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Short communication

Effects of sample preparation methods on the quantification of nucleosides in natural and cultured *Cordyceps*

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ABSTRACT

Sample preparation is the first and very important step, which can greatly influence the repeatability and accuracy of the analysis. To date, several sample preparation methods with different solvents have been used for quantitative determination of nucleosides in *Cordyceps*, but their data are greatly various. In this study, five nucleosides, including adenosine, guanosine, inosine, uridine and cordycepin, in *Cordyceps* were determined using three extraction methods i.e. organic solvent pressurized liquid extraction, boiling water extraction and ambient temperature water extraction and high performance liquid chromatography (HPLC)-diode array detection (DAD). The similar results were obtained when organic solvent pressurized liquid extraction and boiling water extraction were applied. However, the amounts of nucleosides in natural *C. sinensis* and cultured *C. militaris* extracted with ambient temperature water were greatly increased except those of adenosine in natural *C. sinensis* and cordycepin in cultured *C. militaris*. In addition, the amount of investigated nucleosides in cultured *C. sinensis* had no obvious variation among the three extraction methods. The results suggest that sample preparation has significant effect on the quantification of nucleosides in *Cordyceps*.

1. Introduction

Cordyceps, one of the well-known traditional Chinese medicines, has multiple pharmacological activities, which has been widely used for the treatment of various diseases [1,2]. Since 3′-deoxyadenosine or cordycepin with anti-tumor activity was isolated from cultured *C. militaris* in 1964, nucleosides in *Cordyceps* have become a focus. To date, nucleosides are believed to be the active components in *Cordyceps*, and more than ten nucleosides and their related compounds, such as adenine, adenosine, uracil, uridine, guanine, guanosine, hypoxanthine, inosine, thymine, thymidine and deoxyuridine, have been isolated [3]. Indeed, nucleosides are involved in the regulation and modulation of various physiological processes in body, which produce functions through purinergic and/or pyrimidine receptors [4,5]. Therefore, determination of nucleosides is very important for pharmacological study and quality control of *Cordyceps* and their products. To date, among the developed methods for quantification of nucleosides in *Cordyceps*, different solvents, including methanol, ethanol, water and their mixed solutions with different ratio, have been used for sample preparation at different temperature [3]. Their results are also greatly various, which may attribute to the different sample preparation. Actually, decoction is the major administration form in clinic use of traditional Chinese medicine. Therefore, sample preparation using organic solvent may be not available and rational for quality control of Chinese medicine.

In present study, three extraction methods, including organic solvent (methanol) pressurized liquid extraction (OSPLE), boiling water extraction (BWE) and ambient temperature water extraction (ATWE), were optimized and applied for the extraction of nucleosides from natural and cultured *C. sinensis* and cultured *C. militaris*. Their contents of five nucleosides, i.e. uridine, inosine, guanosine, adenosine and cordycepin, were quantitatively evaluated and compared by high performance liquid chromatography (HPLC)-diode array detection (DAD).

2. Experimental

2.1. Materials and chemicals

Adenosine, uridine, guanosine, inosine, cordycepin were purchased from Sigma (St. Louis, MO, USA). Ammonium acetate was from Fluka (Buchs, France). Triethylamine (TEA) and methanol were purchased from Merck (Damstadt, Germany). Deionized water was prepared using a Millipore Milli-Q Plus system (Millipore, Bedford, MA, USA). Reagents not mentioned here were from standard

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sources. The materials of natural *C. sinensis* were obtained from three different regions of China, one from Qinghai, two from Tibet and one from Sichuan. The identities of these natural *Cordyceps* were confirmed by the corresponding author. Cultured *C. sinensis* mycelia were obtained from Hebei, The Hong Kong University of Science and Technology (HKUST), Jiangxi and Wanfeng. Cultured *C. militaris*, a substitute of *C. sinensis*, was obtained from Guobao, Quanxin, Aoli and Xiankang. The species of the cultured *Cordyceps* were certified by State Food and Drug Administration of China or the corresponding author. The voucher specimens of *Cordyceps* are deposited at the Institute of Chinese Medical Sciences, University of Macau, Macao, China.

