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Profiles of nucleosides and nitrogen bases in Chinese medicinal fungus *Cordyceps sinensis* and related species

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Abstract. The profiles of nucleosides and nitrogen bases in fruiting bodies and cultured mycelia of the Chinese medicinal fungus *Cordyceps sinensis* were determined by reversed-phase HPLC. The patterns were compared with those of taxonomically related species. As in other medicinal fungi, such as *Ganoderma lucidum* and *Lentinus edodes*, *C. sinensis* contained a large amount of adenosine. The adenosine content of fruiting bodies was 2.47 mg/g. 3'-Deoxyadenosine (cordycepin), which was originally reported in *C. militaris*, was not detectable in *C. sinensis*. The nucleoside patterns of fruiting bodies and cultured mycelium of *C. sinensis* were very similar. Results also indicated that *C. sinensis* and related fungi, including *Paecilomyces*, contained many modified nucleosides and nitrogen bases in minute quantities. The profiles of nucleosides and nitrogen bases may be useful for the quality control of mycelial culture and commercial products of this medicinal fungus.

Keywords: Adenosine; *Cordyceps sinensis*; Nucleosides; Reversed-phase high performance liquid chromatography.

Introduction

The fruiting bodies of a very limited number of fungi are used in traditional Chinese medicine, although the ancient folk medicine more commonly uses higher plants. *Ganoderma lucidum* (Fr.) Karst (Polyporaceae) and *Cordyceps sinensis* (Berk.) Sacc. (Clavicipitaceae) are two medicinal fungi which have attracted great attention recently for their biological and pharmacological activities. *Cordyceps sinensis* is an entomogenous fungus. Its parasitic hosts belong mainly to the insects of *Coleoptera* and *Lepidoptera*. Recent studies have demonstrated that *Cordyceps* species, and a few medicinal fungi including *Ganoderma* and *Poria cocos*, produce polysaccharides with antitumor activities (Ukai et al., 1983). Most interestingly, these fungi also contain large amounts of adenosine (Ikumoto et al., 1991). The edible mushroom (*Lentinus edodes*) and *Auricularia auricula-judae* have also been demonstrated to contain large doses of adenosine. The adenosine contents of these fungi can be high enough to show a pharmacological effect. Suppression of platelet aggregation through the consumption of these fungi has been reported (Hammerschimdt, 1980; Shimizu et al., 1985), but the contents of adenosine in these medicinal fungi and edible mushrooms have not yet been compared.

We report the profiles of nucleosides and nitrogen bases in *C. sinensis* and several taxonomically related fungi. Quantitation of adenosine and profile analysis were carried out by reversed-phase high performance liquid chromatography (RP-HPLC) (Gehrke and Kuo, 1990). The adenosine contents of several medicinal fungi and edible mushrooms were also determined and compared.

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Materials and Methods

Materials

The LC-grade organic solvents were obtained from Labscan (Dublin, Ireland). Authentic nitrogen bases, nucleosides, and analogues were purchased from Sigma (St. Louis, MO, USA).

The fruiting bodies of *Cordyceps sinensis* were obtained from mainland China. Strains of *C. sinensis* (VGH-CS-1), *C. memorabilis* (ATCC 36743), *C. militaris* (IFO 30377), *C. sphingum* (CBS 114.22), *Paecilomyces cicadae* (TSRI 757.22), *P. javanicus* (TSRI, 757.24), *P. farinosus* (TSRI 757.23), and an unidentified species of *Cordyceps* were obtained from the Taiwan Sugar Research Institute (TSRI), Tainan, Taiwan, ROC. The strains were maintained on PDA slants. The stationary cultures

were maintained at 26±1.5°C for 30 days in 1-L culture flasks (6 flasks for each strain) containing 300 ml medium (Wang, 1989). The culture medium consisted of 2% glucose, 0.5% peptone, 2% malt extract, and potato dextrose broth (24 g/L).

The fresh samples of *Lentinus edodes* and *Auicularis auricula-judae* were purchased from a local market. *Ganoderma lucidum* (TP-1) was cultured in liquid medium according to a previous procedure (Shiao et al., 1987).

HPLC apparatus

A Hewlett-Packard liquid chromatograph (HP-1090) (North Hollywood, CA, USA), which was equipped with a variable-wavelength UV detector (HP-1050) and a Rheodyne 7125 injector (20-μl loop) (Berkeley, CA, USA), was used.

