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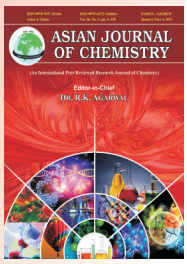

Simultaneous Determination of 13 Nucleosides and Nucleobases in *Ganoderma lucidum* and Related Species by HPLC-DAD

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A simple, sensitive and reliable HPLC-DAD method was performed for the simultaneous determination of 13 nucleosides and nucleobases including cytosine, uracil, cytidine, hypoxanthine, uridine, thymine, 2′-deoxyuridine, adenine, inosine, guanosine, thymidine, adenosine and 2′-deoxyadenosine in *Ganoderma lucidum* and its related species. The analysis was conducted on a phenomenex C_{18} column (250 mm × 4.6 mm i.d., 5 mm) eluting with a gradient of acetonitrile in water at a flow rate of 0.6 mL/min and the detection wavelength was set as 260 nm. The optimized method attained good linear relation ($r^2 \geq 0.9996$ for 13 analytes), satisfactory precision (RSD < 1.52 %) and good recovery (from 98.10 to 101.27 %). The validated method was successfully applied to the simultaneous determination of 13 analytes in 42 samples from *Ganoderma lucidum* and its related species collected from different areas, which could be beneficial to control their quality.

Keywords: *Ganoderma lucidum*, HPLC-DAD, Nucleobase, Nucleoside, Quality control.

INTRODUCTION

The *Ganoderma*, one of the prevalent Chinese medicinal mushrooms, is a multiple species colony of genus *Ganoderma* belonging to the polyporeceae[1] widely distributed in China. The two species have officially been adopted in Chinese Pharmacopoeia under the same crude drug name Lingzhi constituting *Ganoderma lucidum* (leyss. ex Fr.) Karst and *G. sinense* Zhao, Xu et Zhang[2]. Lingzhi has been recognized by medical professions for the treatment of debility and weakness, insomnia, hepatitis, cardiovascular diseases and cancer[3-4], *etc*. Up to now, an abundant variety of chemical component constituents have been authenticated in Lingzhi including triterpenes, polysaccharides, nucleosides, nucleobases, steroids, fatty acids, alkaloids, proteins, amino acids and inor ganic elements[5].

Chemical studies demonstrated that numerous nucleosides and nucleobases were present in the aqueous extract of Lingzhi[6]. Nucleosides and nucleobases were reported to play the certain roles in the regulation and modulation of diverse physiological processes in body through purinergic and/or pyrimidine receptors[7-8]. Recent pharmacological investigations revealed that water extract of *Ganoderma* exert antitumor and immunomodulatory activities[9-10]. Furthermore, adenosine, which is present in *G. lucidum*, was reported to have a profound activity against platelet aggregation[11]. Therefore, nucleosides and nucleobases could be major active components other than triterpenes and polysaccharides in *Ganoderma*.

Although several studies on the quantitative determination of nucleosides and nucleobases in Lingzhi have been reported[6,12-13], they were either time-consuming in preparing samples or unable to detect many compounds. In this study, a sensitive and reliable HPLC-DAD method was developed to analyze 13 nucleosides and nucleobases in the 42 samples of *G. lucidum* and its related species that were collected from 11 areas in China.

EXPERIMENTAL

A total of 42 samples from *G. lucidum* and its related species including *G. duroppora* Lloyd, *G. sinense* and a cultured variety of *G. lucidum* were collected from Sichuan, Yunnan, Anhui, Shandong, Hebei, Hubei, Guizhou, Guangxi, Guangdong, Tibet provinces and Hong Kong. All samples were identified by Professor Qing Songyun according to the morphological and microscopic characteristics. The collection details are given in Table-4.