2.2. Sample preparation

2.2.1. Organic solvent PLE

0.5 g powder of *Cordyceps* were mixed with diatomaceous earth in a proportion (1:1) and placed into an 11 ml stainless steel extraction cells, respectively. The extraction with methanol was performed under optimized conditions [6]: temperature, 160 °C; static extraction time, 5 min; pressure, 1.034×10^4 KPa and one static cycle and one for the number of extraction. The solution was filtered through a 0.45 μm Econofilter (Agilent Technologies, Palo Alto, CA, USA) before HPLC analysis.

2.2.2. Boiling water extraction

0.5 g powder of *Cordyceps* were mixed with 10 ml boiling (95–100 °C) Milli-Q water in a glass tube with stopper, accurately weighted and kept at boiling water bath (95 °C) for 30 min. After extraction, the extract was cooled down to the room temperature, and made up the lost weight with water, then centrifuged at 1.2×10^4 rpm (Centrifuge 5415D, Eppendorf, Germany) for 5 min. The supernatant was filtered through a 0.45 μm Econofilter before HPLC analysis.

2.2.3. Ambient temperature water extraction

0.5 g powder of *Cordyceps* were mixed with 10 ml Milli-Q water (25 °C) in a glass tube with stopper, accurately weighted and kept at room temperature (25 °C) for 18 h, then kept in a boiling water bath (95 °C) for 5 min. The extract was cooled down to the room temperature, and made up the lost weight with water, then centrifuged at 1.2×10^4 rpm for 5 min. The supernatant was filtered through a 0.45 μm Econofilter before HPLC analysis.

2.3. HPLC analysis

All separations were performed on an Agilent Series 1100 (Agilent Technologies, USA) liquid chromatograph, equipped with a vacuum degasser, a quaternary pump, an autosampler, and a diode array detector (DAD) system, connected to a Agilent ChemStation software. A Zobax SB-AQ column (250 mm × 4.6 mm i.d., 5 μm) with a Zobax SB-C_{18} guard column (20 mm × 3.9 mm i.d., 5 μm) was operated at 25 °C. Solvents that constituted the mobile phase were (A) 10 mM aqueous TEA and (B) methanol. The separation was achieved using gradient elution of 0–25% B for 30 min. The flow rate was 1 ml/min, and the injection volume was 10 μl. The analytes were monitored at 254 nm.

3. Results and discussion

3.1. Optimization of extraction conditions

3.1.1. Optimization of BWE

0.5 g powder of natural (from Sichuan) and cultured (from Hebei) *C. sinensis* and cultured *C. militaris* (from Quanxin) were used for extraction as mentioned above. The extraction time was 0.5, 1, 2, 4, 8 h, respectively. After HPLC analysis, the peak areas of the five investigated compounds were used for evaluation of the extraction efficiency. The results showed that their contents were similar though the extraction time was extended. Therefore, extraction time was selected as 0.5 h for BWE.

3.1.2. Optimization of ATWE

0.5 g powder of natural (from Sichuan) and cultured (from Hebei) *C. sinensis* and cultured *C. militaris* (from Quanxin) were used. The extractions were performed at room temperature for 1, 2, 4, 8, 12, 18, 24, 30 h, respectively. The extraction efficiency present as the peak areas of five investigated compounds, i.e. uridine, inosine, guanosine, adenosine and cordycepin, were shown in Fig. 1. As the results showed that, the peak areas of uridine, inosine and guanosine in natural *Cordyceps* increased (Fig. 1A) with the extraction time extension and reached the top at 18 h, and then decreased with the time increase. While its adenosine decreased

