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ScienceDirect
Chinese Journal of Natural Medicines 2021, **19**(4): 295-304
doi: 10.1016/S1875-5364(21)60030-4
Chinese Journal of Natural Medicines

•Research article•

Simple and robust differentiation of *Ganoderma* species by high performance thin-layer chromatography coupled with single quadrupole mass spectrometry QDa

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Available online 20 Apr., 2021

[ABSTRACT] In this study, a high performance thin-layer chromatography/single quadrupole mass spectrometry QDa (HPTLC-QDa) method for robust authentication of *Ganoderma lucidum*, a popular and valuable herbal medicine, has been developed. This method is simple and practical, which allows direct generation of characteristic mass spectra from the HPTLC plates automatically with the application of in situ solvent desorption interface. The HPTLC silica gel plates were developed with toluene-ethyl formate-formic acid (5 : 5 : 0.2, *V*/*V*) and all bands were transferred to QDa system directly in situ using 80% methanol with 0.1% formic acid as desorption solvent. The acquired HPTLC-QDa spectra showed that luminous yellow band b3, containing ganoderic acid B/G/H and ganodenic acid B, the major active components of Ganoderma, could be found only in *G. lucidum* and *G. lucidum* (Antler-shaped), but not in *G. sinense* and *G. applanatum*. Moreover, bands b13 and b14 with *m*/*z* 475/477 and *m*/*z* 475/491/495, respectively, could be detected in *G. lucidum* (Antler-shaped), but not in *G. lucidum*, thus allowing simple and robust authentication of *G. lucidum* with confused species. This method is proved to be simple, practical and reproducible, which can be extended to analyze other herbal medicines.

[KEY WORDS] High performance thin-layer chromatography; Mass spectrometry QDa; *Ganoderma*; Authentication
[CLC Number] R917 **[Document code]** A **[Article ID]** 2095-6975(2021)04-0295-10

Introduction

The safety of Chinese medicine is commonly sourced from adulterant herbs, pesticide residue, and illegal dye addition, especially the herbal drugs which have multiple sources [1]. When the chemical composition and safety of a herb drug are not well investigated, discrimination of the herbal drug with its adulterants heavily rely on methods such as morphological and microscopic identification [2, 3], DNA and LC fingerprint technologies [4-6]. Previously, the phytologist authenticated plants, notably those medicinal ones, depending mainly on the macroscopic [3] along with microscopic [7] properties. This task requires not only profound knowledge, but also results in much misrecognition, e.g. Japanese star anise (*Illicium anisatum*) *vs.* Chinese star anise (*Illicium verum*) [8]. Owing to the rapid development of genetic sequencing techniques, DNA barcoding, an innovative tool to substitute the whole genome sequencing, has been a workhorse in herbal medicine identification [5, 9]. However, this approach faces such critical obstacles as expensive instrumentation and the selection of reliable nucleic acid sequences, and even worse, it is almost impossible to extract desired DNA fragments from target medicines [10]. Fingerprint chromatography is usually used for assessing the quality of herbal medicines that are acquired by capillary electrophoresis (CE) [11], thin-layer chromatography (TLC) [12], gas chromatography (GC) [13], liquid chromatography (LC) [14], and nuclear magnetic resonance (NMR) [15] spectrometry, which is usually ambiguous and difficult to differentiate on various operational conditions. Development of simple and robust methods for authentication of herbal medicines is thus an important and necessary task.

Ganoderma (Lingzhi), a woody Basidinomycetes mush-

[Received on] 30-May-2020
[Research funding] The work was supported by National Key R&D Program of China (Nos. 2019YFC1711000 and 2018YFC1707900) , the National Natural Science Foundation of China (No. 8200140730), and Qi-Huang Scholar of National Traditional Chinese Medicine Leading Talents Support Program (2018).
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These authors have no conflict of interest to declare.

room belonging to the family of Ganodermaceae of the Apgyllophorals, is one of the most popular and valuable herbal medicines [16, 17], and has been used as a folk medicine in China as early as 2000 A.D.. There are approximately 80 *Ganoderma* species in the world, while only about 20 species are used for medical purposes [18, 19]. The majority are widely used as traditional medicines worldwide for enhancing "vital energy" of humans and promoting "longevity" [20], and more broadly, as dietary supplements and functional food to enhance the immunological functions [18]. Polysaccharides and ganoderic acids are abundant and bioactive ingredients, and are reported to be responsible for various biological activities, and for instance, anti-inflammation [21], anti-tumor [21], anti-hypertension [22], anti-bacteria [23], immune system-enhancing activities [23] and hepatoprotective activities [24]. USA Pharmacopoeia (43 edition) officially adopted the fruiting bodies of *G. ludcidum*, which was the most popular used *Ganoderma* species in the market. Owing to the increasing demand of Ganoderma and the limited supply and extremely high prices of wild Ganoderma, some other *Ganoderma* species [25], such as *G. lucidum* (Antler-shaped), *G. sinense* and *G. applanatum*, are commonly found as the adulterants to the official *Ganoderma* species, some of which are even several times higher in prices [16, 26]. It is difficult to distinguish them based on traditional morphological identification, and particularly, most of the commercial Ganoderma products are in slice or powder form, which makes accurate differentiation more difficult. Mislabeling and misuse of *Ganoderma* species would be a potential threat to product safety. Therefore, it is necessary to develop a reliable, simple and robust analytical method to differentiate official species from confused ones.

In Chinese Pharmacopoeia (2015 edition, ChP), thin layer chromatography (TLC) serves as a major vehicle for authentication of Chinese medicines [27]. In general, these TLC-based methods have been widely accepted for medicinal plant authentication through detecting a set of so-called diagnostic bands. However, these techniques also face some critical obstacles, for instance, extra expensive reference substances and the development of many tailored elution programs, due to the co-eluting phenomenon. In the present study, high performance thin layer chromatography mass spectrometry/single quadrupole QDa detector (HPTLC-QDa), a simple and easily accessible technique that requires no reference substance and only one tailored elution program, was developed for rapid and robust differentiation of *Ganoderma* species. Compared to other methods, HPTLC-QDa allows, in an automatic operation, to generate mass spectra in a short period of time that contains chemical compositions of bands and can serve as the fingerprints and biomarkers for characterization of the *Ganoderma* species. As a powerful technique for analysis of herbal medicines, few HPTLC-QDa method has been used for analysis of Chinese medicine yet. Here we demonstrated that the HPTLC-QDa could unequivocally differentiate official and confused *Ganoderma* species based on their TLC chromatograph and mass spectra, and the schematic diagram was shown in Fig.1.

