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Rapid Differentiation of *Ganoderma* Species by Direct Ionization Mass Spectrometry

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Abstract

In this study, direct ionization mass spectrometry (DI-MS) has been developed for rapid differentiation of *Ganoderma* (known as *Lingzhi* in Chinese), a very popular and valuable herbal medicine. Characteristic mass spectra can be generated by DI-MS directly from the raw herbal medicines with the application of a high voltage and solvents. Rapid differentiation of the *Ganoderma* species that are officially stated in the Chinese pharmacopoeia from easily confused *Ganoderma* species could be achieved based on this method, as the acquired DI-MS spectra showed that ganoderic acids, the major active components of *Ganoderma*, could be found only in the official *Ganoderma* species but not in the confused *Ganoderma* species. In addition, classification of wild and cultivated *Ganoderma* and potential differentiation of *Ganoderma* from different geographical locations could be accomplished based on principal component analysis (PCA) or hierarchical clustering analysis (HCA). The method is rapid, simple and reproducible, and can be further extended to analysis of other herbal medicines.

Keywords: Mass spectrometry; Direct ionization; *Ganoderma*; Active components; Differentiation.

1. Introduction

Ganoderma (also known as *Lingzhi* in Chinese) is one of the most popular and valuable herbal medicines, and has been used as folk medicine in China for thousands of years [1]. As early as 200 A.D., *Ganoderma* was cited in the *Shen Nong's Herbal Classics*, which is widely considered as the oldest Chinese book on oriental herbal medicines agriculture and the foundation of traditional Chinese medicines. *Ganoderma* is used for enhancing "vital energy" of peoples and promoting "longevity" [2], and is a valuable medicinal mushroom widely used in countries such as China, Korea and Japan [3]. The global sale of products derived from *Ganoderma* exceeded 2.5 billion US dollars each year [4]. *Ganoderma* is a genus of polypore mushrooms that grow on wood, mostly on deciduous trees such as maple, chestnut and beech in subtropical regions [5]. *Ganoderma* belongs to the Ganodermataceae family of the Fungi kingdom, and there are approximately 80 *Ganoderma* species in the world while only about 20 species are used for medical purposes [6]. In China, only 2 *Ganoderma* species are officially described in Chinese Pharmacopoeia [7], i.e. *Ganoderma lucidum* (Leyss. ex Fr.) (*Chizhi* in Chinese) [8] and *Ganoderma sinense* (*Zizhi* in Chinese) [9], which are the most popularly used *Ganoderma* in the market and are cultivated in many countries due to their health benefits and commercial values [10]. Owing to the increasing demand of *Ganoderma* and the limited supply and extremely high prices of wild *Ganoderma*,

cultivation has become the major source of *Ganoderma* since early 1970s [11]. A variation of cultivated *Ganoderma* is called antler-shaped *Ganoderma ludicum*, which is grown by varying the cultivation conditions such as the carbon dioxide levels, light, humidity or pH, leading to an antler-shaped appearance of the herb [12]. Studies have revealed that *Ganoderma* exhibited therapeutic effects such as anti-inflammation [13], anti-tumor [13], anti-HIV [14], anti-hypertension [15], anti-bacteria [16], anti-diabetic [17], anti-aging [18], immune system-enhancing activities [16], hepatoprotective activities [19] and anti-hypercholesterolemic activities [20]. Ganoderic acids are the major active components of *Ganoderma* with structures closely related to tetracyclic triterpenoids [21].

However, some other *Ganoderma* species [22-24], such as *Ganoderma atrum*, *Ganoderma duropora* Lloyd and *Ganoderma applanatum*, which have appearances similar to *Ganoderma lucidum* and *Ganoderma sinense* (see Fig. S1 in the Supporting Information for photos of various *Ganoderma* species) but few reported significant pharmacological values [25], are commonly found as the adulterants to the official *Ganoderma* species, which are generally several times higher in prices in the market. Under the increased profitability of trading *Ganoderma* and the global demands for *Ganoderma*, the problem of species confusion of *Ganoderma* is getting serious.

Ganoderma of different species, including the official and confused species of *Ganoderma,* have now flooded into the market, and it is difficult to distinguish them based on traditional morphological inspection. Particularly, most of the commercial *Ganoderma* products are in slice form, which makes accurate differentiation more difficult. Mislabeling and misuse of *Ganoderma* species would be a potential threat to product safety [26]. In the other hand, more than 70 % of *Ganoderma* are cultivated in the northern and middle areas in China, including Zhejiang, Hubei and Shandong provinces [25]. *Ganoderma* originating from different geographical locations is believed to have different quality and different curative effects, and thus differ in selling prices. However, it is not easy to determine the geographical origins with the existing analytical tools as well as visible inspection. Therefore, it is necessary to develop a reliable, simple and rapid analytical method to differentiate official species from confused species and wide type from cultivated type, and discriminate the origins of *Ganoderma*.