Cytosine, uracil, cytidine, hypoxanthine, uridine, thymine, 2′-deoxyuridine, adenine, inosine, guanosine, thymidine, adenosine and 2′-deoxyadenosine (Fig. 1) were purchased from Sigma (St. louis, MO, USA). HPLC grade acetonitrile was obtained from Fisher Chemicals (Loughborough, UK). Deionized water was prepared using a PURELAB Option-R system (ELGA, UK).

Standard solution preparation: Standard stock solutions were prepared in water: cytosine, 18 μg/mL; uracil, 100 μg/mL; cytidine, 30 μg/mL; hypoxanthine, 47 μg/mL; uridine, 57 μg/mL; thymine, 35 μg/mL; 2′-deoxyuridine, 76 μg/mL; adenine, 17 μg/mL; inosine, 26 μg/mL; guanosine, 25 μg/mL; thymine, 25 μg/mL; adenosine, 33 μg/mL; 2′-deoxyadenosine, 40 μg/mL. All the solutions were filtered with 0.45 μm membrane filters and stored at 4 °C.

Sample preparation: 1 g of the fruiting body powder was accurately weighed and extracted with 20 mL of water in an ultrasonic water bath for 45 min. The extract was centrifuged at 3000 × g for 5 min. The supernatant was sequentially filtered through a 0.45 μm membrane filter. Then 20 μL of each sample solution was injected for HPLC analysis.

HPLC condition: The Agilent HPLC system was equipped with a G1322A degasser, a G1310A quaternary pump, a G1329A autosampler, a G1316A column temperature controller, a G1315B DAD and linked to an Agilent ChemStation running ChemStation software. Chromatographic separations were achieved on a Phenomenex C_{18} column (250 mm × 4.6 mm i.d., 5 mm). The mobile phases consist of acetonitrile (A) and water (B) with a gradient program as follows: 0-5 min, isocratic 2 % A; 5-17 min, linear gradient 2-3 % A; 17-27 min, linear gradient 3-6 % A; 27-35 min, linear gradient 6-9 % A; 35-45 min, linear gradient 9-20 % A. A pre-equilibration period of 15 min was used between individual runs. The flow rate was held constant at 0.6 mL/min. The detection wavelength was 260 nm and the column temperature was kept at 25 °C.

RESULTS AND DISCUSSION

Optimization of solvent extraction method: In order to obtain quantitative extraction of nucleosides and nucleobases from samples, some variables in the extraction were examined, such as solvent, extract method and extraction time. Considering target analytes with high polarity, different levels of aqueous methanol were used to investigate the effect of solvent on the extraction of tested constituents from samples. The results indicated that pure aqueous had the highest extraction yields of the tested constituents. To find the optimal condition for extraction method, ultrasonic extraction and refluxing were selected. The results testified that ultrasonic extraction is more efficient than refluxing extraction in extracting target elements. Furthermore, different ultrasonic time (30, 45 and 60 min) was tested, as a result, the best extraction time was selected as 45 min. Therefore, it was found that 45 min of ultrasonication with water was sufficient and efficient to extract samples.

Optimization of chromatographic conditions: According to the absorption maxima of 13 nucleosides and nucleobases on the UV spectra with 3-D chromatograms of HPLC-DAD, the detection wavelength was performed at 260 nm. Several columns such as Agilent XDB-C_{18}, Agilent Zorbax SB-AQ, Kromasil C_{18} and Phenomenex C_{18} were tested and the results justified that a Phenomenex C_{18} column could retain cytosine and separate uridine and adenosine better than the other columns tested. The different mobile phases consisting of acetonitrile-water and methanol-water were compared by different gradient elution modes. The results manifested that acetonitrile-water could attain better resolutions. The different flow rates (1 mL/min, 0.8 mL/min and 0.6 mL/min) were investigated and finally the flow rate 0.6 mL/min could separate uridine. Fig. 2 shows the typical HPLC separation of 13 nucleosides and nucleobases standards as well as four different *Ganoderma* species obtained at 260 nm under the optimized chromatographic condition. Authentication of tested analytes was accomplished by comparison of their retention times and their UV spectra with those obtained on injecting standards under the same condition or by spiking the samples with stock standard solutions.

