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Efficient extraction and rapid quantitative determination of nucleoside compounds from *Cordyceps jiangxiensis*, a new *Cordyceps* producing-cordycepin

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***Cordyceps*, a well-known and precious traditional Chinese medicine, has received increasing attention worldwide due to its outstanding curative effects for different diseases. Nucleosides are the main active compounds of *Cordyceps*, and are usually the chemical marker used for the quality control of *Cordyceps* and its bioproduct. In this study, an optimal condition for extracting nucleosides in *Cordyceps jiangxiensis* was achieved by an orthogonal design as follows: 15% ethanol-water extraction solvent, 10 min extraction time, 20:1 solvent to sample ratio, and 1 extraction frequency. Also, a simple, rapid, and reliable method by High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) was successfully used to simultaneously and qualitatively identify seven nucleosides compounds in *C. jiangxiensis*. Determination was achieved on a Shimadzu VP-ODS column (4.6 × 250 mm i.d. 5 µm) using a gradient elution with a methanol/water mobile phase. All calibration curves showed good linearity (R^2>0.99) within the test ranges. The overall relative standard deviations for intra- and inter-day of seven analytes were less than 4.2%. Under the developed method, the findings indicated that uridine was the most abundant nucleoside, adenosine was inferior to uridine, and cordycepin with antitumor activity was also detected in *C. jiangxiensis*. The method developed might be applied as an alternative approach in assessing the quality of other *Cordyceps* species.**

Key words: *Cordyceps jiangxiensis*, nucleosides, extraction, quantitative detection, High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD).

INTRODUCTION

Cordyceps, an entomopathogenic fungus, has received increasing attention worldwide in the past decades due to its medicinal values for curing various diseases in traditional Chinese medicine (Zhong and Xiao, 2009; Xiao et al., 2009a; Liang, 2007). *Cordyceps jiangxiensis*, a medicinal macrofungus native to eastern China, has been collected and identified as a new species of the genus *Cordyceps* several years ago (Liang, 2007; Xiao et al., 2006a). Furthermore, it has been used as a folk recipe of Chinese medicine for centuries. Its pharmacological properties present highly promising prospects for the development of nutraceuticals and new drugs, and have attracted much attention in China (Liang, 2007). However, the natural sources of *Cordyceps* are very limited because of their host specificity and confined environmental requirements for growth, which are also the technology bottleneck for industrialization. Fortunately, cultured *Cordyceps* mycelia possess the same bioactive

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ingredients and similar pharmacological effects as natural *Cordyceps* (Zhong and Xiao, 2009; Xiao and Zhong, 2007). The large-scale production of *Cordyceps* by submerged fermentation has proven to be a promising method to meet human needs and to reduce the pressure on natural sources (Zhong and Xiao, 2009; Xiao and Zhong, 2007). Accordingly, proper conditions for the culture of *C. jiangxiensis* in submerged fermentation to produce mycelia and intra-/exocellular polysaccharides have been developed (Xiao et al., 2004, 2006). Recently, polysaccharides or extracts of *C. jiangxiensis* have been confirmed to possess potent anti-tumor pharmacological properties through the apoptotic pathway of caspase activation (Xiao et al., 2006b, Xiao and Zhong, 2008), and scavenging free-radical activity (Xiao et al., 2011a). Its chemical composition demonstrates that it is a valuable and promising source for the development of healthy foods and drugs (Xiao et al., 2009b, 2011b, 2012). However, limited information is available about the quality control for *C. jiangxiensis* and its bioproduct.

Nucleosides play important roles in the regulation of various physiological processes in body (Gill and Indyk, 2007). Moreover, some nucleosides and their analogs such as 5-fluorouracil, 2'3'-dideoxyadenosine, acyclo-guanosine, have been used as antitumor or antiviral agents (Holliday and Cleaver, 2008; Klubes and Cerna, 1983; Clercq and Field, 2006). 3'-deoxyadenosine (cordycepin) from some *Cordyceps* species such as *Cordyceps militaris, Cordyceps kyushuensis* shows potent antitumor and antimicrobial activities (Sun et al., 2003; Li et al., 2006; Xiao and Zhong, 2007). N^6-(2-hydroxyethyl) -adenosine first separated from *Cordyceps* pruinosa is an efficient calcium antagonist (Liang, 2007). Though the efficacy of *Cordyceps* may not be completely derived from nucleoside compounds, nucleosides have been recognized as major active components in *Cordyceps* (Li et al., 2006; ECPC, 2005; Gong et al., 2004; Holliday and Cleaver, 2008). According to previous reports (Yang et al., 2007; Xiao et al., 2009b; Xiao et al., 2013), approximate 20 nucleosides have been isolated from *Cordyceps*. Also, there are signi-ficant differences in the variety and content of nucleosides among the different *Cordyceps* species (Li et al., 2006; Yang et al., 2007; Xiao et al., 2013). The determination of nucleosides, therefore, is very important for pharmaco-logical study and quality control of *C. jiangxiensis*.

