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Identification and determination of nucleosides in Cordyceps sinensis and its substitutes by high performance liquid chromatography with mass spectrometric detection

Fang-Qiu Guo [a], Ai Li [a], Lan-Fang Huang [a,*], Yi-Zeng Liang [a], Ben-Mei Chen [b]

[a] *Research Center of Modernization of Chinese Herbal Medicines, College of Chemistry and Chemical Engineering, Central South University, Changsha 410083, PR China*
[b] *Analytical Testing Center of Central South University, Changsha 410078, PR China*

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Abstract

Cordyceps sinensis (Cs) is a well-known traditional Chinese medicine (TCM) and Cordyceps mycelia (Cm), a cultured Cordyceps, is a substitute for Cordyceps sinensis. The most important active components in them are nucleosides. A high selective, sensitive and accurate high performance liquid chromatography method with photodiode array detection (DAD) and mass spectrometric detection has been developed for simultaneous separation, identification and quantification of nucleosides in Cs and Cm using a mobile phase including (A) ammonium acetate (40 mM, pH 5.2) and (B) methanol with a gradient system on a 2.0 mm × 150 mm Shimadzu VP-ODS column. The presence of each nucleoside in Cs and Cm was ascertained by comparison of MS data, UV spectra and retention time with standards. LC/ESI-MS in selective ion monitoring (SIM) mode were used for the quantification of nucleosides in Cs and Cm. 2-Chloroadenosine was used as internal standard for this assay. The precisions and accuracies were in the range of 1.5–5.3% and −3.5 to 5.0%, respectively. The limits of detection and quantification for nucleosides were in the order of 0.1–0.6 μg ml^{-1} and 0.5–2.0 μg ml^{-1}, respectively. The recoveries were in the range of 92.0–107.0%. With the developed method, the concentrations of nucleosides in Cs and Cm from different sources were determined. Cs, characterized with far lower concentration of adenosine and cordycepin than Cm, can be very easy to distinguish from Cm. This reliable method would be useful for the study and quality control of Cordyceps sinensis and its substitutes.

Keywords: High performance liquid chromatography; Photodiode array detector; Mass spectrometry; Nucleoside; Cordyceps sinensis; Cordyceps mycelia

1. Introduction

For centuries, Cordyceps sinensis, a caterpillar fungus of the genus Cordyceps, has been used in China as food and herbal medicine for a variety of diseases. Cordyceps sinensis extract has been used for treatment of hyperglycemia, respiratory and liver diseases, renal dysfunction, renal failure [1,2] and as antioxidant [3]. Cordyceps sinensis is also an anticancer agent [4]. In addition to its usage as a crude drug, the Chinese has used it extensively as a folk tonic food or invigorant. In traditional Chinese medicines (TCMs), Cordyceps sinensis plays a very important role due to its function as food and herbal medicine. Because of its scarcity in nature and high price, many studies have been carried out in order to find substitutes for Cordyceps sinensis, and some fruitful progress has been achieved [5–10]. Previous studies have also proven that the most important bioactive constituents in Cordyceps sinensis and cultured Cordyceps are nucleosides, which are soluble [1–10]. For example, the quality control of Cordyceps sinensis and its substitutes depends on the concentration of nucleosides [5]. The structures of the main nucleosides identified in Cordyceps sinensis and cultured Cordyceps are shown in Fig. 1. Several techniques are available for the analysis of nucleosides in Cordyceps sinensis and cultured Cordyceps, such as thin layer chromatography

* Corresponding author. Tel.: +86 731 8836376; fax: +86 731 8879616.
E-mail address: lf18huang@yahoo.com.cn (L.-F. Huang).