Various methods, including taxonomy [27], morphology [28], microscopy [29], DNA technology [30] and fingerprint chromatography [31, 32], have been developed for species differentiation of *Ganoderma*, Among these methods, fingerprint chromatography can provide precise information about the chemical ingredients present

in *Ganoderma*, and thus is commonly adopted for species differentiation of *Ganoderma*. However, this method requires sample homogenization and extraction, separation of components using techniques such as liquid chromatography (LC) [33], gas chromatography (GC) [34] and capillary electrophoresis (CE) [35], and detection using techniques such as diode array detection (DAD) [36] and mass spectrometry (MS) [37-39], and is relatively time-consuming and laborious. In the present study, direct ionization mass spectrometry (DI-MS), a simple and rapid technique that requires no or only minimal sample preparation for analysis of raw samples [40-44], was developed for rapid differentiation of *Ganoderma* species. Compared to other methods, DI-MS allows direct analysis of raw *Ganoderma* samples, and in a short period of time can generate mass spectra that contain chemical compositions of *Ganoderma* species and can serve as the fingerprints for characterization of the species. As a powerful technique for analysis of herbal medicines, DI-MS has not been used for analysis of *Ganoderma* yet. We demonstrated that DI-MS could unequivocally differentiate official and confused *Ganoderma* species based on their mass spectra. Meanwhile, wild and cultivated *Ganoderma* and potentially *Ganoderma* from different geographical origins could also be distinguished based on principal component analysis (PCA) or hierarchical clustering analysis (HCA) of the spectra.

2. Experimental

2.1. Chemicals and materials

Samples of *Ganoderma* species from different geographical origins of China were provided by the local farmers and manufactures or purchased from authentic Chinese herbal medicine pharmacies (see Table S1 for the sample information). The identities of the samples were confirmed by Mr. Xiang Liu, Mr. Songyun Qin, Mr. Zaibo Yu and Mr. Jifeng Zhao, who are experts in species identification of Chinese medicinal herbs. All the *Ganoderma* samples were sealed in bags and stored in an electronic dry cabinet before analysis. Sodium iodide used for external calibration of mass spectrometer was purchased from Panreac Química (Barcelona, Spain). Methanol and all the other solvents used in this study were of HPLC grade and purchased from Tedia (Fairfeild, OH, USA). All chemicals were used directly without further purification.

2.2. Setup for DI-MS analysis of Ganoderma samples

The schematic diagram of experimental setup for DI-MS analysis of *Ganoederma* samples is shown in Fig. 1. A small piece of raw *Ganoederma* sample was cut into a sharp triangular shape (about 1 cm for the height and about 0.5 cm for the base width) and placed orthogonal to the mass spectrometer inlet by using a metal clip, with distances of 1 cm in horizontal and 0.5 cm in vertical from the sample tip to the MS inlet (see Fig. S2 for photo of the experimental setup). A crocodile clip was used to

connect the high voltage supply of the mass spectrometer to the metal clip. With the application of a high voltage (3.5 kV) and some solvents (10 μL) to the center of the *Ganoederma* sample, spray ionization could be induced from the sharp end of the herbal medicine to generate mass spectra.

2.3. Mass spectrometric measurements

Mass spectra were acquired on a QToF II mass spectrometer (Waters, Milford, MA) using positive ion mode. Instrumental control and data acquisition were conducted by using MassLynx 4.1 software. Voltage of microchannel plate (MCP) detector, sample cone voltage and source temperature were set at 2.1 kV, 30 V and 40 ºC, respectively. High voltage applied to the samples was typically set at 3.5 kV, a voltage obtained after optimization. Mass spectra were acquired in an *m/z* range of 100 - 1000 Da with an interscan time of 0.1 s and a scan time of 1 s. Typically, mass spectra were generated by accumulating data from the first minute. Before DI-MS analysis, sodium iodide was used for *m/z* calibration of the instrument.

2.4. Principal component analysis (PCA) and hierarchical clustering analysis (HCA)

Principal component analysis (PCA) and hierarchical clustering analysis (HCA) were carried out using Umetrics SIMCA 14 software. For each DI-MS spectrum, the

normalized intensities (absolute intensity of the peak observed / total absolute intensity of all peaks observed in the mass spectrum) of the monoisotopic peaks with signal intensities higher than 5% were input to the software for the analysis.

