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Article

Analysis of the Nucleoside Content of *Cordyceps sinensis* Using the Stepwise Gradient Elution Technique of Thin-Layer Chromatography

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Nucleoside is the main class of active components in *Cordyceps sinensis*. Thin-layer chromatography (TLC) is one of the most commonly used methods in pharmacopoeias for analyzing chemical components of herbal medicine. Since the isocratic elution method cannot be applied successfully in TLC analysis for separating all the nucleoside components, the stepwise gradient elution has been developed in this work to separate eight nucleoside standards with success. In this way, quantitative analyses of the samples of *Cordyceps sinensis* were achieved via the proposed TLC procedure coupled with the scanning densitometric techniques of CAMAG and TLCQA methods for qualitative and quantitative analysis.

Keywords thin-layer chromatography, *Cordyceps sinensis*, stepwise gradient elution, nucleoside, software, quantitative analysis

Introduction

Traditional Chinese medicine (TCM) has been used for more than five thousand years and proved to be an effective and valuable health care alternative. In recent years, there is a growing trend in investigating Chinese medicine (CM) and CM formulation in order to discover new health products for treating different diseases.

Cordyceps sinensis, also known as Chinese caterpillar fungus or "Dong Chong Xia Cao" (winter worm, summer grass), is one of the most valued Chinese medicinal herbs.[1] In TCM, *Cordyceps sinensis* is relatively non-toxic, has an anti-asthmatic effect and is also a potential anti-cancer agent.[1] The herb regulates and ensures the normal functioning of various parts of the body, improves the immune system, as well as promotes overall vitality and longevity. Therefore, a lot of health care products of *Cordyceps sinensis* are available in the market (Bodyonics, Hicksville, U.S.A., 1997).

Many fungi belonging to the genus *Cordyceps sinensis* have been demonstrated to produce natural products with various biological activities. The active principles of *Cordyceps sinensis* are believed to come from the nucleosides including uracil, hypoxanthine, uridine, inosine, guanosine, adenine, adenosine, cordycepin and others. In 1950, Cunningham *et al.*[2] isolated a nucleoside derivative, cordycepin, from *Cordyceps militaris*. This class of active ingredient has been found to exhibit different biological activities.

Thin-layer chromatography (TLC) is a widely used technique for chemical analysis of CM and CM formulation though it has lower resolving power than GC and HPLC. Various pharmacopoeias such as American Herbal Pharmacopoeia (AHP) (Upton, R., Santa Cruz, US, 2002), Chinese Drug Monographs and Analysis (Wagner, H., Kötzting/Bayer, Wald, Germany, 1997), Pharmacopoeia of the People's Republic of China (Chemical Industry Press, Beijing, 1997), *etc*. use TLC to provide characteristic fingerprints of herbs. Some active components of nucleosides, along with ergosterol and *D*-mannitol have been successfully identified within *Cordyceps sinensis* by using various chromatographic methods.[3] Among these techniques, TLC has the advantages of many-fold possibilities of detection in analyzing herbal medicine. In addition, TLC can be employed for multiple sample analysis. For each plate, more than 30 spots of samples can be studied simultaneously in one time.

TLC study is usually carried out only under isocratic conditions resulting in lower separation capacity. In this way, the eight nucleosides of uracil, hypoxanthine, uridine, inosine, guanosine, adenine, adenosine and cordycepin related to *Cordyceps sinensis* cannot fully be separated by the reported solvent system.[4] In order to overcome this difficulty and enhance the TLC selectivity, the multistep gradient elution (GE) technique has been developed.[5,6] In general, there are two different

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modes of multistep GE. The first one is the stepwise mobile phase GE[7] in which eluent fractions of increasing eluent strength are introduced to the adsorbent layer without interrupting the elution process. The other technique is the programmed multiple development[8,9] or its automated version[10] in which the plate is developed over increasing distances with several eluents of decreasing eluent strength with the mobile phase being evaporated after each development. In this work, a two-step GE was employed to separate the eight nucleoside standards commonly presented in *Cordyceps sinensis* sample. In addition, the developed TLC plates as obtained in this study were analyzed by two different scanning densitometric techniques provided by the CAMAG Company (refer to the operating manual of Videoscan version 1.01 from Camag, Switzerland) and the TLCQA method.[11] Details of the qualitative and quantitative results are given in the following sections.