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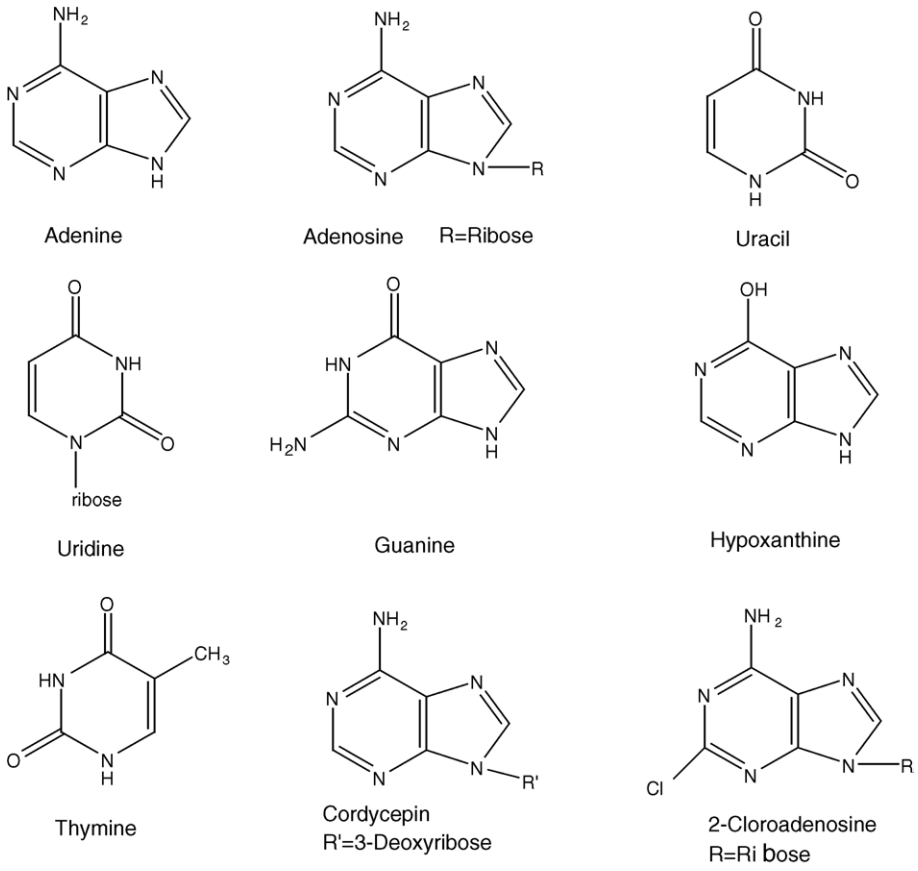

Fig. 1. Structures of the main nucleosides identified in Cordyceps sinensis and Cordyceps mycelia.

(TLC) [6,12], capillary electrophoresis [9–11] and high performance liquid chromatography (HPLC) [7,8,13–17]. However, these methods mentioned have disadvantages, such as low sensitivity and poor selectivity. Some trace amount of nucleosides cannot be detected with above method, but these trace amount of nucleosides is also very important to differentiation and quality control of Cordyceps sinensis and cultured Cordyceps from the view that function of TCM emphasizes its unity. So it is very important to establish a reliable method for identification and determination of more active nucleosides in TCMs mentioned above.

Because LC/MS often provides low limit of detection, high selectivity and short analysis time, it has been proven to be of considerable value for identification and determination of active components in TCMs [18–21]. It also has been used for determination of nucleosides in Cordyceps sinensis and its substitutes [22–24]. But only a part of nucleosides was determined. The present study describes a high performance liquid chromatography with photodiode array detection (DAD) and mass spectrometry coupling with an electrospray ionization interface (MS-ESI) for identification of nucleosides in Cordyceps sinensis and its substitutes from different resources. LC/ESI-MS in selective ion monitoring (SIM) mode was used for quantitative analysis of nucleosides of Cordyceps sinensis and its substitutes. The experimental conditions, such as chromatographic conditions and mass spectrometric detection conditions were optimized. Based on this work, a simple, sensitive and reproducible LC/DAD/MS method for simultaneous separation, identification and determination of active components in various Cordyceps sinensis and its substitutes was developed.

2. Experimental

2.1. Materials and standards

Standards of uracil, hypoxanthine, uridine, thymine, guanine, adenine, adenosine, cordycepin and 2-chloroadenosine were purchased from Sigma (St. Louis, MO, USA). A stock solution of each standard (0.2 mg ml^{-1}) except guanine was prepared in methanol and stored in a refrigerator. Guanine (0.1 mg ml^{-1}) was prepared in 0.1 mol l^{-1} HCl and stored in a refrigerator. Dilutions of 2.0 and 20.0 μg ml^{-1} of each compound were prepared by diluting the stock solution with methanol. Acetate acid and ammonium acetate used were analytical grade. HPLC-grade methanol was obtained from Hanbang Science and Technology Co. (Jiangsu, China) and Milli-Q quality water was used in the preparation of the

mobile phase. Solvents were filtered through a 0.45 μm membrane and degassed.