3. Results and discussion

3.1. Optimization for DI-MS analysis of Ganoderma samples

Stable spray ionization with strong signal is crucial for DI-MS analysis of *Ganoderma* samples. The experimental conditions for this were thus optimized before the sample analysis. Important factors, including the configuration of the experimental setup, the high voltage and the extraction and ionization solvent, were optimized for DI-MS analysis of *Ganoderma* samples.

For the distance between the sample and MS inlet and the high voltage applied onto the sample, it was found that when the sample was placed > 1 cm for the height and > 0.5 cm for the base width from the MS inlet or the applied voltage was lower than 3.5 kV, no or very weak ion signals were obtained, and when the sample was placed < 1 cm for the height and < 0.5 cm for the base width to the MS inlet or the applied voltage was higher than 3.5 kV, electrical discharge was observed. Optimal MS signals were obtained when the sample tips were placed perpendicular to the MS inlet with the

distances of 0.5cm in y-coordinate and 1 cm in x-coordinate (see Fig. S2), and with the high voltage of 3.5 kV in positive ion mode.

The added solvent served for extraction of compounds from the sample and ionization of the extracted compounds during DI-MS analysis. In this study, solvents of different polarities and acidities, including water, methanol/water (1/1), acetonitrile/water (1/1), methanol with 0.1% formic acid, methanol, acetonitrile, ethanol, chloroform, dichloromethane and hexane, were used for DI-MS analysis of a cultivated *Ganoderma lucidum* sample and the resulting spectra are shown in Fig. S3. Ganoderic acids, the major active components of *Ganoderma,* were detected and the signal intensities of the DI-MS spectra for the cultivated *Ganoderma lucidum* are summarized in Table 1. The ion profiles of the spectra varied significantly with the solvents used. Ganoderic acids were abundantly detected as the base peaks when methanol with 0.1% formic acid, methanol and acetonitrile were used. However, the signals of ganoderic acids were significantly suppressed and choline [45] cation was remarkably observed as the base peaks when more polar solvents, i.e. water, methanol/water (1/1) and acetonitrile/water (1/1) were used, indicating that solvents of higher polarity favored the extraction and ionization of ionic compounds. On the other hand, when less polar or non-polar solvents, i.e. ethanol, chloroform, dichloromethane and hexane were used, triglycerides [46]

would be detected as the base peaks and the signal intensities of ganoderic acids were also significantly suppressed, indicating that solvents of lower polarity favored the extraction and ionization of relatively non-polar compounds. Methanol with 0.1% formic acid was eventually chosen as the solvent for subsequent DI-MS analysis of the *Ganoderma* samples because it allowed detection of ganoderic acids with the highest signal intensity. The added low level of formic acid was believed to facilitate the ionization of the analytes [47].

3.2. DI-MS spectra of the Ganoderma samples

Typical DI-MS spectra of various *Ganoderma* species are shown in Fig. 2. Ganoderic acids, the major active components used for characterization of *Ganoderma*, could be observed in the DI-MS spectra. For two official *Ganoderma* species stated in the Chinese pharmacopoeia, i.e. *Ganoderma lucidum* and *Ganoderma sinense*, distinct peaks, with masses corresponding to potassium adducts of different ganoderic acids including ganoderic acids E (*m/z* 551.2), AM_1/C_1/J (*m/z* 553.3), A/B (*m/z* 555.3), C/C_2 (*m/z* 557.3), D/M/N (*m/z* 569.3), G/I (*m/z* 571.3), L (*m/z* 573.3), F (*m/z* 609.2), H (*m/z* 611.3) and K (*m/z* 613.3), were observed (see Table S2 for the summarized accurate mass data of the observed ions and Table S3 for their chemical structures). Choline cation [45] (*m/z* 104.1) and mannitol [48] (*m/z* 221.1) were also detected in the DI-MS

spectra. Isomers of each ganoderic acid were detected as a single peak in the DI-MS spectrum, and their differentiation probably could be achieved by using ion mobility mass spectrometry [49]. The predominance of potassium adduct ions in the DI-MS spectra was believed to be related with the presence of potassium salts in the herbal samples and the use of the relatively polar solvent that favored the extraction of these salts.