A pre-packed reversed phase column (Cosmosil 5C18, 250x4.6-mm, 5-μm) of Nacalai Tesque (Kyoto, Japan) was used. A semi-preparative Cosmosil C18 column (250x8 mm, 10 μm) was also chosen to collect metabolites for spectroscopic identification.

Sample preparation

Cultured mycelia were harvested and gently rinsed with distilled water. Mycelial mats were dried at 45°C in darkness. The mycelia were ground into powder and extracted with distilled water (20 w/v) (Li et al., 1990; Shiao et al., 1989). The aqueous extracts were passed through cartridge columns (reversed phase). After eluting with distilled water, the cartridge column was eluted with a mixture of methanol-water (1:1, v/v) to recover samples for HPLC analysis.

For commercial products of *C. sinensis* from mainland China, only the fruiting bodies were collected. This avoided the interference of nucleosides from the host insects.

Determination of nucleosides and nitrogen bases

The profiles of nucleosides and nitrogen bases were determined by RP-HPLC with gradient elution (Gehrke and Kuo, 1989; Gehrke and Kuo, 1990). Solvents A and B each containing 2.5% MeOH and 20% MeOH in 0.01 M $(NH_4)H_2PO_4$ were prepared. The gradient started with 100% solvent A and was linearly increased to 25% solvent B over 10 minutes. The volume percentage of solvent B was further increased to 40% over 20 minutes. It was finally increased to 100% solvent B over 30 minutes and remained so for another 10 minutes. The flow rate was maintained at 0.9 mL/min. The UV detector was set at 260 nm.

Identification of adenosine and related metabolites

Peaks in RP-HPLC were initially identified by co-injection with authentic samples. Each peak was either confirmed by using a diode array detector (HP-1040M) or further identified, after collection, by spectroscopic methods. The peaks corresponding to adenosine and cordycepin, were recovered by using a semi-preparative RP-HPLC column (250x8-mm). They were completely identified by mass and NMR spectroscopy (Shiao et al., 1989; Shimizu et al., 1985). Quantitation of each metabolite was based on peak area in RP-HPLC. Results were obtained from the calibration curves of authentic standards.

Results and Discussion

Determination of nucleosides and nitrogen bases by RP-HPLC

RP-HPLC was feasible for determination of the profiles of nucleosides and nitrogen bases in *C. sinensis*. To illustrate the separation behavior, the resolution of twenty authentic samples of nucleoside and nitrogen bases were examined. In a multi-step, linear-gradient elution, eighteen standards were well resolved (Figure 1). The nucleosides were eluted ahead of their corresponding nitrogen bases, and of their deoxy-counterparts. In general, the eluting sequences of nitrogen bases and nucleosides followed the trend of C>G>T>A. The mobile phase was particularly designed that the five common nitrogen bases, five common nucleosides, and four common deoxyribonucleosides were well resolved. They were about equally spaced in the chromatogram in a reasonable elution period (40 min). In this condition, guanine and hypoxanthine were not separated. These two metabolites could be better resolved under different elution condition. Since the contents of hypoxanthine were very low in the samples, the quantitative determination of adenosine and profile analysis by RP-HPLC were not affected.

Patterns of nucleosides and nitrogen bases in *Cordyceps* and *Paecilomyces*

To provide mycelial samples on a comparable basis, fungi (except *G. lucidum*) were cultured in liquid medium under identical conditions. The HPLC profiles of *C. sinensis* and of three species of *Cordyceps* are shown in Figure 2. Evidently, adenosine was the predominant nucleoside in *C. sinensis* and related species. The adenosine content of *C. sinensis* (2.47 mg/g dry weight) was also the highest among these four species of *Cordyceps* (Table 1). Several species of *Paecilomyces*, one of the anamorph of *C. sinensis*, were also examined. The profiles of *Paecilomyces farinosus* (the conidial state of *C. memorabilis*) (Pacioni and Frizzi, 1978; Kobayashi, 1982), *P. cicadae*, *P. javanicus*, and an unidentified species of *Cordyceps* are shown in Figure 3. The nucleoside patterns were found similar to that of *C. sinensis*. In these fungi, the predominant nucleosides were adenosine, guanosine, and uridine. The adenosine contents of these fungal species are listed in Table 1. Excepting *C. sphingum*, the *Cordyceps* species all contained high levels of adenosine. A previous study to