Samples of Cordyceps sinensis and Cordyceps mycelia from different resources of the PR China were purchased from local drug stores of Changsha, China.

2.2. Sample preparation

The extraction of nucleosides in Cordyceps sinensis and Cordyceps mycelia with water proved to be best for the qualitative and quantitative analysis of nucleosides [17]. Cordyceps sinensis was dried at 35 °C for 2 h and grinded into powder (approximately 20 meshes) at first. Subsequently, about 1.0 g of Cordyceps sinensis was exactly weighed and added to 20 ml of distilled water for extraction of nucleosides. The sample-water mixture was placed into an ultrasonic bath for 2 h. Then, the sample-mixture was filtered and the filtrate was vacuum-dried. The residue was dissolved in 10 ml methanol. Similar procedure for extraction was also performed on about 1.0 g of other samples. The obtained samples were stored in a refrigerator.

2.3. Instrumentation

The liquid chromatography system (Shimadzu Kyoto, Japan) consisted of a LC-10Advp solvent delivery pump, a FCV-10ALvp low pressure gradient unit, a DGU-14A degasser, a CTO-10Avp column oven and a SPD-M10Avp photodiode array detector. Mass spectrometer was LCMS-2010 (Shimadzu Kyoto, Japan). The column utilized for separation was a 2.0 mm × 150 mm Shimadzu VP-ODS column with a particle size of 5 μm. The analytical column was protected by a C_{18} guard-pak cartridge (Waters, Milford, MA, USA).

2.4. Chromatographic conditions

Mobile phase including (A) ammonium acetate (40 mM, pH 5.2) and (B) methanol was degassed ultrasonically before use. Each component of the mobile phase was filtered through a 0.22 μm membrane. Chromatographic separation was performed at room temperature and a flow-rate of 0.2 ml min^{-1}. The solvent elution program is shown in Table 1.The wavelength of photodiode array detector was 190–300 nm.The amount of injection was 5 μl.

Table 1
Solvent elution program for HPLC

Step	Time (min)	Ammonium acetate (%) (40 mM, pH 5.2)	Methanol (%)
1	0	95	5
2	10	85	15
3	15	85	15
4	18	80	20
5	23	80	20
6	28	95	5

2.5. Mass spectrometric detection conditions

MS coupling with an electrospray ionization (ESI) interface was used. Peaks were detected in both scan and SIM mode. $[M + H]^+$ at *m*/*z* 113, 137, 245, 127, 152, 136, 268, 252 and 302 (IS ion) was selected as the SIM ion in quantitative analysis. Sensitivity optimization was performed by injection of an adenosine standard (20 μg ml^{-1}). Mass spectrometric detection conditions for both scan and SIM are as follows. ESI temperature was 400 °C. Curved desolvation line (CDL) and block temperature was 250 and 200 °C, respectively. Probe voltage was +4.5 kV. Detector voltage was 1.5 kV. CDL voltage was −20 V. Q-array Bios was 50 V. Nebulizing gas flow was 4.5 l min^{-1}.

2.6. Validation of quantitative method

After optimum conditions of separation and ionizing for the mixed standard solution had been established, validation was performed. A set of seven non-zero calibration standards of each component, ranging from 1.0 to 111.5 μg ml^{-1} for uracil, 0.8 to 107.5 μg ml^{-1} for hypoxanthine, 1.0 to 113.0 μg ml^{-1} for uridine, 1.0 to 124.5 μg ml^{-1} for thymine, 1.2 to 118.5 μg ml^{-1} for guanine, 2.0 to 135.0 μg ml^{-1} for adenine, 0.6 to 113.5 μg ml^{-1} for adenosine and 0.5 to 109.5 μg ml^{-1} for cordycepin was prepared in order to calculate the standard curves. LC/ESI-MS in SIM mode were used to exam linearity in these ranges. The calibration curves were calculated by plotting peak area ratio (*Y*) of each analyte and IS in LC/ESI-MS TIC against concentrations (*X*, μg ml^{-1}). The concentration of IS was 10 μg ml^{-1}. Each standard was analyzed in triplicate. The retention times of uracil, hypoxanthine, uridine, thymine, guanine, adenine, adenosine and cordycepin and IS in LC/ESI-MS TIC were 3.24, 4.48, 5.32, 7.79, 8.27, 10.46, 14.26, 18.22 and 19.49 min, respectively. The selectivity, precision, accuracy and recovery of this assay were also validated. Quality control samples of each analyte at concentrations of 2.0, 20.0 and 80.0 μg ml^{-1} were prepared by diluting the stock solution with the mobile phase. Precisions for the determination of nucleosides were calculated from six replicate analyses of quality control samples. Various concentrations of nucleoside standards (see Table 5) at sample level were added to about 1.0 g Cordyceps mycelia from Yunnan Province. After preparing as Section 2.2, the concentrations of nucleosides were determined and recoveries were calculated. Due to using SIM mode for peak detection, no interference from other components with different polarities existed in Cordyceps sinensis and its substitutes at the retention times of each analyte, was observed.

