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Simultaneous Determination of 16 Nucleosides and Nucleobases by Hydrophilic Interaction Chromatography and Its Application to the Quality Evaluation of *Ganoderma*

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ABSTRACT: In order to develop a simple, efficient, and sensitive method for comprehensive analysis of the nucleosides and nucleobases in natural products, a zwitterionic hydrophilic interaction chromatography (ZIC-HILIC) method for the simultaneous determination of 16 nucleosides and nucleobases has been studied. A mechanistic study confirmed that ZIC-HILIC separation showed a mixed-mode effect of both hydrophilic and electrostatic interactions. This method was validated to be precise, accurate, and sensitive with overall precision (intra- and interday) less than 1.8% (RSD), and LOD and LOQ was in the range of 0.005−0.029 μg/mL and 0.018−0.096 μg/mL, respectively. With this method, the nucleosides and nucleobases in *Ganoderma* of different species (*G. atrum*, *G. lucidum*, and *G. sinense*) and origins were quantified. The results showed that the contents varied with the species and origins. With the aid of hierarchical cluster analysis (HCA), cultivated *Ganoderma* from different origins and species were successfully discriminated. It is for the first time that the content of nucleosides and nucleobases in *G. atrum* is reported and compared. Our data showed that HILIC had advantages as a useful and potential tool for the study of the bioactive components in *Ganoderma* as well as their quality control, and could therefore be used for the determination of the analytes in other natural products.

KEYWORDS: *HILIC, nucleosides, nucleobases, Ganoderma, quality evaluation*

INTRODUCTION

Ganoderma lucidum (Leyss.: Fr. Karst), a nutritious mushroom (also known as Reishi or LingZhi), has been used as a functional food and preventive medicines for promoting health and longevity in Asian countries for more than 2000 years and now has also become a popular dietary supplement in Western countries.[1−4] In order to meet the increasing market demand, nowadays *Ganoderma* of different cultivation methods, origins, and species such as *Ganoderma lucidum* (*G. lucidum*), *G. sinense*, and *G. atrum* etc. have flooded into the market. However, studies showed that the chemical constituents and some biological activities are quite different depending on the species, location, and cultivation methods.[5] Thus, in order to elucidate the differences between them and control the quality of *Ganoderma*, many scientists are devoted to developing the qualitative and quantitative methods for the active components in *Ganoderma*. However, many of them were focused on the ethanol soluble constituents such as triterpenoids in *Ganoderma*.[6] Little attention was paid to polar compounds. As the polar constituents, nucleosides and nucleobases have a lot of bioactive functions including antiplatelet aggregation, antiarrhythmic, and antiseizure effects.[7−9] Although nucleosides and nucleobases were selected as the quality control marker in the medicinal higher fungi *Cordyceps sinensis*, *Agrocybe aegerita*, *Termitomyces albuminosus*, and *Lentinus edodes*[10,11] for *Ganoderma*, little information concerning this was found. Gao et al. compared the distribution of six nucleosides and five nucleobases in the pilei and the stipes of *G. lucidum* and *G. sinense* with the method of HPLC-DAD.[12] Yang et al. also determined eight nucleosides and nucleobases in the species of *G. lucidum* and *G. sinense* with capillary electrophoresis−mass spectrometry (CE-MS).[13] However, in those previous studies, a limited number of nucleosides and nucleobases were determined, and *Ganoderma* of other species (such as *G.aturm*) and of different origins were not studied. Because of the remarkable activities of nucleosides and nucleobases and their inadequate research in the *Ganoderma* genus, it seems interesting to qualitatively and quantitatively compare the profiles of nucleosides and nucleobases between *Ganoderma* of different species and origins.

Until now, many studies have been focused on the development of different techniques to separate, characterize and/or quantify these compounds in biological fluids and Chinese medicines, including gas chromatography,[14,15] capillary zone electrophoresis,[16,17] CE-MS,[18,19] and capillary electrochromatography.[20] But these methods have some limitation such as intensive working labor, long analysis time, low sensitivity, and/or limit of analytes.[21,22] However, the versatility, short analysis time, and high resolution of liquid chromatography (LC) have made it the most widely used technique. Thus, reversed-phase high performance liquid chromatography (RP-HPLC) with[23,24] and without ion-pair,[25−27] liquid chromatography−mass spectrometry (LC−

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MS),[28,29] and ultraperformance liquid chromatography (UPLC)[21,30,31] have been the method of choice for the analysis of these compounds. Although these techniques are powerful, hydrophilic interaction chromatography (HILIC) could be an alternative approach for the separation of such compounds. It has been reported that HILIC can retain highly polar compounds that are not retained by RP chromatography with additional benefits, including superior solubility of the polar analytes in the mobile phase with compositions comparable to those employed in RP-LC, better selectivity of the analytes compared to RP chromatography, and enhanced sensitivity for MS detection.[32,33] Recently, several studies confirmed that HILIC was an ideal tool for the analysis of nucleobases and nucleosides. Mora et al. found that HILIC constitutes a valid and reliable method of the analysis of ATP and its metabolites and would be an interesting alternative to other methodologies.[34] Chen et al. reported that the HILIC method was superior to RP-HPLC for the separation of nucleosides and nucleobases in the samples of Geosaurus and Leech, escepially for guanine, hypoxanthine, 2′-deoxyuridine, uridine. and thymidine, which cannot be easily separated by other analytical methods.[35] However, in this method with a TSKgel Amide-80 column only 14 nucleosides and nucleobases with a separation time as long as 110 min were observed. Marrubini et al. describe the behavior of 12 pyrimidines and purines on the ZIC-HILIC and TSKgel Amide-80 column. respectively. and found that only the ZIC-HILIC column provided full resolution of the selected compounds in about 60 min.[36] Most recently, Zhao et al. also developed a HILIC-TOF/MS method for the separation of 16 nucleosides and nucleobases;[37] however, in the HILIC-DAD chromatograms, adenine and uridine/2′-deoxyguanosine and cytidine were coeluted, and the instrumentation was too expensive to be used widely. Thus, in those previous HILIC methods, only a limited number of nucleosides and nucleobases were determined with a long analysis time[35,36] and expensive instrumentation.[37] The establishment of a simple, efficient, and sensitive method is thus urgently required for the identification and quantification of nucleosides and nucleobases in TCMs.

In light of this, as the first attempt, we developed a new HILIC method for the fast simultaneous analysis of 16 nucleosides and nucleobases in *Ganoderma* of different species and origins. Besides, in order to further understand the HILIC mechanism of retention and describe the behavior of 16 nucleosides and nucleobases on a commercial column, the retention of the analytes was systematically investigated by varying the chromatographic conditions and optimized systematically. On the basis of the optimum HILIC analytical conditions, the concentrations of these analytes in natural and cultured *Ganoderma* from different origins were compared with the aid of HCA, which aims to provide an alternative, feasible approach for the quality assessment of *Ganoderma* in addition to the methods using polysaccharides and triterpenoids as the markers.[38,39]

MATERIALS AND METHODS

Reagents and Standard Solutions. HPLC grade acetonitrile (ACN) was purchased from Merck (Darmstadt, Germany). Ammonium acetate, ammonium formate, ammonium bicarbonate, ammonium trifluoroacetate, and ammonia were of analytical grade. Water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA).

