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Determination of nucleosides and nucleobases in different species of *Cordyceps* by capillary electrophoresis–mass spectrometry

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ABSTRACT

In present study, a capillary electrophoresis–mass spectrometry (CE–MS) method was developed for the simultaneous analysis of 12 nucleosides and nucleobases including cytosine, adenine, guanine, cytidine, cordycepin, adenosine, hypoxanthine, guanosine, inosine, 2′-deoxyuridine, uridine and thymidine in natural and cultured *Cordyceps* using 5-chlorocytosine arabinoside as an internal standard (IS). The CE separation conditions and MS parameters were optimized systematically for achieving good CE resolution and MS response of the investigated compounds. The optimum CE electrolyte was 100 mM formic acid containing 10% (v/v) methanol. The optimum MS parameters were as follows: 75% (v/v) methanol containing 0.3% formic acid with a flow rate of 3 μL/min was selected as the sheath liquid; the flow rate and temperature of drying gas were 6 L/min and 350 °C, respectively. The optimized CE–MS method was successfully applied for the simultaneous determination of 12 nucleosides and nucleobases in natural and cultured *Cordyceps*. On the basis of quantitative results, the total content of nucleosides is much higher in cultured *Cordyceps* (9138 ± 4823 μg/g for cultured *C. sinensis*; 3722 ± 1446 μg/g for *C. militaris*) than in natural ones (2167 ± 412 μg/g). However, the hypoxanthine (131 ± 47 μg/g) and inosine (335 ± 90 μg/g) are much higher in natural *C. sinensis*. Cordycepin, which is abundant in cultured *C. militaris* (2276.5 ± 842.6 μg/g), is only found in natural *C. sinensis* with very low content and cannot be detected in the cultured ones.

1. Introduction

As a very precious traditional Chinese medicine (TCM) with the functions of replenishing kidney and soothing lung, *Cordyceps* has been used for treating a wide range of disorders, mainly including, fatigue, night sweating, hyposexualities, hyperglycemia, hyperlipidemia, asthemia after severe illness, respiratory disease, renal dysfunction and failure, arrhythmias and other heart diseases, and liver disease [1,2].

Nucleosides and nucleobases, involved in the regulation and modulation of various physiological processes in body through purinergic and/or pyrimidine receptors [3,4], are recognized to be the main bioactive components in *Cordyceps* [5]. Adenosine has been used as a marker for the quality control of *C. sinensis* in Chinese Pharmacopoeia [6]. To date, more than 10 nucleosides, nucleobases and their related compounds, including adenine, adenosine, cytosine, cytidine, uridine, guanine, guanosine, hypoxanthine, inosine, thymine, thymidine, 2′-deoxyuridine and cordycepin have been isolated and/or identified from *Cordyceps* [5]. Therefore, determination of nucleosides and nucleobases is extremely important for the pharmacological study and quality control of *Cordyceps* and its products.

Until now, several chromatographic methods such as thin layer chromatography (TLC) [7,8], high-performance liquid chromatography (HPLC) [9–11], liquid chromatography–mass spectrometry (LC–MS) [12–14] and ultra-performance liquid chromatography (UPLC) [15] have been applied for the analysis of nucleosides and related compounds in *Cordyceps*. In addition, capillary electrophoresis (CE) has steadily begun to play an increasing important role in various pharmaceutical analyses. Typically, CE has many attractive features for the separation and quantification such as simplicity, high separation efficiency, small sample volume requirement and low organic solvent consumption [16–18]. To date, the nucleoside and nucleobase compounds in *Cordyceps* have been analyzed by capillary zone electrophoresis (CZE) [19–24] and capillary electrochromatography (CEC) [25]. However, in the previous CE methods, only a limited number of nucleosides and nucleobases were determined in most cases [19–24]. Furthermore, these methods were typically lacking of analytical specificity because of the low selectivity of UV detector, which resulted in identification and quantification difficulties for the analysis of real samples.

Mass spectrometry (MS) provides a specific and selective tool, in which a selected ion can be used for the quantification analysis [18].

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As a multidimensional analytical approach, CE–MS coupling combines the advantages of both techniques, which is considered as a powerful method for separation and quantification [26–28]. Until now, CE–MS has been used for analysis of nucleosides in urinary sample [28,29], anti-HIV nucleosides [30] and their active metabolite [27], and beer [31]. Although the migration behaviour of a set of 11 nucleosides and nucleobases in capillary electromigration techniques were investigated [31], there were only a limited number of analytes quantified by CE–MS [27–31]. On the other hand, Chinese medicine such as *Cordyceps* usually has numerous endogenous