Experimental

Chemicals and samples of *Cordyceps sinensis*

Eight nucleoside standards including uracil, uridine, hypoxanthine, inosine, guanosine, adenine, adenosine, and cordycepin were employed in the present study of *Cordyceps sinensis* and were acquired from Wako (Japan). Eleven samples of *Cordyceps sinensis* including eight solid samples and three liquid oral samples were purchased from health food stores of Hong Kong and Korea (Table 1). Details of these samples can be obtained from the authors. Solvents for TLC experimentation were of analytical grade and were utilized without further purification.

Table 1 Information of the eleven *Cordyceps sinensis* samples

	Sample name	Country	Date of manufacture
1	Cordyceps Pill (Guangzhou)	China[b]	1999
2	Superior Quality Dongchong Xiacao (Sichuan)		1999
3[a]	China Cordyceps King (Guangzhou)		1999
4	Dongchunghacho Green Tea	Korea[c]	1999
5	Dongchunghacho Tea		1999
6	Powdered Dongchunghacho		1999
7	Dongchunghacho (cold and dry)		1999
8	Cordyceps Militaris Tablet	China and Japan[d]	1999
9	Cordyceps Militaris (dry)		1999
10[a]	Lung-Nourishing Oral Solution		1999
11[a]	Energetic Oral Solution		1999

[a] Liquid oral sample. [b] The cordyceps samples of China were distributed by Yue Hwa Chinese Products. [c] The cordyceps samples of Korea were manufactured by SLI Korea Bio-Engineering, and distributed by Doowon Farming Co. Ltd., Korea. [d] The cordyceps samples of the joint products of China and Japan cooperation were manufactured by Shen Yang Sunny High Bio. Engineering Co., Ltd, and distributed by Kwong Lee Bio-Product Co. Ltd, Hong Kong.

Sample preparation

The water-soluble constituents, nucleosides, were extracted by the following procedure.[12] 0.5 g of the powder or dried sample was firstly extracted with 20 mL of deionized water and the solution was sonicated for 2 h. Then the liquid layer was filtered off and dried by using a rotary evaporator. The residue was dissolved in 10 mL of methanol. For both the solid and liquid oral samples, the sample solution was then passed through a SEP-PAK C_{18} cartridge (Waters Assoc., US) being pre-washed with 2 mL methanol and then 5 mL of water. After loading, the sample solution was directed through the cartridge and collected in a vial. Then, the resulting solution was filtered through a 0.45 μm Nylon-66 filter (Alltech, US). In our TLC analysis, the volumes of the standards and sample solutions used were 2 and 8 μL respectively.

Stepwise gradient elution

It has been demonstrated that the conventional TLC separation procedures cannot be applied successfully to analyze complex multi-component systems like Chinese herbs. For instance, the isocratic TLC gives overlapping spots for inosine and guanosine.[4] Usually the weaker eluent resolves the less retained solutes whereas the stronger eluent resolves the more polar solutes. Thus, a well-chosen gradient condition may separate more components involved. A two-step gradient procedure has therefore been developed in this work for separating the standards of nucleosides as well as these components present in the cordyceps samples with marked improvement.

