C.

2.3. Procedures

Buffer containing 0.5 M boric acid and 12.2% acetonitrile was adjusted to pH 8.6 (apparent pH) with 1 M sodium hydroxide. The running buffer was filtered through a 0.25 μm membrane before it was transferred to the inlet/outlet vials. The nucleosides were first dissolved in the buffer at ~1 mg/ml as a stock solution. A certain volume of the stock solution was transferred to a 2-ml volumetric flask, which was brought up to its volume with buffer so as to obtain the desired concentration. All solutions were found stable when stored at 4 °C for 2 months. *Cordyceps* materials (1 g) were mixed with 15 ml buffer and then ultrasonic extraction was performed at room temperature for 15 min. After centrifugation at 4000 × g for 30 min, the supernatant was filtered through a 0.25 μm membrane and certain volume of internal standard solution (2 mg/ml) was added before analysis.

Determination of linearity, reproducibility and recovery of six investigated components in *Cordyceps* were performed using adenosine monophosphate as an internal standard (IS). In brief, the stock solution of the internal standard contained 2.00 mg/ml adenosine monophosphate dissolved in running buffer. Increasing concentration of the six analytes and were run with internal standard (0.2 mg/ml at each concentration level). The calibration curve [peak area ratio of analytes to IS (Y) versus concentration of analyte (X), μg/ml] over the studied range of six investigated components were determined.

2.4. Statistical software package

The statistical software package are SAS system for windows release version 6.12 (SAS Institute, Cary, NC, USA) and SPSS 10.0 for windows (SPSS Inc., Chicago, IL, USA). Both SAS and SPSS comprise a number of “procedures”—graphical, statistical, reporting, processing and tabulating procedures—that enable simple and rapid data evaluation.

3. Results and discussion

3.1. Optimization of CE conditions

3.1.1. Preliminary investigations

Cultured *Cordyceps* was used as a sample for optimization of CE conditions. Before specific limits for individual central composite design (CCD) factors were selected, pilot experiments had to be carried out in which the effects of buffer concentration, pH, temperature, voltage, and proportion of acetonitrile as modifier were studied (Fig. 1). Compared with the investigated standards, the separation of adenine, uracil, and uridine are sensitive to the conditions. Therefore, optimization studies were only carried out with these components.

