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Review

Chromatographic and electrophoretic methods for Lingzhi pharmacologically active components

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Abstract

Lingzhi is the Chinese name given to the *Ganoderma* family of mushrooms, which was considered the most valuable medicine in ancient China and was believed to bring longevity, due to its mysterious power of healing the body and calming the mind. Today, Lingzhi is still widely revered as a valuable health supplement and herbal medicine worldwide, as studies (mostly conducted in China, Korea, Japan and the United States) into the medicinal and nutritional values of Lingzhi revealed that it does indeed contain certain bioactive ingredients (such as triterpenes and polysaccharides) that might be beneficial for the prevention and treatment of a variety of ailments, including important diseases such as hypertension, diabetes, hepatitis, cancers, and AIDS. As research into the biological activities of Lingzhi, as well as the quality assurance and quality control of Lingzhi products, require the isolation/purification of active ingredients from Lingzhi, followed by subsequent analytical and/or preparative separations, the present review summarizes the various chromatographic and electrophoretic methods (as well as sample pretreatment methods) typically employed to achieve such extraction/separation procedures.
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Keywords: Lingzhi; Chromatographic/electrophoretic methods; Active ingredients; Triterpenes/triterpenoids; Polysaccharides

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1. Introduction

The fungus *Ganoderma lucidum* – also known as "Lingzhi" in Chinese, "Reishi" in Japanese, and "Youngzhi" in Korean – is a member of the genus *Ganoderma* and has been traditionally used as a popular folk medicine for the promotion of health in the Orient. For example, as early as in 100 B.C., Lingzhi was cited in the Shen Nong's Herbal Classic (widely considered as the oldest book on oriental herbal medicine and the foundation of traditional Chinese medicine) for enhancing "vital energy" and promoting "longevity", and this "mushroom of longevity" has been deemed as the most exalted medicine in ancient China [1]. The genus *Ganoderma*, however, was established in the West by a Finnish botanist, P. Karsten, in 1881 [2], and more than 120 species have been reported in the world since then, a majority of which was found in China [3]. In addition to *G. lucidum* (red), other members of the genus *Ganoderma* that are commonly known to possess medicinal/nutritional values include *G. applanatum* (brown), *G. tsugae* (red-brown), *G. sinense* (dark purple to black) and *G. capense* (dark red).

During the past two decades, modern research has revealed that Lingzhi contains a variety of chemical ingredients, including triterpenes, polysaccharides, nucleosides, steroids, fatty acids, alkaloids, proteins, peptides, amino acids, and inorganic elements [1,4]. Among these ingredients, triterpenes and polysaccharides have attracted considerable attention as they have been shown to possess diverse and potentially significant pharmacological activities. Lingzhi has been used for the treatment of a wide range of ailments and chronic diseases, such as migraine, hypertension, arthritis, bronchitis, asthma, anorexia, gastritis, haemorrhoids, diabetes, hypercholesterolaemia, nephritis, dysmenorrhoea, constipation, lupus erythematosis, hepatitis, and cardiovascular problems [1,5]. Importantly, Lingzhi has been demonstrated by recent scientific studies to possess anti-cancer [6] (including leukemia [7,8]), anti-ageing [9] and anti-microbial/viral activities [10,11], including anti-human immunodeficiency virus (HIV) activity [12,13]. Additionally, the presence of neuroactive compounds in the extracts of Lingzhi that mediated the neuronal differentiation and neuroprotection of rat PC12 cells has also been demonstrated recently [14].

Progress in Lingzhi research relies heavily on the development and application of chromatographic and other separation techniques, as current research is focused on the isolation, purification, identification and characterization of bioactive ingredients present in Lingzhi, as well as on the quality assurance and quality control of Lingzhi products, all of which require the qualitative and/or quantitative determination of active ingredients present in the raw herbs, during the manufacturing process, and/or in the final Lingzhi products. This review summarizes the different types of chromatographic (as well as electrophoretic) separation techniques that have been used in the research and development of Lingzhi, including sample pretreatment methods employed prior to analytical or preparative separation, as well as the use of hyphenated techniques, such as gas chromatography–mass spectrometry, for the separation and characterization of bioactive components present in Lingzhi and related species.