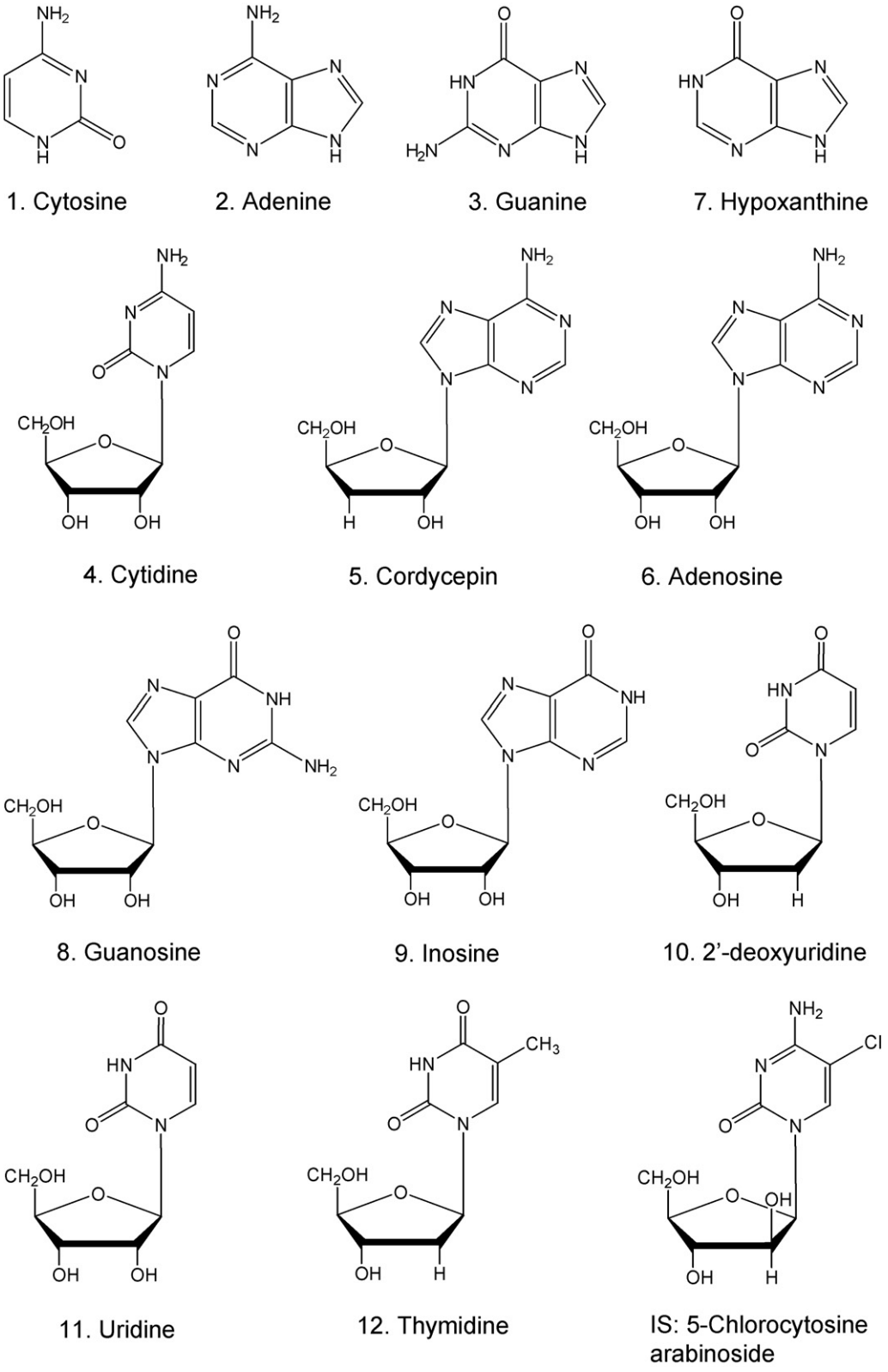

Fig. 1. Chemical structures of investigated nucleosides and related compounds.