Adenine (Ade), adenosine (Ado), cytosine (Cyt), cytidine (Cyd), uracil, uridine (Urd), guanine, guanosine (Gua), inosine (Ino), thymine, thymidine, 2′-deoxyuridine, 2′-deoxyadenosine, 2′-deoxycytidine, 2′-deoxyganosine, and 2′-deoxyinosine were purchased from Sigma (St. Louis, MO, USA). ACN−water (1:1) was used as the solvent for stock solution preparation, and the concentrations for each standard were 0.2 mg/mL. All of the standard solutions were filtered through a 0.45 μm membrane filter and stored at 4 °C in brown glass bottles. Before injection, a certain volume of stock solution was transferred to a 10 mL volumetric flask and diluted with the solvent to the desired concentration.

Natural and cultivated Lingzhi samples, belonging to *G. lucidum*, *G. aturm*, and *G. sinense*, were collected from different locations in China. The samples were collected in the period of May 2007−May 2008.

Table 1. *Ganoderma* Test Samples

species	commercial name (China)	origin (China)	sample no.
G. atrum	Black Lingzhi	Ganzhou, Jiangxi	1−4
G. lucidum	Korean Lingzhi	Jiaxiang, Shandong	5,6
	Taishan Lingzhi	Wangdangpu, Shandong	7
	Lingzhi	Longquan, Zhejiang	8−9
	G. lucidum No. 6	Huangshan, Anhui	10,11
	Lingzhi G8	Jinzhai, Anhui	12
	Golden Lingzhi	Yinshan,Hubei	13,14
G. sinense[a]	Zizhi	Lijiang,Yunnan	15−17
G. lucidum[a]	Lingzhi	Lijiang, Yunnan	18,19
G. sinense	Zizhi	Wuyishan, Fujian	20

[a]Natural samples.

Their origins are listed in Table 1. Morphological species identification was carried out by Dr. Zhihong Fu (Jiangxi University of Traditional Chinese Medicine, China). The collected specimens were dried with a vacuum dryer (Senxin Equipment Co., Ltd., Shanghai, China) at 60 °C for 12 h and cut into small pieces.

Sample Preparation. *Ganoderma* materials (1 g) were mixed with 20 mL of bidistilled water solution, and then ultrasonic extraction was performed at room temperature for 10 min. After centrifugation at 4000*g* for 30 min, the supernatant was filtered through a 0.20 μm-membrane filter prior to injection into HPLC.

HILIC Analysis of Nucleosides and Nucleobases. Analysis was performed on an Agilent Series 1200 liquid chromatograph, equipped with a vacuum degasser, a quaternary pump, an autosampler, and a diode-array detector (DAD), connected to an Agilent ChemStation running ChemStation software. A ZIC HILIC column (3.5 μm, 100·4.6 mm) (SeQuant, Umea, Sweden) and a guard column (20·2.1 mm) were used for separation. The mobile phase consisted of solvent A (ACN modified with 3 mM ammonium acetate) and solvent B (pH 6; 15 mM ammonium acetate in water). The following linear gradient procedure was used: 0−18 min, 3−5% B; 18−19 min, 5−10% B; and 19−30 min, 10−20% B. The flow rate was 1.0 mL/min, and finally, the column was washed with 50% B for 10 min. The column was equilibrated for 15 min under initial conditions before each injection. The column compartment temperature was kept at 25 °C, and the injection volume was 2 μL. Separations were monitored using a diode array detector at a wavelength of 254 nm. All injections were repeated three times. The identifications of nucleosides and nucleobases were achieved by comparing their retention time and spectrum against known standards. The external standard method was used for the determinations of nucleosides and nucleobases.

Method Validation. Water stock solutions containing reference compounds were prepared and diluted to appropriate concentrations for calibration. Six concentrations of the solution were analyzed in triplicate, and then the calibration curves were constructed by plotting the corresponding peak areas versus the concentration of each analyte. The limits of detection (LOD) and quantification (LOQ) under the

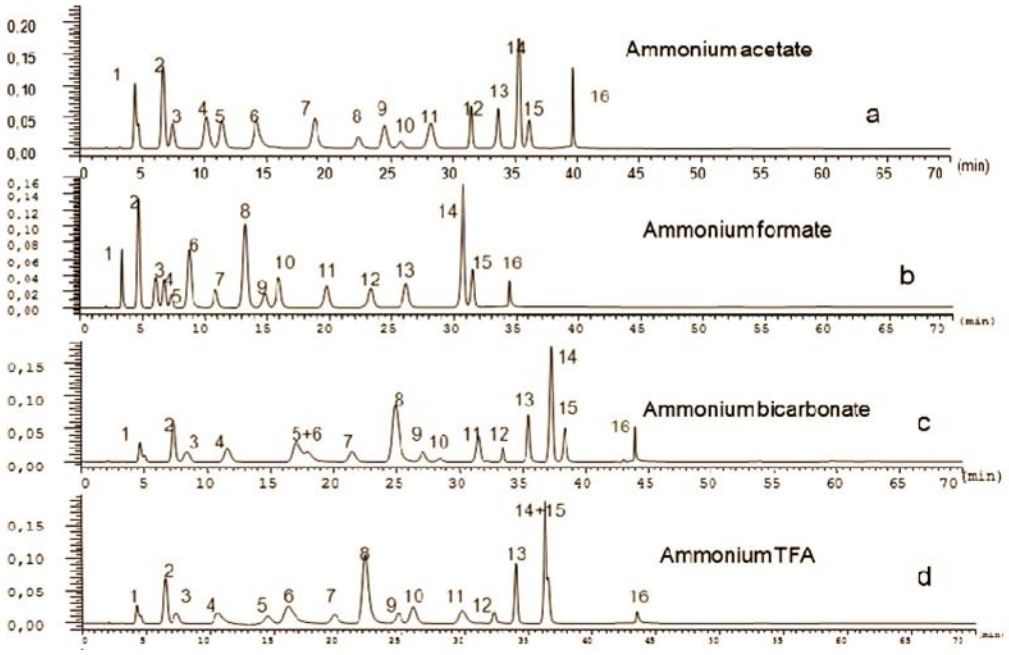

Figure 1. Effects of different salts on the separation of standard mixtures on ZIC-HILIC. Types of salts were as indicated. 1, thymine; 2, uracil; 3, thymidine; 4, 2′-deoxyadenosine; 5, 2′-deoxyuridine; 6, adenine; 7, adenosine; 8, uridine; 9, guanine; 10, 2′-deoxyinosine; 11, cytosine; 12, 2′-deoxycytidine; 13, inosine; 14, 2′-deoxyguanosine; 15, cytidine; 16, guanosine.

present chromatographic conditions were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively.

Known volumes of standard solutions were added to a *G. atrum* sample, and sample stability was tested by periodically analyzing this spiked sample solution kept at room temperature for various periods (0, 1, 2, 4, 8, 12, and 18 h). The RSDs were used as the indicator of stability.