2. Triterpenes/triterpenoids

Triterpenes/triterpenoids are bitter components of Lingzhi that have received considerable attention owing to their well-known pharmacological activities [15]. Since the first isolation of two new bitter triterpenes, ganoderic acids A and B, from the dried epidermis of *G. lucidum* in 1982 by Kubota et al. [16], more than 130 oxygenated triterpenes (mostly lanostane-type triterpenes) have subsequently been isolated from the fruiting bodies, spores, mycelia and culture media of Lingzhi [15,17]. It should be noted that Lingzhi is the only known source of these bioactive ganoderic acids. In general, triterpenes have been reported to possess significant bioactivities, such as anti-oxidation [18], hepatoprotection [19], anti-allergy [20], anti-hypertension [21], cholesterol reduction [22], as well as inhibiting platelet aggregation [23], due to the inhibition of enzymes such as β-galactosidase, angiotension coverting enzyme, cholesterol synthase, etc.

Recent research work that demonstrated the anti-cancer, anti-oxidative and anti-viral activities of triterpenes/triterpenoids included: (1) a triterpene fraction from the mycelia of *G. lucidum* was reported by Lin et al. [24] to inhibit the growth of human hepatoma cells via suppressing protein kinase C and activating mitogen-activated protein kinases and C2-phase cell cycle arrest; (2) Zhu and co-workers [25] recently studied the anti-oxidative activities of *G. lucidum* extracts and found that the triterpene fraction exhibited the highest effect by testing the ingredients against pyrogallol induced oxidation on erythrocyte membrane and Fe (II)-ascorbic acid induced lipid peroxidation in liver mitochondria. The major ingredients of the triterpene fraction consisted of ganoderic acids A, B, C and D, lucidenic acid B and ganodermanotriol; and (3) Min et al. [26] reported that a number of triterpenes isolated from the spores of *G. lucidum*, such as ganoderic acid β, luciumol B, ganodermanodiol, ganodermanontriol and ganolucidic acid, showed significant anti-HIV-1 protease activity.

2.1. Extraction and isolation

In general, there are two solvent extraction approaches employed for the isolation of triterpenes from Lingzhi. The first one involved the extraction of total triterpenes with organic solvents and water [26–42]. For example, in the isolation of triterpenes that possess anti-HIV activity, Min et al. [26,35,39] extracted triterpenes from the spores of *G. lucidum* with methanol by refluxing. Similarly, a new triterpenoid, named ganolactone, as well as three triterpenoids, ganoderiol A, ganoderiol B and ganodermatriol, were recently identified (with the aid of silica gel chromatography) from the CH_2Cl_2 soluble fraction of the fruiting body of *G. lucidum* [42].

In the second approach, the selective isolation of the acidic triterpenes from the total triterpenes fraction [16,43–50] was carried out. For example, in the work described by Ma et al. [49], the fruiting bodies of *G. lucidum* were extracted with 95% aqueous ethanol under reflux and the ethanol extracts were evaporated under reduced pressure. The residue was then suspended in water and extracted with chloroform. A saturated $NaHCO_3$ aqueous solution was added to the concentrated chloroform extract. The water layer was extracted with chloroform again after the solution was acidified (pH 3–4) with 6N HCl.

After solvent extractions, the isolated triterpene fractions were usually subjected to additional purification by repeated silica gel column chromatography. The mobile phases that have been used included methanol–chloroform [16,26,27,30–32,35–37,39,48,50,51], dichloroform–methanol [30], hexane–acetone [26,33,35,37,39,52], ethyl acetate–petroleum ether [28,29,48], benzene–methanol–water [32], ethyl acetate–chloroform [31], ethyl acetate–dichloromethane [34], petroleum ether–ethyl acetate [29], benzene–methanol–water [32] and chloroform–methanol–water [32,52]. Also, other researchers have reported the methylation of extracted acidic triterpenes with ethereal diazomethane and then the obtained derivatives were subjected to chromatography on silica gel column and preparative HPLC [32,43,44].

As an alternative to organic solvent extractions for analytical separations, the use of a small Sep-Pak C_{18} cartridge for the extraction of triterpenes (ganoderic acids) have been shown to be a rapid and efficient method [53]. In this work, the reversed-phase (RP)-HPLC profiles of extracted triterpenoids from *G. lucidum* were found to be similar between the use of conventional solvent extraction and Sep-Pak C_{18} cartridge extraction methods, with the latter method requiring significantly less time and volumes of organic solvents. In a more recent study [54], the use of a C_{18} Sep-Pak cartridge for rapid and efficient sample pretreatment prior to the RP-HPLC separation of various triterpenoids present in seven commercial *Ganoderma* products were also reported. To further reduce the consumption of organic solvents, the use of supercritical CO_2 (with a small amount of ethanol added as modifier) has been demonstrated to be an environmentally friendly and effective approach for the extraction of triterpenes from *G. lucidum* [55], with higher extraction yield and lower extraction temperature compared to those of conventional extraction methods. Table 1 shows a list of various bioactive triterpenes/triterpenoids that have been extracted/isolated from Lingzhi and related species as reported in the literature.