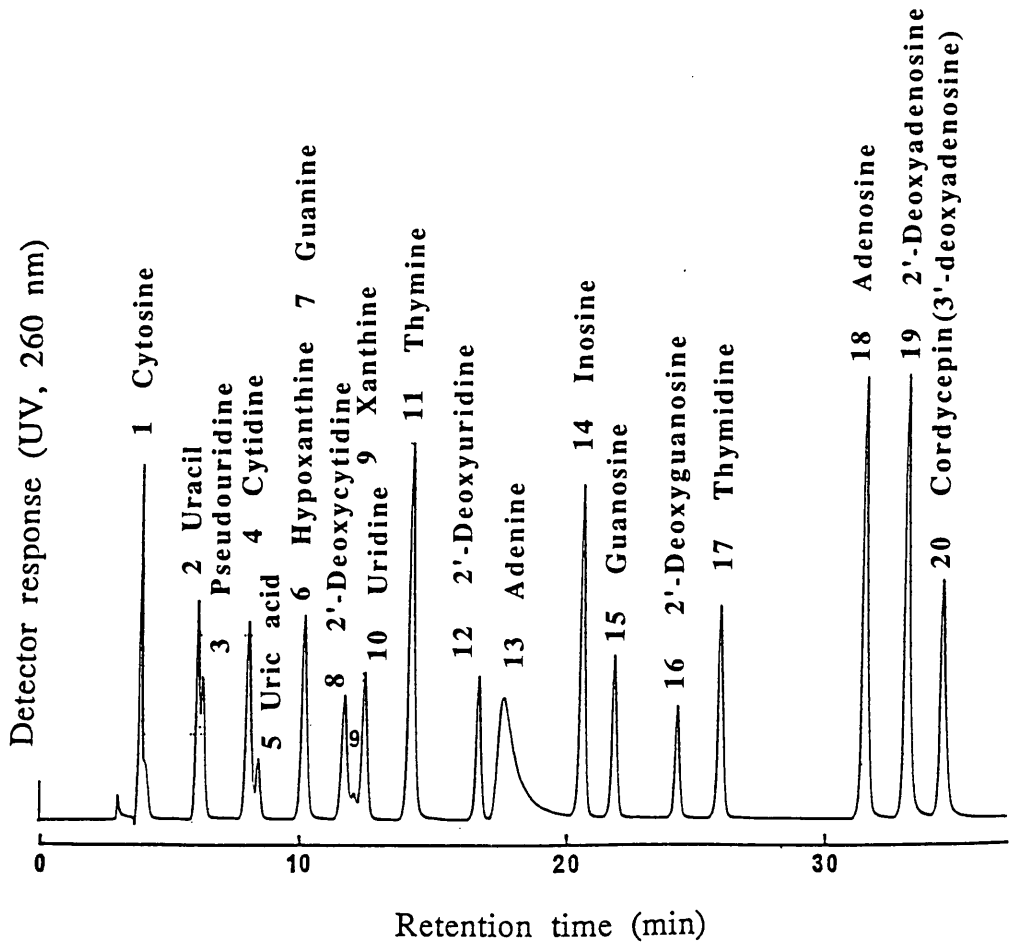

Figure 1. Reversed-phase liquid chromatogram of twenty standards of nucleosides and nitrogen bases.

A reversed phase column (Cosmosil 5C18, 250x4.6-mm, 5-μm) was used. Mobile phase was prepared from two solvent systems (Solvent A, 2.5% MeOH in 0.01 M $(NH_4)H_2PO_4$; Solvent B, 20% MeOH in 0.01 M $(NH_4)H_2PO_4$). The gradient started with 100% A and was linearly increased to 25% B over 10 min. The volume percentage of B was further increased to 40% over 20 min. It was finally raised to 100% B over 30 min and remained so for another 10 min. The overall elution time was 40 min. The flow rate was maintained at 0.9 mL/min and the wavelength of UV detector was set at 260 nm. Peak identification: 1, cytosine; 2, uracil; 3, pseudouridine; 4, cytidine; 5, uric acid; 6, hypoxanthine; 7, guanine; 8, 2'-deoxycytidine; 9, xanthine; 10, uridine; 11, thymine; 12, 2'-deoxyuridine; 13, adenine; 14, inosine; 15, guanosine; 16, 2'-deoxyguanosine; 17, thymidine; 18, adenosine; 19, 2'-deoxyadenosine; 20, cordycepin (3'-deoxyadenosine).