In recent years, the simultaneous determination of multiple components, also frequently considered as quality control standards, have become the trend in the quantitative analysis of Chinese medicine. For example, chromatographic fingerprinting of nucleosides is one of the quality control standards for *Cordyceps* products (Li et al., 2006; Yu et al., 2007; Xiao et al., 2013), which may also be used as a marker in identifying authentic *Cordyceps* from imitations in the market (Hsu et al., 2002). To date, many methods are available for the quantification of nucleosides in *Cordyceps*, including thin layer chromatography, high performance liquid chromate-graphy (HPLC), capillary electrophoresis, liquid chromatography–mass spectrometry, and ion-pair reverse-phase chromatography (Yang et al., 2007; Xiao et al., 2013). However, the quality control standards of *Cordyceps* still remain controversial. In an attempt to establish a reliable quality control system for *Cordyceps*, it is first necessary to select a reliable analytical method to identify the profile of nucleosides in *Cordyceps*. Based on significant differences of nucleosides among the different *Cordyceps* species, further, for certain *Cordyceps* species, selecting major and specific nucleosides for quality control is also necessary. Among the above methods mentioned, HPLC might be the most available determiner of nucleosides in *Cordyceps* products because of its specificity, sensitivity, convenience, economy, and availability (Yu et al., 2007; Yang et al., 2007; Li et al., 2004; Xiao et al., 2013). However, the separation of the different nucleosides and their analogs is very difficult during *Cordyceps* preparations because of the similarities in their chemical structures. Determining high-efficiency HPLC conditions for the simultaneous separation and determination of nucleosides with good resolution for adjacent peaks within a short analysis time is essential. For wavelength detection, diode array detection (DAD), with the additional UV-vis spectral information, allows for easy qualitative analysis of peaks in a fingerprint chromatogram compared with common UV detectors. DAD can also record a series of chromatograms along a wide range of wavelengths, which allows fast and simple wavelength optimization (Liang et al., 2009; Xiao et al., 2013).

In the present study, HPLC-DAD detection of seven major nucleoside compounds in *C. jiangxiensis* (uracil, uridine, inosine, guanosine, adenine, adenosine, and cordycepin) was conducted for their simultaneous separation and determination, after a simple ultrasonic extraction procedure. It is the first to develop a method for the simultaneous determination of the seven main nucleoside compounds in *C. jiangxiensis*, which is important in pharmacological studies and quality control of *Cordyceps* products.

MATERIALS AND METHODS

Chemicals

Standards of uracil, adenine, uridine, inosine, guanosine, adenosine, and cordycepin were purchased from Sigma (St. Louis, MO, USA). LC-grade methanol was purchased from Tedia Company, Inc. (OH, USA). Ultrapure water was prepared using a Millipore Milli-Q Plus system (Millipore, Bedford, MA, USA). The other reagents were of analytical grade.

Sample

The voucher specimens of *C. jiangxiensis* were deposited at the Institute of Fungal Resource, University of Guizhou, Guiyang, China. As previously described (Liang, 2007; Xiao et al., 2004,

Table 1. Factors and levels of nucleosides extraction for the $L_9(3^4)$ orthogonal design.

Factors level	A	B (min)	C (v/m)	D(times)
1	ultrapure water	30	20:1	2
2	5% methanol water-solution	20	10:1	3
3	15% methanol water-solution	10	30:1	1

A, B, C, and D represent correlation factors of nucleoside extraction: extraction solvent, extraction time, solvent to sample ratio, and frequency, respectively. Numbers 1-3 in the first column represent levels of each factor in the orthogonal design.

Table 2. $L_9(3^4)$ orthogonal test results of nucleosides extraction and its analysis for *Cordyceps jiangxiensis* ($\bar{x}$±s.d., n=3).

Runs	A	B	C	D	Total peak areas of nucleosides tested
1[#]	1[†]	1	1	1	5950251[‡]
2	1	2	2	2	2639998
3	1	3	3	3	4421425
4	2	1	2	3	4550169
5	2	2	3	1	2102561
6	2	3	1	2	4468190
7	3	1	3	2	3607988
8	3	2	1	3	6829044
9	3	3	2	1	5300164
K1[§]	13011674	14108408	17247485	13352976	
K2	11120920	11571603	12490331	10716176	
K3	15737196	14189779	10131974	15800638	
R[*]	4616276	2618176	7115511	5084462	
optimal level	3	3	1	3	

[†] The arrangement of columns A-D was decided by orthogonal design for $L_9(3^4)$, and A, B, C, and D represents extraction time, temperature, extractant, and frequency, respectively. [#] Each row of the experimental run number represents one experimental replicate, and each run was replicated thrice. [‡]Values are mean of triple determinations. [§] K_i^X the peak area of nucleosides tested in *Cordyceps jiangxiensis* in thrice experiment at X_i. [*]Symbol *R* means the maximum value of K_i^X minus the minimum value of its. symbol *X* in the above represents A, B, C, and D, respectively, and symbol *i* represents each level.