Experimental condition, data acquisition and processing

An aliquot of 500 mg/L nucleoside standards was used in our TLC study. Aliquots (2 μL) were spotted onto a TLC plate of Silica gel 60 F_{254} (Merck KGaA, Darmstadt, Germany) using the CAMAG Linomat IV semi-automatic sampler (CAMAG, Switerland). The TLC plates were developed within the CAMAG flat bottom chamber (20 cm×10 cm) (CAMAG, Switerland) at ambient temperature. All the nucleoside standards and samples were studied with three duplicated runs within three weeks to scrutinize the quality of the data and results obtained. Since the spots of nucleosides can only be observed in the UV region, their images were captured by the CAMAG video store system (CAMAG, Switerland) at 254 nm. Images of the TLC plates developed and 2D cross-section profiles of the nucleosides were obtained by using the CAMAG TLC system (CAMAG, Switerland) through which the R_f values and peak areas of the TLC spots were determined.

The components of the samples under study were identified according to the R_f values of the correspond-

ing TLC spots. As for quantification, it is based on relevant information of the TLC spot obtained via different methodologies such as the total intensity or optical density of pixels within the spot[13,14] or peak area[15,16] of the spot in its two dimensional (2D) fluorescence spectra and 2D intensity or optical density graph. Nowadays, the advancement of image analysis techniques enhances the accuracy of this kind of measurement based on the 2D profile of the TLC spot by using the peak area and the intensity volume. In this investigation, the nucleoside standards and *Cordyceps sinensis* samples were studied by the methods provided by the CAMAG Videoscan (ver 1.01) (Method A) and TLCQA-UV (Method B) for image and data analysis. The performances of both Method A and B were compared in this study and would be discussed in detail later.

Results and discussion

Qualitative analysis of the nucleoside standards

The TLC plates as purchased from the supplier were found not suitable for studying nucleosides because it caused strong tailing upon development. This might affect the quality of separation and the quality of the results obtained. To minimize this effect, it was recommended in reference[17] that the TLC plate should be prior treated by a mixture of 0.05 mol/L Na_2HPO_4 and 0.05% carboxymethyl cellulose (4∶1, *V*/*V*). Then it was put into an oven (60 ℃) for about 8 min for activation. Isocratic development was employed by using the composition of chloroform-ethylacetate-isopropanol-water (8∶2∶6∶0.6, *V*/*V*) with two drops of ammonia solution in each 10 mL of mixtures as recommended in the literature.[4] However, inosine and guanosine appeared to migrate with a shorter distance and their TLC spots overlapped with each other.

In order to separate inosine and guanosine, the proposed two-step gradient elution procedure was employed. In the first step, a weak solvent of solvent A (chloroform-ethylacetate-isopropanol-water-triethylamine with a ratio of 10∶2∶8∶0.5∶2, *V*/*V*) was used over a development distance of 18 cm. The distance was determined by optimizing the separation of the TLC spots. In this way, hypoxanthine, uridine, adenine, adenosine, uracil and cordycepin were separated in the upper part of the chromatogram. In the second step, the plate was developed over 9 cm with a more polar solvent, solvent B (chloroform-ethylacetate-isopropanol-water-dimethylformamide with a ratio of 10∶2∶8∶0.5∶2, *V*/*V*), to isolate the remaining two nucleosides of guanosine and inosine. Owing to different polarities of triethylamine and dimethylformamide, the eight nucleoside standards were well separated via our two-step GE procedure. Thin layer chromatogram with five different concentrations of the eight nucleoside standards as observed under 256 nm wavelength is shown in Figure 1. Lanes 1 to 5 represent mixtures of the standards with different concentrations of 50, 100, 500, 750, 1000 mg/L respectively for each nucleoside. It can be seen that the eight nucleosides are well separated in these lanes via our two-step gradient elution procedure. The 2D and 3D TLC profiles of lane 3 (Figure 1) are given in Figure 2a and 2b respectively. The profiles 1 and 2 which corresponds to inosine and guanosine are separated from each other. To the best of our knowledge, the eight nucleosides under study have never been separated by the TLC technique before.