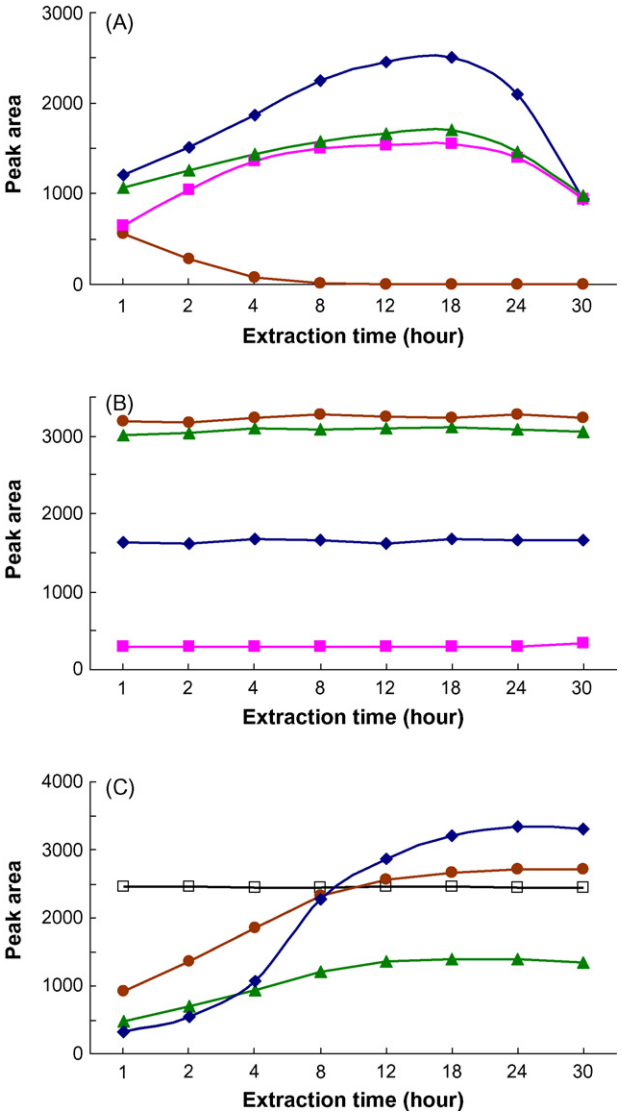

Fig. 1. Effects on the amounts of uridine, inosine, guanosine and adenosine in (A) natural (from Sichuan) and (B) cultured (from Hebei) *Cordyceps sinensis* and cultured (C) *C. militaris* (from Quanxin) extracted by ambient temperature water with different extraction time. (♦) Uridine; (■) inosine; (▲) guanosine; (●) adenosine and (□) cordycepin.

Table 1
Linear regression data, limit of detection (LOD), limit of quantification (LOQ), intra- and inter-day repeatability of the investigated compounds

Analytes	Linear regression data			LOD (μg/ml)	LOQ (μg/ml)	Intra-day (n = 6), R.S.D. (%)			Inter-day (n = 6), R.S.D. (%)		
	Regression equation	r^2	Test range (μg/ml)			H[a]	M[b]	L[c]	H	M	L
Uridine	$y = 24.89x - 5.17$	0.9999	7.62–152.33	0.03	0.08	0.20	0.16	0.14	0.51	0.22	0.45
Inosine	$y = 13.64x - 0.20$	0.9999	9.28–176.38	0.03	0.07	0.22	0.08	0.18	0.58	0.21	0.66
Guanosine	$y = 23.76x + 14.14$	0.9999	8.86–177.20	0.04	0.10	0.14	0.08	0.21	0.56	0.20	0.31
Adenosine	$y = 28.13x - 19.12$	0.9999	15.42–246.72	0.09	0.19	1.47	1.15	0.80	1.79	2.09	1.95
Cordycepin	$y = 35.45x - 122.54$	0.9999	27.30–518.80	0.12	0.28	0.41	0.75	0.72	2.66	0.92	1.44

[a] H, the concentrations of uridine, inosine, guanosine, adenosine and cordycepin were 0.105, 0.134, 0.114, 0.215 and 0.400 μg/mL, respectively.
[b] M, the concentrations of uridine, inosine, guanosine, adenosine and cordycepin were 0.053, 0.067, 0.057, 0.108 and 0.200 μg/mL, respectively.
[c] L, the concentrations of uridine, inosine, guanosine, adenosine and cordycepin were 0.026, 0.034, 0.029, 0.054 and 0.100 μg/mL, respectively.

with extraction time prolonged, and even disappeared when the extraction time was more than 8 h. However, these changes in cultured *C. sinensis* have not been investigated (Fig. 1B). For cultured *C. militaris*, the detected compounds, including uridine, guanosine and adenosine were increased with the extraction time prolonged, but cordycepin kept stable within the investigated extraction time range (Fig. 1C). Considering the three types of samples, the extraction time was finally selected as 18 h.

3.2. Calibration curves

Water stock solutions containing five reference compounds were prepared and diluted to appropriate concentrations for the construction of calibration curves. At least six concentrations of the solution were analyzed in triplicate, and then the calibration curves were constructed by plotting the peak areas versus the concentration of each analyte. The results were shown in Table 1.