2006a), *C. jiangxiensis* was prepared through liquid fermentation technology. Previous studies have indicated that the extraction of nucleosides from *C. sinensis* and its substitutes using water is the best selection method for the determination of nucleosides (Yang and Li, 2008). Thus, water was used as extractant for the extraction of nucleosides in the preliminary experiment. Up to 1.0 g of dried powder *Cordyceps* (60-100 mesh) was mixed with 10 mL ultrapure water, and then extracted at room temperature for 30 min using an ultrasonic processor. The procedure was repeated twice. After the combined extract was centrifuged at 4500 g for 15 min, the supernatant (extract sample) was stored at 4°C in a refrigerator before HPLC analysis.

Orthogonal design and procedure for sample preparation

Based on previous reports (Yang and Li, 2008) and consistence with our preliminary experimental results, ultrasonic extraction was more suitable for the preparation of nucleosides compounds of *Cordyceps*, not microwave-assisted extraction or hot-water extraction. The extraction conditions were further optimized by $L_9(3^4)$ orthogonal design (Zhao, 2006). In this study, extraction solvent, extraction time, solvent to sample ratio, and frequency were the orthogonal factors for the extraction of nucleosides from the *Cordyceps* samples. The design factors and the levels of orthogonal layout are shown in Table 1. The experimental conditions of each experimental run are listed in Table 2, including the experimental results in the last column. For the extraction treatment, 0.4 g of sample was placed in a 50 mL disposable centrifuge tube, and mixed with a determined volume of extraction solvent for each of the experimental runs. Thereafter, the mixture was allowed to stay overnight at 4ºC, and then it was placed in an ultrasonic water bath to be treated at room temperature according to the orthogonal design conditions. After extraction, the tubes were centrifuged at 5000 rpm for 10 min. The extract was collected, its total volume measured, and then it was stored at 4ºC for future use. Each experimental run was repeated three times.

Preparation of standard solutions

The nucleosides were dissolved in ultrapure water until a 1.0 mg/mL solution was achieved. A certain amount of the stock solution was transferred to a 10 mL volumetric flask, and filled up to its volume with the same solvent to obtain the desired concentration. All solutions were found stable when stored at 4°C for three weeks. The extract sample was then transferred to a 25 mL volumetric flask, filled up to its volume with the extraction solvent, and filtered through a 0.22 µm Millipore filter prior to injection into the HPLC system.

HPLC analysis

Chromatographic analysis was performed using a Shimadzu Series LC-20A HPLC (Shimadzu Kyoto, Japan), equipped with a binary high-pressure pump, a CTO-10Avp column oven, and a photodiode array detector connected to an LCsolution Software. The column used for separation was the Shimadzu VP-ODS column (4.6 × 250 mm i.d. 5 µm) and the Shimadzu VP-ODS C18 guard column (4.6 × 12.5 mm). As previously described (Yu et al., 2007; Hsu et al., 2002; Li et al., 2004; Yang and Li, 2008), phosphate buffer, acetate, and acetonitrile have often been used as the mobile phases for the HPLC analysis of nucleosides. The optimized chromatographic conditions involving two mobile-phases systems with ultrapure water (W) and methanol (M), as well as elution conditions, were determined. The elution conditions were as follows: 0 min to 3.0 min, isocratic 15% M; 3.0 min to 3.5 min, linear gradient 15% to 24% M; 3.5 min to 8.5 min, isocratic 24% M; 8.5 min to 9.0 min, linear gradient 24 to 35% M; 9.0 min to 15.0 min, isocratic 35% M. Finally, the column was washed with 100% B for 10 min before reconditioning the steps of the column using 15% M isocratic for 15 min. The flow-rate was 1 mL/min and the injection volume was 20 µL. All injections were repeated three times to ensure reproducibility. The system was operated at 35°C. The detecting wavelength of the photodiode array detector was set to the range of 190 to 500 nm, and the nucleosides were monitored and quantified at 260 nm. The nucleosides were identified by comparing their retention time, purity coefficient, and spectrum against known standards. The external standard method was used to determine the nucleosides.

RESULTS AND DISCUSSION

Screening of conditions for the separation and determination of different nucleosides

In the present study, an optimized strategy for HPLC conditions was performed on the extract sample of *C. jiangxiensis*. Based on the absorption maxima of seven analytes and the stable baseline of the UV spectra with 3D chromatograms of HPLC-DAD detection, a 260 nm detection wavelength was used. The results suggest that the separation was improved when the column temperature was raised to 35°C, and the mobile phase was delivered at a flow rate of 1.0 mL/min.