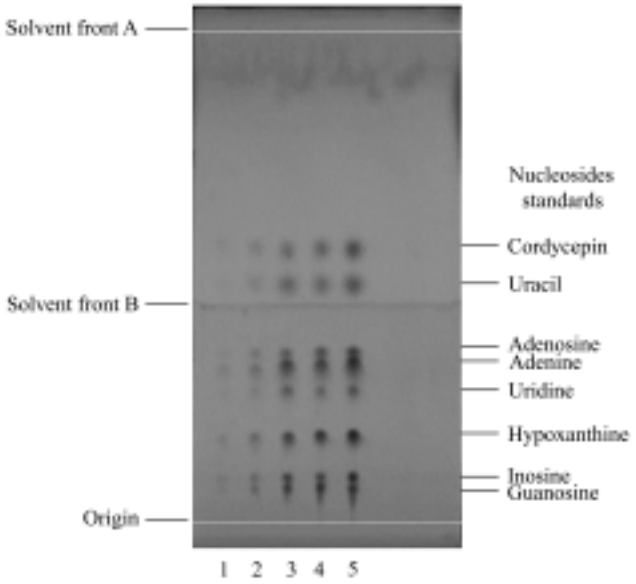

Figure 1 Thin layer chromatogram of the nucleoside standards with different concentrations by using the proposed two-step TLC gradient elution method. Lanes 1, 2, 3, 4 and 5 represent respectively mixtures of all the eight standards with 50, 100, 500, 750 and 1000 mg/L for each component in each mixture.

Table 2 lists the retention factors (R_f values), peak areas and intensity volumes of the eight nucleoside standards investigated in this work. The relative percentage deviations of these quantities are also included for reference.

It can be seen that the relative deviation of R_f as shown in Table 2 as well are in the range of 1.42% to 3.66%. This indicates that the reproducibility of the TLC system as well as the methods proposed is good. Also, the standard deviations of the peak area obtained from Method A (Table 2) are slightly higher than those of Method B.

Quantitative analysis of *Cordyceps sinensis* samples by using Methods A and B

Calibration curves of the nucleoside standards Two methods were employed in this investigation for preparing the calibration curves of the nucleoside standards. Method A is based on the peak areas obtained from the TLC chromatogram (*e.g.* Figure 2a), while Method B is made use of the intensity volumes of the TLC spots (*e.g.* Figure 2b). Two sets of calibration

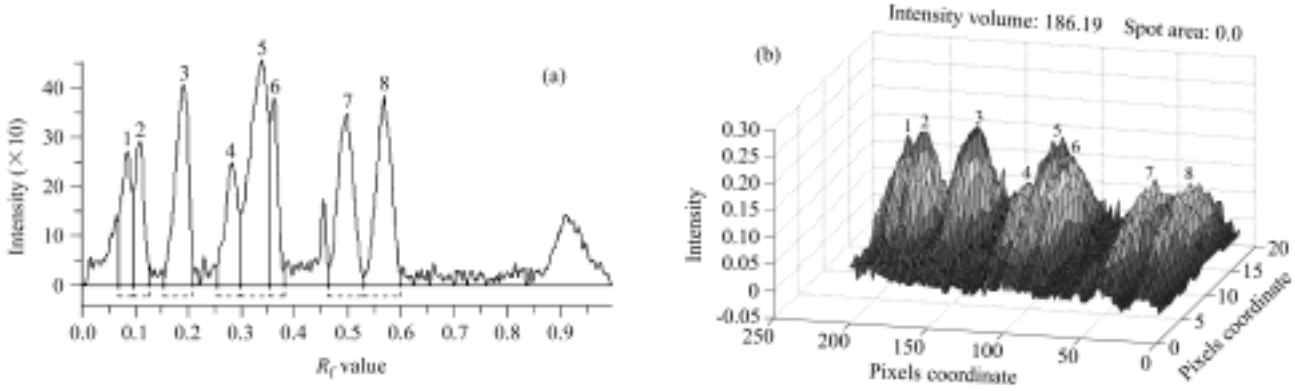

Figure 2 (a) The 2D cross-section profile and (b) the 3D plot of the eight nucleoside standards as obtained from two-step TLC gradient elution (see Experimental section for detail) and acquired by Method A and B respectively. Peaks 1, 2, 3, 4, 5, 6, 7 and 8 correspond to guanosine, inosine, hypoxanthine, uridine, adenine, adenosine, uracil and cordycepin, respectively.