3. Results and discussion

3.1. Optimization of chromatographic conditions

The selection of mobile phase should consider both separation and effect on ionizing. The separation of nucleosides

Table 2
The values of t_R, UVλ_{max}, $[M+H]^+$, $[M+Na]^+$ and other MS ions of nucleoside standard compounds

Peak number	Compound	t_R (min)	UVλ_{max} (nm)	$[M+H]^+$ (m/z)	$[M+Na]^+$ (m/z)	$[2M+H]^+$ (m/z)	$[2M+Na]^+$ (m/z)
1	Uracil	3.24	203,259	113			
2	Hypoxanthine	4.48	198,252	137		273	295
3	Uridine	5.32	205,262	245	267		
4	Thymine	7.79	203,264	127			
5	Guanine	8.27	203,261	152		303	325
6	Adenine	10.46	207,261	136			
7	Adenosine	14.26	206,260	268	290		
8	Cordycepin	18.22	207,260	252	274		

Table 3
Regression equation, correlation coefficients (R), linear range and limit of detection

Compound name	Regression equation	R	Liner range ($\mu g\,ml^{-1}$)	Limit of detection ($\mu g\,ml^{-1}$)
Uracil	$Y = 0.1162X + 0.0124$	0.9982	1.0–111.5	0.2
Hypoxanthine	$Y = 0.08647X + 0.00902$	0.9974	0.8–107.5	0.2
Uridine	$Y = 0.04715X + 0.00396$	0.9967	1.0–113.0	0.3
Thymine	$Y = 0.06537X + 0.00614$	0.9962	1.0–124.5	0.2
Guanine	$Y = 0.08436X + 0.00904$	0.9985	1.2–118.5	0.4
Adenine	$Y = 0.1438X + 0.0136$	0.9987	2.0–135.0	0.6
Adenosine	$Y = 0.1292X + 0.0113$	0.999	0.6–113.5	0.1
Cordycepin	$Y = 0.2146X + 0.0194$	0.9993	0.5–109.5	0.1

was very difficult due to their similar structure if an isocratic HPLC method was used. For the separation of nucleosides in Cordyceps sinensis and its substitutes, some reports [13,14] used the mobile phase including (A) KH_2PO_4 buffer and (B) methanol with a gradient elution system. However, the solvent system was not available for the LC/MS analysis due to the presence of potassium ion and high-concentration salts. In our study, ammonium acetate was chosen to control the acidity of the mobile phase. Using (A) ammonium acetate and (B) methanol as the mobile phase and a gradient elute program, eight nucleosides being studied were successfully separated. Chromatographic conditions, such as concentration of ammonium acetate, pH and solvent elution program, were optimized on a 2.0 mm × 150 mm Shimadzu VP-ODS column. The optimum chromatographic conditions are summarized in Section 2.4. HPLC chromatogram of a mixed working standard solution detected with a photodiode array detector set at 260 nm is shown in Fig. 2A and their retention time (t_R), UVλ_{max} values are shown in Table 2.