For the mobile phase system, the results showthat these compounds cannot be separated by an isocratic elution system, such as acetonitrile, methanol, or buffer solution (water-KH_2PO_4), or by two mobile-phase isocratic elution system, such as acetonitrile/water, acetonitrile/buffer solution, methanol/water, and methanol/buffer solution, because of their similar chemical structures, as shown in Figure 1. Thus, the above four double mobile-phase systems with linear gradient elution condition (A: 0% to 15%; B: 100% to 85%) were further used for their separation. Most of the compounds were separated within 35 min, except for inosine, guanosine, and adenine, in which the analytical duration of the methanol/water system was less than 20 min (Figure 2A). Furthermore, taking into account the causticity of the salt solution in the HPLC system and the high cost of acetonitrile, the methanol/water system was used for further study. For the effective separation of each component of interest from the sample matrix, complex gradient HPLC systems are usually required (Yang et al., 2007; Liang et al., 2009). Thus, to obtain the desired separation effects for these compounds, the methanol/water system, with alternating isocratic and gradient elution strategies, was used in this study. Relatively good peak profiles and resolutions for inosine, guanosine, and adenine were obtained by prolonging the initial elution time from 0 min to 3 min (Figure 2B, C). The initial concentrations of the mobile phase increased from 8 to 15%, then from 15 to 24%, resulting in the desired separation profile with higher peak profile and resolution, and shorter analytical time of about 12 min, as shown in Figure 2D. As a result, the final elution conditions used for the methanol (M)/water (W) mobile phase were as follows: 0 to 3.0 min, isocratic 15% M; 3.0 to 3.5 min, linear gradient 15 to 24% M; 3.5 to 8.5 min, isocratic 24% M; 8.5 to 9.0 min, linear gradient 24 to 35% M; 9.0 to 15.0 min, isocratic 35% M. The analytical time was much less than those in similar reports (Yu et al., 2006, 2007).

Optimal extraction conditions for nucleosides in cultured *C. jiangxiensis*

High-efficiency extraction is necessary for the quantitative determination of specific compounds in *Cordyceps* products before analysis. To obtain a relatively high yield of the total investigated nucleosides, an optimized extraction procedure of nucleosides in *C. jiangxiensis* was developed using a $L_9(3^4)$ orthogonal layout (Tables 1 and 2). The peak areas were used as tested indices in this study. Among the nine experimental groups, the highest peak area of the total investigated nucleosides in *C. jiangxiensis* reached 6,829,044, as shown in Table 2. The eighth group (A3B2C1D3) obtained the highest peak area, whose levels of homologous factors involved 15% ethanol water-solution extraction solvent, 20 min extraction time, 20:1 solvent to sample ratio, and one extraction frequency. The fifth group obtained the lowest total peak areas of nucleosides with 2,102,561, whose corresponding factors and levels were A2B2C3D1, including 5% ethanol water-solution extraction solvent, 20 min extraction time, 30:1 solvent to sample ratio, and two extraction frequencies.

As to the effect of the extraction factors and levels on the extraction yield of nucleosides in *C. jiangxiensis*, the degree among the factors was C>D>B>A (extraction time > extractant > extraction frequency > extraction temperature), according to the order of magnitude of the R values (maximum difference) (Table 2). This order was also demonstrated by the F and/or P value in the variance analysis in Table 3. Among the four factors, only extraction time (B) was not associated with the extraction yield of nucleosides in *C. jiangxiensis* (Table 3). The other three factors have extremely significant effect, with $p<0.01$. Based on the maximum K value (the gross extraction yield of nucleosides in three instances) of each