Calibration curves, LODs and LOQs: The aqueous stock solution composing 13 analytes were prepared and diluted with water to appropriate concentrations for the construction

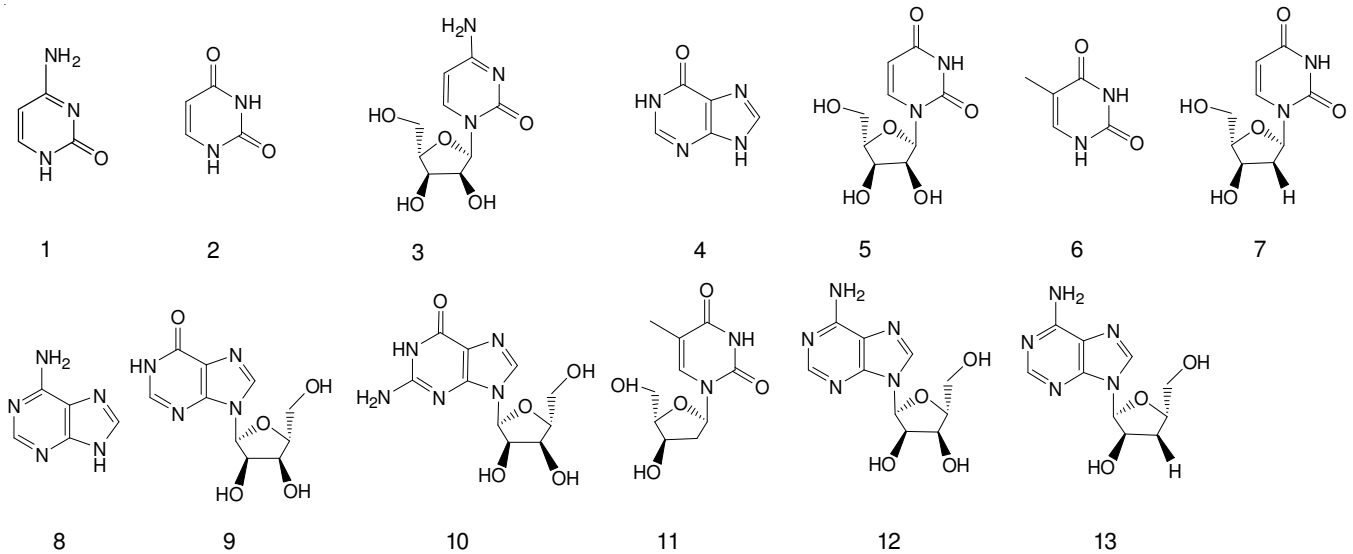

Fig. 1. Chemical structures of the nucleosides and nucleobases. 1,cytosine; 2, uracil; 3, cytidine; 4, hypoxanthine; 5, uridine; 6, thymine; 7, 2′-deoxyuridine; 8, adenine; 9, inosine; 10, guanosine; 11, thymidine; 12, adenosine; 13, 2′-deoxyadenosine