Intra- and interday variations were chosen to determine the precision of the developed method. For the intraday variability test, the mixed standards solution was analyzed for six replicates within 1 day, while for the interday variability test, the solution was examined in duplicate for consecutive 3 days. Variations of the peak area were taken as the measures of precision and expressed as percentage relative standard deviations (RSD).

For recovery studies, known volumes of standard solutions were added to *G. atrum* sample at three levels. The spiked samples were extracted and analyzed using the method described above.

Hierarchical Clustering Analysis. Hierarchical clustering analysis (HCA) of samples was performed using Number Cruncher Statistical System software, version 07.1.16 (NCSS, Kaysville, UT, USA). The group average technique (nonweighted pair group) was applied for data agglomeration, and Euclidean distance measure was used to establish clusters.

RESULTS AND DISCUSSION

Effects of Mobile Phase on the Separation. During method development here, a higher proportion of organic solvent in the mobile phase was found to increase the retention of the analytes. With ACN% lower than 90%, regardless of the other method parameters (salt concentration, temperature, and pH), the analytes could not be separated and coeluted in early elution time; while when its concentration amounted to 95%, the retention times of analytes were greatly prolonged but too long for guanosine (compound 16) as it was retained over 30 min. Thus, higher ACN amounts were used in the first minutes of the chromatogram, the concentration being decreased later to shorten the chromatogram and to improve the peak shape.

Effect of Salt Type and Concentration on the Separation. Several ammonium salts, namely, acetate, formate, bicarbonate, and trifluoroacetate were investigated to determine the most adequate buffer regarding its solubility in organic solvents and its interaction with the studied compounds. The results in Figure 1 showed, ammonium acetate was the salt of choice because it provides the best results in selectivity and reproducibility, presents excellent solubility even at high concentrations of organic solvents, and is highly volatile, making it suitable for eventual further mass spectrometry analysis.

Retention and peak shape were influenced by the presence of ammonium acetate at different concentrations. An increase of ammonium acetate concentration from 0 to 50 mM in solution B was evidenced by a reduction of retention and deterioration in peak shape (Figure 2). Further increase in the salt concentration was not possible due to solubility limitation in

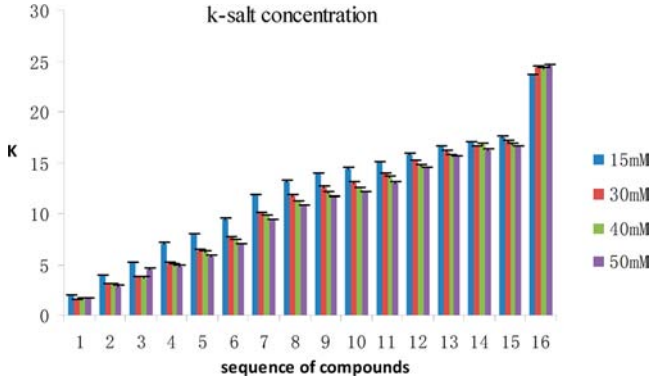

Figure 2. Influence of the concentration of ammonium acetate in mobile phase on the capacity factor (k) of 16 standard compounds on the ZIC-HILIC column. The data were presented as average of three replicates; error bars represent the standard deviations.

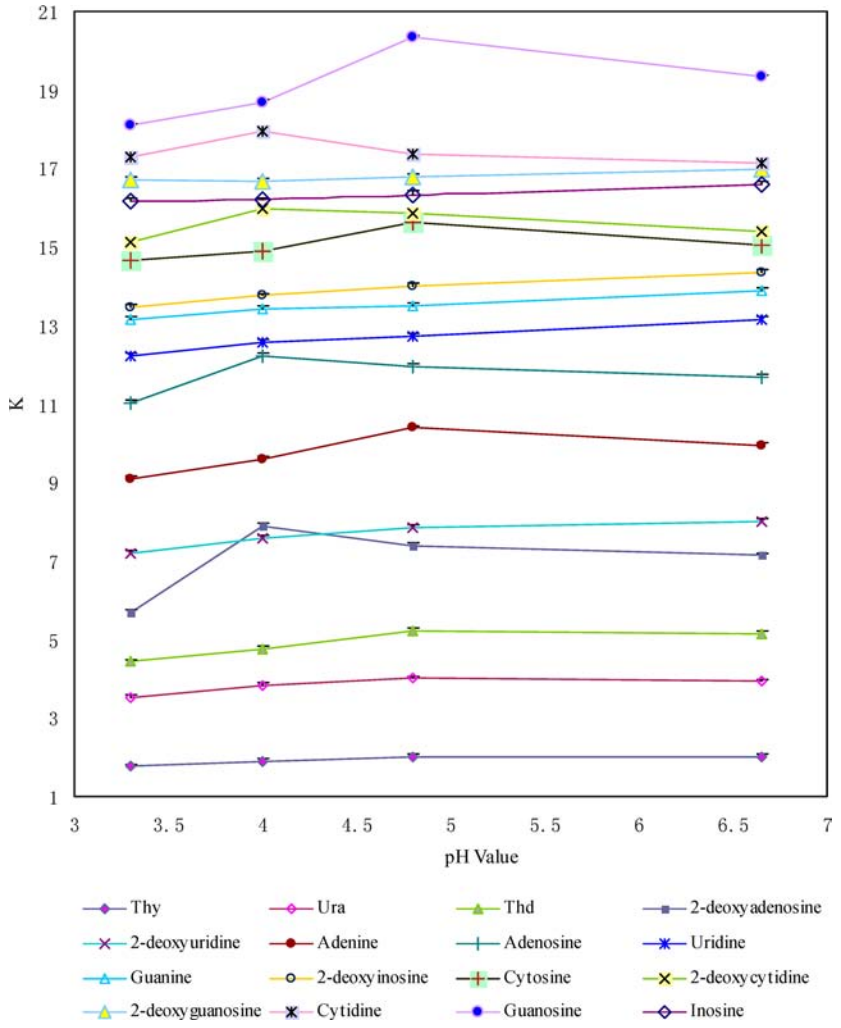

Figure 3. Influence of the pH of mobile phase on the capacity factor (k) of 16 standard compounds on the ZIC-HILIC column. The data are presented as the average of three replicates; error bars represent the standard deviations.

the mobile phase. This was different with the prevailing characteristics of a hydrophilic partition mechanism, according to which the increase in salt concentration usually leads to an increase in retention.

However, this was in agreement with the results of some previous studies.[36,40] On the basis of those results, we speculated that the strong retention on the column when no salt is present in the eluent comes from the complex interactions between the analytes, the mobile phase, and the sorbent of the aqueous layer (which results in hydrophilic partition), and the analytes and the stationary phase (which led to retention through adsorption). When salt is added to the mobile phase, a decrease in retention occurs. This maybe due to the fact that ion exchange sites and dipole−dipole interactions are shielded by the presence of solvated ions supplied by the salt.

However, when the concentration was lower than 15 mM, the reproducibility of the retention time and peak area could not be guaranteed owing to an insufficient buffering effect of the mobile phase. Thus, an initial concentration of 15 mM ammonium acetate in solution B (overall salt concentration in the mobile phase is 3.36 mM) was selected, providing an optimum analysis separation.