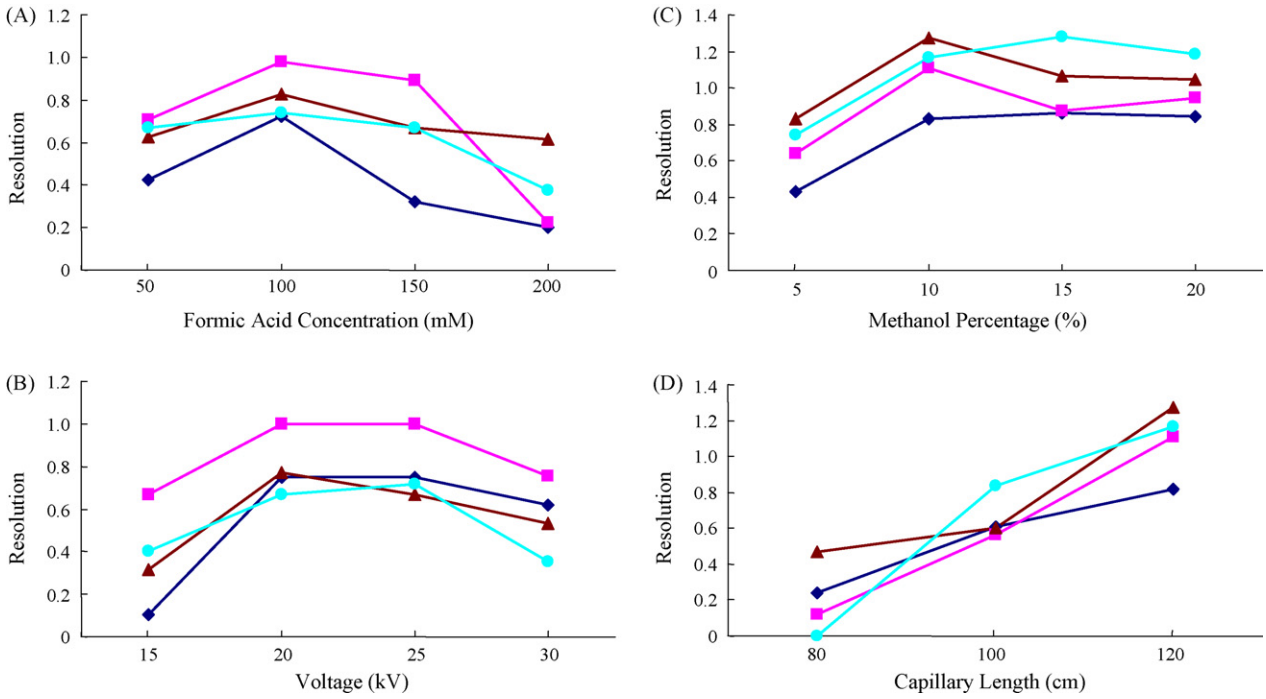

Fig. 2. Effect of electrolyte concentration (A), applied voltage (B), methanol percentage (C) and capillary length (D) on the resolutions of cytidine and cordycepin (◆), cordycepin and adenosine (■), hypoxanthine and guanosine (●), inosine and uridine (▲). Experimental conditions: 75% (v/v) methanol containing 0.3% formic acid with the flow rate of 3 μL/min as the sheath liquid; the nebulizer gas pressure, drying gas flow rate, and drying gas temperature for the ESI source were set at 15 psi, 6 L/min and 350 °C, respectively. For other experimental conditions please refer to Section 2.3. Each point was repeated three times. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

compounds. Therefore, CE–MS should be extremely suitable for analyzing nucleosides and nucleobases in *Cordyceps*, as the compounds with similar CE migration time can be differentiated using mass-to-charge information from the MS detection. Unfortunately, most of the common buffer systems used in CZE cannot be applied to the CE–MS analysis directly, due to the fact that MS sensitivity may deteriorate as the bulk flow of non-volatile buffer (e.g. borate buffer) enters the ion source.

As the first attempt, we developed a new CE–MS for the analysis of nucleosides and nucleobases in *Cordyceps*. The various parameters affecting the resolution of CE separation, namely the electrolyte concentration, applied voltage, organic modifier and capillary length were investigated and optimized systematically. After the optimum electrophoretic separation conditions were selected, the ESI-MS conditions were also optimized to obtain high MS responses. Based on the optimum CE–MS analytical conditions, the contents of these nucleoside compounds in natural and cultured *Cordyceps* were measured and compared.

2. Materials and methods

2.1. Materials and chemicals

Cytosine, cytidine, guanine, uridine, hypoxanthine, 2′-deoxyuridine, inosine, guanosine, thymidine, adenine, adenosine, cordycepin and 5-chlorocytosine arabinoside (Fig. 1) were purchased from Sigma (St. Louis, MO, USA). Formic acid and acetic acid were purchased from Fisher Scientific (Hanover Park, IL, USA); ammonia solution (28%) was purchased from APS Finechem (NSW, Australia); methanol was purchased from Merck (Darmstadt, Germany). Ultra-pure water was obtained using a Millipore Milli-Q Plus system (Millipore, Bedford, MA, USA). The materials of natural *C. sinensis* were obtained from four different regions of China, namely Qinghai (1 sample), Tibet (2 samples), Sichuan (1 sample) and Macau (Zhongqiao, 1 sample). The identities of these natural *Cordyceps* were confirmed by Dr. Shao-Ping Li, University of Macau, Macau SAR, China. Cultured *C. sinensis* mycelia were obtained from Hebei, Anhui, Jiangxi, Huadong and Wanfeng. Cultured *C. militaris*, one of the substitutes for *C. sinensis*, was obtained from Quanxin, Aoli and Xiankang. The species of the cultured *Cordyceps* were certified by State Food and Drug Administration of China or manufacturer. The voucher specimens of *Cordyceps* were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macau, China.

2.2. Sample preparation

Each accurately weighed *Cordyceps* material (0.2 g) was mixed with 10 mL hot (95 °C) Milli-Q water, and then immediate ultrasonic extraction of nucleosides and nucleobases was performed at 75 °C for 30 min. After cooling to room temperature, the supernatant was filtered through a 0.25 μm membrane, and then diluted 1:1 by internal standard solution (5-chlorocytosine arabinoside, 0.14 mg/mL) prior to analysis.

2.3. CE–ESI-MS analysis

All CE–MS analysis were performed on an Agilent CE system in conjunction with an Agilent Trap XCT mass spectrometer equipped with an Agilent CE–ESI-MS sprayer kit (G1607A) and an Agilent CE–ESI-MS adapter kit (G1603A) (Agilent Technologies, Waldbronn, Germany). The CE–ESI-MS adapter kit includes a capillary cassette, which facilitates thermostating of the capillary, and the CE–ESI-MS sprayer kit, which simplifies coupling the CE system with MS system equipped with an electrospray source. The design of the sprayer consists of a triaxial flow arrangement, in which the CE eluent is mixed with a sheath liquid at the sprayer tip, and then nebulized with nitrogen gas. Unless stated otherwise, sheath liquid with the flow rate of 3 μL/min was delivered using a Hewlett Packard 1100 series isocratic pump equipped with a 1:100 splitter. All system