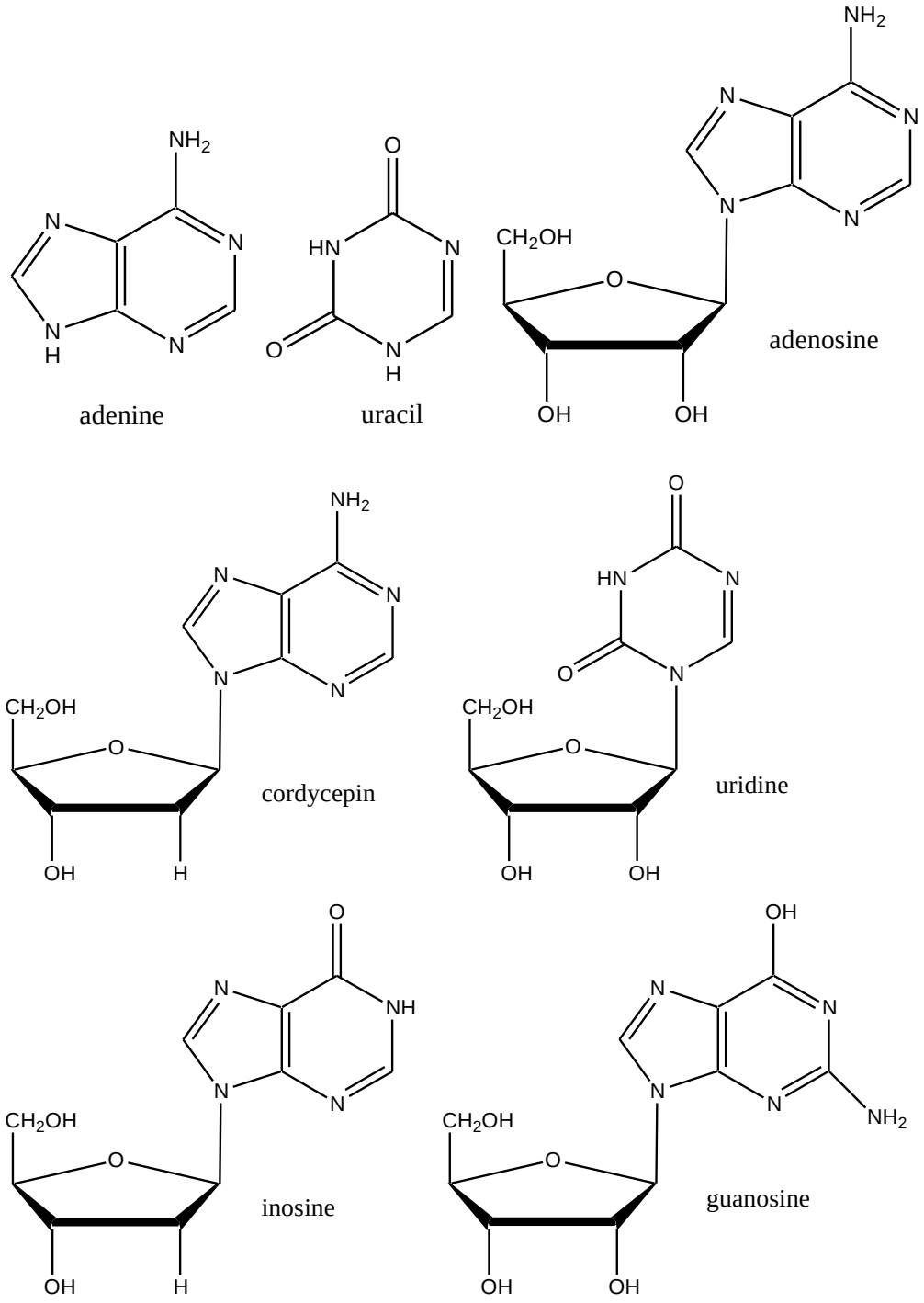

Figure 1. Chemical structures of nucleosides and nucleobases.

column in Table 2, the optimal level of each factor for extracting nucleosides in *C. jiangxiensis* (A3B3C1D3) was confirmed. The optimal condition for extracting nucleosides in *C. jiangxiensis* are as follows: 15% ethanol water-solution extraction solvent, 10 min extraction time, 20:1 solvent to sample ratio, and 1 extraction frequency.

Validation procedure

The stock standard of nucleosides was prepared using 1 mg/mL solution. Additional calibration levels were prepared by serial gradient dilution with ultrapure water. A standard calibration curve was created using these nucleo-