Effect of pH on Separation. In general, the pH value of the mobile phase has a significant impact on retention and selectivity in hydrophilic chromatography by influencing solute ionization. The nature of the stationary phase in ZIC-HILIC restricts the range that can be used to pH 3−8. Thus, we assessed its performance from pH 3.3 to 6.6 with intermediate pH levels at 4.0 and 4.8. The pH value of salt stock solution was only adjusted for solution B with acetic acid before being mixed with solution A. The results are shown in Figure.3. As can be seen, the retention of the solutes is related to their pK_a values which were compiled by R. Williams.[41]

For the solutes with pK_a values either above or below the pH range of mobile phase (3.3−6.6), its retention time and elution order remained relatively constant with increasing pH because they were not charged in the pH range covered. However, for Cyt, Gua, Ade, Ado, and Cyd with pK_a values within the pH range being examined, an increase in retention occurred with the increasing pH of mobile phase and reached maximum at moderate pH, indicating that if analytes are in their charged form, an electrostatic interaction occurs along with the hydrophilic interactions because the charged form will become more hydrophilic due to better solubility in the aqueous phase retained on the stationary phase. Likewise, the electrostatic interactions with the residue of sulfobetaine of the stationary

phase would be greater in the charged form than in their neutral form. The electrostatic interactions would thus occur mainly with the terminal sulfonic group and not with the interior ammonium group, which is clearly sterically hindered.

It was also found that 2-deoxyadenosine, 2-deoxycytidine, Gua, and Ado reached the maximum retention prior to Ade, Cyt, and Cyd, which is ascribed to the earlier attainment of complete neutrality due to lower pK_a values of 2-deoxyadenosine, 2-deoxycytidine, Gua, and Ado.

In all cases, 6.6 > pH >4.5 offered a good separation and selectivity of these compounds. It could be concluded that the method would be more robust in this pH range. That is why a pH of 6.0 was chosen for further experiments.

Effect of Column Temperature on the Separation. Column temperature is another important parameter that affects the retention of polar compounds in HILIC. The effect of column temperature on retention was investigated in the temperature range of 20−70 °C. As shown in Figure 4, increase

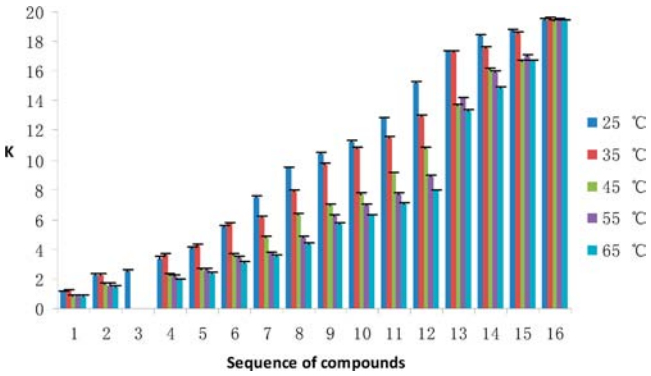

Figure 4. Influence of the temperature on the capacity factor (k) of 16 standard compounds on the ZIC-HILIC column. The data are presented as the average of three replicates; error bars represent the standard deviations.

in the temperature caused a decrease in retention time for all those compounds, which resulted in poor resolution among compounds, especially for compounds 2 and 3. They were coeluted when the column temperature was higher than 35 °C. Therefore, 25 °C was used for the column.

Effect of Injection Solvent on Separation. The injection solvent is also an important parameter in HILIC because as in reverse-phase chromatography, the injection solvent can strongly influence the peak shape of the analytes. A high proportion of water in the injection solvent could result in lower retention, worse separation, and poorer efficiency, but the analytes would not be easily solubilized in highly organic solutions. In our case, good solubilization of all the studied compounds was achieved by using 50% acetonitrile/50% water solution as injection solvent.

The final chromatographic conditions described in the Materials and methods section resulted in a good separation of the 16 studied compounds as can be observed in Figure 5f.

Validation of the Method. As shown in Table 2, the correlation coefficients (R^2) ranged from 0.993 to 0.9998. The different ratios of LOQ and LOD may be derived from the difference of HPLC response to the analytes, and the LODs were between 0.005 and 0.029 μg/mL, and the LOQs were between 0.018 and 0.096 μg/mL, respectively. Especially the LOD and LOQ for thymine, thymidine, uridine, inosine, cytidine, and guanosine were much lower than that of the previous RP-HPLC methods (10 and 12), which demonstrated that this developed HILIC method was more sensitive than those RP-LC methods.

For high throughput analysis, samples usually have to be stored in an autosampler for a long time, which should be considered carefully for the unstable analytes. The storage stability (RSD) of the 16 nucleosides and nucleobases were 0.56−3.2%, as determined from the peak areas (Table 3). These confirm that the sample solution was stable for 18 h.

As shown in Table 3, the RSDs of the intraday assay were 0.34−3.99% (n = 6), while the RSDs of the interday assay were 0.41−1.75% (n = 6). For every test, the calibration concentrations were also back-calculated from the peak area of the analytes. The deviation from the nominal concentration defined as accurate was better than 88% for both of short-term and long-term assays.

The recoveries were found within 85.98−102.04%. All of the above indicated that the performance of the established method was satisfactory.

Application for Analysis of Real Samples. *G. atrum*, one member of the genus *Ganoderma*, could also be used to promote health and longevity and has been developed into a variety of foods, such as *G. atrum* tea. But in the past 3 decades, most of the scientific research has been focused on the species of *G. lucidum* and *G. sinense*, and very few reports could be found on *G. atrum*. Previous studies on *G. lucidum* and *G. sinense* reported that contents of triterpenes, polysaccharides and nucleosides in these two species were distinctive.[12,13,38] However, a limited number of nucleosides and nucleobases were determined, and few are concerning the contents of nucleosides and nucleobases in *G. atrum* and its comparison to the species of *G. lucidum* and *G. sinense*. Therefore, the contents of those target compounds in these three species of *Ganoderma* (including cultured and natural samples) were investigated. Representative HILIC chromatograms of the standards and extracts from cultured and natural *G. lucidum*, *G. sinense*, and *G. atrum* are shown in Figure 5. The identification of investigated compounds was carried out by comparison of their retention time and their UV spectra with those obtained by injecting standards and spiked samples in the same conditions. The chromatographic profiles for different species varied significantly (Figure 5). Therefore, the chromatograms with the profiles of each analyzed species would be helpful to control the quality of *Ganoderma*, indicating the importance of nucleoside profiles in the discrimination of the species.