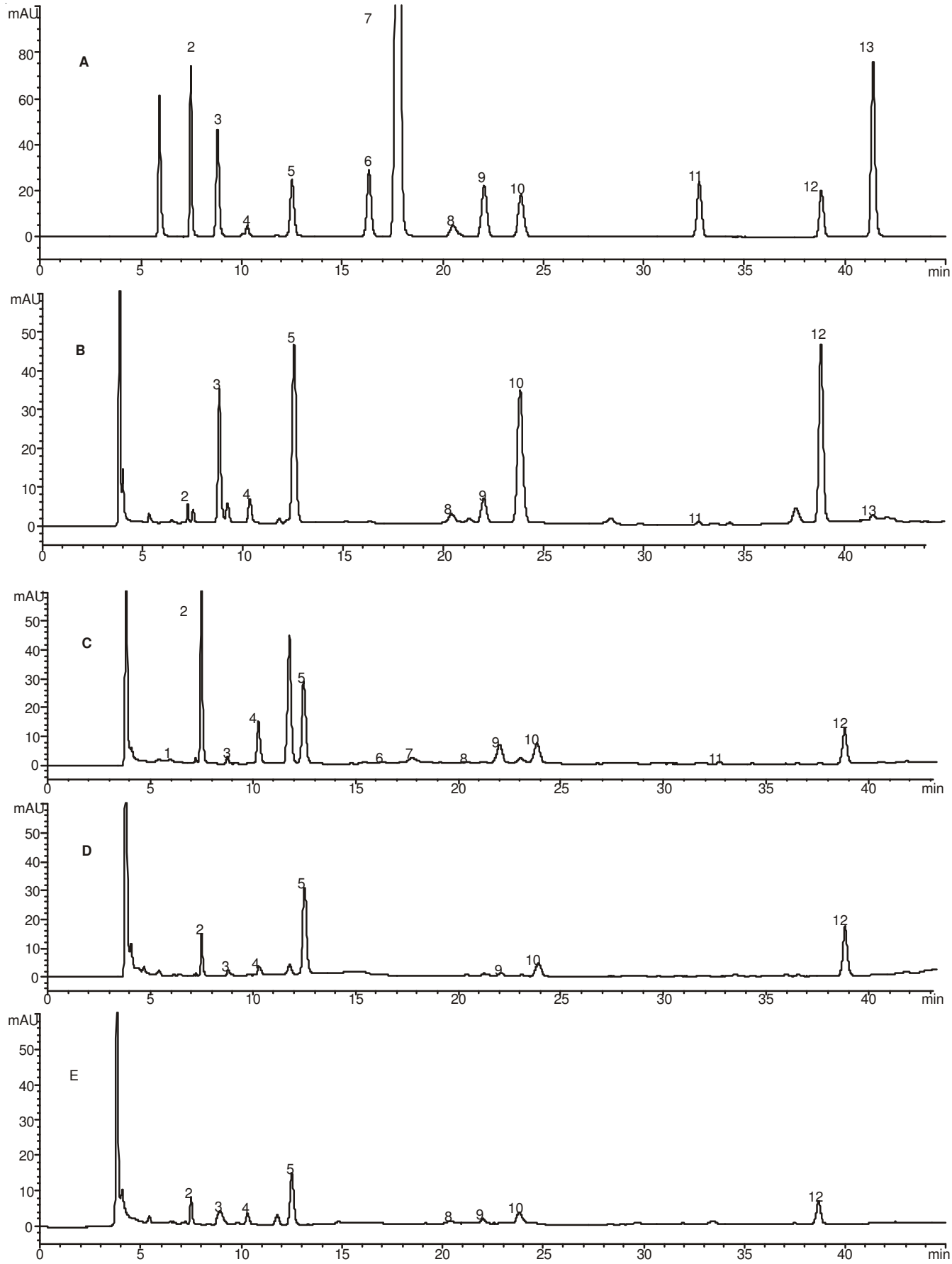

Fig. 2. Representative HPLC chromatograms of mixed standards and four *Ganoderma* species. The denotations from 1 to 13 are the corresponding chemicals as listed in Fig. 1. (A) Mixed nucleosides and nucleobases standards, (B) *G. lucidum*, (C) *G. sinense*, (D) *G. duroppora,* (E) Cultured variety of *G. lucidum*

of calibration curves. At least six concentrations of the 13 analytes solutions were injected in triplicate and then the calibration curves were established by plotting the peak areas against the concentration of each analyte. All target constituents showed good linearity ($r^2 = 0.9996$) (Table-1). The stock solutions containing 13 reference compounds were further diluted with water to appropriate concentrations for determining the LODs and LOQs. The limits of LOD and LOQ under the optimized chromatographic conditions were determined at a S/N of 3 and 10, respectively. The result showed that the LODs and the LOQs for the 13 nucleosides and nucleobases were less than 0.075 and 0.242 μg/mL, respectively (Table-1).