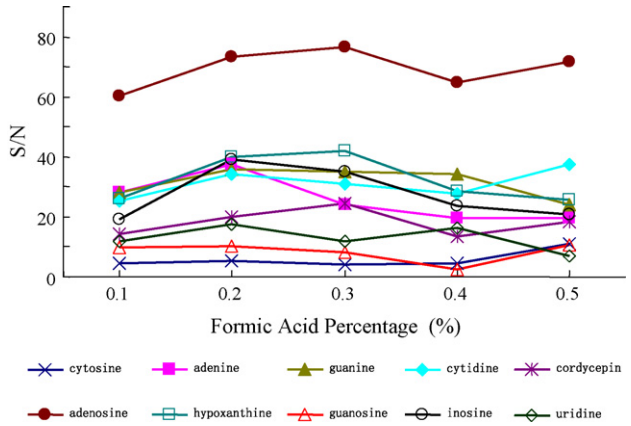

Fig. 3. Effect of formic acid percentage (v/v) in sheath liquid on the sensitivity of nucleosides. Experimental conditions: 100 mM formic acid containing 10% methanol as CE electrolyte, 120 cm length capillary was used for separation with the temperature at 25 °C, and 25 kV as the applied voltage. For other experimental conditions please refer to Section 2.3. Each point was repeated three times.

control, data acquisition and data analysis were performed with the Agilent CE ChemStation and Agilent LC/MSD Trap software. Separations were carried out using a fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) with 50 μm i.d. × 120 cm total length using a suitable electrolyte (100 mM formic acid and 10% (v/v) methanol), unless stated otherwise. The capillary temperature was thermostated to 25 °C. Prior to first use, a new capillary was flushed with sodium hydroxide (0.1 M) for 10 min and water for 10 min consecutively. Before each injection, the capillary was preconditioned for 5 min by flushing with the CE electrolyte. While after every 10 runs, the capillary was flushed with sodium hydroxide (0.1 M) for 10 min, water for 10 min and CE electrolyte for 10 min consecutively. Sample was injected into the capillary with an injection time of 6 s at 50 mbar.

ESI-MS analysis was performed in the positive mode, and the full scan mode was set at *m*/*z* 50–350. The capillary voltage was set at 4500 V. For quantitative analyses, selected ion monitoring (SIM) mode was used.

2.4. Calibration curves

Stock solutions containing 12 analytes were prepared and diluted to appropriate concentration with ultrapure water, then mixed with 0.14 mg/mL 5-chlorocytosine arabinoside solution in the ratio 1:1 to establish the calibration curves. Six concentrations of the 12 analytes' solution were analyzed in triplicate, and then the calibration curves were constructed by plotting the relative peak areas *versus* the concentrations of each analyte.

2.5. LOD and LOQ

Stock solution containing 12 reference compounds was diluted to a series of appropriate concentrations with ultrapure water, and an aliquot of the diluted solutions were injected into CE–MS for analysis. The limits of detection (LOD) and quantification (LOQ) under the optimized CE–MS conditions were determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively.

2.6. Precision, repeatability and recovery

Intra- and inter-day variations were chosen to determine the precision of the developed assay. For intra-day variability test, the mixed standards solution was analyzed for six replicates ($n = 6$) within 1 day, while for inter-day variability test, the solution was examined in duplicates for consecutive 3 days ($n = 6$). Variations were expressed by the relative standard deviations (RSD) for intra- and inter-day.

The recovery was preformed by adding known amount of individual standards into a certain amount (0.1 g) of cultured *Cordyceps* (from Hebei) material. Six replicates were performed. The mixture was extracted and analyzed using the method mentioned above.

$$\text{Recovery}(\%) = \frac{\text{amount found} - \text{original amount}}{\text{amount spiked}} \times 100$$

3. Results and discussion

3.1. Optimization of CE parameters

In this study, the separation conditions of 12 nucleosides and nucleobases by CE–MS were investigated and optimized thoroughly. Typically, the separation resolution ($R_{A,B}$) of two peaks *A* and *B* is defined here as [32]:

$$R_{A,B} = \frac{2(t_B - t_A)}{W_B + W_A}$$

where t_A, t_B represent the migration time and W_A, W_B represent the width of the peaks *A* and *B* measured by extrapolating the relatively straight sides to the baseline, respectively.

During the preliminary investigation on the resolution of separation, the effect of two volatile organic acids, i.e. acetic acid and formic acid at the concentration of 100 mM with different pH (2.2–4.8) adjusted by ammonia was compared. It was observed that more favorable separation resolutions of most compounds could be achieved at lower pH values. Therefore, formic acid without ammonia adjustment was selected for further optimization, in order to obtain the best resolutions of all the neighboring peaks in a short analytical time. As the resolutions of cytidine and cordy-

Table 1
Linear regression data, LOD and LOQ of the investigated compounds.

Analytes	Base peak (*m*/*z*)	Linear regression data			LOD (μg/mL)	LOQ (μg/mL)
		Regressive Equation	Test range (μg/mL)	R^2		
Cytosine	112	$y = 16.8950x + 0.0159$	1.17–25.00	0.9992	0.31	1.04
Adenine	136	$y = 31.3950x + 0.0301$	0.64–20.63	0.9913	0.16	0.43
Guanine	152	$y = 15.3240x + 0.0136$	0.59–18.75	0.9944	0.13	0.42
Cytidine	244	$y = 7.7185x + 0.0178$	0.90–28.75	0.9956	0.27	0.78
Cordycepin	252	$y = 46.7530x + 0.0865$	0.98–31.25	0.9923	0.12	0.45
Adenosine	268	$y = 25.4060x + 0.1019$	1.11–71.25	0.9936	0.28	0.62
Hypoxanthine	137	$y = 24.2320x + 0.0257$	0.90–14.38	0.9900	0.23	0.75
Guanosine	284	$y = 8.2395x + 0.0408$	3.98–63.75	0.9890	0.50	2.00
Inosine	137	$y = 11.0960x + 0.0210$	2.27–36.25	0.9976	0.57	2.06
2′-Deoxyuridine	229	$y = 2.1620x - 0.0023$	50.63–135.00	0.9915	12.66	38.94
Uridine	245	$y = 2.8112x - 0.0147$	24.38–168.75	0.9924	9.75	20.83
Thymidine	243	$y = 9.6757x - 0.3883$	53.75–215.00	0.9970	7.88	18.53