Three independent measurements were conducted for different slices of the same *Ganoderma* sample to investigate the reproducibility of the technique. Taking sample 5 (wild *Ganoderma lucidum*) and sample 6 (cultivated *Ganoderma lucidum*) as examples, similar peak profiles with comparable ion intensities were obtained in the DI-MS spectra from the three independent analyses (see Fig. S4), demonstrating a high level of reproducibility of the DI-MS technique.

3.3. Differentiation of official and confused Ganoderma species

DI-MS was applied to analyze official *Ganoderma* species stated in the Chinese pharmacopoeia, i.e. *Ganoderma lucidum* and *Ganoderma sinense*; and three easily confused species, i.e. *Ganoderma atrum*, *Ganoderma duropora* Lloyd and *Ganoderma applanatum*. As shown in Fig. 2, the three confused *Ganoderma* species gave spectral

profiles that were obviously different from those for the official *Ganoderma* species. Ganoderic acids, the major active components of *Ganoderma*, could not be detected for the three confused species but were abundantly detected for the two official species (Fig. 2), allowing unambiguous differentiation of the confused species from the official species.

3.4. Differentiation of different species, and wild and cultivated species

PCA plot of the two official *Ganoderma* species was generated from the first and second principal components based on their DI-MS data. As shown in Fig. 3a, the clusters of different *Ganoderma lucidum* and *Ganoderma sinense* species (wild, cultivated and antler-shaped) are well separated in the PCA plot. The clusters of wild *Ganoderma lucidum* and wild *Ganoderma sinense* samples are located in quadrant I and right-hand side of quadrant IV of the PCA plot, respectively, while cultivated *Ganoderma lucidum*, *Ganoderma lucidum* (antler-shaped) and cultivated *Ganoderma sinense* samples are located in quadrant II, III and left-hand side of quadrant IV of the PCA plot, respectively, allowing unequivocal differentiation among them.

Similar PCA results were obtained for the confused *Ganoderma* samples, as shown in Fig. 4a. The clusters of *Ganoderma applanatum*, *Ganoderma duropora* Lloyd and

Ganoderma atrum samples were located separately in quadrant I, III and IV of the PCA plot, respectively. For *Ganoderma applanatum*, only the wild samples were analyzed in this study, since *Ganoderma applanatum* is seldom cultivated. For *Ganoderma duropora* Lloyd, although the clusters of the wild and cultivated samples were located at the same quadrant in the PCA plot, they were well separated to allow the differentiation. However, the clusters of the wild and cultivated samples of *Ganoderma atrum* were not clearly separated, due to the high degree of similarity of their spectra as shown in Figs. 2f and 2g.

HCA, another commonly used statistics tool [50], was also employed to analyze the DI-MS results. As shown in Figs. 3b and 4b, results similar to those obtained from PCA analysis were obtained by using HCA. The dendrograms used in the HCA plots allowed clear groupings of the *Ganoderma* samples.

3.5. Differentiation of species from different origins

As shown in Fig. 3b, the HCA plot of the two official *Ganoderma* species shows that the sample data from the same geographical origins are grouped together within the brackets, indicating that samples from different geographical origins could be differentiated for both wild and cultivated *Ganoderma lucidum* and *Ganoderma sinense*

samples. For example, seven cultivated *Ganoderma lucidum* samples from Anhui (samples 6-12) are grouped together, showing clear difference from the others. Similar results could also be observed from the HCA plot of the confused *Ganoderma* species (Fig. 4b) and the PCA plots of both official and confused *Ganoderma* species (Figs. 3a and 4a). Although only limited numbers of samples were available for each sample source, it can be seen that the sample points of the same sources are clustered together. For example, for wild *Ganoderma lucidum*, two samples from Sichuan (samples 2 & 3) and two samples from Zhejiang (samples 4 & 5) are each clustered together and separate from each other in the PCA plot (Fig. 3a), suggesting that the origins of these wild *Ganoderma* samples could be distinguished by DI-MS. However, in general, more samples are needed to further validate differentiation of the sample origins by the method.

4. Conclusions

In this study, a DI-MS-based method has been developed for rapid differentiation of *Ganoderma* species. Official *Ganoderma* species stated in the Chinese pharmacopoeia and confused *Ganoderma* species could be unambiguously differentiated based on the detection of the major active components in the DI-MS spectra. Differentiation between wild and cultivated species, and potentially discrimination of species from different

geographical origins could also be achieved based on the DI-MS analysis combining with PCA or HCA analysis. The method is simple, rapid and reproducible, and can be easily adopted by researchers in relevant fields. Considering the large market and high analytical demand of *Ganoderma*, this method is expected to have positive impact on the *Ganoderma* industry. The method can also be further expanded for analysis of other herbal medicines as well as other plant and animal samples [51-54], and has great potential applications in the future.