Precision, repeatability and accuracy: Intra- and inter-day variations were employed to mensurate the precision of the method. The intra-day precision was determined on the mixed standards solution for six replicates during a single day

TABLE-1
CALIBRATION CURVES, LOD AND LOQ FOR THE NUCLEOSIDE AND NUCLEOBASES STANDARDS

Analytes[a]	Linear regression data			LOD (μg/mL)	LOQ (μg/mL)
	Regression equation	Linear range (μg/mL)	r^2		
1	y = 50798x-4.6148	0.25-143	1.0000	0.021	0.063
2	y = 71746x-22.5640	0.25-100	0.9999	0.042	0.135
3	y = 28489x-2.6890	0.50-250	1.0000	0.075	0.242
4	y = 64928x-13.7390	0.07-25	0.9996	0.011	0.038
5	y = 34180x-11.3920	0.25-100	0.9999	0.041	0.142
6	y = 60018x-6.8973	0.20-54	1.0000	0.039	0.136
7	y = 41844x-8.7466	0.25-77	0.9999	0.067	0.211
8	y = 93551x-15.371	0.35-19	0.9996	0.020	0.069
9	y = 27365x-2.0231	0.50-236	1.0000	0.041	0.138
10	y = 18056x+9.6044	0.50-250	1.0000	0.037	0.115
11	y = 32421x+5.3993	0.25-80	1.0000	0.049	0.157
12	y = 55580x-9.9152	0.35-174	0.9999	0.052	0.171
13	y = 54445x-3.7556	0.50-69	1.0000	0.038	0.125

[a]The notation for analytes (nucleosides and nucleobases) refers to Fig. 1.

TABLE-2
PRECISION AND REPEATABILITY OF THE NUCLEOSIDE AND NUCLEOBASES STANDARDS

Analytes[a]	Precision				Repeatability (n = 6)	
	Intra-day (n = 6)		Inter-day (n = 6)		Mean (mg/kg)	RSD (%)
	Mean (μg/mL)	RSD[b] (%)	Mean (μg/mL)	RSD (%)		
1	35.53	0.14	35.51	0.20	10.57	3.74
2	25.30	0.11	25.35	0.21	108.4	3.29
3	62.47	0.15	62.37	0.13	54.73	2.95
4	5.83	1.39	5.74	1.35	50.97	3.26
5	25.22	0.18	25.14	0.61	95.91	4.03
6	26.89	0.05	26.86	0.22	9.05	3.01
7	38.42	0.05	38.06	1.23	2.34	2.23
8	4.79	1.46	4.82	1.52	14.70	3.73
9	59.05	0.12	59.09	0.21	55.96	4.34
10	62.82	0.10	62.58	0.23	202.05	4.09
11	39.91	0.08	38.91	0.55	5.15	4.08
12	21.86	0.09	21.79	0.55	48.50	2.58
13	69.10	0.15	69.01	0.13	7.25	4.43

[a]Notation for analyte (nucleosides and nucleobases) refers to Fig. 1, [b]RSD (%) = (SD/mean) × 100 %

TABLE-3
RECOVERIES OF THE NUCLEOSIDE AND NUCLEOBASES STANDARDS

Analytes[a]	Original (μg)	Spiked (μg)	Found (μg)	Recovery[b] (%)	RSD[c] (%)
1	9.90	10.00	19.80	98.96	2.12
2	104.88	100.00	204.50	99.62	3.15
3	54.40	55.00	108.70	98.73	3.45
4	50.45	50.00	99.50	98.10	2.16
5	91.13	90.00	181.70	100.63	2.83
6	2.31	2.50	4.79	98.98	3.21
7	8.87	10.00	19.10	101.27	3.24
8	14.05	15.00	29.10	100.32	2.56
9	45.48	50.00	105.20	99.43	2.48
10	190.68	200.00	389.70	98.88	1.89
11	4.85	5.00	97.79	100.85	2.54
12	46.68	50.00	97.10	98.89	2.35
13	7.41	7.00	14.40	99.82	2.67