Table 4 gave a summary of the nucleobase and nucleoside analytes from these samples. The contents of theses nucleosides and nucleobases in *Ganoderma* collected from different locations varied significantly (Figure 5 and Table 4). Total nucleoside and nucleobase contents ranged from 127.11 to 1333.11 μg/g dry matter. Typically, the total amounts of the nucleosides and nucleobases in *G. lucidum* (>362 μg/g dry matter) were evidently higher than those in *G. sinense*, which was in accordance with the previous studies.[12,13]

Among the individual nucleosides, the content (measured in peak area) of thymidine, 2′-deoxyuridine, and adenine (peaks 3, 5, and 6) was significantly higher in *G. lucidum* and *G. sinense* (Figure 5b−e) than that in *G. atrum*. In contrast, the contents of uridine, guanine, 2′-deoxyinosine, and 2′-deoxyguanosine (peaks 8, 9, 10, and 14) were typically abundant in *G. atrum* (Figure 5a) and cultured *G. sinense* (Figure 5d), while they were almost not found in *G. lucidum* samples (Figure 5b). Besides, for the samples of *G. lucidum* from different origins, the

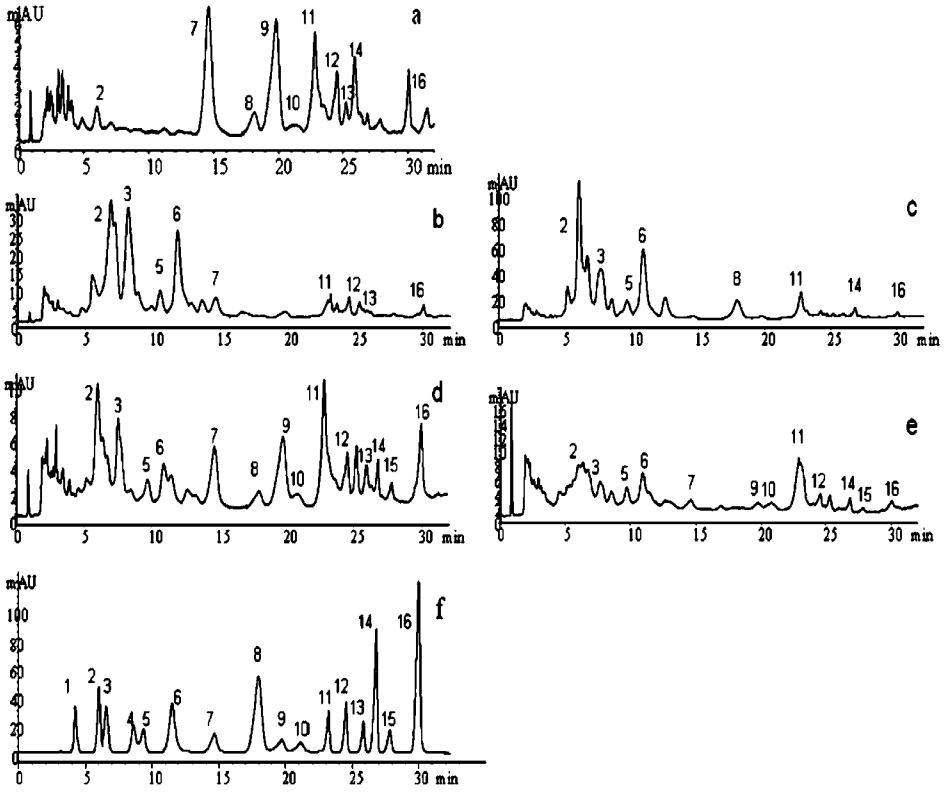

Figure 5. Representative HILIC chromatograms for the samples and mixed-standards. Cultured *G. atrum* from Ganzhou city, Jiangxi province (a); cultured *G. lucidum* from Longquan city, Zhejiang province (b); natural *G. lucidum* from Lijiang, Yunnan province (c); cultured *G. sinense* from Wuyi Mountain, Fujian province (d); natural *G. sinense* from Lijiang, Yunnan province (e); and mixture of 16 standard compounds (f).

contents of analytes were also determined. The contents of each analyte in *G. lucidum* from different origins were similar, except that uridine and guanine were more abundant in *G. lucidum* from Shandong Province (Table 4). However, the differences in the total nucleoside and nucleobase contents were quite considerable. The highest level was found for a sample from Hubei province, and the lowest was found for samples from Anhui province.

Interestingly, there were significant differences in the total amount of nucleosides and bases between natural and cultured samples. As shown in Figure 5c, for *G. lucidum* samples, the contents of both total nucleosides and nucleobases and the individual analytes, such as uracil, uridine, and cytosine, in natural samples were more abundant than those in cultured ones. However, for *G. sinense*, the levels were much lower in natural species (Figure 5e), especially for adenosine, guanine, inosine, and guanosine.

To further visualize the relationships of various *Ganoderma* samples from different origins and species, hierarchical cluster analysis (HCA) was performed on the peak areas of analytes. Hierarchical cluster analysis is a multivariate analysis technique, which is used to sort samples into groups. A row dendrogram is produced to visualize the result of hierarchical clustering calculation. Figure 6 clearly shows that total 20 *Ganoderma* samples were divided into 3 clusters according to their species. Cluster-I was formed by samples 1−4 collected from the same origin, which belonged to the species *G. atrum*. Similar results were also observed for cluster II, which consisted of samples 5−14 collected from various locations, but all belonged to species *G. lucidum*. Typically, in cluster II some subclusters were also formed along with their regions. For instance, samples 5−6 coming from the same region Jiaxiang was clustered together, which indicated that the profiles of nucleosides and bases were similar when they were cultured under the same conditions. Interestingly, cluster III mainly consisted of natural *G. sinense* and natural *G. lucidum*, and samples of the two species were clustered in two subclusters. It indicated that the natural *G. lucidum* and nutral *G. sinense* could be preliminarily discriminated with HCA. Besides, we also observed that sample 20 (cultured *G. sinense*) was far from the natural *G. sinense*, although they all belonged to cluster III. This result indicated that the cultured *Ganoderma* were quite different from the natural samples from the viewpoint of nucleosides and nucleobases. This agreed very well with the comparisons of their chromatogram profile and contents (Figure 6). Therefore, the characteristic peaks of nucleosides and nucleobases in the HILIC profile could be used as potential markers for discrimination and quality control of different *Ganoderma*, as already suggested.

The study provided valuable insight into distribution in different species of *Ganoderma*, which could also be developed as a reliable means of differentiating other various fungi.

Concluding Remarks and Future Perspectives. Analytical methods for the routine analysis of bioactive chemical compounds have to be sensitive and specific, and insofar as possible, more compounds must be analyzed in one analytical run with less time. Methods based on chromatographic separation with or without MS detection seem optimal to meet these requirements. For nucleosides and nucleobases, two approaches using LC or LC-MS can be found in the literature: RP-LC, and HILIC. For example, Guo et al.[29] reported the use of RP-LC for the determination of 11 nucleosides and bases in *Cordyceps* samples. A HPLC−ESI−MS/MS method was also developed for natural and cultured *Cordyceps*, as presented by