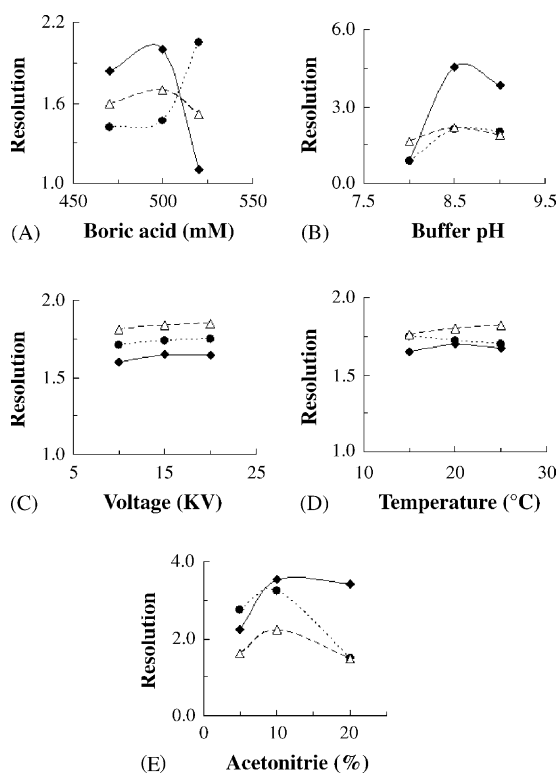


Fig. 1. Influence of selected factors on resolution (R_S) of adenine (●), uracil (◆), and uridine (Δ). Condition: pressure injection 50 mbar for 10 s, 56 cm × 75 μm i.d. capillary (48 cm effective length, Agilent fused-silica), detected at 254 nm. (A) Running buffer 450–550 mM boric acid–sodium hydroxide (pH 8.6) without acetonitrile as organic modifier, voltage 20 kV at temperature 20 °C. (B) Running buffer 0.5 M boric acid–sodium hydroxide (pH 7.5–9.5) without acetonitrile as organic modifier, voltage 20 kV at temperature 20 °C. (C) Running buffer 0.5 M boric acid–sodium hydroxide (pH 8.5) without acetonitrile as organic modifier, voltage 10–20 kV at temperature 20 °C. (D) Running buffer 0.5 M boric acid–sodium hydroxide (pH 8.5) without acetonitrile as organic modifier, voltage 20 kV at temperature 15–25 °C. (E) Running buffer 0.5 M boric acid–sodium hydroxide (pH 8.5) with acetonitrile (5–20%) as organic modifier, voltage 20 kV at temperature 20 °C.

Out of the five factors, three were selected, which displayed the most pronounced effect on the quality of separation expressed as resolution (R_S), the factors being: buffer concentration, pH and proportion of acetonitrile. The effect of voltage and temperature within the range permitted was insignificant for the separation. Take into account the quality and time of analysis, the voltage and temperature were set at 20 kV and 20 °C, respectively.

3.1.2. Central composite design

The central composite design was selected for the optimization of three parameters. From the preliminary experiments, the following upper and lower limits were selected: the parameter boric acid concentration (X_1) range from 470 mM (lowest level) to 520 mM (highest level), pH (X_2) from 8.0 (lowest level) to 9.0 (highest level), and acetonitrile proportion (X_3 , v/v) from 5% (lowest level) to 20% (highest level). The experimental conditions for the central composite design

Table 1
Optimization method parameters for central composite design and response results for average resolution of adenine, uracil, and uridine

Experiment	Boric acid (mM)	Buffer pH	Acetonitrile (% v/v)	Average resolution (R_S)
1	480	8.21	8.17	1.43
2	510	8.21	8.17	1.59
3	480	8.79	8.17	2.10
4	510	8.79	8.17	2.33
5	480	8.21	16.83	1.10
6	510	8.21	16.83	1.14
7	480	8.79	16.83	1.61
8	510	8.79	16.83	3.00
9	470	8.5	12.5	1.78
10	520	8.5	12.5	1.65
11	495	8	12.5	1.49
12	495	9	12.5	2.01
13	495	8.5	5	2.25
14	495	8.5	20	1.57
15–20	495	8.5	12.5	2.92

The mean values of three determinations are presented. The variation is less than 6% of the mean.

and average resolution of adenine, uracil, and uridine were presented in Table 1. The experimental design required 15 experiments. Five additional experiments were carried out at the center point to estimate the overall error. The experiments were performed in random order to avoid systematic error.

Using SAS program, experimental results were fitted to a second-order model relating average resolution of adenine, uracil, and uridine to the factors. The model was described as follow:

$$Y = -614.99 + 1.51X_1 + 57.92X_2 - 1.39X_3 - 0.0019X_1^2 - 4.60X_2^2 - 0.0176X_3^2 + 0.0408X_1X_2 + 0.002X_1X_3 + 0.096X_2X_3$$

Since the quadratic response surface is calculated in ($N + 1$) dimensions, where N is the number of factors in the CCD, the quadratic response surface for the three factors involved generates a four-dimensional response surface, which can be readily visualized in the three-dimensional (3-D) response surface. The response model is mapped against two experimental factors while the third is held constant at its optimum. That way, 3-D response function is depicted in Fig. 2. Three variables at the optimum value of average R_S (3.015) proposed were determined. And the following optimum CE conditions have been selected: buffer (boric acid) concentration, 500 mM; pH: 8.6; proportion of acetonitrile: 12.2%; voltage 20 kV at the temperature 20 °C. A comparison of electrophoretic traces of separation for cultured *Cordyceps* before and after optimization was given in Fig. 3.