[a]Notation for analytes (nucleosides and nucleobases) refers to Fig. 1, [b]Recovery (%) = (amount found – original amount)/amount spiked × 100 %, [c]RSD (%) = (SD/mean) × 100 %

TABLE-4
CONTENTS IN mg/100 g OF THE 13 NUCLEOSIDES AND NUCLEOBASES IN FOUR DIFFERENT *Ganoderma* SPECIES

No	Species	Sources [a]	Collection time	1 [b]	2	3	4	5	6	7	8	9	10	11	12	13	Total
1	*G. lucidum*	Emen, Sichuan	2008.12	tr [c]	1.70	0.86	0.81	5.54	nd [d]	nd	0.43	0.59	2.97	tr	3.14	nd	16.04
2	*G. lucidum*	Hongya, Sichuan	2008.12	tr	0.44	1.48	1.30	2.99	nd	nd	0.31	0.27	4.14	tr	2.29	0.17	13.39
3	*G. lucidum*	Heze, Shandong	2008.12	tr	0.51	2.20	0.39	6.30	tr	nd	1.23	tr	6.65	tr	1.86	nd	19.14
4	*G. lucidum*	Huoshan, Anhui	2008.12	tr	1.10	3.08	0.55	11.74	tr	tr	0.33	1.11	12.00	tr	9.66	tr	39.57
5	*G. lucidum*	Dabieshan, Anhui	2008.03	0.29	4.45	3.25	1.30	7.79	nd	tr	0.89	tr	14.03	tr	8.62	tr	40.62
6	*G. lucidum*	Dabieshan, Anhui	2008.03	0.23	3.34	5.74	1.58	5.73	tr	tr	tr	3.61	18.87	tr	12.33	0.21	51.64
7	*G. lucidum*	Shimian, Sichuan	2008.08	tr	5.63	16.55	3.43	55.39	tr	0.79	2.60	8.92	89.01	1.24	32.10	1.28	216.94
8	*G. lucidum*	Shennongjia, Hubei	2008.11	0.39	2.63	6.37	6.94	16.11	nd	tr	0.87	1.46	19.21	tr	11.35	tr	65.33
9	*G. lucidum*	Tiquan, Sishuan	2008.11	tr	0.45	7.75	0.73	12.62	nd	tr	0.40	1.93	22.54	0.09	12.35	0.54	59.40
10	*G. lucidum*	Lushan, Sichuan	2008.12	nd	0.83	1.26	0.85	4.19	tr	tr	0.50	0.66	3.88	tr	2.60	nd	14.77
11	*G. lucidum*	Mianyang, Sichuan	2008.12	tr	1.36	4.33	0.73	11.24	nd	nd	tr	2.63	12.84	tr	9.04	nd	42.17
12	*G. lucidum*	Hebei	2008.12	0.74	6.04	1.86	1.52	39.01	tr	tr	5.81	0.68	31.94	0.67	6.58	nd	94.85
13	*G. lucidum*	Hebei	2008.12	tr	0.62	11.84	1.33	18.59	nd	nd	0.74	4.00	5.42	tr	12.38	0.39	55.31
14	*G. lucidum*	Anhui	2009.05	1.18	14.40	0.85	5.30	5.34	0.52	1.18	0.69	5.25	18.49	0.15	1.18	0.17	54.70
15	*G. lucidum*	Hubei	2009.05	0.44	5.16	6.61	2.01	12.55	0.28	0.37	1.29	2.45	21.99	0.36	7.04	0.39	60.94
16	*G. lucidum*	Hongkong market	2009.06	nd	1.35	3.59	0.57	11.88	nd	nd	0.32	2.10	11.80	tr	8.01	nd	39.62
17	*G. lucidum*	Tibet	2009.08	0.34	7.53	4.63	2.82	9.46	nd	0.34	0.54	2.10	16.05	0.48	4.58	0.50	49.37
18	*G. sinense*	Sichuan	2008.12	tr	9.77	1.31	5.70	20.49	0.27	0.65	0.58	5.48	11.75	0.37	2.13	nd	58.50
19	*G. sinense*	Yunnan	2009.04	0.31	6.84	0.65	3.96	15.24	tr	tr	0.60	2.14	6.31	0.31	5.52	nd	41.88