3.3. Limits of detection and quantification

The water stock solution containing five reference compounds was diluted to a series of appropriate concentrations with the same solvent, and an aliquot of the diluted solutions were injected into HPLC for analysis. The limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively. The data of LOD and LOQ for each investigated compounds were shown in Table 1.

3.4. Precision

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 within 1 day, while for inter-day variability test, the solution was examined in duplicates for consecutive 3 days. Variations were expressed by

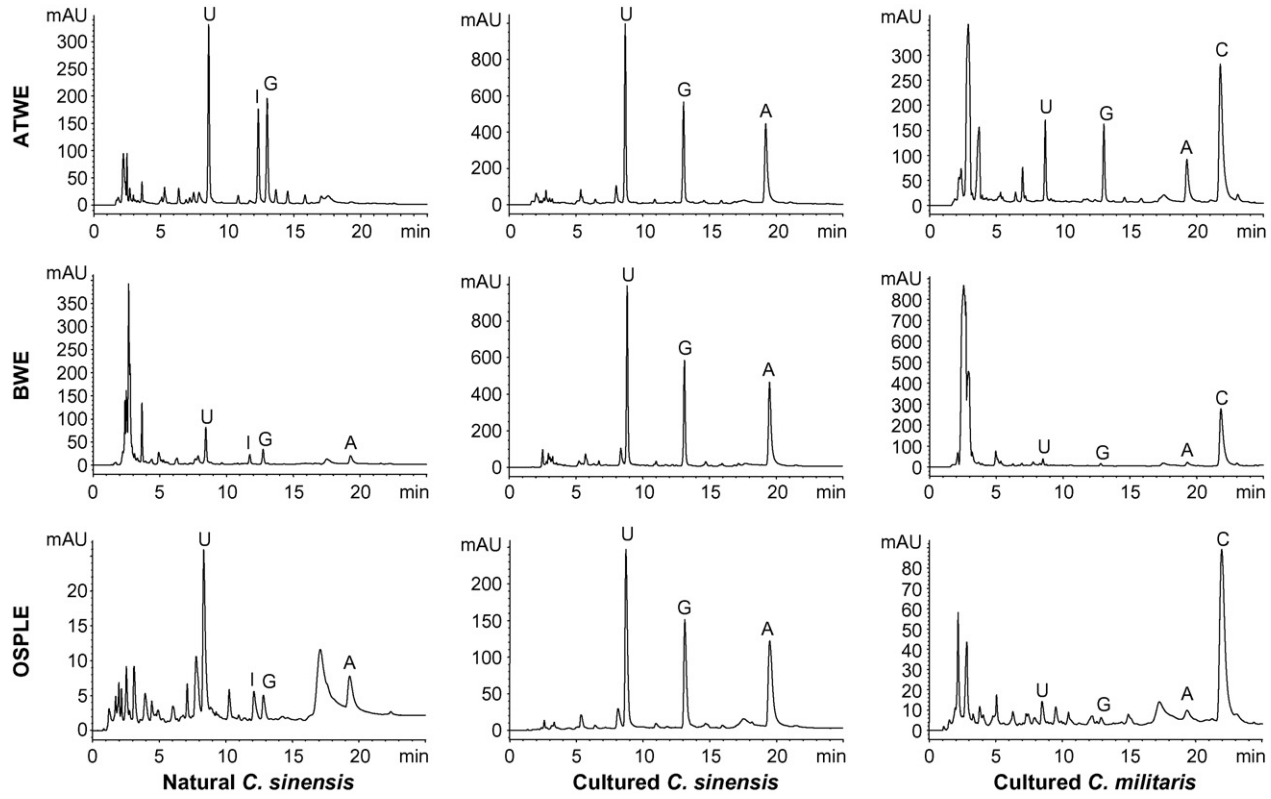

Fig. 2. Typical HPLC-DAD chromatograms of natural (Tibet 1) and cultured *C. sinensis* (Wanfeng) and cultured *C. militaris* (Aoli) extracted by ambient temperature water extraction (ATWE), boiling water extraction (BWE) and organic solvent pressurized liquid extraction (OSPLE). U, uridine; I, inosine; G, guanosine; A, adenosine and C, cordycepin.