Table 2 Retention factors (R_f), peak areas and intensity volumes of the eight nucleoside standards (500 mg/L) as obtained from the two-step TLC gradient elution studies by using Method A and Method B

Peak No.	Nucleoside	R_f[a]		Peak area[b]	Intensity volume[b]
		Method A	Method B	Method A	Method B
1	Guanosine	0.079 (3.66)	0.082	2105.37 (1.47)	14.77 (0.31)
2	Inosine	0.099 (2.06)	0.097	1633.83 (0.66)	13.95 (0.38)
3	Hypoxanthine	0.183 (2.23)	0.179	3581.41 (0.99)	23.69 (0.39)
4	Uridine	0.286 (1.78)	0.281	2003.35 (1.66)	15.96 (0.35)
5	Adenine	0.323 (3.19)	0.313	5332.97 (0.94)	33.23 (0.94)
6	Adenosine	0.343 (1.78)	0.337	2095.64 (0.99)	14.99 (0.57)
7	Uracil	0.497 (1.84)	0.488	3488.35 (1.82)	21.92 (1.12)
8	Cordycepin	0.570 (1.42)	0.562	3045.25 (1.19)	18.68 (0.77)

[a] The quantity within the parenthesis represents the relative (%) deviation of R_f which was calculated using the expression [R_f(Method A) − R_f(Method B)/R_f(Method B)]×100%. [b] The quantity within the parenthesis represents the standard deviation of the peak area and intensity volume as obtained from triplicate experiments.

curves were thus obtained and depicted in Figure 3a and 3b. The correlation coefficients "R^2" of the calibration curves of the eight standards are given in Table 3. It is obvious that very good linear relationship is obtained and the linear region is laid on this calibration curve. Those based on the intensity volumes (Method B) perform slightly better in view of the magnitudes of the correlation coefficients obtained. The detection limit of the TLC technique can only be up to mg/L level. Thus, the same limit applies to that of the nucleosides studied and the lower detection limit was found to be 50 mg/L as given in Figure 3a and 3b.

Performance of Methods A and B in quantification In order to evaluate the quality of the two sets calibration curves, they were applied to control experiment to determine the concentrations of nucleosides with known values. In Table 2 the calculated values of each standard and their applied concentrations of 500 mg/L are listed. The deviations of the two sets of concentration obtained are given in Table 4.

Referring to Table 4, the deviations of the concentrations as determined via peak area (Method A) is in the range of 3.15% to 9.41% while those of Method B employing intensity volume is 1.41% to 7.57%. In general, Method B gives results with lower discrepancies. For guanosine, uridine and adenosine, Method B outperforms Method A with much lower deviations of 1.5% to 3%, nearly three times more precise than Method A.