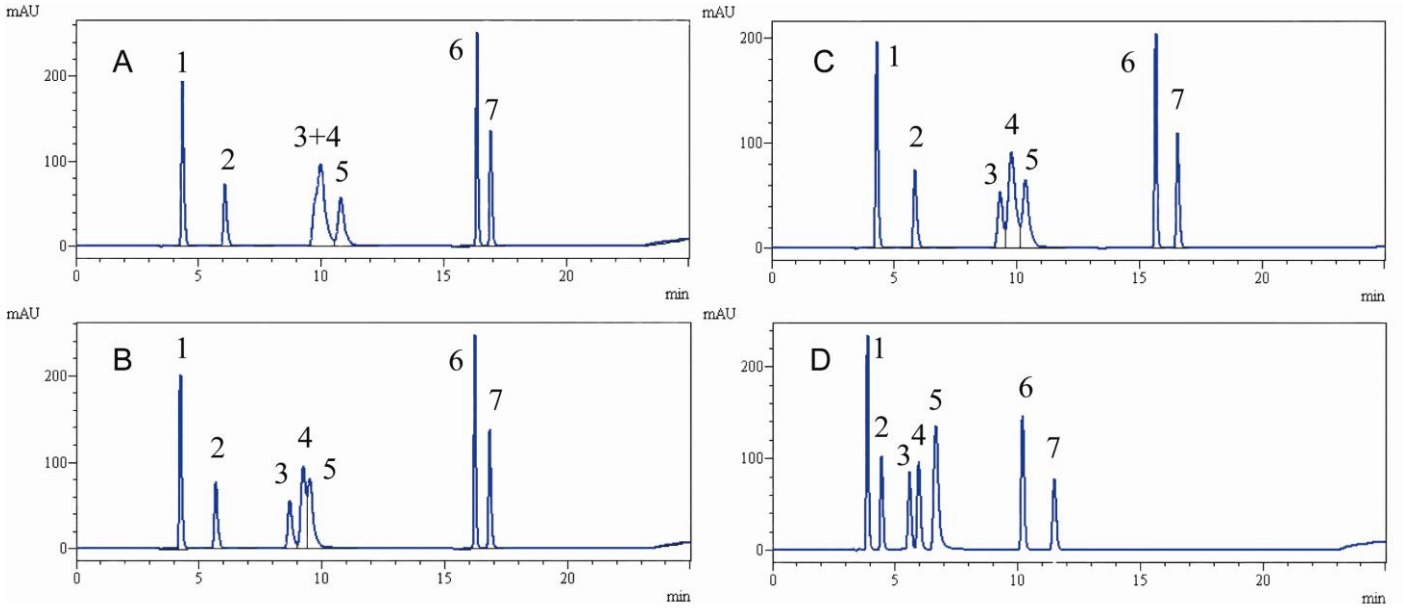

Figure 2. Chromatograms of mixed standard water solutions in different mobile phases. **A**. The elution condition for the mobile-phases, methanol (M) (0 to 15% within 20 min) and ultrapure water (W) (100 to 85% within 20 min). **B**. The elution condition for the M/W mobile-phases, 0 to 1.0 min, isocratic 8% M; 1.0 to 3.5 min, linear gradient 8 to 15% M; 3.5 to 8.5 min, isocratic 15% M; 8.5 to 9.0 min, linear gradient 15 to 35% M; 9.0 to 15.0 min, isocratic 35% M. **C**. The elution condition for the M/W mobile-phases, 0 to 2.0 min, isocratic 8% M; 2.0 to 3.5 min, linear gradient 8 to 15% M; 3.5 to 8.5 min, isocratic 15% M; 8.5 to 9.0 min, linear gradient 15 to 35% M; 9.0 to 15.0 min, isocratic 35% M; **D.** The elution condition for the M/W mobile-phases, 0 to 3.0 min, isocratic 15% M; 3.0 to 3.5 min, linear gradient 15 to 24% M; 3.5 to 8.5 min, isocratic 24% M; 8.5 to 9.0 min, linear gradient 24 to 35% M; 9.0 to 15.0 min, isocratic 35% M .**1,** uracil; **2,** uridine; **3,** inosine; **4,** adenine; **5,** guanosine; **6,** adenosine;**7:,**cordycepin.

Table 3. Variance analysis of the $L_9(3^4)$ orthogonal test results on nucleosides extraction for *Cordyceps jiangxiensis*[§].

Variance Source	Sum of Squares	Degree of Freedom	Mean Square	*F* value	Significance Level
Corrected model	36273032984774.440[#]	8	4534129123096.800	10.638	.001
Intercept	353244443369987.600	1	353244443369987.600	828.813	.000
A	7180764367283.100	2	3590382183641.551	8.424	.009
B	2954854213157.442	2	1477427106578.721	3.466	.077
C	17516186613900.450	2	8758093306950.220	20.549	.000
D	8621227790433.430	2	4310613895216.719	10.114	.005
Errors	3835848293516.000	9	426205365946.222		

[§]Statistically significant at 95% confidence level; [#] *R* Squared =0.904; Adjusted *R* Squared = 0.819.

Table 4. Regression curves, linearity, limit of quantification (LOQ), and limit of detection (LOD).

Analyte	Linear regression data			LOD	LOQ
	Linear range (μg/mL)	Regression equation	R^2	(μg/mL)	(μg/mL)
Uracil	0.95–92.00	Y=83653.97X+51089.60	0.9915	0.010	0.05
Adenine	0.96–96.00	Y=110193.50X+76283.66	0.9998	0.001	0.01
Uridine	0.88–175.00	Y=46749.92X-8299.70	1.0000	0.003	0.01
Inosine	0.88–175.00	Y=48799.90X-4571.77	1.0000	0.005	0.01
Guanosine	4.40–176.00	Y=57174.02X+30802.4	0.9997	0.003	0.01
Adenosine	1.00–100.00	Y=68802.19X-16891.63	0.9998	0.001	0.01
Cordycepin	1.13–91.00	Y=89476.15X-15045.62	0.9999	0.050	0.01

The data was present as average of three determinations. R^2, squares of correlation coefficients for the standard curves; LOD, limit of detection; LOQ, limit of quantification.

Table 5. Intra- and inter-day repeatability of the investigated analytes.

Analyte	Intra-day (n=5) R.S.D. (%)			Inter-day (n=5) R.S.D. (%)		
	H-conc. †	M-conc. ‡	L-conc. §	H-conc.	M-conc.	L-conc.
Uracil	0.65	1.43	1.30	0.48	1.00	3.99
Adenine	1.09	2.49	0.76	1.31	2.61	4.16
Uridine	0.79	1.56	0.41	2.01	1.67	2.22
Inosine	1.52	0.92	0.30	0.89	0.61	1.03
Guanosine	0.58	1.20	0.65	0.50	0.70	2.12
Adenosine	0.69	0.70	0.44	0.58	0.72	0.77
Cordycepin	0.59	0.70	0.66	0.62	0.63	1.03

R.S.D. means relative standard deviations. †H-conc. represents that the concentrations of uracil, adenine, uridine, inosine, guanosine and adenosine, cordycepin were 10.23, 10.56, 12.03, 13.22, 8.68, 10.50, and 8.10µg/mL, respectively. ‡M-con, represents that the concentrations of uracil, adenine, uridine, inosine, guanosine and adenosine, cordycepin were 1.45, 1.49, 2.64, 2.75, 2.18, 2.31, and 1.77 µg/mL, respectively. §L-conc. represented that the concentrations of uracil, adenine, uridine, inosine, guanosine and adenosine, cordycepin were 0.25, 0.29, 0.68, 0.64, 0.88, 0.67, and 0.49 µg/mL, respectively.

side water-methanol solutions at a concentration range of 0.5 to 200 µg/mL. A linear regression analysis was performed by plotting the peak areas against concentrations of nucleosides. The regression equations obtained by the least square method are listed in Table 4. Using the determined chromatographic condition, the values of the correlation coefficient R^2 of the calibration curves of the seven analyses were all more than 0.99. These results suggest good relationship between the nucleoside concentrations and their peak areas within the tested range.