20	*G. sinense*	Yunnan	2009.04	tr	4.39	1.27	2.44	18.73	0.30	0.57	1.03	7.11	11.84	0.32	3.55	nd	51.55
21	*G. sinense*	Yunnan	2009.04	0.31	2.44	0.97	0.95	17.91	tr	tr	0.65	4.24	6.05	0.02	5.11	nd	38.65
22	*G. sinense*	Guangdong	2009.08	tr	2.77	0.45	2.48	8.05	tr	tr	0.51	2.07	3.68	0.05	1.60	nd	21.66
23	*G. sinense*	Guizhou	2009.08	0.38	2.58	1.29	1.28	12.96	tr	tr	0.74	4.69	7.49	0.23	5.85	nd	37.49
24	*G. sinense*	Guangxi	2009.08	tr	2.51	0.57	1.41	7.53	0.22	0.43	0.43	1.92	4.54	tr	3.47	nd	23.03
25	*G. sinense*	Sichuan	2009.08	tr	7.76	0.86	2.79	11.53	0.27	0.48	0.45	4.86	8.09	0.21	3.43	nd	40.73
26	*G. duroppora*	Hebei	2008.12	nd	0.73	0.27	0.88	8.06	nd	nd	0.36	0.94	1.76	nd	2.36	nd	15.36
27	*G. duroppora*	Guizhou	2009.05	nd	1.04	1.79	0.92	5.85	nd	nd	0.49	0.71	5.74	nd	2.32	nd	18.86
28	*G. duroppora*	Guizhou	2009.05	tr	1.80	0.73	0.83	25.04	nd	nd	0.34	1.97	8.15	0.14	5.69	nd	44.69
29	*G. duroppora*	Guizhou	2009.05	tr	5.90	7.76	2.29	38.83	0.61	tr	0.71	4.80	31.56	tr	19.46	nd	111.92
30	*G. duroppora*	Yunnan	2009.05	tr	5.47	0.67	0.91	5.40	tr	nd	0.89	tr	2.44	tr	2.42	nd	18.20
31	*G. duroppora*	Guizhou	2009.05	tr	1.69	0.66	0.81	1.67	nd	nd	0.30	1.37	4.92	tr	6.62	nd	18.04
32	*G. duroppora*	Guangxi	2009.06	tr	6.22	15.72	8.25	49.16	tr	0.76	1.17	7.67	50.49	1.93	9.00	0.19	150.56
33	*G. duroppora*	Hongkong market	2009.06	nd	1.21	tr	1.12	1.85	nd	nd	0.61	0.73	1.39	tr	1.19	nd	8.10
34	Cultured variety of *G. lucidum*	Shandong	2008.12	tr	1.35	0.69	0.88	2.98	nd	nd	0.36	0.64	2.07	nd	2.84	nd	11.81
35	Cultured variety of *G. lucidum*	Guangdong	2009.8	tr	2.91	1.81	2.91	3.96	tr	nd	1.21	1.47	8.30	0.12	1.21	0.34	24.24
36	Cultured variety of *G. lucidum*	Guangdong	2009.8	nd	1.36	0.42	1.08	2.16	0.27	tr	0.41	0.72	2.43	tr	1.64	nd	10.49
37	Cultured variety of *G. lucidum*	Hongkong market	2010.6	tr	1.73	1.00	1.20	8.54	nd	nd	0.46	1.67	5.17	tr	2.58	nd	22.35
38	Cultured variety of *G. lucidum*	Anhui	2010.6	tr	1.06	0.55	0.80	6.21	0.25	nd	0.40	1.03	2.80	tr	1.88	nd	14.98
39	Cultured variety of *G. lucidum*	Shandong	2010.6	tr	0.82	0.41	0.66	5.37	tr	nd	0.33	1.16	1.87	tr	1.56	nd	12.18
40	Cultured variety of *G. lucidum*	Guizhou	2010.8	tr	0.82	0.09	0.60	5.35	tr	nd	0.33	1.15	1.86	0.08	1.60	nd	11.88
41	Cultured variety of *G. lucidum*	Guangxi	2010.8	tr	2.80	0.72	2.44	5.43	nd	nd	0.33	1.72	3.39	nd	3.00	nd	19.83
42	Cultured variety of *G. lucidum*	Yunnan	2010.8	tr	2.03	0.09	1.92	1.29	nd	nd	nd	1.27	1.67	nd	1.91	nd	10.18