Material and Methods

Plant materials and chemicals

The *G. lucidum* (GL), *G. sinense* (GS), *G. lucidum* (Antler-shaped) (GLA), *G. applanatum* (GA), and were respectively collected in 2016 from Anhui, Guangxi, Shandong and Heilongjiang provinces, China. The plant materials were identified by Prof. GUO De-An, National Engineering Laboratory for TCM standardization Technology, Shanghai Institute of Materia, Chinese Academy of Sciences, where these voucher specimens (Nos. 2016001, 2016002, 2016003, 2016004, respectively) were deposited. The information of other 12 batches of *G. lucidum* was listed. The solvents (analytical grade) of toluene, ethyl formate, formic acid and ethanol for the extraction and derivatization process were pur-

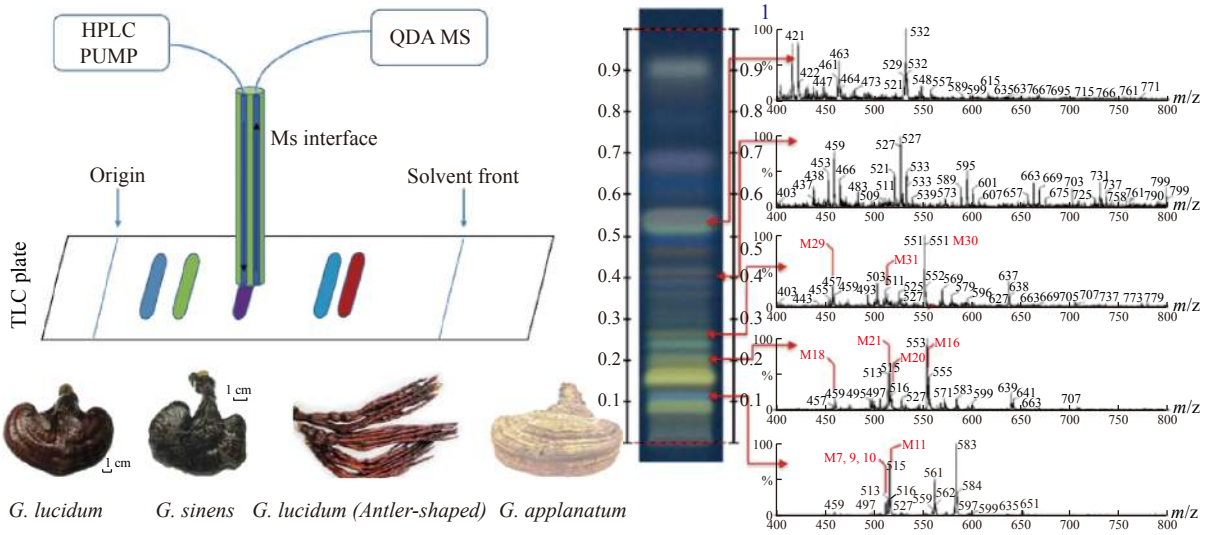

Fig. 1 Schematic diagram of HPTLC-QDa MS analysis of *Ganoderma* samples

chased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Acetonitrile and Methanol of HPLC-grade used in the mobile phase were supplied by Merck KGaA (Darmstadt, Germany). Ultrapure water (18.2 MΩ·cm^{-1} at 25 °C) was prepared by Millipore Alpha-Q water purification system (Millipore, Bedford, MA, USA). HPTLC plates used for HPTLC-QDa were obtained from Merck KGaA (Darmstadt, Germany). A total of nine reference standards, Ganoderic acid C (GC, purity: HPLC, ≥ 98.2%), Ganoderic acid A (GA, purity: HPLC, ≥ 99.3%), Ganoderic acid G (GG, purity: HPLC, ≥ 99.1%), Ganoderenic acid B (GEB, purity: HPLC, ≥ 98.7%), Ganoderic acid B (GB, purity: HPLC, ≥ 99.3%), Ganoderic acid H (GH, purity: HPLC, ≥ 98.1%), Ganoderenic acid D (GED, purity: HPLC, ≥ 98.5%), Ganoderic acid D (GD, purity: HPLC, ≥ 97.7%), and Ganoderic acid F (GF, purity: HPLC, ≥ 98.7%) were purchased from Shanghai Standard Biotech Co., Ltd..

Sample preparation

An aliquot of 1.0 g of accurately weighted fine powder (10 mesh) of *Ganoderma* species fruiting body was immersed in 50.00 mL alcohol with ultrasound assistance (37 kHz, 1130 W) for 15 min. The suspension was centrifuged at 10 000 r·min^{-1} for 5 min, and the supernatant was evaporated to dryness under reduced pressure at 50 °C. The obtained residue was dissolved in 2.00 mL of alcohol before injection. Test solutions of nine ganoderic acid compounds were prepared in alcohol at a concentration of approximately 0.5 mg·mL^{-1}.

HPTLC Chromatographic analysis

Regarding HPTLC domain, CAMAG TLC system (Muttenz, Switzerland), consisting of an automatic TLC Sample application, a TLC visualizer, an automatic development chamber 2, a chromatogram immersion device III, and a TLC plate heater III, was employed. The *G. lucidum*, *G. sinense*, *G. applanatum*, and *G. lucidum* (Antler-shaped) samples and reference standards were directly deposited onto HPTLC plate (20 cm × 10 cm) by an automatic TLC sample application apparatus controlled by visionCATS software. The injection volume was set as 2 μL for standard solution, and 4 μL for sample solution with bands interval of 8 mm. The HPTLC plate was pre-saturated in the automatic development chamber 2 with the help of a filter paper for 20 min and then developed with toluene-ethyl formate-formic acid (5 : 5 : 0.2, *V*/*V*) as the developing reagent for 10 min until the solvent front moved to 8 cm, which was controlled by visionCATS software. During the course of experiments, the relative humidity was maintained at 33% and the room temperature was 25 °C. After that, the developed HPTLC plates were removed from the chamber, and allowed to air-dry for 10 min. The post-chromatographic derivatization was performed by dipping the plates into the derivatization reagent by a CAMAG immersion device III, and followed by heated at 105−110 °C on a CAMAG TLC plate heater III for about 5 min. The derivatization reagent was prepared by mixing a solution of 10% sulfuric acid in alcohol (NOTE-prepare fresh. Slowly and gradually add sulfuric acid to ice-cold alcohol, and mix well). Ultimately, these plates were immediately examined under both white light and the long-wave UV light (366 nm) by TLC Visualizer with 12 bit CCD digital camera (CAMAG) controlled by visionCATS software.