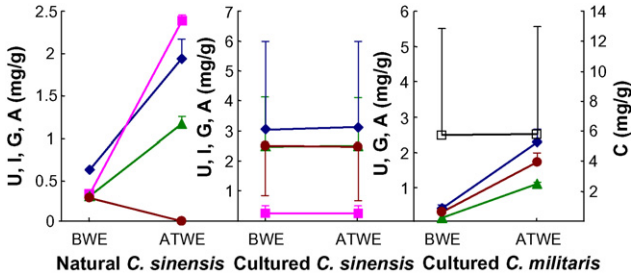

Fig. 3. Effects of boiling water extraction (BWE) and ambient temperature water extraction (ATWE) on the contents of five investigated compounds in natural and cultured *C. sinensis* and cultured *C. militaris*. U, uridine (♦); I, inosine(■); G, guanosine (▲); A, adenosine (●); C, cordycepin (□).

Table 2
Contents (mg/g) of five investigated compounds in natural and cultured *Cordyceps* extracted by different methods

Sample	Extraction	Analytes				
		Uridine	Inosine	Guanosine	Adenosine	Cordycepin
Natural *C. sinensis*						
Sichuan	OSPLE[a]	0.78[b]	0.23	0.08	0.31	–[c]
	BWE	0.71	0.32	0.30	0.28	–
	ATWE	2.02	2.27	1.00	–[c]	–
Qinghai	OSPLE	0.60	0.22	0.09	0.29	–
	BWE	0.57	0.35	0.32	0.28	–
	ATWE	2.10	2.46	1.24	–	–
Tibet 1	OSPLE	0.75	0.23	0.07	0.24	–
	BWE	0.72	0.29	0.23	0.22	–
	ATWE	1.50	2.32	1.09	–	–
Tibet 2	OSPLE	0.71	0.22	0.08	0.31	–
	BWE	0.69	0.31	0.35	0.30	–
	ATWE	2.08	2.40	1.27	–	–
Cultured *C. sinensis*						
Hebei	OSPLE	1.45	0.55	2.53	2.39	–
	BWE	1.47	0.54	2.47	2.31	–
	ATWE	1.53	0.55	2.53	2.34	–
HKUST	OSPLE	0.51	–	0.21	0.25	–
	BWE	0.55	–	0.20	0.25	–
	ATWE	0.55	–	0.23	0.26	–
Jiangxi	OSPLE	2.79	–	2.71	2.26	–
	BWE	2.80	–	2.69	2.26	–
	ATWE	2.81	–	2.70	2.24	–
Wanfeng	OSPLE	7.40	–	4.49	5.06	–
	BWE	7.46	–	4.51	5.09	–
	ATWE	7.44	–	4.51	5.05	–
Cultured *C. militaris*						
Guobao	OSPLE	0.68	–	0.05	0.16	16.71
	BWE	0.66	–	0.05	0.15	16.34
	ATWE	2.21	–	1.17	1.53	16.47
Aoli	OSPLE	0.34	–	0.08	0.26	3.31
	BWE	0.35	–	0.08	0.23	3.31
	ATWE	1.17	–	1.15	0.97	3.26
Xiankang	OSPLE	1.47	–	0.36	1.37	1.76
	BWE	1.47	–	0.36	1.50	1.71
	ATWE	3.14	–	1.21	2.43	1.71
Quanxin	OSPLE	0.56	–	0.89	0.63	1.63
	BWE	0.58	–	0.90	0.67	1.63
	ATWE	2.56	–	0.82	1.88	1.63

[a] OSPLE, BWE and ATWE represent for organic solvent pressurized liquid extraction, boiling water (95 °C) extraction and ambient temperature water (25 °C) extraction, respectively.
[b] Average of duplicates.
[c] Undetectable.

the relative standard deviations (R.S.D.s) for intra- and inter-day (Table 1).

For the repeatability of the extraction of the five investigated compounds from *Cordyceps* by ATWE or BWE, 0.5 g powder of natural (from Sichuan) and cultured (from Hebei) *C. sinensis* and cultured *C. militaris* (from Quanxin) were extracted by ATWE or BWE triplicates and analyzed by HPLC as mentioned above. The overall R.S.D.s of ATWE and BWE for uridine, inosine, guanosine, adenosine and cordycepin were less than 1.29, 2.93, 1.05, 2.31 and 0.11%, respectively.