Table 1. Major nucleosides and nitrogen bases (mg/g dry weight)[a] in *Cordyceps sinensis* and several related fungi.

Fungus	Uracil	Guanine	Uridine	Guanosine	Adenosine
Cordyceps sinensis	0.17	0.13	1.30	1.10	2.47
C. memorabilis	0.05	trace	3.16	1.50	2.16
C. militaris	0.19	0.11	1.33	0.96	1.44
C. sphingum	0.92	0.70	0.42	0.17	0.09
Cordyceps sp.	0.30	trace	2.07	1.03	1.34
Paecilomyces cicadae	trace	trace	1.99	0.98	1.22
P. javanicus	trace	trace	2.06	0.93	1.37
P. farinosus	0.29	0.36	2.17	0.73	1.12

[a]Results were obtained by RP-HPLC analysis. Data represent the mean values of three determinations.

Table 2. Adenosine contents of *Cordyceps sinensis* and several medicinal and edible fungi.

Fungus	Adenosine content (mg/g dry weight)
Cordyceps sinensis (VGH-CS-1)	1.71
Cordyceps sinensis (Qinghai, China)[a]	2.47
Cordyceps sinensis[b]	0.63
Cordyceps militaris[b]	2.26
Ganoderma lucidum (TP-1)	0.13
Ganoderma lucidum[c]	0.40
Lentinus edodes	2.33
Auricularia auricula-judae	0.32

[a]Analysis was performed on the fruiting bodies only. Results from this study are expressed as mean values of three determinations.

[b]Result reported by Ikumoto et al., 1991. The fruiting bodies and parasitic host larva were used. Samples were obtained from mainland China.

[c]Results reported by Shimizu et al., 1985.