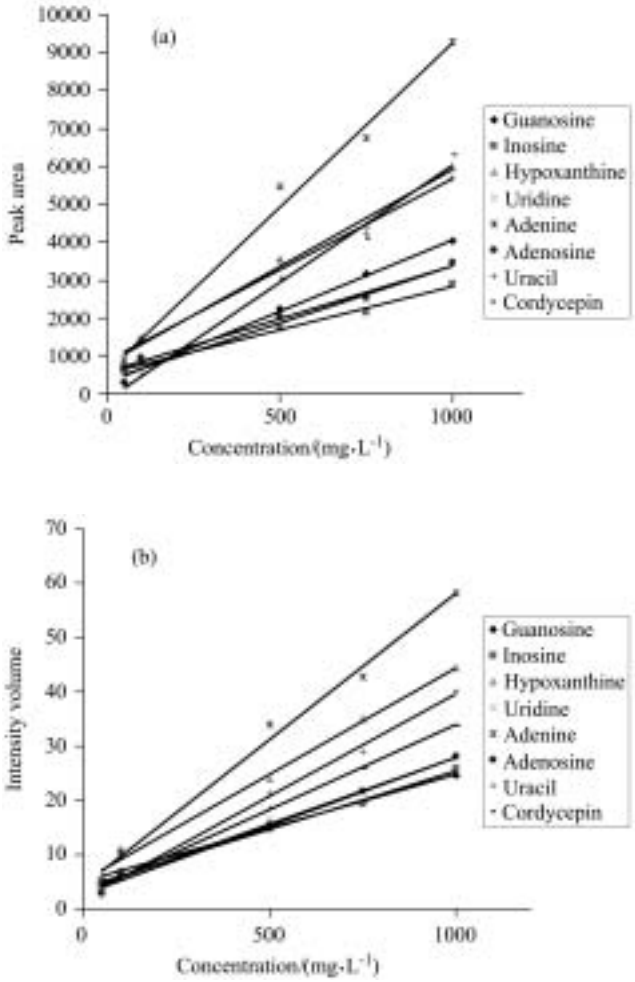

Figure 3 Calibration curves of the eight nucleoside standards as obtained using (a) Method A and (b) Method B. The symbols ◆, ■, ▲, ×, ∗, ●, + and − denote guanosine, inosine, hypoxanthine, uridine, adenine, adenosine, uracil and cordycepin respectively.

Table 3 The values of correlation coefficients "R^2" obtained for the eight nucleoside standards using Method A and Method B

Nucleoside	Correlation coefficients "R^2"	
	Method A	Method B
Guanosine	0.9932	0.9956
Inosine	0.9936	0.9959
Hypoxanthine	0.9917	0.9943
Uridine	0.9906	0.9936
Adenine	0.9913	0.9922
Adenosine	0.9932	0.9942
Uracil	0.9904	0.9954
Cordycepin	0.9905	0.9951

Yet, both methods yield similar results for cordycepin. The reason why Method B performs better than Method A is most probably the higher reliability in using the intensity volume containing more relevant information extracted from the TLC spot compared to that using the peak area. It is because the most intense part of a TLC spot might not locate at the central region of a TLC spot where the 2D peak profile is chosen in most cases. Therefore, it is recommended to apply Method B for TLC quantitative analysis as demonstrated in this work.

Chemical analyses of different sources of *Cordyceps sinensis* samples The nucleoside contents of eleven *Cordyceps sinensis* samples were studied and determined by both Method A and Method B. As their calibration curves are available, the concentration and the amount of each nucleoside within the sample can be easily determined. In Table 5 the quantitative results of all samples under study are summarized. It can be seen that almost all the samples contain uridine and adenosine. This explains why adenosine is usually regarded as

Table 4 Concentrations and deviations (dev)[a] of eight nucleoside standards determined by Methods A and B

Nucleoside	Method A			Method B		
	Peak area	Concentration/ (mg · L^{-1})	Dev/%	Intensity volume	Concentration/ (mg · L^{-1})	Dev/%
Guanosine	2105.37	480.12	3.98	14.77	492.96	1.41
Inosine	1633.83	473.69	5.26	13.95	468.60	6.28
Hypoxanthine	3581.41	544.09	8.82	23.69	472.16	5.57
Uridine	2003.35	544.61	8.92	15.96	514.19	2.84
Adenine	5332.97	547.06	9.41	33.23	537.84	7.57
Adenosine	2095.64	538.31	7.66	14.99	487.81	2.44
Uracil	3488.35	547.03	9.40	21.92	533.37	6.67
Cordycepin	3045.25	515.75	3.15	18.68	514.23	2.85

[a] The percentage deviation of the nucleoside standards was calculated using the expression (calculated concentration−applied concentration/applied concentration)×100%.