3.2. Identification and quantitation of investigated compounds in *Cordyceps*

Electrophoretic profiles of water-soluble extracts from natural and cultured *Cordyceps* were shown in Fig. 4. The

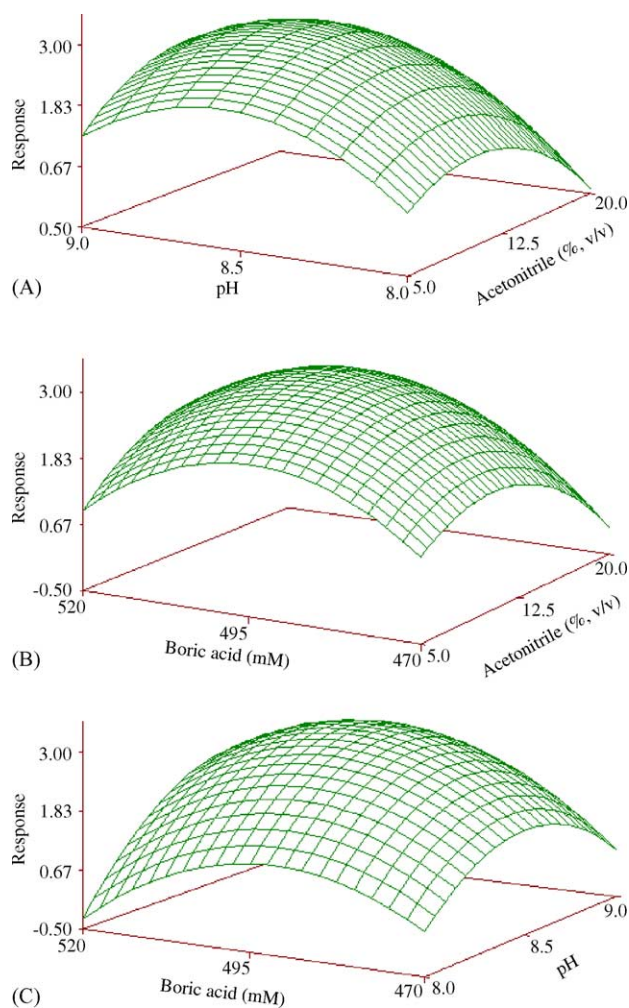


Fig. 2. Response surface for resolution response function of adenine, uracil, and uridine. (A) The buffer (boric acid) concentration was held at its optimum predicted value (500 mM). (B) The buffer pH was held at its optimum predicted value (8.63). (C) The proportion of acetonitrile (% v/v) was held at its optimum predicted value (12.23%). Response was presented as the average resolution of adenine, uracil, and uridine.

identification of investigated compounds was carried out by comparison of their migration time and UV spectra with those obtained injecting standards in the same conditions or by spiking *Cordyceps* samples with stock standard solutions.

The linearity, regression, reproducibility and recovery study of six investigated components in *Cordyceps* were performed using adenosine monophosphate as an internal standard mentioned above. The migration time repeatability of six components for short-term (1 day) and long-term (3 days) were good, and R.S.D. were less than 0.62% and 0.81%, respectively. The area repeatability was also calibration by using internal standard. Both short-term and long-term repeatability (R.S.D.) of six peaks area detected for the investigated components were less than 1%. The linearity of each standard was confirmed by plotting the peak area ratio of individual standard and adenosine monophosphate (internal standard) versus the concentration of the standard. The high correlation