The limit of detection (LOD) and the limit of quantification (LOQ) were determined, using the chromatographic conditions previously developed, based on the signal-to-noise ratios (S/N) of 3:1 and 10:1. The lowest concentration of the working solution containing seven reference analytes were diluted with ultrapure water to desired concentrations, and the aliquots were injected into the HPLC for analysis. In this study, five replicates of blank samples were analyzed. Three and ten average standard deviations of the blank responses to the corresponding slope of the calibration curve were regarded as LOD and LOQ, respectively. The LOD and LOQ for all tested analytes were all lower than 0.05 µg/mL, as shown in Table 4. This finding suggests that the quantification of each analyte investigated was at full capacity.

Intra- and inter-day variations were used to verify the precision of the method developed. For the intra- and inter-day variability tests, the standard solutions were determined in triplicate, five times daily, for three consecutive days. As shown in Table 5, precisions for all the analytes were calculated using the relative standard deviation (%RSD). The computed RSDs were 0.30 to 2.49% (n = 5) for the intra-day assay and 0.48 to 4.16% (n = 5) for the inter-day assay on three different concentrations. The %RSD was within the acceptable limit of 5%.

A recovery test was used to evaluate the accuracy and stability of the method. The *C. jiangxiensis* materials were spiked with known amounts of mixed standards at two concentration levels before extraction. The spiked samples were extracted with 15% ethanol water-solution following the procedure above-mentioned. Three replicates were performed for the test, and background levels were subtracted in all recovery determinations. The recovery of the investigated analytes ranged from 85.23 to 104.36%, and their %RSD values were all less than 5.5%, as shown in Table 6. The findings indicate the accuracy and reliability of the method developed. Likewise, the accuracy of the method was independent from the compound concentration and chemical structure.

In addition, selectivity was validated using an extract of *C. jiangxiensis* and a mixture of available standards optimizing separation and detection. The purity of the peaks was checked using a diode array detector through multivariate analysis. The three spectra of each peak, corresponding to the upslope, apex, and down slope, were normalized using a computer and then super-imposed. The peaks were considered pure when the three spectra coincided (match factor was ≥95%).

The above results show that the quantitative analysis of the seven investigated nucleosides from *C. jiangxiensis* materials by HPLC-DAD is satisfactory and feasible.

Application identification and quantitative determination of the investigated nucleoside compounds in *C. jiangxiensis*

The HPLC-DAD analysis presented a very detailed characterization of the investigated analytes in cultured *C. jiangxiensis* qualitatively and quantitatively. The typical HPLC chromatogram of methanol-water extracts from *C. jiangxiensis* detected at 260 nm is shown in Figure 3. Comparing the retention time and UV spectra with known standards, identification of the peaks of the investigated analytes was performed in *C. jiangxiensis* using the developed chromatographic method. The known analytes for peaks 1 to 7 were identified as uracil, uridine, inosine, adenine, guanosine, adenosine, and cordycepin, respectively, as shown in Figure 3. Based on the typical

Table 6. Recoveries from the assay of seven investigated analytes.

Analyte	Orignial (μg)	Spiked (μg)	Found (μg)	Recovery[†] (%)	R.S.D.[§] (%)
Uracil	4.30	10.21	13.94[†]	94.42	5.49
	4.45	1.47	5.95	102.04	
Adenine	1.60	10.73	12.30	99.72	2.28
	2.56	1.45	3.96	96.55	
Uridine	39.01	12.36	51.22	98.79	3.34
	40.81	2.60	43.26	94.23	
Inosine	-[#]	13.16	13.15	99.92	3.07
	-	2.75	2.87	104.36	
Guanosine	1.80	8.64	10.52	100.93	0.01
	1.88	2.18	4.05	99.54	
Adenosine	13.53	10.48	23.69	96.95	0.01
	14.14	2.29	16.36	96.09	
Cordycepin	0.85	8.09	8.05	89.00	3.06
	0.93	1.76	2.43	85.23	

[†] The data was present as average of three determinations. [†] Recovery (%) = (found amount −original amount)/ spiked amount×100. [§] R.S.D. (%) = 100× (S.D./mean). [#] Under the limit of quantitation.