HPTLC-QDa MS measurements

An optimal HPTLC-MS method was developed to comprehensively profile the bands in plates. The MS data was acquired on a HPTLC-QDa system involving a CAMAG TLC-MS interface 2, Waters HPLC pump and a single quadruple MS detector QDa (ACQUITY QDa; Waters). The chromatographic bands were eluted from the plate into the mass spectrometer using 80% methanol with 0.1% formic acid at a flow rate of 0.2 mL·min^{-1}. HPTLC chromatogram was achieved in both positive and negative mode within a single run by HPTLC-QDa, due to the rapid switching between ES^- and ES^+. A capillary voltage of 0.8 kV, cone voltage of 15 V and the desolation temperature of 600 °C were utilized in both ionization modes. The mass range of *m*/*z* 400−1100 was set for full-scan acquisition with a sampling rate of 10 points/s.

High-resolution centroid MS data of the solution of HPTLC chromatographic bands collected from CAMAG MS interface were further acquired on a Thermo LTQ-Orbitrap Velos Pro hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). And the data was processed by Xcalibur software (Thermo Fisher Scientific, Inc., Waltham, MA, USA).The ESI source parameters were set as follows: source spray voltage, 3.2 V; capillary temperature, 350 °C; source heater temperature, 300 °C; sheath gas (N_2), 40 arbitrary units; and auxiliary gas (N_2), 10 arbitrary units.

Results and Discussion

Optimization of HPTLC-QDa method for authentication of Ganoderma samples

To obtain good separation and high sensitivity for both triterpene acids and sterols at the same time, the HPTLC method for robust authentication of *Ganoderma* species was optimized in the first step. Three important factors, i.e. developing solvent system for separation, sample preparation used for extraction from samples, and the configuration of the experimental setup used for the chromatographic system.

In HPTLC, developing system serves for the good separation of target components, and is thus crucial for the observation of desired band signals. Four developing solvent systems, including petroleum ether-ethyl formate-formic acid, dichloromethane-methanol, 1-hexane-ethyl acetate-methanol-formic acid and toluene-ethyl formate with different additives, were tested for HPTLC analysis of *Ganoderma* samples. And the resulting plates (Fig. S1−2) showed that the spectra varied significantly with the solvent system used. Detection of both triterpene acids and sterols could be achieved by the last system with formic acid added, and then solvent burden radio, formic acid concentration and effects of additional solvent from different polarities were investigated systematically (Fig. S3). Toluene-ethyl formate-formic acid (5 : 5 : 0.2)

was eventually chosen as the developing solvent for HPTLC-QDa analysis of Ganoderma because the corresponding profile of chromatogram allowed detection of all possible triterpene acids and sterols with ideal separation.

Different sample extraction solvents of different polarities including chloroform, ethyl acetate and alcohol were tested for HPTLC-QDa analysis of *Ganoderma* and it was found that solvents of different polarities had no influence on the profile and background of chromatograms. Alcohol was selected as the extraction solvent considering its environment-friendly nature (Fig. S4). Different volumes of extraction (15, 25, 50, 100 mL) and extraction time (15, 30, 45, 60 min) were also tested. Similar results were obtained, and 50 mL and 15 min were selected ultimately in view of sample powder filtration and time-saving property, respectively (Fig. S5−6).

Moreover, configuration of the experimental setup used for HPTLC analysis, i.e. application volume, band width, and developing distance was also optimized. When the volume of application was close to maximum injection, trailing signals and co-eluting bands were generated, but when the volume was too little, a lot of non-obvious signals could not be observed. A configuration with application volume of 4 μL, band width in 8 mm, and developing distance of 8 cm were found to allow relatively stable, distinct and easy observation signals for samples and thus used for analysis (Fig. S7−8).

For QDa system, the mobile phase of HPTLC-QDa, a key parameter that greatly affected the generation of ions in terms of relative intensity of the adduct ions and product ions due to in-source fragmentation, was optimized using the reference standards. Three mobile phase system, including MeOH with addition of 0.1% formic acid (FA), MeOH : 2-proanol = 3 : 1 (0.1% FA) and 80%MeOH (0.1% FA), were evaluated by comparing the $[M-H]^-$, $[M-H-H_2O]^-$ and $[M+HCOO]^-$ ion responses of six index components. As illustrated in Fig. S10, background noises were observed for most of triterpene acids in MeOH system, and 80%MeOH (0.1%FA) was selected as mobile phase considering that 2-proanol was not appropriate for MS system. Other parameters that had only modest effects on MS signals were selected as recommended values provided in section 2.4.

HPTLC spectra of Ganoderma species samples

Typical HPTLC spectra of various *Ganoderma* species are shown in Fig. 2 and Fig. S9. Maximum number of bands could be observed at UV light (366 nm) after derivatization. In the upper third of the plate, four intense different color bands, corresponding to sterols, were observed (violet-brown band R_F = 0.98, blue band R_F = 0.89, blue-green band R_F = 0.77, red-violet R_F = 0.66). Several less intense blue bands were also detected in the middle of HPTLC spectra. The predominance of four bands, including two yellowish bands (R_F = 0.34, R_F = 0.19), an intense orange band (R_F = 0.24), less intense orange band (R_F = 0.24) were reckoned to be the diagnostic signals of *Ganoderma* species.