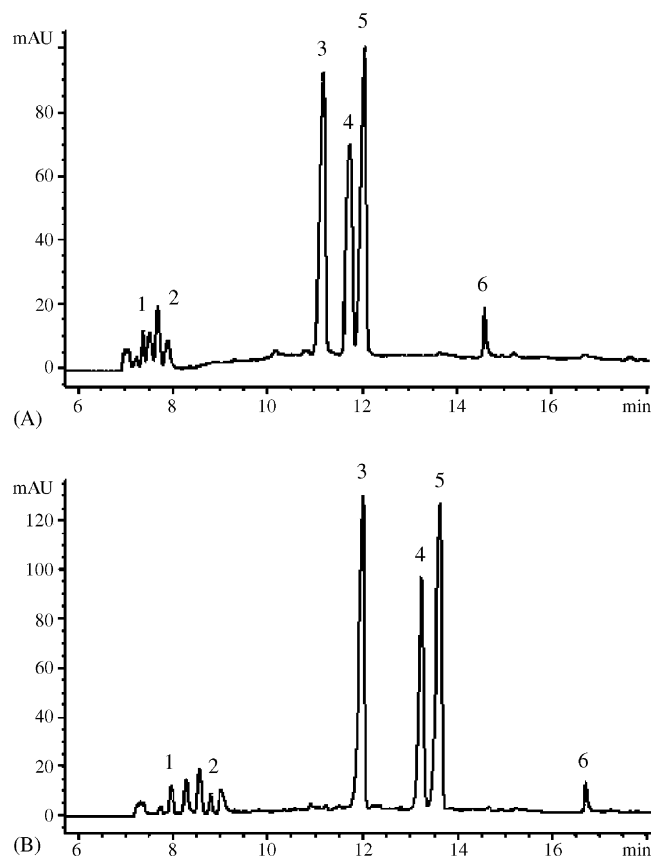


Fig. 3. The comparison of electrophoretic traces of separation for a cultured *Cordyceps* before and after optimization. Condition: pressure injection 50 mbar for 10 s, 56 cm \times 75 μ m i.d. capillary (48 cm effective length, Agilent fused-silica), voltage 20 kV at temperature 20 $^{\circ}$ C, detected at 254 nm. (A) Running buffer 0.5 M boric acid–sodium hydroxide (pH 8.6) without acetonitrile as organic modifier. (B) Running buffer 0.5 M boric acid–sodium hydroxide (pH 8.6) with 12.2% acetonitrile as organic modifier. (1) Adenine, (2) uracil, (3) adenosine, (4) guanosine, (5) uridine, and (6) inosine.

coefficient (r) values indicated good correlations between investigated compounds concentrations and their peak areas ratio. The recovery was performed by adding 0.3, 0.3, 2.5, 2.5, 4.0, 0.3 mg of adenine, uracil, adenosine, guanosine, uridine and inosine into an accurately weighed (0.5 g) Jiangxi cultured *Cordyceps* mycelia, respectively. The mixture was extracted and analyzed using the mentioned method above. The reproducibility ($n = 3$) was also determined by using Jiangxi cultured *Cordyceps* mycelia. The linearity, recovery and reproducibility of the investigated components in *Cordyceps* were reported in Table 2.

By using the calibration curve of each investigated compound, natural and cultured *Cordyceps* were analyzed. Table 3 shows the summary results. In general, the amount of investigated components except inosine (less than 0.55 mg/g) in cultured *Cordyceps* mycelia was higher than those in natural *Cordyceps* (more than 2.36 mg/g). Especially, the amount of adenosine (less than 0.27 mg/g) in natural *Cordyceps* was much lower than that in cultured *Cordyceps* (more than 3.40 mg/g). The results are corresponding with those of our