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Table 1. Summary of the detectability of ganoderic acids and signal intensities of the DI-MS spectra obtained with the cultivated *Ganoderma lucidum* and various solvents.

Solvent	Ganoderic acids	Base peak	Signal intensity
Methanol with 0.1% formic acid	✓	Ganoderic acid	Strongest
Methanol	✓	Ganoderic acid	Strong
Acetonitrile	✓	Ganoderic acid	Medium
Water	✓	Choline	Strong
Methanol/water (1/1)	✓	Choline	Strong
Acetonitrile/water (1/1)	✓	Choline	Strong
Ethanol	✓	Triglyceride	Medium
Chloroform	✓	Triglyceride	Weak
Dichloromethane	✓	Triglyceride	Weak
Hexane	✓	Triglyceride	Weak

FIGURE CAPTIONS

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

Fig. 2. DI-MS spectra of the official *Ganoderma* species: (a) wild *Ganoderma lucidum*, (b) cultivated *Ganoderma lucidum*, (c) *Ganoderma lucidum* (antler-shaped), (d) wild *Ganoderma sinense,* (e) cultivated *Ganoderma sinense*; and DI-MS spectra of three easily confused species: (f) wild *Ganoderma atrum*, (g) cultivated *Ganoderma atrum*, (h) wild *Ganoderma duropora* Lloyd, (i) cultivated *Ganoderma duropora* Lloyd, and (j) wild *Ganoderma applanatum*.

Fig. 3. (a) PCA plot and (b) HCA plot of *Ganoderma lucidum* (labeled in red) and *Ganoderma sinense* (labeled in purple) samples based on their DI-MS data. Samples 1-5 are wild *Ganoderma lucidum* samples, samples 6-24 are cultivated *Ganoderma lucidum* samples, samples 25-26 are *Ganoderma lucidum* (antler-shaped) samples (labeled in orange), samples 27-28 are wild *Ganoderma sinense* samples, and samples 29-31 are cultivated *Ganoderma sinense* samples.

Fig. 4. (a) PCA plot and (b) HCA plot of the three easily confused species, i.e. *Ganoderma atrum* (labeled in black), *Ganoderma duropora* Lloyd (labeled in brown) and *Ganoderma applanatum* (labeled in green) samples, based on their DI-MS data. Sample 32 is wild *Ganoderma atrum* sample, samples 33-35 are cultivated *Ganoderma atrum* samples, sample 36 is wild *Ganoderma duropora* Lloyd sample, samples 37-41 are cultivated *Ganoderma duropora* Lloyd samples, and samples 41-42 are wild *Ganoderma applanatum* samples.

Fig. 1.

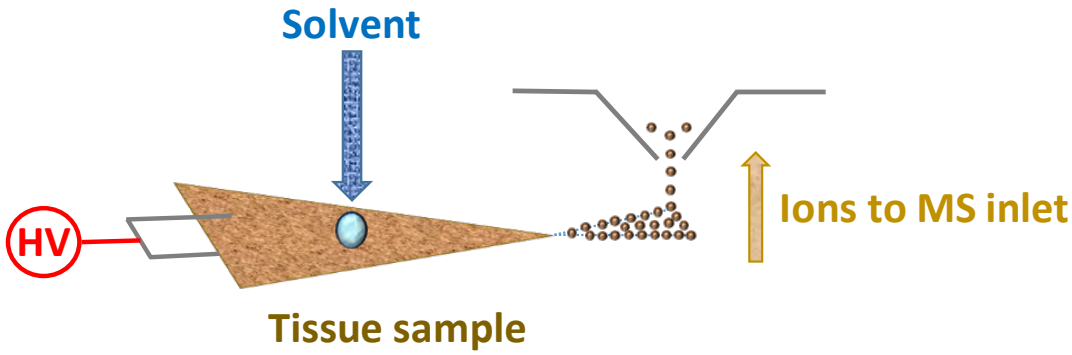


Fig. 2.