Table 2
Short term and long term repeatability of the investigated compounds.

Analytes	Short term (n = 6), RSD (%)		Long term (n = 6), RSD (%)	
	RPA[a]	Rt_R	RPA	Rt_R
Cytosine	3.5	0.4	4.5	0.7
Adenine	1.9	0.4	1.6	0.7
Guanine	1.3	0.4	4.6	0.7
Cytidine	2.7	0.3	4.5	0.4
Cordycepin	3.6	0.3	4.0	0.4
Adenosine	2.1	0.1	2.6	0.4
Hypoxanthine	2.3	0.1	2.2	0.2
Guanosine	2.1	0.2	1.7	0.2
Inosine	2.1	0.3	2.4	0.4
2′-Deoxyuridine	2.7	0.4	4.9	0.8
Uridine	2.3	0.4	4.3	0.8
Thymidine	2.4	0.4	3.8	0.8

[a] RPA, relative peak area to internal standard; Rt_R, relative retention time to internal standard.

cepin, cordycepin and adenosine, hypoxanthine and guanosine, and inosine and uridine were the poorest as compared to other analytes, they were chosen as optimization markers. Then, the effect of different concentrations including 50, 100, 150 and 200 mM of formic acid on the separation of nucleosides and nucleobases was investigated with 25 kV separation voltage, 25 °C capillary temperature and 120 cm capillary length. As shown in Fig. 2A, 100 mM formic acid concentration was found to be a suitable compromise in resolving all of the tested compounds. Therefore, 100 mM was selected as the optimized concentration of formic acid.

For the applied voltage, 15, 20, 25 and 30 kV were investigated, while the formic acid concentration was kept at 100 mM, capillary temperature was set at 25 °C with the length of capillary as 120 cm. The voltage of 20 and 25 kV had similar effect on the resolutions of the investigated compounds, but compared to 20 kV, the analytical time was reduced when 25 kV was used as applied voltage (Fig. 2B). Thus, a voltage of 25 kV was considered to be the suitable voltage, as the 12 nucleosides and nucleobases were well separated within a shorter analytical time.

As organic modifier (e.g. methanol) can improve the resolution, peak shape and baseline separation of some analytes [33], different percentages of methanol (5%, 10%, 15% and 20%, v/v) in the electrolyte were also investigated, when the formic acid concentration, applied voltage, capillary temperature and capillary length were kept at 100 mM, 25 kV, 25 °C and 120 cm, respectively. The results showed that the effect of the methanol on the resolutions were relatively higher when the percentage of methanol reached 10% (Fig. 2C). Consequently, 10% (v/v) methanol was selected as organic modifier.

The separation of compounds should be based on the appropriate length of capillary, thus different lengths of capillary (80, 100 and 120 cm) were tested for the separation of nucleosides and nucleobases, when 100 mM formic acid containing 10% methanol, 25 °C, and 25 kV were used as CE electrolyte, capillary temperature and applied voltage, respectively. As it can be seen from the results (Fig. 2D), long capillary (120 cm) led to better resolutions of investigated compounds, and was finally chosen as the optimum capillary length.

In addition, different capillary temperatures (15, 20, 25 and 30 °C) were also investigated with 100 mM formic acid containing 10% methanol as CE electrolyte, 120 cm as capillary length and 25 kV as the applied voltage (data not shown). As the temperature of the capillary had no significant effect on the separation, 25 °C was selected. Considering both the resolutions of 12 compounds and total analytical time, the optimum conditions of CE were concluded to be: 100 mM formic acid containing 10% (v/v) methanol as

Table 3
Contents (μg/g) of the investigated compounds in natural and cultured *Cordyceps*.