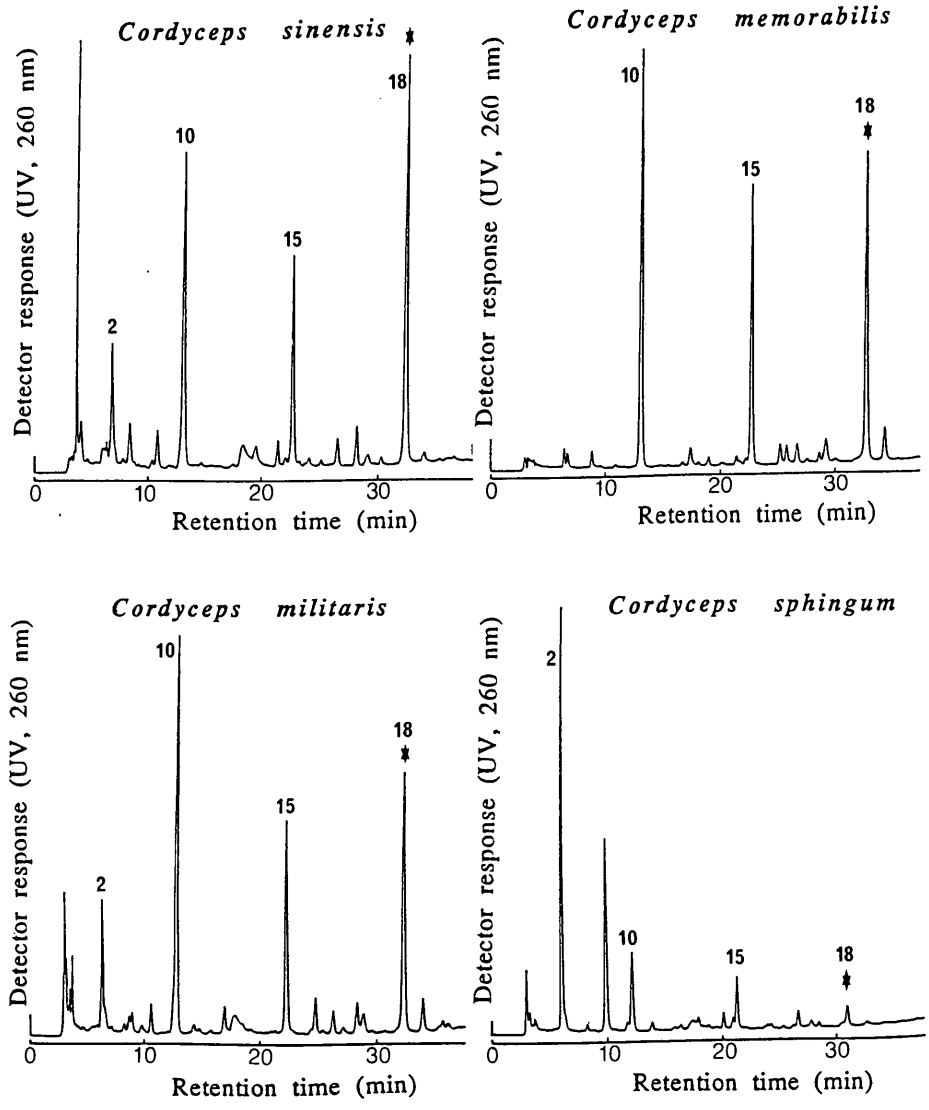

Figure 2. RP-HPLC profiles of nucleosides and nitrogen bases of *Cordyceps sinensis* and three *Cordyceps* species.

The HPLC condition was identical to that of Figure 1. The fruiting bodies of a commercial sample of *C. sinensis* were used for analysis. The mycelia of the other three species (*Cordyceps memorabilis*, *C. militaris*, and *C. sphingum*) were obtained from 30-day-old liquid cultures in identical condition. The peak corresponding to adenosine is marked by an asterisk (*) in the chromatograms.