HPTLC for authentication of Ganoderma species samples

As shown in Figs. 2−3, the HPTLC results clearly demonstrated that samples of *G. lucidum* and *G. applanatum* could be differentiated from that of *G. sinense* and *G. lucidum* (Antler-shaped). An intense yellow band at R_F value of 0.15, corresponding to ganoderic acid G, B, H and ganoderenic acid B, was prominently dominated in the lower third of plate in *G. lucidum* and *G. applanatum*. In addition, the bands of ganoderic acid F, ganoderic acid D and ganoderenic acid D located above the yellow intense band could also be observed in the chromatograms of *G. lucidum* and *G. applanatum*. The HPTLC fingerprint of *G. lucidum* was similar to that of *G. applanatum*, however, a light blue band at R_F value of 0.11 due to ganoderic acid A was visible only in that of *G. lucidum*, and a violet band at R_F value of 0.60 was formed only in *G. applanatum*, which can be used for the authentication of *G. lucidum* and *G. applanatum*.

HPTLC-QDa spectra of Ganoderma species samples

Typical HPTLC-QDa spectra of *G. lucidum* was displayed in Fig. 4. Eleven MS spectra, corresponding to the main bands in HPTLC plate, could be detected in *G. lucidum*. Ganoderic acids, the active component in *G. lucidum*, were concentrated on the bands in the lower HPTLC spectra, while sterols, the diagnostic components in *G. sinense*, were pre-

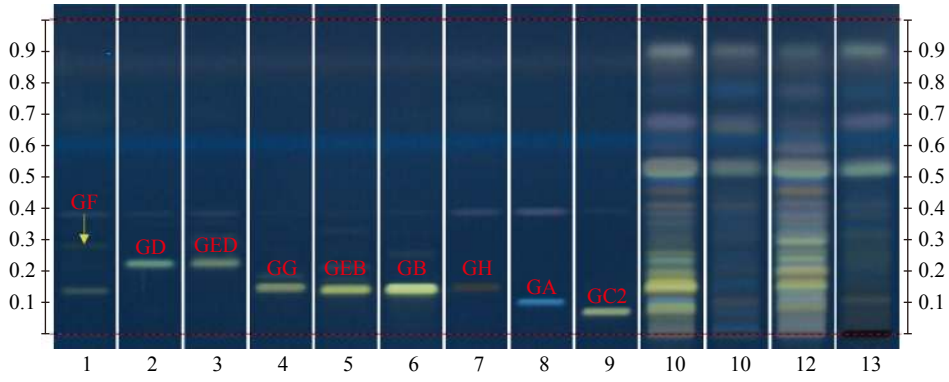

Fig. 2 HPTLC chromatogram of *Ganoderma* species and nine standards under UV light at 366 nm after derivatization on the TLC Visualizer instrument (1-GF; 2-GD; 3-GED; 4-GG; 5-GEB; 6-GB; 7-GH; 8-GA; 9-GC2; 10-*G. lucidum*; 11-*G. sinense*; 12-*G. lucidum* (Antler-shaped); 13-*G. applanatum*)

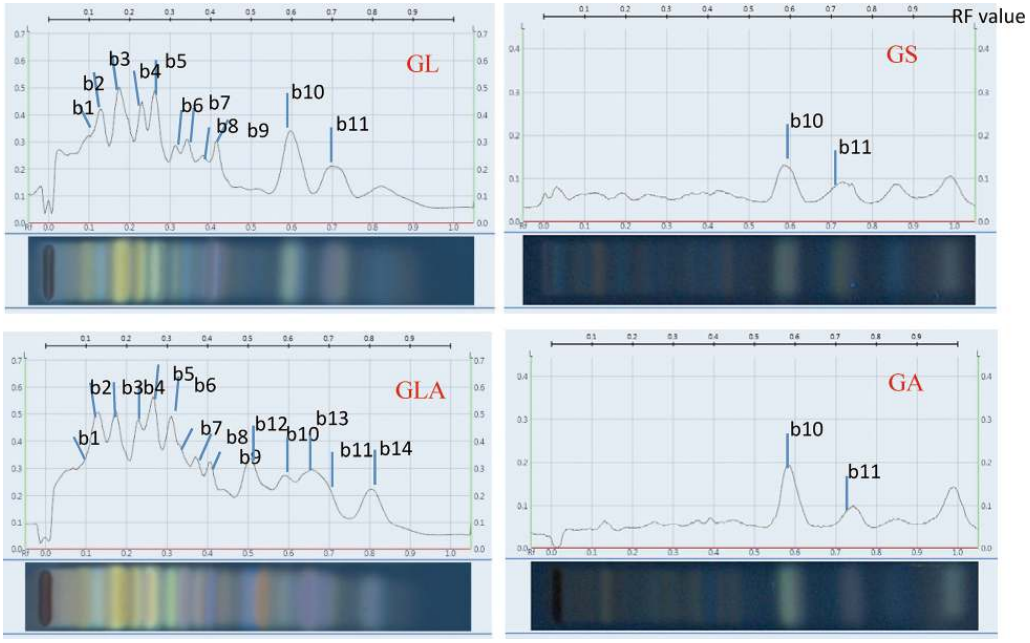

Fig. 3 The HPTLC chromatographic profiles of *G. lucidum* (GL), *G. sinense* (GS), *G. lucidum* (Antler-shaped) (GLA), *G. applanatum* (GA), b1-b14: chromatographic bands obtained UV light at 366 nm after derivatization