2.2. Qualitative and quantitative analysis

2.2.1. Thin-layer chromatography (TLC)

Silica gel TLC plates were frequently used for the qualitative and semi-quantitative determination of triterpenes isolated from different species of *Ganoderma*. Development solvents employed included chloroform–methanol–water (30:4:1) [32,56,57], hexane–ethyl acetate (1:1) [58,59], hexane–ethyl acetate–diethyl ether (1:1:1) [58,59], chloroform–diethyl ether–methanol (9:1:1) [58], chloroform–diethyl ether–ethyl acetate (9:1:1) [59] and methylbenzene–ethyl acetate–acetic acid (12:4:0.5) [59]. Considering that many triterpenes extracted from Lingzhi contained carboxyl groups, solvent systems that consisted of a small quantity of acid are preferred for TLC separations, i.e., chemically neutral solvent systems usually lead to band tailing [59]. Visualization of the sample spots for triterpenes was commonly achieved by spraying with detection reagent such as 10% sulfuric acid in ethanol [32,56,57].

The differentiation of Lingzhi from several other fungal crude drugs, such as hoelen, omphalia and polyporus (frequently used in traditional Chinese medicine), has been reported by Kohda et al. [32] using a qualitative TLC method. It was found that ganoderic acids B and C are unique constituents of Lingzhi, and these substances showed characteristic bright red spots on a TLC plate after spraying with H_2SO_4 and followed by heating. Also, TLC has been applied for the

Table 1
Bioactive triterpenes/triterpenoids isolated from various Lingzhi species

Triterpene/ triterpenoid	Source	References
Ganoderic acid A	Fruiting body of *G. lucidum*	[16,28,32,37,49, 51,52]]
	Spores of *G. lucidum*	[26,39]
Ganoderic acid B	Fruiting body of *G. lucidum*	[16,43,44,49, 51,52]
	Spores of *G. lucidum*	[26,39]
Ganoderic acid C1	Fruiting body of *G. lucidum*	[32,37,43,46,51]
	Spores of *G. lucidum*	[26]
Ganoderic acid C2	Fruiting body of *G. lucidum*	[43,49]
	Spores of *G. lucidum*	[35]
Ganoderic acid D	Fruiting body of *G. lucidum*	[32,47]
Ganoderic acid E	Fruiting body of *G. lucidum*	[36,43,47]
Ganoderic acid F	Fruiting body of *G. lucidum*	[45,47]
Ganoderic acid G	Fruiting body of *G. lucidum*	[49,52]
	Spores of *G. lucidum*	[39]
Ganoderic acid H	Fruiting body of *G. lucidum*	[47,49,51,52]
Ganoderic acid R	Cultured mycelia of *G. lucidum*	[27]
Ganoderic acid S	Cultured mycelia of *G. lucidum*	[27]
Ganoderic acid LM2	Fruiting body of *G. lucidum*	[50]
Ganoderic acid α	Fruiting body of *G. lucidum*	[51]
Ganoderic acid β	Spores of *G. lucidum*	[26]
Ganoderic acid ε	Fruiting body of *G. lucidum*	[50]
Ganolucidic acid A	Spores of *G. lucidum*	[26]
	Fruiting body of *G. lucidum*	[47]
Ganolucidic acid D	Spores of *G. lucidum*	[35]
Ganoderiol A	Fruiting body of *G. lucidum*	[29,51]
Ganoderiol B	Fruiting body of *G. lucidum*	[29,45,49,51]
Ganoderiol F	Fruiting body of *G. lucidum*	[51]
	Spores of *G. lucidum*	[26]
Ganodermanondiol	Spores of *G. lucidum*	[26]
	Fruiting body of *G. lucidum*	[37]
Ganodermanontriol	Fruiting body of *G. lucidum*	[29,37,45,51]
	Spores of *G. lucidum*	[26]
Lucidenic acid A	Fruiting body of *G. lucidum*	[36,47,48]
Lucidenic acid N	Fruiting body of *G. lucidum*	[36]
Lucidumol A	Spores of *G. lucidum*	[26]
Lucidumol B	Spores of *G. lucidum*	[26]
Lucialdehyde B	Fruiting body of *G. lucidum*	[37]
Lucialdehyde C	Fruiting body of *G. lucidum*	[37]
Methyl ganoderate A	Fruiting body of *G. lucidum*	[43]
Tsugaric acid A	Fruiting body of *G. tsugae*	[31]
Tsugaric acid B	Fruiting body of *G. tsugae*	[31]
Tsugaric acid C	Fruiting body of *G. tsugae*	[34]

differentiation of different species of *Ganoderma* by Su et al. [56]. They found that different species of Lingzhi exhibited unique triterpene patterns on the TLC chromatograms. Based on these TLC patterns, 64 samples can be grouped into 18 different groups, similar to grouping patterns obtained using HPLC. Similarly, distinctive TLC patterns of various triterpenes obtained from the extracts of the fruiting bodies of *G. lucidum* and *G. tsugae* have also been reported by other researchers [60].