Table 5 Amounts of nucleosides (mg/g) ($\times 10^{-2}$) of the *Cordyceps sinensis* samples as determined by Methods A and B[a]

Sample	Country	Guanosine	Inosine	Hypoxanthine	Uridine	Adenine	Adenosine	Uracil	Cordycepin
1	China	11.98 (12.31)	4.89 (5.47)	—	64.71 (65.13)	—	78.22 (78.51)	6.63 (7.24)	—
2		5.33 (5.52)	9.79 (10.40)	—	97.27 (97.50)	—	95.58 (96.33)	8.44 (9.22)	—
3[b]		—	—	—	—	—	—	—	—
4	Korea	—	—	—	—	—	—	—	—
5		—	—	—	—	—	—	—	—
6		—	—	—	9.11 (9.21)	0.93 (1.22)	3.09 (3.37)	—	—
7		2.27 (2.63)	1.39 (1.92)	—	34.18 (34.52)	—	37.71 (37.97)	—	—
8	China & Japan cooperation	—	—	—	3.41 (3.79)	—	4.38 (4.79)	3.24 (3.96)	23.62 (24.01)
9		—	—	—	27.43 (27.82)	1.36 (1.66)	27.95 (28.45)	29.03 (29.24)	36.98 (37.08)
10[b]		—	—	—	1.44 (1.53)	0.10 (0.23)	0.68 (0.95)	0.07 (0.11)	2.88 (3.12)
11[b]		—	—	—	0.23 (0.34)	0.59 (0.73)	1.07 (1.26)	1.25 (1.54)	5.14 (5.50)

[a] The quantity within the parenthesis denotes the amount as determined by Method B. [b] The amount is represented in mg/mL for the liquid oral sample.

the marker component of *Cordyceps sinensis*, and uridine is also common in the *Cordyceps* samples. Since the contents of hypoxanthine are usually very low as reported,[18] no hypoxanthine can be found in all the samples studied. Besides, Table 6 shows the precision and recovery results for extraction of the standard mixture and Sample 9. Three extractions were made and Method B was employed for quantitative analysis in this measurement. The precision of the extraction method gave a mean R.S.D. lower than 3% and mean value for recovery was 98.02%.

Table 6 Precision and recovery measurements obtained for eight nucleoside standards and Sample 9 ($n=3$)[a]

Nucleoside	Relative standard deviation/%	Recovery/%
Guanosine	2.34	98.84
Inosine	1.86	97.63
Hypoxanthine	2.64	98.06
Uridine	2.75	96.95
Adenine	1.60	97.96
Adenosine	2.23	97.88
Uracil	2.49	98.77
Cordycepin	2.32	98.09
Mean value	**2.28**	**98.02**

[a] Method B was employed for the quantitative analysis.

By comparing the content of *Cordyceps sinensis* samples from different sources (Table 5), it can be found that the three products from China except Sample 3 show no cordycepin but contain the highest amounts of uridine and adenosine. It is obvious that the liquid oral samples such as Samples 3, 10 and 11 contain lower amounts or nonucleosides present. Moreover, among all samples, only guanosine and inosine are present in the two Chinese and one Korean products in lower quantity. For the Korean samples, the nucleosides amount is low except those in Sample 7. The most valuable component cordycepin as described previously are found in all the four samples of the China and Japan joint products and the amounts are relatively high in Samples 8 and 9. Therefore, it is concluded that these four samples should have better therapeutic effects than the others.

Conclusions

The two-step gradient elution TLC method proposed in this work has been applied successfully to separate and analyze nucleosides of *Cordyceps sinensis*. The eight nucleosides of uracil, uridine, hypoxanthine, inosine, guanosine, adenine, adenosine and cordycepin are well separated. With the help of the CAMAG and TLCQA-UV methods, it is easy to get useful qualitative and quantitative information from the developed TLC plate. As can be seen from the quantitative results reported, the four samples of *Cordyceps sinensis* from the joint products of China and Japan cooperation have

more valuable medical effect compared to others as they contained the most effective component—cordycepin. Finally, this study has showed the advantages in applying the proposed chromatographic elution system combined with effective scanning densitometry systems for quantitative analysis of the herbal medicine.

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