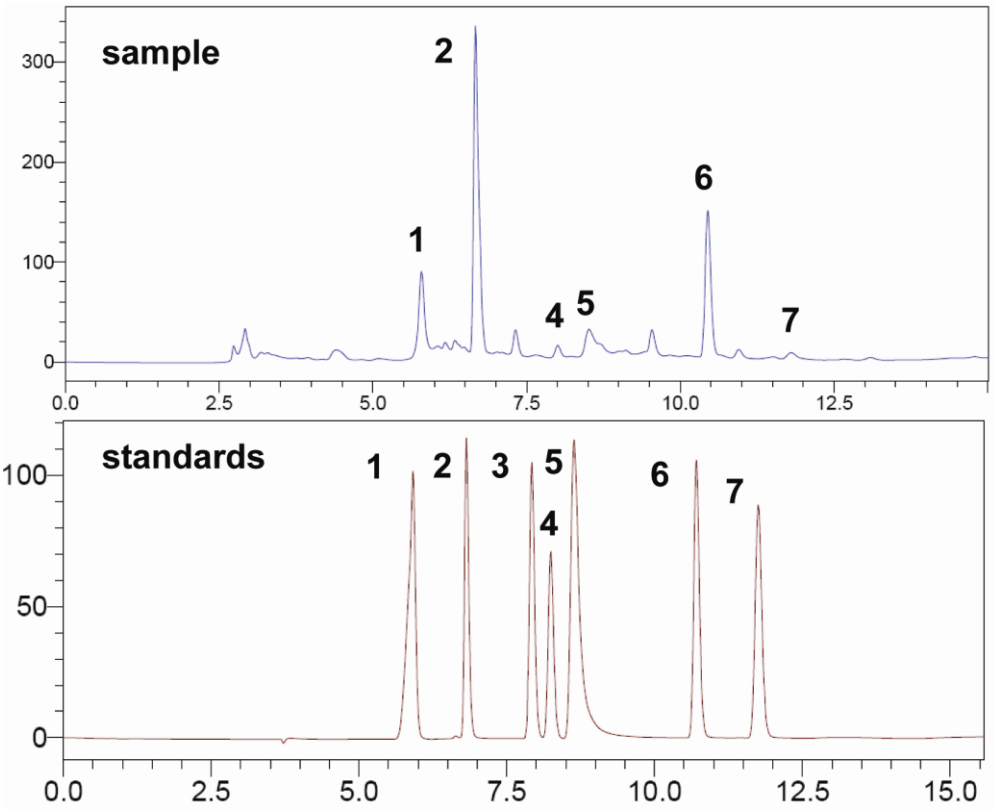

Figure 3. Typical HPLC chromatogram at 260 nm for mixed standards and cultured *C. jiangxiensis*. 1, uracil; 2, uridine; 3, inosine; 4, adenine; 5, guanosine; 6, adenosine; 7, cordycepin.

chromatogram in Figure 3, uridine and adenosine are the main nucleoside compounds in cultured *C. jiangxiensis*. Similar to cultured *C. militaris* (Yu et al., 2006), inosine (peak 3) was not detected in the cultured *C. jiangxiensis*. By contrast, natural *C. sinensis* contains rich inosine ingredient (Yu et al., 2006). Cordycepin is a considerable active component in *Cordyceps*, with various pharmacological activities, such as inhibition of protein kinase acti-

activity, enhancement of cell differentiation, anti-tumor, and anti-microbial properties (Sun et al., 2003; Xiao and Zhong, 2007). However, not all *Cordyceps* species contain cordycepin. For example, *C. militaris* and *C. kyushuensis* possess abundant amounts of cordycepin (Sun et al., 2003; Yang et al., 2007; Yu et al., 2007), whereas cordycepin is not found in many *Cordyceps* preparations, such as *Cordyceps taii*, and *Cordyceps gunnii* (Xiao et al., 2009b). Herein, trace amount of cordycepin (peak 7) was found and verified in cultured *C. jiangxiensis* by comparing the retention time and the UV spectra. Using the calibration curves of the known analytes, the analytes tested were quantitatively studied. The contents of these analytes were determined as follows: uracil (890.12 µg/g), adenine (513.48 µg/g), uridine (7802.14 µg/g), guanosine (376.14 µg/g), adenosine (2705.92 µg/g), and cordycepin (177.52 µg/g). The concentrations of uridine and adenosine were significantly higher than those of natural *C. sinensis* and cultured *C. militaris* (Li et al., 2006). Although only trace amounts of cordycepin was detected in cultured *C. jiangxiensis*, the amount was markedly higher than in natural and cultured *C. sinensis* (Li et al., 2006; Hsu et al., 2002). Currently, nucleosides are believed to be the major active components in *Cordyceps* (Hsu et al., 2002; Gong et al., 2004; ECPC, 2005; Li et al., 2004, 2006; Yu et al., 2007; Yang et al., 2007; Liang et al., 2009). Furthermore, nucleoside profiles, especially those of adenosine, cordycepin, and inosine, have been considered chemical markers for the quality control of *C. sinensis* and *C. militaris* (Li et al., 2006; Yu et al., 2006). Thus, adenosine, uridine, and cordycepin can be used for markers of quality control in *C. jiangxiensis*.

Conclusions

In the present study, a rapid HPLC-DAD method was successfully devised for the first time, which can be used to simultaneously separate and identify the nucleosides in *C. jiangxiensis* and other *Cordyceps* spp. The results show that the method developed has good separation and repeatability. Due to its high selectivity and sensitivity, HPLC-DAD is a powerful tool for the qualitative and quantitative analyses of nucleosides in Chinese medicines. HPLC-DAD is also useful in the study and quality control of *Cordyceps*. Uridine, adenosine, and cordycepin can be used as markers for the quality control of *C. jiangxiensis* bioproducts.

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