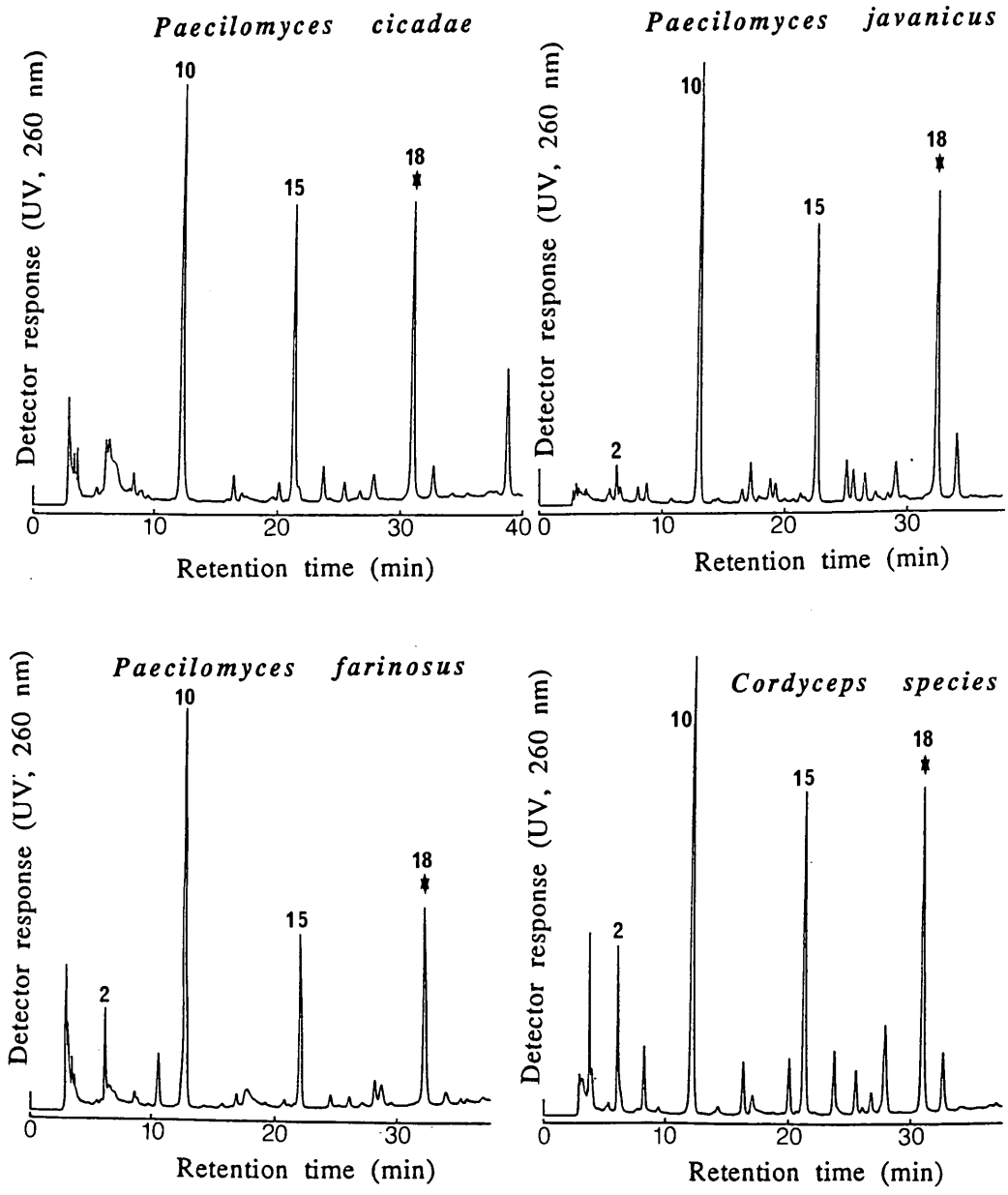

Figure 3. RP-HPLC profiles of nucleosides and nitrogen bases of *Paecilomyces cicadae*, *P. javanicus*, *P. farinosus*, and a species of *Cordyceps*.

The HPLC condition was identical to that of Figure 1. The mycelia of the four species were obtained from 30-day-old liquid cultures. The peak corresponding to adenosine is marked by an asterisk (*) in the chromatograms.

compare the amino acid compositions and TLC profiles of crude extracts of *C. sinensis* and *Paecilomyces tenuipes* also demonstrated that their profiles were very similar (Chen, 1992).

Cordycepin (3'-deoxyadenosine) is a modified nucleoside originally isolated from *C. militaris* (Cunningham et al., 1950; Glazer and Kuo, 1977; Deitch and Sawicki, 1979). Since then, several other modified nucleosides were identified from various species of *Cordyceps* (Kredich and Guarino, 1961; Furuya et al., 1983). We have carefully examined the production of cordycepin in these fungi. Evidently, the fruiting bodies and cultured mycelia of *C. sinensis* did not produce cordycepin to a detectable level. The strain of *C. militaris* used in this study produced a trace amount of this modified nucleoside.

The speculation that medicinal fungi in traditional Chinese medicine contain large amount of adenosine

gains further support from this study. The adenosine contents of *Ganoderma lucidum* (Shimizu et al., 1985), *Lentinus edodes*, and *Auricularia auricula-judae* were compared with that of *C. sinensis*. As listed in Table 2, the adenosine contents of *C. sinensis*, *C. militaries*, and *L. edodes* were particularly high (Ikumoto et al., 1991; Shimizu et al., 1985). Pharmacological effects of adenosine, such as inhibition of platelet aggregation, can be expected even when moderate amounts of these fungi are consumed (Hammerschmidt, 1980). Adenosine in fungus *C. sinensis* can be derived from *de novo* synthesis or the host insects. The accumulation of large quantity of adenosine and the occurrence of modified nucleosides in *Cordyceps* deserves attention. The majority of modified nucleosides, which include N^6-(2-hydroxyethyl)adenosine (Furuya et al., 1983) and homocitrullylaminoadenosine (Kredich and Guarino, 1961), in the genus *Cordyceps* are biogenetically derived from adenosine.

The application of secondary metabolites as markers for mycelial culture and as quality control for products of *C. sinensis* deserves exploration. Ergosterol was suggested for this purpose in the identification of products containing *C. sinensis* (Li and Li, 1991). Since ergosterol is a common metabolite in fungi, the applicability as a marker is disputable. Nucleosides and nitrogen bases occur in microorganisms including *C. sinensis* and other fungi. The overall profiles, however, instead of any single metabolite, and occurrence of uniquely modified nucleosides can be feasible for these purposes.

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藥用眞菌冬蟲夏草與相關菌株的核苷與氮鹼譜型

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本研究採用逆相式高效液相層析法分析了中國藥用眞菌冬蟲夏草子實體與液相培養菌絲中的核苷與氮鹼譜型，並與一些分類相近菌株進行比較。有如與其它藥用眞菌靈芝一般，冬蟲夏草含有高量腺苷，於子實體中其值達 2.47 mg/g，原獲自蛹蟲草的 3-去氧腺苷則無法自冬蟲夏草子座和菌絲中測得。野生冬蟲夏草與液相培養菌絲之核苷與氮鹼組成相近。逆相層析譜亦顯示冬蟲夏草之核苷與氮鹼亦與其無性世代擬青霉相近，且亦均含多種但少量之修飾型核苷與氮鹼，推測核苷與氮鹼譜型或可做爲冬蟲夏草菌絲培養與成品之品質指標。

關鍵詞：腺苷；核苷；逆相式高效液相層析；冬蟲夏草。