Analytes	Natural *C. sinensis*					Cultured *C. sinensis*					Cultured *C. militaris*		
	Sichuan	Qinghai	Zhongqiao	Tibet 1	Tibet 2	Anhui	Hebei	Huadong	Jiangxi	Wanfeng	Aoli	Quanxin	Xiankang
Cytosine	44.3 ± 1.3[a]	+[b]	+	+	+	+	+	+	129.3 ± 6.9	+	+	+	+
Adenine	100.8 ± 0.9	44.2 ± 2.6	79.0 ± 3.2	34.8 ± 1.3	83.7 ± 2.5	601.5 ± 18.3	212.1 ± 8.1	70.7 ± 3.6	755.7 ± 24.2	283.0 ± 8.7	191.0 ± 5.6	+	26.3 ± 1.0
Guanine	19.2 ± 1.2	+	+	147.4 ± 7.2	+	506.8 ± 24.6	117.8 ± 3.2	+	400.4 ± 17.5	554.5 ± 23.1	59.3 ± 2.6	+	+
Cytidine	324.0 ± 4.7	121.4 ± 5.3	33.2 ± 1.4	+	146.3 ± 5.2	162.7 ± 5.4	227.3 ± 5.2	74.3 ± 3.0	821.8 ± 25.8	105.8 ± 4.0	+	+	35.7 ± 1.5
Cordycepin	32.8 ± 1.8	+	+	+	+	–[c]	–	–	–	–	3240.9 ± 53.3	1682.9 ± 55.1	1905.7 ± 48.0
Adenosine	431.2 ± 2.5	309.0 ± 3.9	263.7 ± 8.9	232.7 ± 7.7	353.0 ± 16.2	1197.8 ± 49.8	2251.6 ± 108.0	2764.9 ± 119.8	2945.0 ± 71.1	5569.6 ± 54.8	279.9 ± 14.2	534.1 ± 25.4	1452.0 ± 22.5
Hypoxanthine	186.1 ± 7.0	108.0 ± 1.9	64.5 ± 2.7	134.7 ± 5.2	162.0 ± 4.7	72.9 ± 4.7	65.6 ± 4.2	+	+	+	+	+	+
Guanosine	343.8 ± 12.7	399.9 ± 10.1	196.9 ± 5.9	278.7 ± 3.5	403.7 ± 13.4	2063.5 ± 52.3	2651.2 ± 88.7	2341.1 ± 93.4	3409.9 ± 123.3	4304.3 ± 165.6	260.1 ± 8.7	170.6 ± 5.1	402.5 ± 15.7
Inosine	444.8 ± 14.7	401.3 ± 10.6	215.1 ± 6.1	311.4 ± 8.4	300.3 ± 10.3	+	514.3 ± 22.8	–	+	+	–	–	–
2′-Deoxyuridine	+	–	+	+	+	+	+	+	+	+	+	+	+
Uridine	1170.4 ± 40.1	932.5 ± 30.8	767.2 ± 34.0	1236.8 ± 31.0	1142.7 ± 31.7	1346.1 ± 38.5	1604.6 ± 48.2	1278.5 ± 15.8	4368.8 ± 61.1	6938.0 ± 208.9	+	+	1498.7 ± 49.5
Thymidine	–	–	–	–	+	+	–	–	+	+	–	–	–

[a] Average of triplicates ± SD.
[b] Under the limits of quantification.
[c] Undetectable.