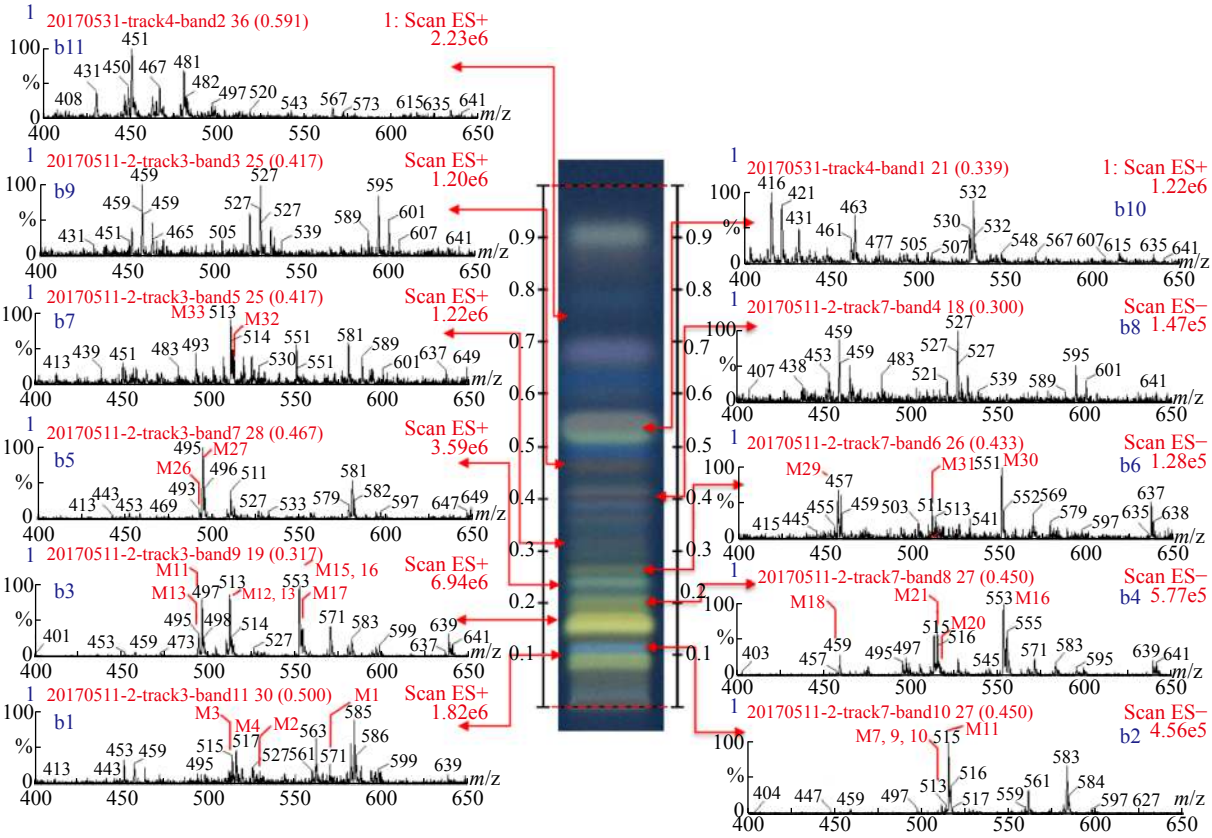

Fig. 4 Typical HPTLC-QDA MS spectra of *G. lucidum*

dominated in the upper spectra, which was consistent with the results after derivatization. And mass data of fourteen bands were recorded in *G. applanatum*. Ganoderic acid A/ B/C2/ D/H/F/G and ganoderenic acid B/D, nine active components used for characterization of *G. lucidum*, all could be observed in the HPTLC-QDa spectra. These compounds were

detected as deprotonated precursor ions at *m/z* 515/515/5 17/513/571/569/531 and 513/511, respectively, in the spectra with the mass consistent with the literatures, and further confirmed with MS/MS in the Orbitrap and the reference substances (Fig.S11). It could be found in the HPTLC-QDa MS chromatogram that co-eluting of the chemical components was ubiquitous in all bands. Taking yellowish band b3 as example, a total of eight ions including two ions at *m/z* 513 $[M-H]^-$, *m/z* 495 $[M-H-H_2O]^-$, two ions at *m/z* 515 $[M-H]^-$, *m/z* 497 $[M-H-H_2O]^-$, *m/z* 571 $[M-H]^-$ and *m/z* 553 $[M-H-H_2O]^-$, were detected in the HPTLC-QDa MS spectra. Among these ions, four were unambiguously identified by comparing with reference standards as ganoderic acid G (*m/z* 513), ganodeneric acid B (*m/z* 495), ganoderic acid B (*m/z* 497) and ganoderic acid H (*m/z* 513), respectively, as illustrated in Fig.S11 and Fig. 5.

A total of 52 compounds, containing 42, 45, 5 and 5 separately exposed from *G. lucidum*, *G. lucidum* (Antler-shaped), *G. applanatum*, and *G. sinense,* and their detailed information was given in Table 1 [28, 29]. Four botanical origins of Ling-zhi only contained 5 common components, while the proportions of characteristic components in *G. lucidum* and *G. lucidum* (Antler-shaped) were 69.8% and 75.4%, respectively. The results demonstrated high chemical diversity

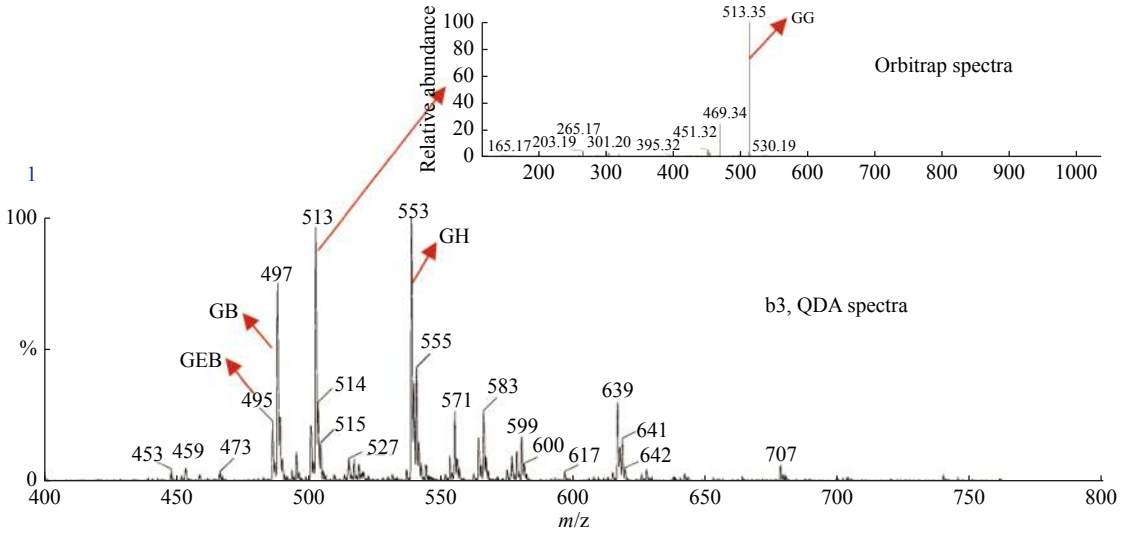

Fig. 5 HPTLC-MS spectra of band b3 obtained by QDa and Orbitrap

Table 1 The HPTLC-QDa and Orbitrap MS/MS data of *G. lucidum* and its counterfeits