Table 2. Linear Regression Data, LOD, and LOQ, of the Investigated Compounds

analytes	linear regression data[a]		LOD (μg/mL)	LOQ (μg/mL)
thymine	$y = 2605x + 4.2532$	$R^2 = 0.9997$	0.008	0.025
uracil	$y = 4543.3x + 15.389$	$R^2 = 0.9983$	0.019	0.062
thymidine	$y = 3850.1x - 2.372$	$R^2 = 0.9998$	0.006	0.021
2-deoxyadenosine	$y = 1221x + 3.5638$	$R^2 = 0.993$	0.019	0.063
2-deoxyuridine	$y = 2432.1x + 3.3472$	$R^2 = 0.9996$	0.009	0.030
adenine	$y = 6087.3x - 12.655$	$R^2 = 0.9998$	0.006	0.020
adenosine	$y = 2787.6x + 5.1572$	$R^2 = 0.9994$	0.011	0.036
uridine	$y = 7436.6x - 21.579$	$R^2 = 0.9998$	0.006	0.018
guanine	$y = 2624.4x - 14.608$	$R^2 = 0.9963$	0.014	0.046
2-deoxyinosine	$y = 1041.1x - 9.6026$	$R^2 = 0.9936$	0.012	0.040
cytosine	$y = 3081.8x + 20.863$	$R^2 = 0.9959$	0.029	0.096
2-doxycytidine	$y = 3336x - 0.481$	$R^2 = 0.9992$	0.006	0.021
inosine	$y = 2129.5x - 0.131$	$R^2 = 0.9994$	0.005	0.018
2-deoxyguanosine	$y = 8722.2x + 2.7449$	$R^2 = 0.9988$	0.008	0.026
cytidine	$y = 1831.4x + 1.6518$	$R^2 = 0.999$	0.007	0.023
guanosine	$y = 5469.2x + 1.756$	$R^2 = 0.9994$	0.006	0.019

[a]Calculated from three calibration lines representing concentration (mg/mL) vs area (mAU·s). For each analyte, six data points were used.

Fan et al.[28] However, on this traditional RP-LC, they had low retention for some of the highly polar or hydrophilic analytes such as cytosine and uracil, resulting in low selectivity due to high matrix interference or thus lower sensitivity of the MS detection due to high water percentage in the mobile phase. As an alternative to such methods, the analysis with HILIC has been demonstrated as a valuable complementary approach to RP-LC. The use of a low aqueous and high organic mobile phase in HILIC separation is almost ideal for electrospray ionization, leading to increased sensitivity. For example, Chen et al.[35] reported that a significant increase in separation efficiency for some analytes, especially for guanine, hypoxanthine, 2′-deoxyuridine, uridine, and thymidine, was found when HILIC was compared to RP-HPLC. Most recently, Zhao et al.[37] also developed a HILIC-TOF/MS method for separation in marine substances, which presents clear advantages for the analysis of nucleosides and bases with a LOD (low to 0.6 ng/L) that is 1000-fold lower than that obtained by RP-LC−UV and UP-LC. However, those previous HILIC methods still showed some limitations as well, such as limited analytes detected, long analysis time, and poor selectivity. In the method developed by Chen,[35] only 14 nucleosides and nucleobases were separated on a TSKgel Amide-80 column with the separation time as long as 110 min, and the selectivity between adenosine and adenine, and 2′-deoxycytidine and inosine was poor. Similarly, in the HILIC−TOF/MS method,[37] adenine and uridine, and 2′-deoxyguanosine and cytidine were coeluted with a separation time of 65 min; thus, some of those compounds cannot be detected with above method. But these undetected nucleosides and bases are also very important to differentiation and quality control of the real samples. Therefore, it is very important to establish a new and efficient HILIC method for identification and determination of more active nucleosides and bases in the natural products mentioned above.

In the current work, on the basis of these above results, a new HILIC method was developed for the simultaneous determination of 16 nucleosides and bases in *Ganoderma*. The retention mechanism under the optimum conditions was determined to be a combination of adsorption, partition, and electrostatic interactions. With this method, baseline separation of all 16 analytes was obtained within 35 min. The LOQ and LOD were more than 10 times lower than that achieved by RP-LC and comparable with the LOQ reported for the previous HILIC method, but it was more informative and showed clear advantages, such as faster (shorter time), simpler, and multianalyte simultaneous determination. In addition, it was

Table 3. Stability, Intra-, and Inter-Day Precision of the Investigated Compounds

			intraday precision ($n = 6$)		interday precision ($n = 6$)	
analytes	recovery (%)	stability (RSD, %)25 °C	accuracy (%)	RSD (%)	accuracy (%)	RSD (%)
thymine	93.45	0.56	102.23	0.37	101.76	0.78
uracil	87.88	0.84	88.89	0.34	90.74	1.52
thymidine	89.43	0.81	99.34	0.65	97.93	1.75
2-deoxyadenosine	90.73	1.88	96.50	1.44	95.94	1.09
2-deoxyuridine	91.31	1.43	104.81	1.77	102.39	1.10
adenine	95.58	0.73	103.71	1.27	104.06	0.90
adenosine	97.23	0.78	99.55	0.99	99.63	0.58
uridine	96.28	0.9	93.83	1.10	92.64	1.48
guanine	95.87	1.84	111.06	0.54	112.49	1.25
2-deoxyinosine	85.98	3.2	94.44	0.62	89.81	0.77
cytosine	96.45	0.58	91.61	3.99	91.65	1.26
2-doxycytidine	98.56	1.32	113.20	0.38	113.02	0.41
inosine	98.32	0.56	101.43	1.47	100.22	0.85
2-deoxyguanosine	97.45	1.21	99.26	0.98	96.68	1.48
cytidine	96.89	0.68	106.79	1.42	107.85	0.69
guanosine	102.04	1.63	102.02	1.46	101.82	0.69

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Table 4. Contents (μg/g) of 16 Nucleosides and Nucleobases in 8 Batches of *Ganoderma* Samples (n = 3)

samples	*G. atrum*		*G. lucidum*				*G. sinense*	
analytes	Jiangxi[a]	Zhejiang[a]	Anhui[a]	Shandong[a]	Hubei[a]	natural	Fujian[a]	natural
thymine	–	–[b]	–	–	–	–	–	–
uracil	1.26[c]	145.40	62.87	210.65	406.12	645.43	25.91	30.67
thymidine	–	138.23	117.90	260.22	474.44	235.96	23.77	15.57
2-deoxyadenosine	–	–	–	–	–	–	–	–
2-deoxyuridine	–	25.15	28.75	52.83	173.05	105.66	6.10	10.99
adenine	–	56.54	46.80	67.65	147.29	202.80	12.27	11.25
adenosine	39.76	26.32	8.17	17.14	53.87	0.00	25.67	7.40
uridine	3.64	–	–	8.40	–	41.42	4.36	–
guanine	44.26	–	–	21.03	–	–	45.65	6.38
2-deoxyinosine	10.23	–	–	–	–	–	24.78	18.49
cytosine	10.94	8.31	43.39	19.65	–	88.41	34.00	34.01
2-doxycytidine	6.05	12.36	23.30	9.99	–	–	7.91	13.97
inosine	3.25	13.48	27.55	42.65	–	–	13.23	0.00
2-deoxyguanosine	3.36	–	–	–	–	6.36	2.25	1.29
cytidine	–	–	–	–	–	–	5.39	2.91
guanosine	4.36	4.89	3.28	14.00	25.47	7.05	11.37	1.60
Total	127.11	430.69	362.01	724.21	1270.24	1333.11	242.66	154.53

[a]The origin (the province in China) of the cultured samples. [b]Undetectable. [c]The data are presented as the average of three replicates (RSDs <10%).