Table 4
Summary of precision of each nucleoside

Analyte	Concentration added ($\mu g\,ml^{-1}$)	Concentration found ($\mu g\,ml^{-1}$)	R.S.D. (%, $n = 6$)
Uracil	2.0	1.95	5.1
	20.0	20.32	3.4
	80.0	80.61	1.9
Hypoxanthine	2.0	1.96	4.9
	20.0	19.47	3.5
	80.0	81.20	1.8
Uridine	2.0	2.08	4.6
	20.0	19.72	3.2
	80.0	79.36	1.6
Thymine	2.0	1.93	4.7
	20.0	20.27	2.9
	80.0	82.16	2.3
Guanine	2.0	2.10	5.3
	20.0	20.38	3.2
	80.0	79.12	2.1
Adenine	2.0	2.09	5.7
	20.0	19.74	3.2
	80.0	78.76	2.0
Adenosine	2.0	1.94	4.3
	20.0	20.47	3.6
	80.0	81.45	1.5
Cordycepin	2.0	2.05	5.2
	20.0	20.62	2.9
	80.0	79.56	1.8

3.2. Optimization of the ESI-MS conditions

On-line molecular mass information in the analysis of nucleoside standards was provided by the use of LC/ESI-MS. In order to obtain optimum ionizing conditions, both an atmospheric pressure chemical ionization (APCI) and ESI interface were tested in positive and negative ion mode by scanning between m/z 50 and 350 s^{-1}. Then, ESI interface and positive ion mode were chosen. Peaks were detected in both scan and SIM mode, respectively. The optimum ESI-MS conditions are summarized in Section 2.5. Mass spectra for individual nucleoside in scan mode are shown in Fig. 3. The MS spectra of the uracil, hypoxanthine, thymine, guanine, adenine, adenosine and cordycepin obtained from scan mode was characterized by an $[M+H]^+$ as base peak. However, $[M+Na]^+$ of uridine proved to have a more intense peak than its $[M+H]^+$. In some case, $[2M+H]^+$ could be observed, which may confirm the molecular mass,

Table 5
Summary of recovery of each nucleoside[a]

Analyte	Concentration added (mg g^{-1})	Concentration measured (mg g^{-1})	Concentration found (mg g^{-1})	Recovery (%)
Uracil	0	0.0172		
	0.020	0.0357	0.0185	92.5
	0.030	0.0482	0.0310	103.3
Hypoxanthine	0	n.d.		
	0.020	0.0187	0.0187	93.5
	0.030	0.0276	0.0276	92.0
Uridine	0	0.0854		
	0.050	0.137	0.0513	102.6
	0.10	0.189	0.0964	96.4
Thymine	0	0.0410		
	0.030	0.0715	0.305	101.7
	0.050	0.0936	0.0526	105.2
Guanine	0	n.d.		
	0.050	0.0517	0.0517	103.4
	0.10	0.0946	0.0946	94.6
Adenine	0	0.0290		
	0.020	0.0501	0.0211	105.5
	0.030	0.0571	0.0281	93.7
Adenosine	0	0.324		
	0.30	0.610	0.286	95.3
	0.50	0.834	0.510	102.0
Cordycepin	0	0.150		
	0.10	0.253	0.103	103.0
	0.20	0.364	0.214	107.0

n.d.: not detected.
[a] Nucleoside standards were added to about 1.0 g Cordyceps mycelia from Yunnan Province.