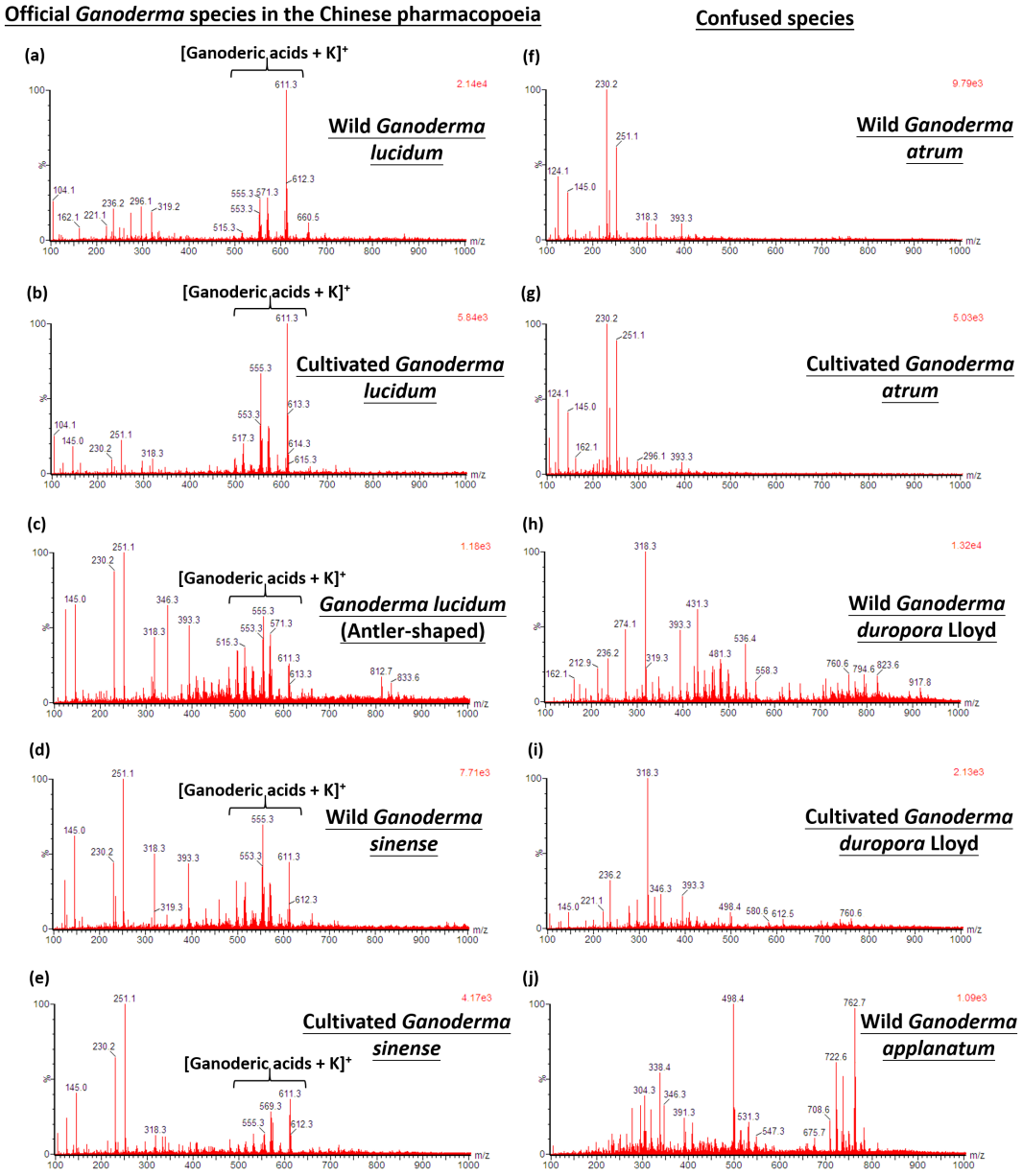

Official Ganoderma species in the Chinese pharmacopoeia
Confused species
(a)
[Ganoderic acids + K]+
Wild Ganoderma lucidum
(b)
[Ganoderic acids + K]+
Cultivated Ganoderma lucidum
(c)
[Ganoderic acids + K]+
Ganoderma lucidum (Antler-shaped)
(d)
[Ganoderic acids + K]+
Wild Ganoderma sinense
(e)
Cultivated Ganoderma sinense
[Ganoderic acids + K]+
(f)
Wild Ganoderma atrum
(g)
Cultivated Ganoderma atrum
(h)
Wild Ganoderma duropora Lloyd
(i)
Cultivated Ganoderma duropora Lloyd
(j)
Wild Ganoderma applanatum
m/z

Fig. 3.