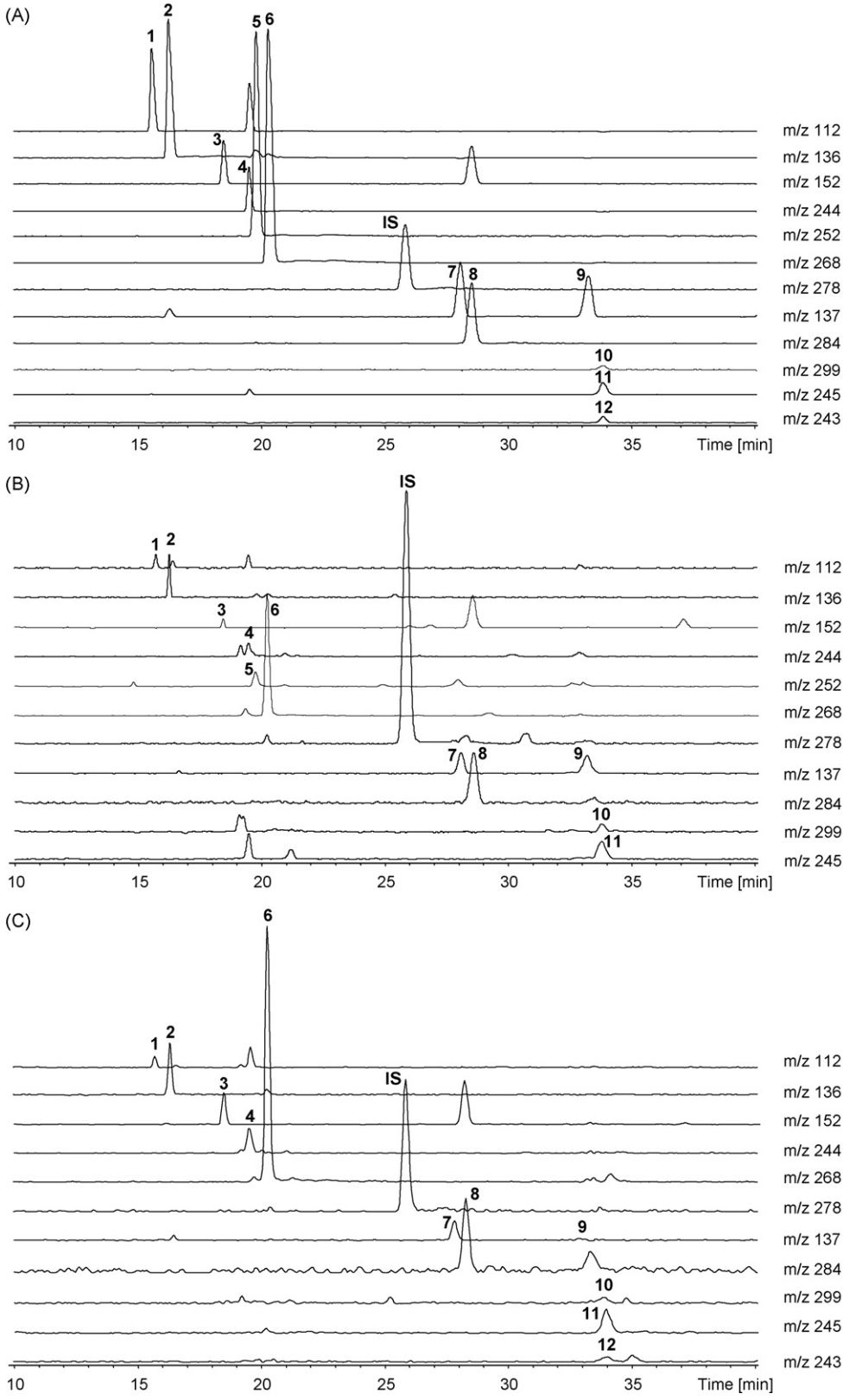

Fig. 4. Selected ion electropherograms of mixed standards (A), natural *Cordyceps sinensis* from Sichuan (B), cultured *C. sinensis* from Jiangxi (C) and cultured *C. militaris* from Aoli (D). CE–MS conditions: 100 mM formic acid containing 10% methanol as CE electrolyte, 120 cm length capillary was used for separation with the temperature at 25 °C, and 25 kV as the applied voltage. 75% methanol containing 0.3% formic acid as the sheath liquid and the flow rate was at 3 μL/min; the nebulizer gas pressure, drying gas flow rate, and drying gas temperature for the ESI source were set at 15 psi, 6 L/min and 350 °C, respectively: (1) cytosine; (2) adenine; (3) guanine; (4) cytidine; (5) cordycepin; (6) adenosine; (7) hypoxanthine; (8) guanosine; (9) inosine; (10) 2′-deoxyuridine; (11) uridine; (12) thymidine; IS: 5-chlorocytosine arabinoside.

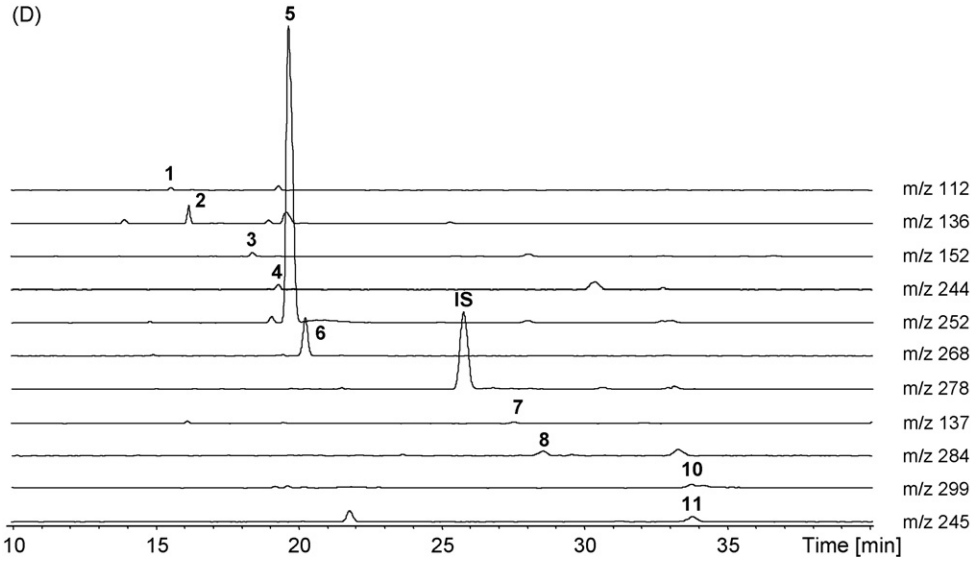

Fig. 4. (*Continued*).

CE electrolyte, 25 kV as applied voltage, and 120 cm capillary was used for separation with 25 °C as capillary temperature.

3.2. Optimization of MS conditions

In current study, it was observed that the effects of most MS parameters on migration time and separation resolution were generally negligible, except for nebuilzed gas pressure.

The sheath liquid, which affects the analyte transfer from the liquid phase into the gas phase together with the CE electrolyte, is generally employed to ensure a stable electrospray, an electrical contact between the capillary outlet and electrosprayer, as well as to boost the flow through the ESI needle [33]. The compositions, including the concentrations of methanol and formic acid in the sheath liquid were optimized to achieve good MS responses of the base peak (i.e. the most abundant ion) for investigated compounds. Formic acid (0.3%) solutions containing different methanol (25%, 50%, 75% and 100%, v/v) were tested as the sheath liquid. It was found that the MS responses for all the investigated compounds were similarly higher with the methanol concentration of 50% and 75%. However, higher concentration of organic solvent is benefit for the evaporation in electrospray. Therefore, the 75% methanol was selected. The concentration of formic acid in the sheath liquid was changed from 0.1% to 0.5% with the raise step of 0.1% to investigate its effect on the response of MS signal. The MS response was the best at 0.3% formic acid (Fig. 3). Finally, 0.3% formic acid in 75% (v/v) methanol solution was used as the sheath liquid. Furthermore, different flow rates (2, 3, 4 and 5 μL/min) of the sheath liquid were also tested, but it had no significant effect on the response of MS. As a result, 3 μL/min was selected due to the relative stability of MS signal.