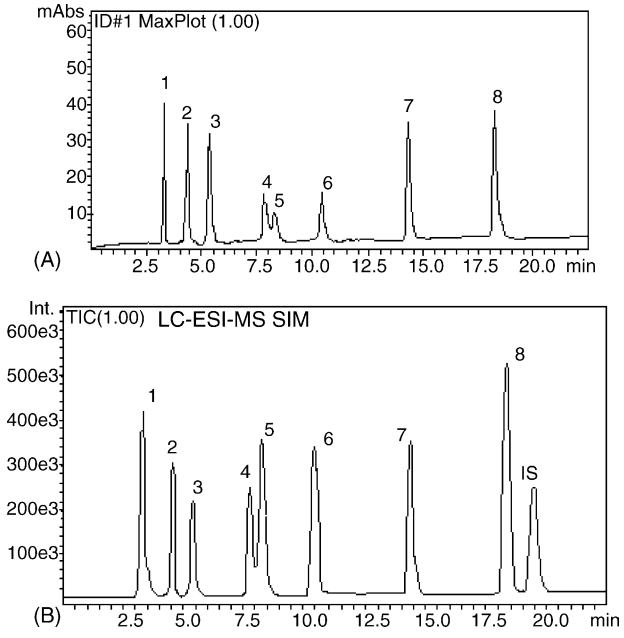

Fig. 2. Simultaneous HPLC-DAD chromatogram (A) and LC/ESI-MS TIC (B) of nucleoside standard mixture in SIM mode with the solvent elution program as in Table 1 and SIM mode parameters listed in Section 2.5. (1) Uracil, (2) hypoxanthine, (3) uridine, (4) thymine, (5) guanine, (6) adenine, (7) adenosine and (8) cordycepin. The concentration of each compound is 10 μg ml^{-1} in HPLC-DAD and 20 μg ml^{-1} in LC/ESI-MS, and IS concentration is 10 μg ml^{-1}.

because a stable compound of two molecular was formed. The intensity of Na adduct will depend on the quality of the water, solvent and other conditions, but forming of $[M + Na]^+$ and $[2M + Na]^+$ ion may also confirm the molecular mass. However, sensitivity will also decreased in MS when many adducts come out. In additional to $[M + H]^+$ as a base peak, the MS spectra of hypoxanthine, uridine, guanine, adenosine and cordycepin obtained from scan mode had obvious other M^+ peaks, such as $[M + H]^+$, $[2M + H]^+$, $[M + Na]^+$ or $[2M + Na]^+$. MS data of eight nucleosides is also summarized in Table 2. As shown in Table 2, Figs. 2 and 3, UV spectrum of each nucleoside standard was very similar, these data cannot be used for identification of nucleosides in Cordyceps sinensis and its substitutes. However, each nucleoside standard showed significant and distinctive MS data and quite different retention times, consequently, the characterization of each standard can be used for comparison with the chromatograms of various resources of Cordyceps sinensis and its substitutes.

3.3. Analytical properties

LC/ESI-MS in SIM mode was proved to provide better defined peaks for each analyte, especially for the minor compounds, which was consistent with its low limits of detection and quantification. Another considerable advantage was the good selectivity, and there was little interference from other