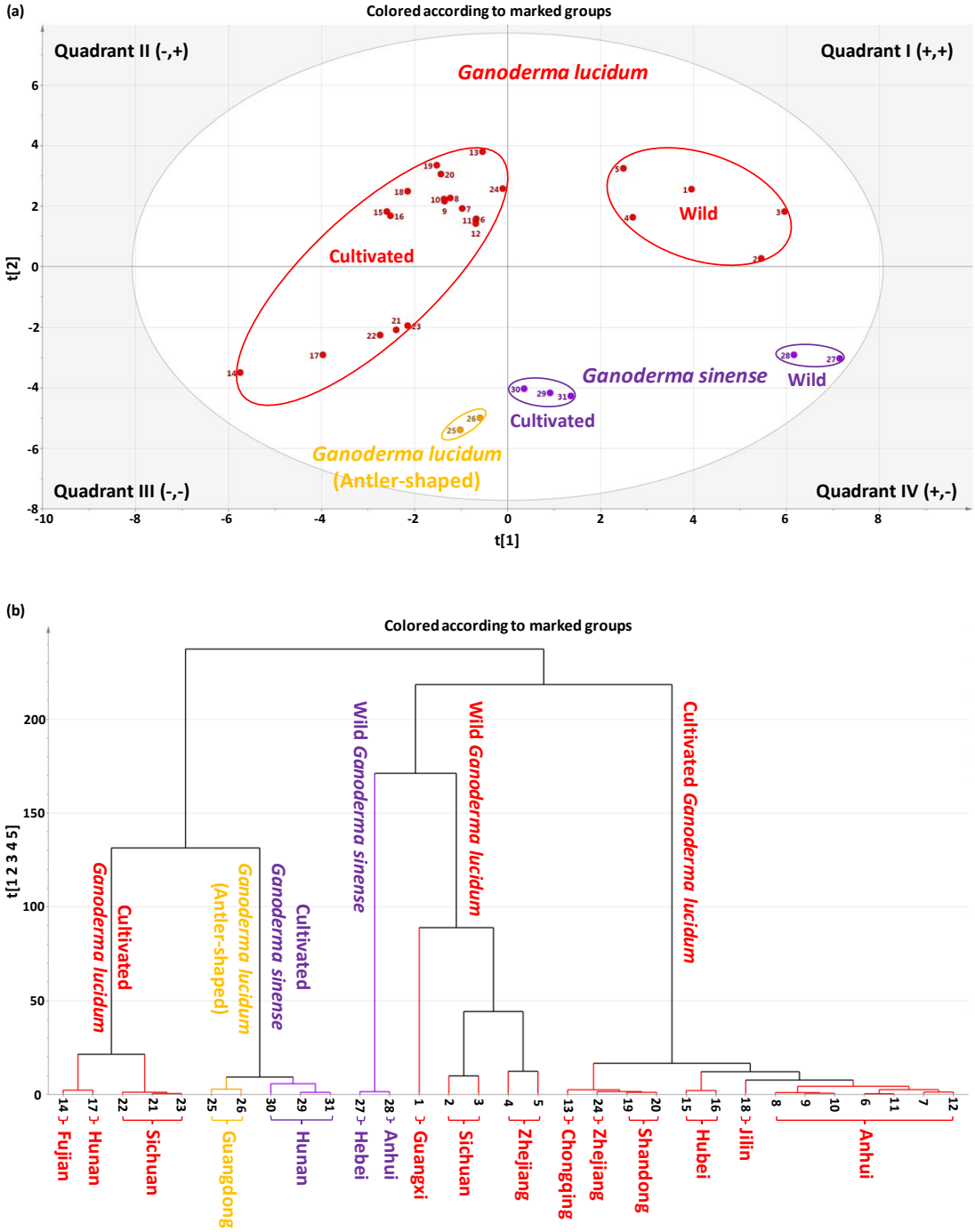
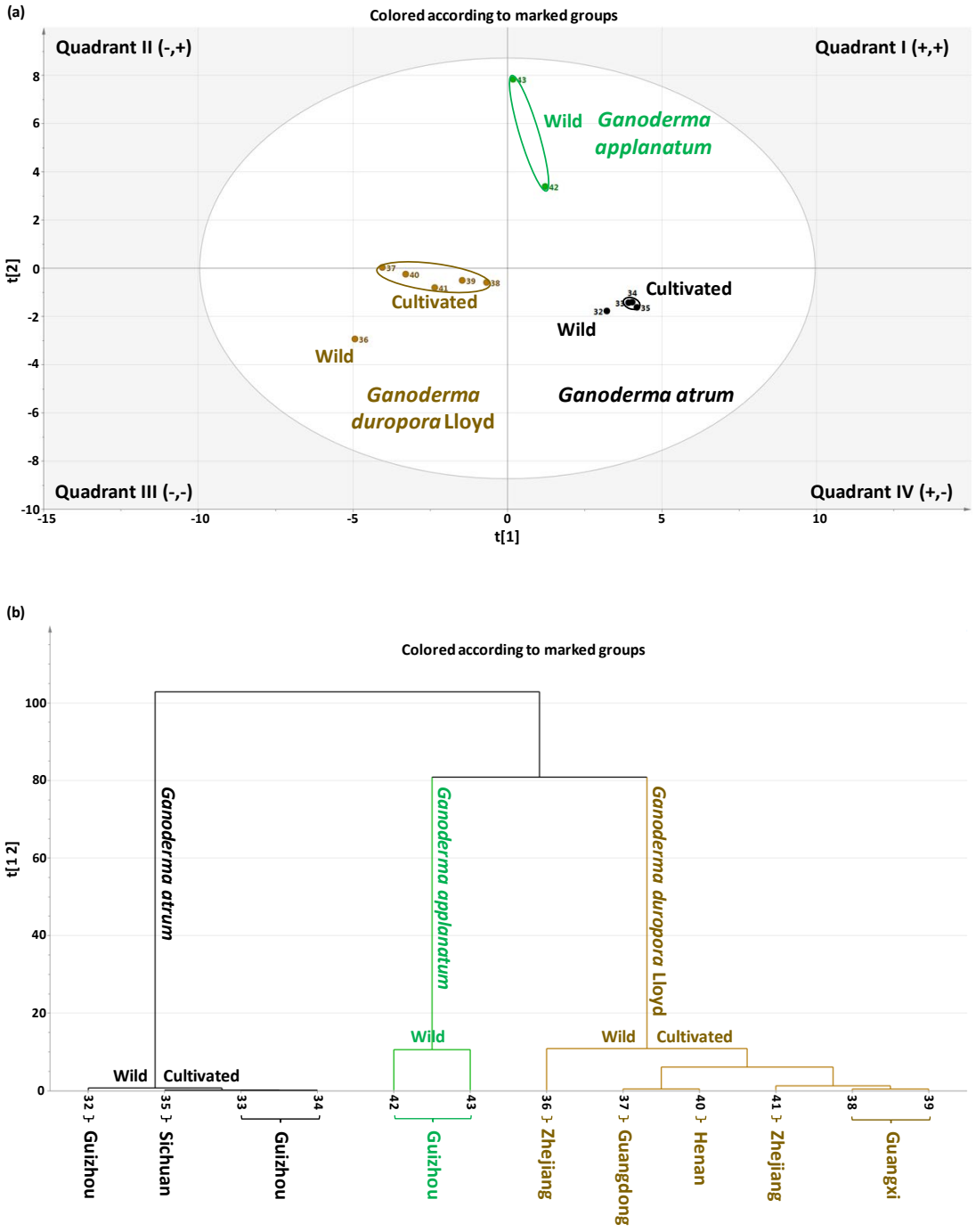