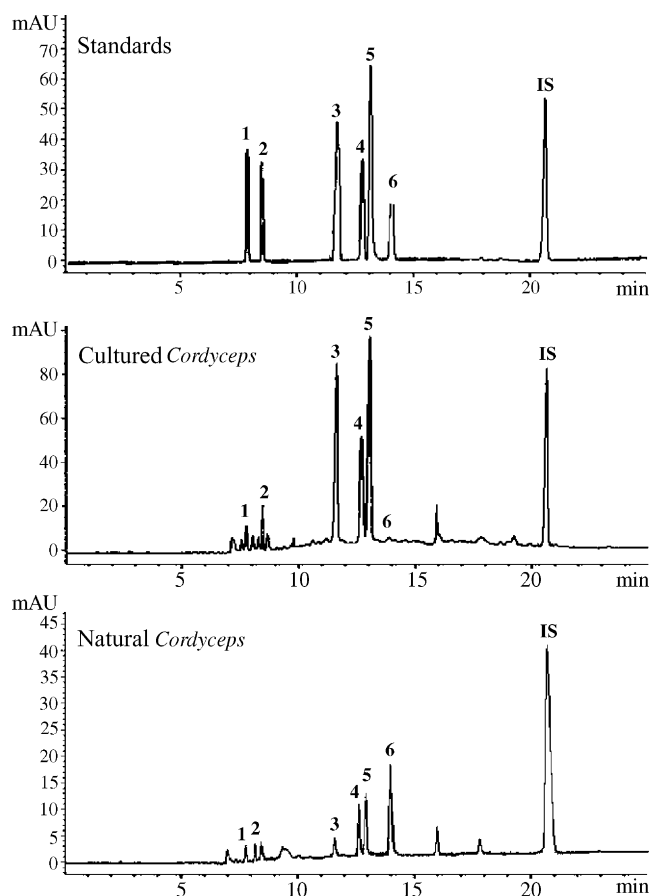


Fig. 4. The capillary electrophoresis profiles of natural and cultured *Cordyceps*. Condition: pressure injection 50 mbar for 6 s, 56 cm \times 75 μ m i.d. capillary (48 cm effective length, Agilent fused-silica), running buffer 0.5 M boric acid–sodium hydroxide (pH 8.6) with 12.2% acetonitrile as organic modifier, voltage 20 kV, temperature 20 °C, detected at 254 nm. Standards, natural and cultured *Cordyceps* are shown. Several individual samples were tested, and similar profiles were achieved. (1) Adenine, (2) uracil, (3) adenosine, (4) guanosine, (5) uridine, (6) inosine. IS adenosine monophosphate.

previous study [19]. Otherwise, it is very interesting that natural *Cordyceps* contain much higher amount of inosine which stimulates axon growth in vitro and in the adult central nerve system [22], than the cultured ones. Actually, the content of inosine in most cultured *Cordyceps* mycelia was too low to be determined.

Table 2
Linear regression data and recoveries of investigated components from *Cordyceps*

Analyte	Linear regression data				Recovery (%)	Reproducibility (%)
	Linear range (μ g/ml)	Slope	Intercept	r^2 ($n = 6$)		
Adenine	3.0–29.6	0.3	–1.2	0.9984	96.7	1.3
Adenosine	48.0–484.0	2.1	–2.6	0.9981	97.1	0.8
Guanosine	57.5–575.0	0.2	–0.8	0.9992	97.5	0.7
Uracil	4.8–48.0	0.2	2.0	0.9987	97.4	1.1
Uridine	91.7–917.0	0.3	–20.1	0.9988	96.3	1.4
Inosine	42.0–420.0	0.3	–11.0	0.9990	97.6	2.1

r^2 , squares of correlation coefficients for the standard curves. Reproducibility is presented as R.S.D. of three replicates.

Table 3
The contents (mg/g) of six components in natural and cultured *Cordyceps*

	Adenine	Uracil	Adenosine	Guanosine	Uridine	Inosine
Natural <i>Cordyceps</i>						
Tibet 1	0.16	0.14	– ^a	1.78	3.40	2.36
Tibet 2	0.16	0.15	–	2.74	4.29	3.48
Qinghai	0.16	0.04	0.27	2.60	4.40	3.45
Cultured <i>Cordyceps</i>						
Jiangxi	0.43	0.67	5.44	5.17	7.61	0.55
Huadong	0.13	0.16	4.15	5.49	5.34	+ ^b
Wanfeng	0.41	0.40	9.71	6.73	15.80	–
Boding	0.94	0.30	5.12	4.71	4.97	0.51
Hiyeh	0.26	0.26	5.27	4.31	8.92	–
Huade	+	0.08	3.40	2.22	2.95	–
Liangji	0.29	0.33	3.77	2.29	7.14	–
Yuxi	0.26	0.33	3.79	2.38	7.43	–
Wanji	0.72	0.53	5.96	1.08	12.05	–

The mean values of three determinations are presented. The variation is less than 10% of the mean.

^a Undetectable.

^b Below lower limit of linear range.

3.3. Discrimination of natural and cultured *Cordyceps*

In Asia, most of the commercial products are being claimed to be derived from natural *Cordyceps*, therefore, the exact identity is assurance of safety and efficacy of medication. In order to discriminate natural and cultured *Cordyceps*, hierarchical cluster analysis was performed based on 32 peaks characteristics from electrophoretic profiles of natural and cultured *Cordyceps*. A method named as average linkage between groups was applied, and Squared Euclidean distance was selected as measurement. Fig. 5A shows the results on the tested 12 samples of *Cordyceps*, which are divided into two main clusters. Cultured *Cordyceps* are in cluster one, and natural *Cordyceps* are in cluster two. Among the peaks of electropherograms for cluster analysis of samples, two typical peaks of adenosine and inosine were optimized based on cluster analysis of 32 peaks and then principle component analysis. Using the peaks characteristics of adenosine and inosine, hierarchical cluster analysis of the tested 12 samples was performed as before. The result was very similar to the one derived from 32 peaks characteristics (Fig. 5B). Therefore, the characteristics of peaks, especially adenosine and inosine, from electrophoretic profiles of