Band	R_F value	Compounds	Identification	Molecule formula	MS/MS data				GL[b]	GS[b]	GLA[b]	GA[b]
					m/z	Ion types	Mass error[d]	Orbitrap MS^n				
b1	0.07	M1	12-acetyl-15-hydroxy-3, 7, 11, 23- tetracarbonyllanosta-8-ene-26-acid [28]	$C_{32}H_{44}O_9$	571.2909	$[M-H]^-$	0.551	571, 529, 455, 425	−	−	+	−
		M2	3, 7, 20- trihydroxy -11, 15, 23- tricarbonyllanosta-8-ene-26-acid [28]	$C_{30}H_{44}O_8$	531.2907	$[M-H]^-$	1.145	531, 513, 451, 251	+	−	−	−
		M3	unknown	$C_{30}H_{44}O_7$	515.3031	$[M-H]^-$	5.375	515, 441, 426, 408	+	−	−	−
		M4	ganoderic acid C2[a]	$C_{30}H_{46}O_7$	517.3170	$[M-H]^-$	1.751	517, 499, 437, 407	+	−	+	−
		M5	methyl ganoderic acid K [28]	$C_{32}H_{44}O_{10}$	587.2861	$[M-H]^-$	1.696	587, 569, 551, 509	+	−	+	−
		M6	unknown	$C_{30}H_{44}O_6$	499.3063	$[M-H]^-$	1.771	499, 481, 437, 285	+	−	+	−
b2	0.10	M7	3, 7, 12-trihydroxy-11, 15-dicarbonyllanosta-8, 16, 24-triene-26-acid [28]	$C_{30}H_{42}O_7$	513.2856	$[M-H]^-$	1.792	513, 469, 265, 237	+	−	+	−
		M8	ganodeneric acid C6 [28]	$C_{30}H_{42}O_8$	529.2806	$[M-H]^-$	1.900	529, 511, 467, 437	+	−	+	−
		M9	ganoderic acid AM1	$C_{30}H_{42}O_7$	513.2860	$[M-H]^-$	2.572	513, 451, 436, 421	+	−	−	−
		M10	unknown	$C_{30}H_{42}O_7$	513.2856	$[M-H]^-$	1.792	513, 439, 424, 409	+	−	+	−
		M11	ganoderic acid A[a]	$C_{30}H_{44}O_7$	515.3014	$[M-H]^-$	2.076	515, 497, 435, 405	+	−	+	−

Continued

Band	R_F value	Compounds	Identification	Molecule formula	MS/MS data				GL[b]	GS[b]	GLA[b]	GA[b]
					m/z	Ion types	Mass error[d]	Orbitrap MS^n				
b3	0.15	M12	ganoderic acid G[a]	$C_{30}H_{44}O_8$	531.2960	$[M-H]^-$	1.422	531, 513, 469, 305	+	−	+	−
		M13	ganodeneric acid B[a]	$C_{30}H_{42}O_7$	513.2859	$[M-H]^-$	2.377	513, 495, 451, 287	+	−	+	−
		M14	ganoderic acid B[a]	$C_{30}H_{44}O_7$	515.3007	$[M-H]^-$	0.718	515, 497, 453, 249	+	−	+	−
		M15	ganodeneric acid K [28]	$C_{32}H_{44}O_9$	571.2916	$[M-H]^-$	2.522	571, 553, 467, 290	+	−	+	−
		M16	ganoderic acid H[a]	$C_{32}H_{44}O_9$	571.2915	$[M-H]^-$	2.522	571, 553, 511, 467	+	−	+	−
		M17	ganoderic acid K [28]	$C_{32}H_{46}O_9$	573.3066	$[M-H]^-$	1.379	573, 555, 469, 451	+	−	+	−
		M18	3, 7, -dihydroxy-4, 4, 14-trimethyl-11, 15-dicarbonyllanosta-8-ene-24-acid [28]	$C_{28}H_{42}O_8$	505.2812	$[M-H]^-$	3.711	505, 459, 441	−	−	+	−
b4	0.20	M19	3, 24, 25-trihdroxy- 7-carbonyllanosta -8-ene [28]	$C_{27}H_{40}O_7$	475.2694	$[M-H]^-$	0.779	475, 457, 439, 411	+	−	+	−
		M18	3, 7-dihydroxy-4, 4, 14-trimethyl-11, 15-dicarbonyl cholestane -8-ene-24-acid [28]	$C_{27}H_{40}O_6$	459.2747	$[M-H]^-$	1.273	459, 441, 423, 379	+	−	+	−
		M20	unknown	$C_{29}H_{42}O_8$	517.2794	$[M-H]^-$	−0.376	517, 499, 457, 439	+	−	+	−
		M21	lucidenic acid E [28]	$C_{29}H_{40}O_8$	515.2647	$[M-H]^-$	1.466	515, 473, 443, 425	+	−	+	−
		M22	ganolucidic acid A [28]	$C_{30}H_{44}O_6$	499.3060	$[M-H]^-$	1.171	499, 437, 285, 269	+	−	+	−
b5	0.22	M23	3, 12, 20-trihydroxy-7, 11, 15-tricarbonyl- cholestane -8, 16, 24-triene-26-acid [28]	$C_{30}H_{40}O_8$	527.2646	$[M-H]^-$	1.243	527, 509, 465, 435	+	−	+	−
		M24	ganoderic acid D2 [28]	$C_{30}H_{42}O_8$	529.2802	$[M-H]^-$	1.144	529, 511, 467, 449	+	−	+	−
		M25	unknown	$C_{30}H_{38}O_7$	509.2544	$[M-H]^-$	2.003	509, 465, 435, 417	+	−	−	−
		M26	ganoderenic acid D[a]	$C_{30}H_{40}O_7$	511.2700	$[M-H]^-$	1.897	511, 493, 449, 285	+	−	+	−
		M27	ganoderic acid D[a]	$C_{30}H_{42}O_7$	513.2852	$[M-H]^-$	1.013	513, 495, 451, 247	+	−	+	−
		M28	ganoderic acid J [28]	$C_{30}H_{42}O_7$	513.2852	$[M-H]^-$	1.013	513, 451, 436, 421	+	−	+	−
b6	0.26	M29	lucideric acid A [28]	$C_{27}H_{38}O_6$	457.2596	$[M-H]^-$	2.482	457, 439, 421, 323	+	−	+	−
		M30	ganoderic acid F[a]	$C_{32}H_{42}O_9$	569.2756	$[M-H]^-$	1.916	569, 551, 509, 465	+	−	+	−
		M31	ganoderic acid E [28]	$C_{30}H_{40}O_7$	511.2700	$[M-H]^-$	1.897	511, 449, 434, 419	+	−	−	−
		M28	ganoderic acid J [28]	$C_{30}H_{42}O_7$	513.2859	$[M-H]^-$	1.220	513, 451, 436, 421	+	−	−	−
b7	0.25	M30	unknown	$C_{27}H_{36}O_7$	471.2381	$[M-H]^-$	0.785	471, 441, 423, 379	+	−	+	−
		M31	ganodermanontriol [28]	$C_{27}H_{38}O_7$	473.2537	$[M-H]^-$	0.676	473, 455, 437, 422	+	−	+	−
		M32	12, acetyl-4, 4, 14-trimethyl-3, 7, 11, 15-tetracarbonyl-Cholestane-8-ene-24-acid [28]	$C_{29}H_{38}O_8$	513.2492	$[M-H]^-$	7.164	513, 471, 441, 423	+	−	+	−
		M33	unknown	$C_{27}H_{38}O_7$	473.2537	$[M-H]^-$	0.676	573, 555, 511, 307	+	−	+	−
		M34	ganolucidic acid E [28]	$C_{30}H_{44}O_5$	483.3106	$[M-H]^-$	0.205	483, 287, 271, 256	+	−	+	−
b9	0.40	M35	ganodeneric DM [28]	$C_{30}H_{44}O_4$	467.3158	$[M+H]^+$	0.457	467, 423, 407, 269	+	−	−	−
		M36	3, 15- dihydroxy -7, 11, 23-tricarbonyl Cholestane-8-ene-26-acid [28]	$C_{30}H_{44}O_7$	515.3010	$[M-H]^-$	1.300	515, 497, 435, 405	+	−	−	−
		M37	11- hydroxy -3, 7-dicarbonyl-Cholestane-8, 24-diene-26-acid[c] [28]	$C_{30}H_{42}O_5$	483.3108	$[M+H]^+$	0.619	519, 483, 385, 345	−	−	+	−
		M38	15- acetyl -3, 11-dicarbonyl-Cholestane-8, 24-diene-26-acid[c] [28]	$C_{32}H_{44}O_6$	525.3213	$[M+H]^+$	0.466	525, 483, 287, 271	−	−	+	−