In addition, the nebulizer gas pressure, drying gas flow rate and temperature were also optimized. Different nebulizer gas pressure (15, 20 and 25 psi), flow rates (5, 6 and 7 L/min) and temperature (300 and 350 °C) were compared (data not shown). Initially, the nebulizer gas pressure was set at 15 psi. At the higher nebulizer gas pressures (20 and 25 psi), the runtime became faster and signal intensities increased slightly accompanied with the worse separation resolutions, which could probably due to that a high nebulize gas pressure creates a suction effect at the end of the CE capillary inducing a flow towards the outlet side. The experimental results also indicated that the MS response increased with the increasing of flow rate from 5 to 6 L/min, while no significant effect was found when the flow rate was increased to 7 L/min. So the flow rate of drying gas was set at 6 L/min. Since the high temperature of drying gas was favorable to the electrolyte evaporation and sensitivity improvement, the temperature was set at 350 °C.

Finally, the optimum MS conditions were: (i) 75% (v/v) methanol containing 0.3% formic acid with the flow rate of 3 μL/min was used as sheath liquid; (ii) the nebulizer gas pressure, drying gas flow rate, and drying gas temperature for ESI source were set at 15 psi, 6 L/min and 350 °C, respectively.

3.3. Validation of method

Quantitative analysis of 12 nucleosides and nucleobases was performed on the SIM mode, in which the base peak of mass spectrum was selected. The base peak of internal standard (5-chlorocytosine arabinoside) was *m*/*z* 278, while the base peaks for the 12 analytes were listed in Table 1. As shown in Table 1, the most protonated molecular ions were the base peaks in the MS spectra, except for inosine, which exhibited the fragment ion (*m*/*z* 137) as the base peak.

The linearity, regression, and linear ranges of 12 analytes were shown in Table 1. Typically, R^2 values of regression equations were in the range of 0.9890 to 0.9992, which were considered to be acceptable. LOD and LOQ for all investigated compounds were less than 12.66 and 38.94 μg/mL, respectively (Table 1). The overall intra- and inter-day variations (RSD) of relative peak area (RPA) and relative retention time (RtR) for 12 analytes were less than 3.6% and 4.9%, and 0.4% and 0.6%, respectively (Table 2). The developed method had good accuracy with overall recovery of 92.7–103.4% for the 12 analytes.

3.4. Determination of nucleosides in Cordyceps by CE–MS

By using the calibration curves of the analytes, natural and cultured *Cordyceps* were analyzed. Table 3 showed the summary of the results determined by CE–MS, which are in accordance with the previous reports [9,11,14,15,25]. In conclusion, the overall content of nucleosides is much higher in cultured *Cordyceps* than in natural ones. However, the hypoxanthine and inosine are much higher in natural *C. sinensis*. Cordycepin, which is abundant in cultured *C. militaris*, is only found in natural *C. sinensis* with very low content and cannot be detected in the cultured ones. These phenomena may be resulted from their different cultured processes and growing envi-

ronments. As the cultured samples were grown in nutrient-rich conditions and matured in a very short period of time, the fungi were vigorous and produced more nucleosides, which are the key materials to produce DNA. Furthermore, the synthesis pathways for inosine and its base hypoxanthine, and cordycepin are different form the normal nucleosides (adenosine, guanosine, uridine, thymidine, etc.). Therefore, variation of the enzymes in different samples produced difference in the content of those compounds.

As shown in Fig. 4, the nucleosides and nucleobases were unequivocal identified by MS detection and their migration times. CE–MS showed the advantages on the separation of some nucleosides and nucleobases that cannot be separated in previous reports by HPLC or LC–MS, such as hypoxanthine and guanine [14], adenosine and adenine [9]. Therefore, it is obviously that CE–MS could be an alternative method for HPLC or LC–MS for the separation of nucleosides and nucleobases. However, the separation of 2′-deoxyuridine, uridine and thymidine is a problem for CE analysis, which can be compensated by MS detection in current study. Nevertheless, more work is still needed to further improve on the LOD and LOQ of the present CE–MS approach for measuring nucleosides and nucleobases present in traditional Chinese medicinal materials. The higher LOD of the current CE–MS method will be compensated by further developments of new CE technology (e.g. on-column transition of capillary isotachophoresis to CZE [34]) and/or by novel on-line pre-concentration method (e.g. field-amplified sample stacking [35]) in future works. It is expected that combined with pre-concentration techniques the CE–MS method can also be applied for the determination of nucleosides and nucleobases on a wide range of biological fluids, such as urine and blood as the levels of nucleosides and their metabolic compounds have been proposed as markers for diagnosis of cancers, AIDS, myocardial cellular energy status, diseases progress and therapy responses [36–39]. On the other hand, CEC, a rapidly evolving hybrid technique between HPLC and CE, had demonstrated its advantages on the analysis of nucleosides and nucleobases in *Cordyceps* [25]. Therefore, it would be interesting to couple the CEC approach to MS for the analysis of nucleosides and nucleobases in the future.

4. Conclusion

CE–MS, to the best of our knowledge, is reported for the first time as an analytical tool for simultaneous determination of the 12 nucleosides and nucleobases. The effectiveness of this new analytical CE–MS method was evaluated by the unequivocal identification and quantification of nucleosides and nucleobases in natural and cultured *Cordyceps*. The results presented in current work were comparable with the more established techniques, such as LC–MS, with lower sample and solvent consumption.

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