(a)
Colored according to marked groups
Quadrant II (-,+)
Quadrant I (+,+)
Quadrant III (-,-)
Quadrant IV (+,-)
Ganoderma lucidum
Cultivated
Wild
Ganoderma sinense
Wild
Cultivated
Ganoderma lucidum
(Antler-shaped)
t[1]
t[2]
(b)
Colored according to marked groups
t[1 2 3 4 5]
Ganoderma lucidum Cultivated
Ganoderma lucidum (Antler-shaped)
Ganoderma sinense Cultivated
Wild Ganoderma sinense
Wild Ganoderma lucidum
Cultivated Ganoderma lucidum
Fujian
Hunan
Sichuan
Guangdong
Hunan
Hebei
Anhui
Guangxi
Sichuan
Zhejiang
Chongqing
Zhejiang
Shandong
Hubei
Jilin
Anhui

Fig. 4.


(a)
Colored according to marked groups
Quadrant II (-,+)
Quadrant I (+,+)
Quadrant III (-,-)
Quadrant IV (+,-)
Wild
Ganoderma applanatum
Cultivated
Wild
Cultivated
Wild
Ganoderma duropora Lloyd
Ganoderma atrum
t[1]
t[2]
(b)
Colored according to marked groups
Ganoderma atrum
Ganoderma applanatum
Ganoderma duropora Lloyd
Wild
Cultivated
Wild
Wild
Cultivated
32 35 33 34 42 43 36 37 40 41 38 39
Guizhou
Sichuan
Guizhou
Guizhou
Zhejiang
Guangdong
Henan
Zhejiang
Guangxi
t[1 2]