Continued

Band	R_F value	Compounds	Identification	Molecule formula	MS/MS data				GL[b]	GS[b]	GLA[b]	GA[b]
					m/z	Ion types	Mass error[d]	Orbitrap MS^n				
		M43	unknown[c]	$C_{30}H_{42}O_3$	451.3190	$[M+H]^+$	−3.704	451, 433, 415	+	+	+	+
b10	0.53	M44	3, 15-dioxo-(24E)-lanosta-7, 9(11), 24-trien-26-oate de methyle[c]	$C_{31}H_{44}O_4$	481.3318	$[M+H]^+$	1.171	481, 355, 323, 225	+	+	+	+
		M45	(24E)-3β-acetoxylanosta-7, 9(11), 24-trien-26-oic acid[c]	$C_{32}H_{48}O_4$	497.3637	$[M+H]^+$	1.164	497, 465, 433, 377	+	+	+	+
b11	0.60	M46	unknown[c]	$C_{25}H_{46}O_8$	475.3271	$[M+H]^+$	1.168	475, 301, 287	−	−	+	−
		M47	lucidenic lactone[c]	$C_{27}H_{40}O_7$	477.2852	$[M+H]^+$	1.089	477, 445, 317, 197	−	−	+	−
		M40	unkown[c]	$C_{32}H_{46}O_3$	479.3485	$[M+H]^+$	−8.620	479, 461, 447	−	−	+	−
b12	0.46	M41	methyl lucidenate C[c]	$C_{28}H_{42}O_7$	491.3009	$[M+H]^+$	1.160	491, 459, 427, 395	−	−	+	−
		M42	ganodermic acid TQ[c] [29]	$C_{32}H_{46}O_4$	495.2990	$[M+H]^+$	1.037	495, 463, 431, 413	−	−	+	−
b13	0.69	M48	unknown[c]	$C_{22}H_{38}O_7$	415.2701	$[M+H]^+$	2.577	415, 302, 190, 172	+	+	+	+
		M49	unknown[c]	$C_{30}H_{38}O_4$	463.2808	$[M+H]^+$	−3.486	463, 355, 229	+	+	+	+
		M50	unknown[c]	$C_{28}H_{46}O_6$	478.3174	$[M+H]^+$	−3.232	479, 461, 447	−	−	+	−
b14	0.79	M51	methyl lucidenate C[c]	$C_{32}H_{42}O_4$	491.3119	$[M+H]^+$	−3.636	491, 459, 427, 395	−	−	+	−
		M52	unknown[c]	$C_{27}H_{42}O_8$	495.2990	$[M+H]^+$	3.795	495, 463, 431	−	−	+	−

a Compounds confirmed by comparing with standards; b GL = G. lucidum; GS = Ganoderma sinensis; GLA = Ganoderma lucidum antler; GA = Ganoderma applanatum; c positive ion mode d ppm

among four botanical origins of Ganoderma.