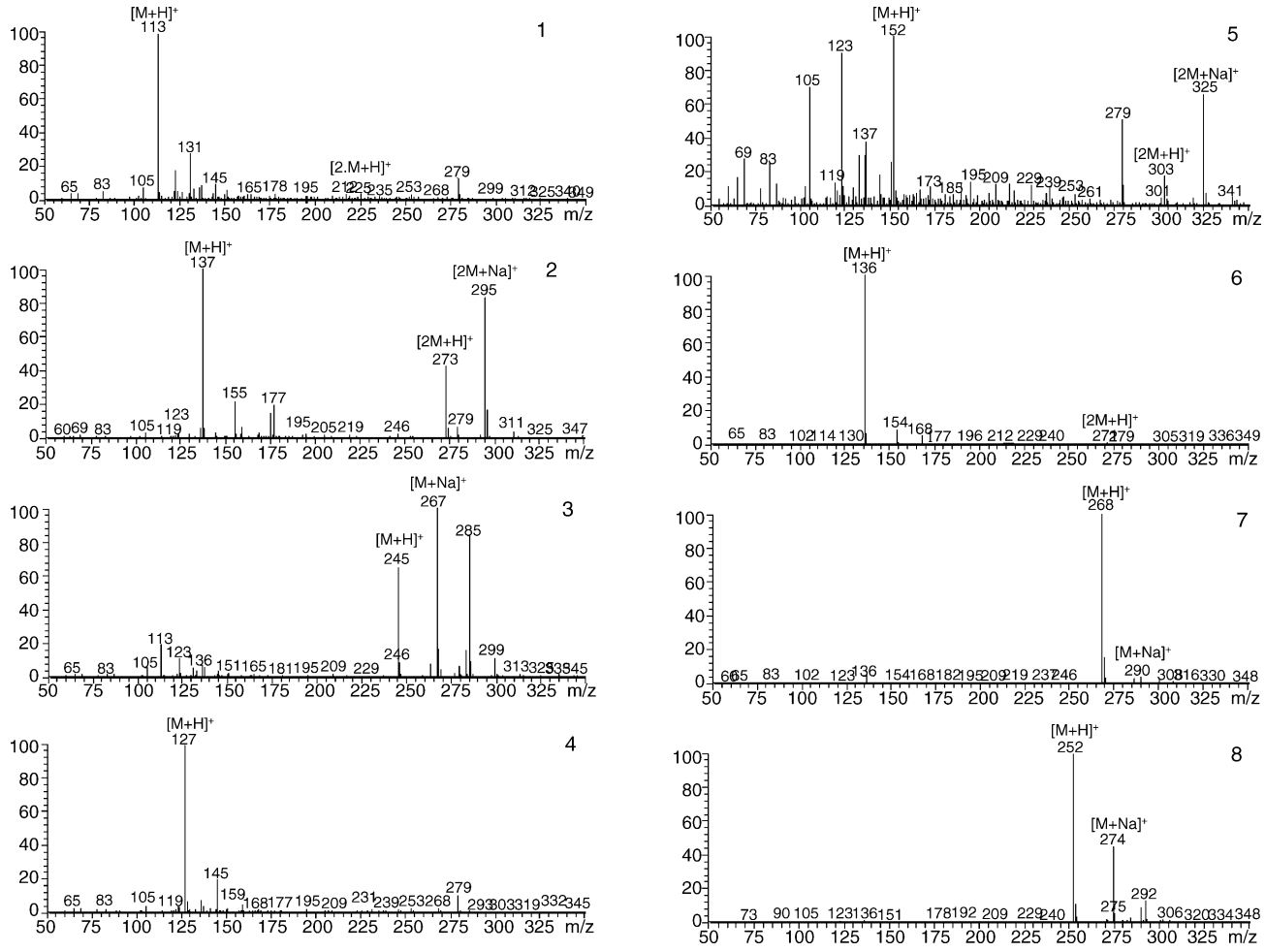

Fig. 3. Mass spectrum of each nucleoside in positive ion and scan mode between m/z 50 and 350 s^{-1} with the solvent elution program as in Table 1 and optimum ESI-MS parameters listed in Section 2.5. Letters refer to the same as in Fig. 2.

components. These peculiarities of this assay have made it a reliable method for quantitative analysis of nucleosides. LC/ESI-MS TIC in SIM mode of a mixed working standard solution is shown in Fig. 2B. Results of regression equation, correlation coefficients and linear range are summarized in Table 3. The correlation coefficients were in the range of 0.9962–0.9993. Because extracts of Cordyceps sinensis and its substitutes contain analytes, no real blank was available for preparation of standards or controls. A solvent blank was analyzed and no peaks at m/z 113, 137, 245, 127, 152, 136, 268, 252 and 302 were observed in the blank. The limits of detection for nucleosides with LC/ESI-MS in SIM mode was in the order of 0.1–0.6 μg ml^{-1} (see Table 3), which were determined from signal to noise ratios of 3. Nevertheless, after applying the method to actual samples, the limits of quantification for nucleosides were considered more important. The lower limits of the range of the calibration curves can be considered the limits of quantification of the assay. The limits of detection and quantification of this assay was lower than those observed of HPLC [13–17]. These limits were even lower than capillary electrophoresis [9–11]. The results of precisions are listed in Table 4. The precisions were in the range of 1.5–5.7%. The recoveries were in the range of 92.0–107.0% when added concentrations were in the range of 0.020–0.50 mg g^{-1} (see Table 5). Experimental results showed that the precision, accuracy and recovery were satisfactory.

3.4. Identification and determination of nucleosides

HPLC with DAD and MS detection gave a very detailed characterization of the investigated nucleosides in Cordyceps sinensis and its substitutes with regard not only to the qualitative but also to the quantitative analysis. HPLC chromatogram detected with photodiode array detection set at 260 nm and LC/ESI-MS TIC (in both scan and SIM mode) of water extracts of a representative real sample are shown in Fig. 4. Based on the comparison of retention time, mass data with those of the standards, nucleosides in various samples can be identified. The results are listed in Table 6. The concentrations of nucleosides in Cordyceps sinensis and its substitutes of different resources were also determined with the developed LC/ESI-MS in SIM mode method. The results are also summarized in Table 6. The concentrations

Table 6
The concentrations of nucleosides in natural Cordyceps sinensis (Cs) and cultured Cordyceps mycelia (Cm) (mg g^{-1})