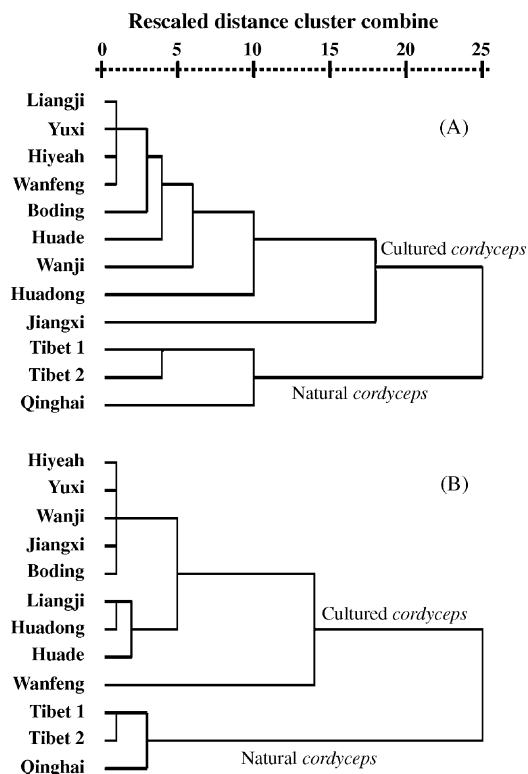


Fig. 5. Dendrograms resulting from average linkage between groups hierarchical cluster analysis. The hierarchical clustering was done by SPSS software. A method named as average linkage between groups was applied, and Squared Euclidean distance was selected as measurement. (A) Dendrogram resulting from 32 peaks, their migration times and peak intensities, derived from CE fingerprints of the tested 12 *Cordyceps* samples. (B) Dendrogram resulting from the characteristics of two peaks, adenosine and inosine, derived from CE profiles of the tested 12 *Cordyceps* samples.

nucleosides could be used as markers for discrimination and quality control of natural and cultured *Cordyceps*. Cordycepin, one of the main compounds in *Cordyceps militaris*, has shown multiple pharmacological activities [23–25]. It is not used as marker for quality control because it is rare in *C. sinensis* [18]. In addition, mannitol [26], ergosterol [27], sterols [28], protein constituent [29] and polysaccharide [6,30] are active components in *C. sinensis*. They could also be as markers for evaluating the quality of *Cordyceps* [18,26,31].

4. Conclusion

It was reported in this study that simultaneous determination of six main nucleosides in natural and cultured *Cordyceps* by using CE based on optimization of CE conditions. The results showed that the developed method have a good separation and repeatability. Discrimination of natural and cultured *Cordyceps* was performed based on hierarchical cluster analysis of the peaks characteristics from CE profiles. Furthermore, adenosine and inosine were optimized. Clus-

ter analysis confirmed that adenosine and inosine could be used as markers for quality control of natural and cultured *Cordyceps*.