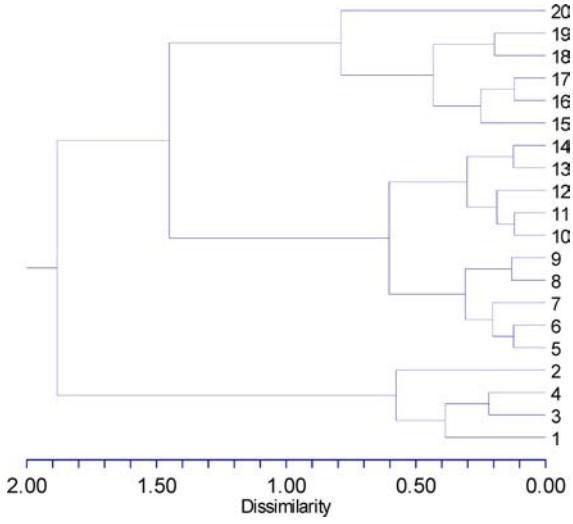

Figure 6. Dendrogram as a result of the hierarchical cluster analysis (HCA) to visualize the relationships of various *Ganoderma* samples from different origins and species. HCA was performed on the peak areas of analytes.

cheaper compared with the above HILIC-MS method. With this method, nucleosides and bases in *G. atrum* were for the first time quantified and discriminated from other two species of *Ganoderma* (*G. lucidum* and *G. sinense*). Thus, our results in the present study clearly suggest that this developed HILIC method is simple, rapid, and sensitive. Therefore, it could be an alternative for the older nonspecific techniques, which are laborious and time-consuming, and which cannot detect all those nucleosides and bases simultaneously. In the future, we can expect continuing development of new HILIC materials or methods and applications for various natural samples with simultaneous separations of strongly and weakly polar sample components in a single run.

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Notes

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ABBREVIATIONS USED

ACN, acetonitrile; Ade, adenine; Ado, adenosine; Cyd, cytidine; Cyt, cytosine; DAD, diode-array detector; *G. lucidum*, *Ganoderma lucidum*; Gua, guanosine; HCA, hierarchical cluster analysis; HILIC, hydrophilic interaction chromatography; Ino, inosine; LC, liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; LOD, limits of detection; LOQ, limits of quantification; RP-HPLC, reversed-phase high performance liquid chromatography; RSD, relative standard deviations; S/N, signal-to-noise ratio; TCMs, traditional Chinese medicines; UP-LC, ultraperformance liquid chromatography; Urd, uridine

REFERENCES

(1) Chen, Y.; Xie, M. Y.; Wang, Y. X.; Nie, S. P.; Li, C. Analysis of the monosaccharide composition of purified polysaccharides in *Ganoderma atrum* by capillary gas chromatography. *Phytochem. Anal.* **2009**, *20*, 503–510.

(2) Zhang, M.; Cui, S. W.; Cheung, P. C. K.; Wang, Q. Antitumor polysaccharides from 383 mushrooms: a review on their isolation process, structural characteristics and antitumor activity. *Trends Food Sci. Technol.* **2007**, *18*, 4–19.

(3) Mau, J. L.; Lin, H. C.; Chen, C. C. Antioxidant properties of several medicinal mushrooms. *J. Agric. Food Chem.* **2002**, *50*, 6072–6077.

(4) Wu, T. S.; Shi, L. S.; Kuo, S. C. Cytotoxicity of *Ganoderma lucidum* triterpenes. *J. Nat. Prod.* **2001**, *64*, 1121–1122.