3.5. Determination of five analytes in Cordyceps

Typical chromatograms of natural and cultured *C. sinensis* and *C. militaris* extracted with OSPLE, BWE and ATWE were shown in Fig. 2. The identification of peaks for investigated compounds was carried out by comparison of their retention time and UV spectra with those obtained injecting standards in the same conditions, as well as by spiking *Cordyceps* samples with stock standard solutions.

By using the calibration curves of the analytes, the contents of the five analytes in natural and cultured *C. sinensis* and *C. militaris* were determined. Table 2 shows the summary results.

3.6. Comparison of different extraction methods on the quantification of nucleosides in Cordyceps

The effects of extraction methods on the quantification of nucleosides in different types of *Cordyceps* samples are various. Generally, the data of natural and cultured *C. sinensis* and cultured *C. militaris* extracted using OSPLE and BWE were similar. While ATWE greatly increased the contents of uridine, guanosine and inosine in natural *C. sinensis*, and uridine, guanosine and adenosine in cultured *C. militaris*, but decreased and even eliminated adenosine in natural *C. sinensis*. Furthermore, the content of cordycepin in *C. militaris*, has no obvious variation among three extraction methods (Fig. 3). In addition, the contents of uridine, guanosine and adenosine, in cultured *C. sinensis*, had no significant variation among the three extraction methods. Therefore, the chemical profiles of natural and cultured *C. sinensis* and cultured *C. militaris* are significantly different between the extracts of BWE (or OSPLE) and ATWE, which could be used for discriminate the source of *Cordyceps*.

In order to elucidate the effect of sample preparation methods on the quantification of nucleosides in *Cordyceps*, further experiments were performed. Two mixtures were obtained by mixing 0.5 g cultured *Cordyceps* (from Hebei) and 0.5 g natural *Cordyceps*

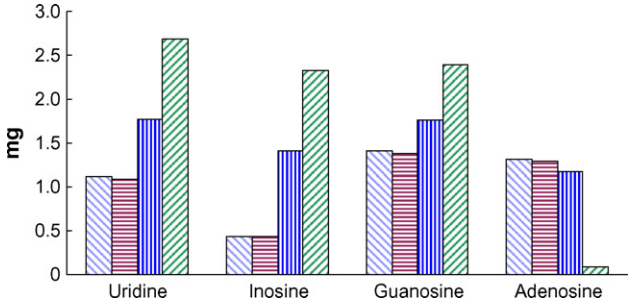

Fig. 4. The effect of different treated natural *Cordyceps* on the amounts of the investigated nucleosides in the mixture of natural (from Sichuan) and cultured (from Hebei) *C. sinensis*. The sum of investigated nucleosides in natural (0.5 g) and cultured (0.5 g) *Cordyceps sinensis* separately extracted with boiling water extraction (BWE, ▨) and ambient temperature water extraction (ATWE, ▥), respectively, and the amount of investigated nucleosides in the mixtures of natural (0.5 g) dried at 100 °C for 30 min (▤) or 30 °C for 30 min (▨) and cultured (0.5 g) *Cordyceps sinensis* extracted with ATWE, respectively.

(from Sichuan) dried at 100 °C for 30 min (Mix 1) or at 30 °C for 30 min (Mix 2), respectively. Then the mixtures were extracted by ATWE. It is interesting that the amounts of investigated compounds determined in two mixtures were significantly different (Fig. 4). In brief, the amounts of uridine, inosine and guanosine determined in Mix 2 were much higher than their sum in natural and cultured *Cordyceps* separately extracted under the same conditions. The phenomenon could be eliminated when natural *Cordyceps* treated at 100 °C for 30 min, which were similar to their sum when the samples separately treated with BWE. The results suggested that natural *Cordyceps* may contain some enzymes which can transfer some compounds into uridine, inosine and guanosine or decompose adenosine. Further study is needed in future.

4. Conclusion

Extraction methods greatly influence the quantitation of nucleosides in *Cordyceps*. Therefore, sample preparation is very important, which should be carefully optimized. In addition, combined with bioassay, it is helpful to develop a rational method for quality control of *Cordyceps*.

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