HPTLC-QDa for authentication of G. lucidum

It could be found in the HPTLC-QDa detection results (Table 1) that numbers of active components of *G. lucidum* and *G. lucidum* (Antler-shaped) were significantly higher than *G. applanatum* and *G. sinense*. The acquired HPTLC-QDa spectra showed that band b3, containing ganoderic acid B/G/H and ganodeneric acid B, the major active components of Ganoderma, could be found only in *G. lucidum* and *G. lucidum* (Antler-shaped), but not in *G. sinense* and *G. applanatum*. Moreover, bands b13 and b14 with *m/z* 475/477 and *m/z* 475/491/495, respectively, were observed in *G. lucidum* (Antler-shaped), but not in *G. lucidum*, thus allowing simple and robust authentication of *G. lucidum* from its counterfeits.

HPTLC quantitative analysis of ganoderic acids in G. lucidum from different origins.

To quantitatively compare the contents of ganoderic acids of *G. lucidum* from different geographical origins, a semi-quantitative method was proposed to evaluate the total content of ganoderic acids with ganoderic acid A as the external standard based on HPTLC instrument. These quantitative bands were detected as ganoderic acids by comparing R_F with reference standards, and further confirmed with MS data in HPTLC-QDa MS spectra. Then the HPTLC spectrum were processed to obtain HPTLC chromatograph by CAMAG VideoScan software Version 2.1.17207.2. After background correction, smoothing, noise reduction and data shearing procedures, the peak areas of all targeted bands were picked from chromatograph and total area of ganoderic acids was used to determine the content by external standard method. Conversion factors were set as 1. Ultimately, the chromatograph converted by software was shown in Fig. 6, and the content results were listed in Table 2. Considering co-eluting phenomenon observed in all bands, the HTPLC semi-quantitative results were further compared to the content obtained by HPLC method in our previous investigation recorded in Diet-

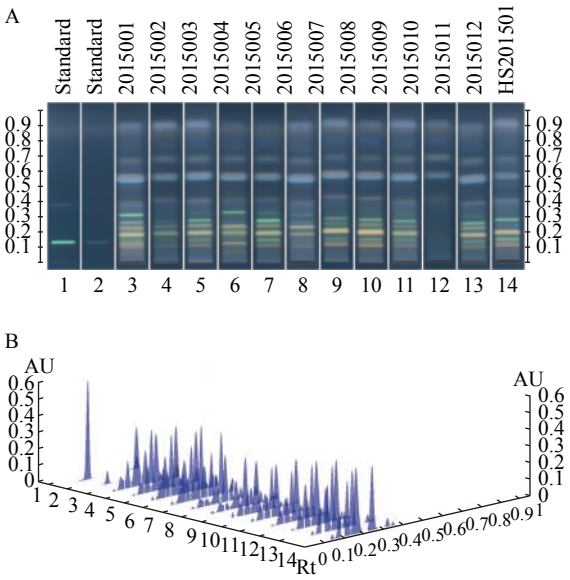

Fig. 6 HPTLC semi-quantitative results of ganoderic acids: A, the HTPLC spectra of twelve *G. lucidum* samples, B, Peaks of ganoderic acids extracted by CAMAG VideoScan software

Table 2 HPTLC semi-quantitative and HPLC quantitative results of ganoderic acids in twelve *Ganoderma* samples (n = 2)

No.	Collection	Source	HPTLC	HPLC	SD
2015001	Shanxi Provinces	*Ganoderma lucidum*	0.37%	0.41%	0.03%
2015002	Anhui Provinces	*Ganoderma lucidum*	0.16%	0.12%	0.02%
2015003	Shandong Provinces	*Ganoderma lucidum*	0.33%	0.27%	0.04%
2015004	Shandong Provinces	*Ganoderma lucidum*	0.26%	0.19%	0.05%
2015005	Dongbei Provinces	*Ganoderma lucidum*	0.36%	0.37%	0.01%
2015006	Shandong Provinces	*Ganoderma lucidum*	0.21%	0.12%	0.06%
2015008	Yunnan Provinces	*Ganoderma lucidum*	0.38%	0.37%	0.01%
2015009	/	*Ganoderma lucidum*	0.42%	0.44%	0.02%
2015010	Jiangsu Provinces	*Ganoderma lucidum*	0.27%	0.25%	0.02%
2015011	Guangxi Provinces	*Ganoderma sinense*	0.00%	0.00%	0.00%
2015012	Anhui Provinces	*Ganoderma lucidum*	0.29%	0.26%	0.02%
HS001	Anhui Provinces	*Ganoderma lucidum*	0.40%	0.41%	0.01%

ary Supplements of United States Pharmacopeia (Table 2 and Fig.S12), and it was found that as an innovative tool to substitute, at least in part, the HTPLC semi-quantitative method could determine the content of ganoderic acids in *G*. lucidum. As shown in Fig. S12, the total content of ganoderic acids from the same geographical origins were not consistent, indicating that samples from different geographical origins could not be differentiated only by their content of total ganoderic acids. Moreover, the content of ganoderic acids of *G. lucidum* were no less than 0.1%, except sample 10, indicating that it may be confused *Ganoderma* species which was further confirmed by HPTLC-QDa method.

Conclusion

In this study, a HPTLC-QDa based analytical method was developed for robust authentication of *Ganoderma* species. *G. lucidum* could be unambiguously differentiated with *G. applanatum*, *G. sinense* and *G. lucidum* (Antler-shaped) based on the detection of the major bands and metabolite makers that presented in HTPLC plates. In addition, HPTLC-QDa was proven to be an alternative powerful mean in profiling and characterization of multi-components in the four *Ganoderma* species. As a result, a total of 52 compounds were identified or tentatively characterized, including 9 identified by direct comparison with reference standards. The method is attested as simple, robust, and can be further extended to analyze other herbal medicines.

Supplementary Materials

Supplementary materials are available as Supporting Information, and can be requested by sending E-mail to the corresponding author.

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Cite this article as: YAO Shuai, ZHANG Jian-Qing, HOU Jin-Jun, HU Xiao-Su, WANG Ling, DA Juan, RAO Wei, WANG Dan-Dan, HUANG Yong, WU Wan-Ying, GUO De-An. Simple and robust differentiation of *Ganoderma* species by high performance thin-layer chromatography coupled with single quadrupole mass spectrometry QDa [J]. *Chin J Nat Med*, 2021, **19**(4): 295-304.