Sources	Uracil	Hypoxanthine	Uridine	Thymine	Guanine	Adenine	Adenosine	Cordycepin
Cs								
Qinghai	0.0862	0.0426	0.617	0.0113	0.0682	0.220	0.093	0.0103
Sichun	0.136	0.0843	0.925	0.0265	0.0739	0.271	0.124	0.00756
Tibet	0.0568	0.0126	0.492	n.d.	0.0474	0.328	0.246	0.00892
Cm								
Jiangxi	0.154	0.0406	1.07	0.0872	0.0327	1.42	0.495	0.542
Hebei	0.0765	0.0243	0.324	0.0364	0.118	0.924	0.842	0.247
Shandon	0.0472	n.d.	0.152	0.0632	0.0571	1.23	0.714	0.240

n.d.: not detected.

of nucleosides obtained from different sources, both in Cordyceps sinensis and its substitutes, Cordyceps mycelia, varied considerably from locality to locality. However, the concentrations of cordycepin in Cordyceps mycelia are much higher than in natural Cordyceps sinensis and the concentrations of adenosine is, on average, about 1.6 times higher in Cordyceps mycelia than in Cordyceps sinensis. This may be as "maker compounds" for the quality control of Cordyceps sinensis and cultured Cordyceps. Since nucleosides are important biologically active components, therefore, determination of nucleosides is very important for the identification, differentiation and quality evaluation of Cordyceps sinensis and its substitutes.

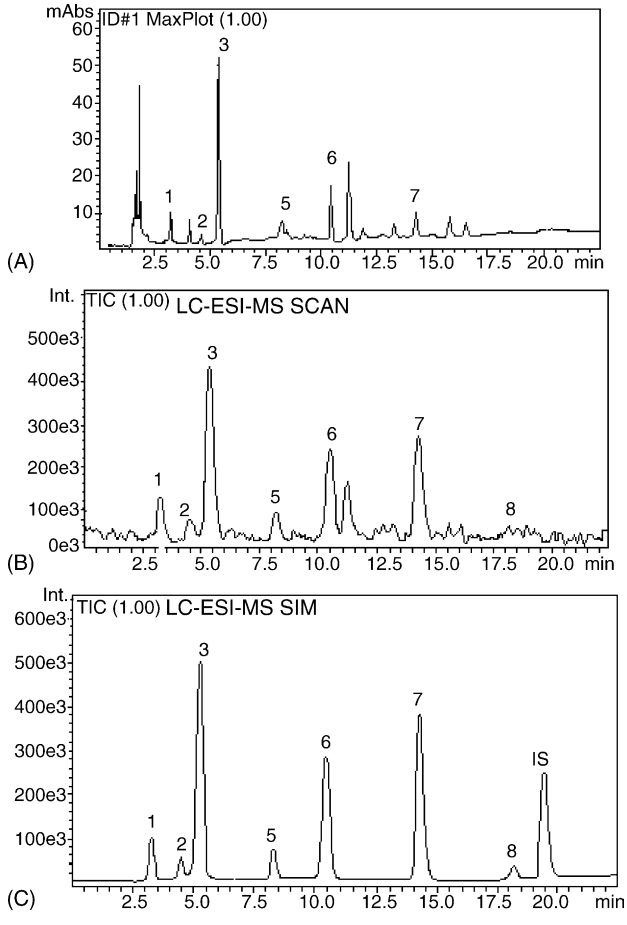

Fig. 4. Simultaneous HPLC-DAD chromatogram (A) and LC/ESI-MS TIC of water extract of Cordyceps sinensis from Tibet of China. Peaks are detected with scan mode in (B) and with SIM mode in (C), respectively. Chromatographic conditions are described in Section 2.4 and ESI-MS parameters for both scan and SIM mode are listed in Section 2.5. Peaks are identified as in Fig. 2.

4. Concluding remarks

Suitable HPLC-DAD and LC/ESI-MS methods have been established, which can be used to separate, identify and determine nucleosides in Cordyceps sinensis and its substitutes simultaneously. Due to high selectivity and sensitivity, LC/ESI-MS in SIM mode proves to be a powerful tool for qualitative analysis and quantitative analysis of nucleosides in TCMs mentioned above. It also would be useful for the study and quality control of Cordyceps sinensis and its substitutes, which does aim at comprehensive revealing the quality and quantity of chemical constituents of traditional medicines for effective evaluation of similarity or differences of analytical samples.

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