(5) Chen, Y.; Xie, M. Y.; Nie, S. P.; Li, C.; Wang, Y. X. Quality control and original discrimination of *Ganoderma lucidum* based on high-performance liquid chromatographic fingerprints and combined chemometrics methods. *Anal. Chim. Acta* **2008**, *623*, 146−156.
(6) Paterson, R. R. *Ganoderma*: A therapeutic fungal biofactory. *Phytochemistry* **2006**, *67*, 1985−2001.
(7) Gao, J. L.; Leung, K. S.; Wang, Y. T.; Lai, C. M.; Li, S. P.; Hu, L. F.; Lu, G. H.; Jiang, Z. H.; Yu, Z. L. Qualitative and quantitative analyses of nucleosides and nucleobases in *Ganoderma spp.* by HPLC-DAD-MS. *J. Pharm. Biomed. Anal.* **2007**, *44*, 807−11.
(8) Chen, T. H.; Wang, M. F.; Liang, Y. F.; Komatsu, T.; Chan, Y. C.; Chung, S. Y.; Yamamoto, S. A nucleoside-nucleotide mixture may reduce memory deterioration in old senescence- accelerated mice. *J. Nutr.* **2000**, *130*, 3085−3089.
(9) Carmen, W. H.; Xin, D. Chromatographic and electrophoretic methods for Lingzhi pharmacologically active components. *J. Chromatogr., B.* **2004**, *812*, 241−257.
(10) Yuan, J. P.; Zhao, S. Y.; Wang, J. H.; Kuang, H. C.; Liu, X. Distribution of nucleosides and nucleobases in edible fungi. *J. Agric. Food Chem.* **2008**, *56*, 809−15.
(11) Li, S. P.; Yang, F. Q.; Tsim, K. W. K. Quality control of Cordyceps sinensis, a valued traditional Chinese medicine. *J. Pharm. Biomed. Anal.* **2006**, *41*, 1571−1584.
(12) Gao, J. L.; Leung, K. S. Y.; Wang, Y. T.; Lai, C. M.; Li, S. P.; Hu, L. F.; Lu, G. H.; Jiang, Z. H.; Yu, Z. L. Qualitative and quantitative analyses of nucleosides and nucleobases in *Ganoderma* spp. by HPLC-DAD-MS. *J. Pharm. Biomed. Anal.* **2007**, *44*, 807−811.
(13) Yang, F. Q.; Zhang, X. M.; Ge, L. Y.; Tan, S. N. Determination of nucleosides and related compounds in *Ganoderma* by CE-MS. J. *China Pharm. Univ.* **2011**, *42*, 337−341.
(14) Stillwell, W. G.; Xu, H. X.; Adkins, J. A.; Wishnok, J. S.; Tannenbaum, S. R. Analysis of methylated and oxidized purines in urine by capillary gas chromatography-mass spectrometry. *Chem. Res. Toxicol.* **1989**, *2*, 94−99.
(15) Teixeira, A. J. R.; Gommers-Ampt, J. H.; van de Werken, G.; Westra, J. G.; Stavenuiter, J. F. C.; de Jong, A. P. J. M. Method for the analysis of oxidized nucleosides by gas chromatography/ mass spectrometry. *Anal. Biochem.* **1993**, *214*, 474−483.
(16) Li, S. P.; Li, P.; Dong, T. T. X.; Tsim, K. W. K. Determin- ation of nucleosides in natural *Cordyceps sinensis* and culture *Cordyceps mycelia* by capillary electrophoresis. *Electrophoresis* **2001**, *22*, 144−150.
(17) Gong, Y. X.; Li, S. P.; Li, P.; Liu, J. J.; Wang, Y. T. Simultaneous determination of six main nucleosides and bases in natural and cultured *Cordyceps* by capillary electrophoresis. *J. Chromatogr., A* **2004**, *1055*, 215−221.
(18) Yeh, C.; Jiang, S. Determination of monophosphate nucleotides by capillary electrophoresis inductively coupled plasma mass spectrometry. *Analyst* **2002**, *127*, 1324−1327.
(19) Yang, F. Q.; Ge, L.; Yong, H. J. W.; Tan, S. N.; Li, S P. Determination of nucleosides and nucleobases in different species of *Cordyceps* by capillary electrophoresis−mass spectrometry. *J. Pharm. Biomed. Anal.* **2009**, *50*, 307−314.
(20) Yang, F. Q.; Li, S.; Li, P.; Wang, Y. T. Optimization of CEC for simultaneous determination of eleven nucleosides and nucleobases in *Cordyceps* using central composite design. *Electrophoresis.* **2007**, *28*, 1681−1688.
(21) Yang, F. Q.; Guan, J.; Li., S. P. Fast simultaneous determination of 14 nucleosides and nucleobases in cultured *Cordyceps* using ultra-performance liquid chromatography. *Talanta* **2007**, *73*, 269−273.
(22) Hou, S. J.; Ding., M. Y. Simultaneous separation and determination of eleven nucleosides and bases in beer, herring sperm DNA and RNA soft capsule by high-performance liquid chromatography. *Anal. Sci.* **2010**, *26*, 1111−1114.
(23) Fürst, W.; Hallström, S. Simultaneous determination of myocardial nucleotides, nucleosides, purine bases and creatine phosphate by ion-pair high-performance liquid chromatography. *J. Chromatogr.* **1992**, *578*, 39−44.
(24) Liu, L.; Ouyang, J.; Baeyens, W. R. G. Separation of purine and pyrimidine bases by ion chromatography with direct conductivity detection. *J. Chromatogr., A* **2008**, *1193*, 104−108.
(25) Guo, F. Q.; Li, A.; Huang, L. F.; Liang, Y. Z.; Chen, B. M. Identification and determination of nucleosides in *Cordyceps sinensis* and its substitutes by high performance liquid chromatography with mass spectrometric detection. *J. Pharm. Biomed. Anal.* **2006**, *40*, 623−630.
(26) Yu, L.; Zhao, J.; Li, S. P.; Fan, H.; Hong, M.; Wang, Y. T.; Zhu, Q. Quality evaluation of *Cordyceps* through simultaneous determination of eleven nucleosides and bases by RP-HPLC. *J. Sep. Sci.* **2006**, *29*, 953−958.
(27) Fan, H.; Yang, F. Q.; Li, S. P. Determination of purine and pyrimidine bases in natural and cultured *Cordyceps* using optimum acid hydrolysis followed by high performance liquid chromatography. *J. Pharm. Biomed. Anal.* **2007**, *45*, 141−144.
(28) Fan, H.; Li, S. P.; Xiang, J. J.; Lai, C. M.; Yang, F. Q.; Gao, J. L.; Wang, Y. T. Qualitative and quantitative determination of nucleosides, bases and their analogues in natural and cultured *Cordyceps* by pressurized liquid extraction and high performance liquid chromatography−electrospray ionization tandem mass spectrometry (HPLC−ESI−MS/MS). *Anal. Chim. Acta* **2006**, *567*, 218−228.
(29) Guo, F. Q.; Li, A.; Huang, L. F.; Liang, Y. Z.; Chen, B. M. Identification and determination of nucleosides in *Cordyceps sinensis* and its substitutes by high performance liquid chromatography with mass spectrometric detection. *J. Pharm. Biomed. Anal.* **2006**, *40*, 623−630.
(30) Swartz, M. E. UPLCTM: An introduction and review. . *J. Liquid Chromatogr. Relat. Technol.* **2005**, *28*, 1253−1263.
(31) Novakova, L.; Matysova, L.; Solich, P. Advantages of application of UPLC in pharmaceutical analysis. *Talanta* **2006**, *68*, 908−918.
(32) Cubbon, S.; Bradbury, T.; Wilson, J.; Thomas-Oates, J. Hydrophilic interaction chromatography for mass spectrometric metabonomic studies of urine. *Anal. Chem.* **2007**, *79*, 8911−8918.
(33) Zhou, T.; Lucy, C. A. Hydrophilic interaction chromatography of nucleotides and their pathway intermediates on titania. *J. Chromatogr., A* **2008**, *1187*, 87−93.
(34) Mora, L.; Hernández-Cázares, A. S.; Aristoy, M. C.; Toldrá, F. Hydrophilic interaction chromatographic determination of adenosine triphosphate and its metabolites. *Food Chem.* **2010**, *123*, 1282−1288.
(35) Chen, P; Li, W; Li, Q; Wang, Y. H.; Li, Z. G.; Ni, Y. F.; Koike, K. Identification and quantification of nucleosides and nucleobases in Geosaurus and Leech by hydrophilic-interaction chromatography. *Talanta* **2011**, *85*, 1634−1641.
(36) Marrubini, G.; Mendoza, B. E. C.; Massolini, G.. Separation of purine and pyrimidine bases and nucleosides by hydrophilic interaction chromatography. *J. Sep. Sci.* **2010**, *33*, 803−816.
(37) Zhao, H. Q.; Chen, J. H.; Shi, Q.; Li, X.; Zhou, W. H.; Zhang, D. L.; Zheng, L.; Cao, W.; Wang, X. R.; Lee, F. S. C. Simultaneous determination nucleosides in marine organism using ultrasound-assisted extraction followed by hydrophilic interaction liquid chromatography−electrospray ionization time-of-flight mass spectrometry. *J. Sep. Sci.* **2011**, *34*, 2594−2601.
(38) Chen, Y.; Bicker, W.; Wu, J. Y.; Xie, M. Y.; Lindner, W. *Ganoderma* species discrimination by dual-mode chromatographic fingerprinting: A study on stationary phase effects in hydrophilic interaction chromatography and reduction of sample misclassification rate by additional use of reversed-phase chromatography. *J. Chromatogr., A* **2010**, *1217*, 1255−1265.
(39) Chen, Y.; Xie, M. Y.; Nie, S. P.; Li, C.; Wang, Y. X. Quality control and original discrimination of *Ganoderma lucidum* based on high-performance liquid chromatographic fingerprints and combined chemometrics methods. *Anal. Chim. Acta* **2008**, *623*, 146−156.
(40) Rodríguez-Gonzalo, E.; García-Gómez, D.; Carabias-Martínez, R.. Study of retention behaviour and mass spectrometry compatibility in zwitterionic hydrophilic interaction chromatography for the separation of modified nucleosides and nucleobases. *J. Chromatogr., A* **2011**, *1218*, 3994−4001.

(41) Jencks, W. P.; Westheimer, F. H.; Williams, R. Aqueous pKa values. *J. Chem. Educ.* (Digital Library) http://research.chem.psu.edu/brpgroup/pKa_compilation.pdf