[a]Samples having same sources were collected from different cultivated farms in the same regions, [b]The denotation from 1 to 13 are the corresponding chemicals as listed in Fig. 1, [c]tr: below the limit of quantification, [d]nd: not detected

and inter-day precision was examined in duplicates on three consecutive days. The result manifested that the relative standard deviation (RSD) of intra- and inter-day variations were both less than 1.52 % (Table-2). To test for the repeatability of the method, the same sample solution was repeatedly analyzed. The RSD was taken as a measure of precision. The results of the repeatability of the method were less than 4.43 % (Table-2). The recovery was tested by adding accurately amount of

individual standards into a certain amount of *G. lucidum* material. Six replicates were executed for the test. The mixture was extracted and analyzed as is described above. The results of the recoveries of target analytes ranged from 98.10 to 101.27% (Table-3).

Quantitative analysis of investigated compounds in *Ganoderma*: The developed HPLC-DAD method was applied to simultaneously quantify 13 nucleosides and nucleobases in 42 samples from *G. lucidum* and its related species that were collected from different regions in China. The results (Table-4) showed that total nucleosides and nucleobases contents ranged from 8.1 to 216.94 mg/100 g and the contents of total nucleoside and nucleobases showed distinct differences in the different *Ganoderma* species. Among the 13 analyzed nucleosides and nucleobases, inosine, guanosine and adenosine were determined as the main nucleosides, which accounted for more than 60 % of total nucleosides and nucleobases in *Ganoderma* species. The contents of guanosine accounted for 30 % of total nucleosides and nucleobases in *G. lucidum*, while the contents of inosine have 30 % of total nucleosides and nucleobases in other investigated *Ganoderma* species. The contents of total nucleoside and nucleobases in cultured variety of *G. lucidum* were much less than *G. lucidum*, except four samples from *G. lucidum* deviated out of the original contents. This could be accounted for variations of cultivated conditions, climate condition and environment.

Conclusion

A simple and accurate HPLC-DAD method was established for the simultaneous determination of 13 nucleosides and nucleobases in different *Ganoderma* species. The validation results showed that the method have good accuracy, repeatability and precision. Inosine, guanosine and adenosine were determined as the main nucleosides, which accounted for more than 60 % of total nucleosides and nucleobases in *Ganoderma* species. This study suggests that this method was suitable for the analysis of nucleosides and nucleobases in *Ganoderma* species and setting a minimum limit of the three nucleosides could be beneficial for evaluating the quality of *Ganoderma* species.

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