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References

- [1] J.S. Zhu, G.M. Halpern, K. Jones, J. Altern. Complement. Med. 4 (1998) 289.
- [2] J.S. Zhu, G.M. Halpern, K. Jones, J. Altern. Complement. Med. 4 (1998) 429.
- [3] Y. Yamaguchi, S. Kagota, K. Nakamura, K. Shinozuka, M. Kunitomo, Phytother. Res. 14 (2000) 647.
- [4] S.P. Li, P. Li, T.T.X. Dong, K.W.K. Tsim, Phytomedicine 8 (2001) 207.
- [5] S.P. Li, Z.R. Su, T.T.X. Dong, K.W.K. Tsim, Phytomedicine 9 (2002) 319.
- [6] S.P. Li, K.J. Zhao, Z.H. Song, T.T.X. Dong, C.K. Lo, J.K.H. Cheung, S.Q. Zhu, K.W.K. Tsim, Life Sci. 73 (2003) 2503.
- [7] J.H. Koh, K.W. Yu, H.J. Suh, Y.M. Choi, T.S. Ahn, Biosci. Biotechnol. Biochem. 66 (2002) 407.
- [8] K. Nakamura, Y. Yamaguchi, S. Kagota, K. Shinozuka, M. Kunitomo, Jpn. J. Pharmacol. 79 (1999) 505.
- [9] J.H. Chiu, C.H. Ju, L.H. Wu, W.Y. Lui, C.W. Wu, M.S. Shiao, C.Y. Hong, Am. J. Chin. Med. 26 (1998) 159.
- [10] T.W. Balon, A.P. Jasman, J.S. Zhu, J. Altern. Complement. Med. 8 (2002) 315.
- [11] T. Kiho, A. Yamane, J. Hui, S. Usui, S. Ukai, Biol. Pharm. Bull. 19 (1996) 294.
- [12] T. Kiho, K. Ookubo, S. Usui, S. Ukai, K. Hirano, Biol. Pharm. Bull. 22 (1999) 966.
- [13] D.H. Yin, X.M. Tang, China J. Chin. Materia Medica 20 (1995) 707.
- [14] D.M. Chen, Chung Hsi I Chieh Ho Tsa Chih 5 (1985) 42.
- [15] Y.H. Sun, Chung Yao Tung Pao 10 (1985) 3.
- [16] D.J. Du, Chung Yao Tung Pao 11 (1986) 51.
- [17] M.S. Shiao, Z.N. Wang, L.J. Lin, J.Y. Lien, J. Wang, J. Bot. Bull. Acad. Sin. 35 (1994) 261.
- [18] S.P. Li, P. Li, C.M. Lai, Y.X. Gong, K.K.W. Kan, T.T.X. Dong, K.W.K. Tsim, Y.T. Wang, J. Chromatogr. A 1036 (2004) 239.
- [19] S.P. Li, P. Li, T.T.X. Dong, K.W.K. Tsim, Electrophoresis 22 (2001) 144.
- [20] G.D. Zhang, Y.X. Li, J. Pharm. Anal. 7 (1987) 6.
- [21] S.P. Li, P. Li, H. Ji, T.T.X. Dong, K.W.K. Tsim, Q. Zhu, J. Chin. Pharm. Sci. 10 (2001) 175.
- [22] L.I. Benowitz, D.E. Goldberg, N. Irwin, Prog. Brain Res. 137 (2002) 389.
- [23] X. Zhou, C.U. Meyer, P. Schmidtke, F. Zepp, Eur. J. Pharmacol. 453 (2002) 309.
- [24] Y.J. Ahn, S.J. Park, S.G. Lee, S.C. Shin, D.H. Choi, J. Agric. Food Chem. 48 (2000) 2744.
- [25] E.N. Kodama, R.P. McCaffrey, K. Yusa, H. Mitsuya, Biochem. Pharmacol. 59 (2000) 273.

- [26] S.P. Li, T.X. Dong, Z.R. Su, Q. Zhu, K.W.K. Tsim, in: China Pharmaceutical Association (Ed.), *Forum of Modern Pharmaceutical Analysis*, Xinhua Press, Beijing, 2001, p. 149.
- [27] Y.C. Kuo, S.C. Weng, C.J. Chou, T.T. Chang, W.J. Tsai, *Br. J. Pharmacol.* 140 (2003) 895.
- [28] J.W. Bok, L. Lermer, J. Chilton, H.G. Klingeman, G.H. Towers, *Phytochemistry* 51 (1999) 891.
- [29] W.F. Chiou, P.C. Chang, C.J. Chou, C.F. Chen, *Life Sci.* 66 (2000) 1369.
- [30] J.H. Xiao, Z.Q. Liang, A.Y. Liu, *Yao Xue Xue Bao* 37 (2002) 589.
- [31] S.P. Li, P. Zhang, Q. Xia, Z.G. Huang, Q. Zhu, *Chin. J. Pharm. Anal.* 23 (2003) 20.