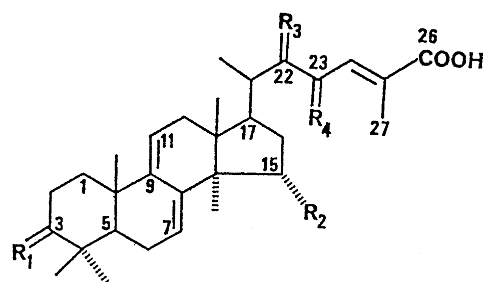
2.2.2. High-performance liquid chromatography (HPLC)

An efficient reversed-phase (RP)-HPLC method was developed by Lin and Shiao [58] for the complete separation of fourteen triterpenes isolated from the mycelia of *G. lucidum*, including five pairs of stereoisomers and one pair of positional isomers. Separations were performed on LiChrosorb C_{18} column with a gradient elution of methanol–acetic acid (100:0.5) and methanol–water–acetic acid (80:20:0.5). The results indicated that the total number of hydroxy and acetoxy groups within the triterpenes played an important role in governing the polarity of these compounds.

In a follow-up paper, Shiao et al. [61] employed RP-HPLC and a binary solvent system consisting of acetonitrile and water under gradient elution for the separation of 24 oxygenated triterpenoids, including eight pairs of stereoisomers and five pairs of positional isomers, and they were able to correlate the molecular polarities with retention characteristics due to the presence of multiple oxygenated functional groups in these oxygenated triterpenoids. In another paper, Shiao and co-workers [62] were able to obtain very good correlation between partition coefficients in *n*-octanol–water and the RP-HPLC separation characteristics (capacity factors) of eight oxygenated triterpenes (paired stereo and positional isomers). Note that *n*-octanol partition coefficients is widely recognized as one of the quantitative physical properties highly suitable for the characterization of the interaction between chemical substances (e.g., drugs) and biological systems (e.g., cell membranes) [63]. A comprehensive review article (with 33 references) that describes the chromatographic analysis of triterpene/triterpenoid natural products in *G. lucidum* has been published by Shiao [64]

2.2.3. HPLC profiling of triterpenes/triterpenoids and quality control/validation

Based on RP-HPLC, Chyr and Shiao [65] have demonstrated the feasibility of utilizing the triterpenoid profiles for the chemical taxonomy of *G. lucidum* and related species (comparable to morphological classification), as well as for the determination and comparison of production yields of several desired triterpenoids in the genus *Ganoderma*. Fig. 1 shows the chemical structures of 25 reference triterpenoids selected for compositional comparison by Chyr and Shiao [65]. The RP-HPLC separation of these triterpenoids present in a standard solution and in an extract of *G. lucidum* was shown in Fig. 2A and B, respectively. Similarly, HPLC was demonstrated by Su et al. [56] to be a simple and reliable method for the differentiation of different members of *Ganoderma*. In this study, the 64 samples examined could be divided into 18 groups based on characteristics of the HPLC pattern of triterpenoids, which were in good agreement with those of morphological examination. For the rapid and efficient characterization of Lingzhi, Sye [53] reported that triterpenoid patterns obtained from different strains of *G. lucidum* can be obtained by RP-HPLC based on the use of a small Sep-Pak C_{18} cartridge for the extraction of ganoderic acids.



	R_1	R_2	R_3	R_4
1	OAc H	OAc	H_2	H_2
2	H OAc	OAc	H_2	H_2
3	OH H	OAc	H_2	H_2
4	OAc H	OH	H_2	H_2
5	H OH	OAc	H_2	H_2
6	H OAc	OH	H_2	H_2
7	=O	OAc	H_2	H_2
8	OH H	OH	H_2	H_2
9	H OH	OH	H_2	H_2
10	OAc H	OH	H OAc	H_2
11	H OH	OAc	H OAc	H_2
12	OAc H	OAc	H OAc	H_2
13	H OAc	OAc	H OAc	H_2
14	OH H	OAc	H_2	O
15	OAc H	OAc	H_2	O
16	OAc H	OH	H_2	O
17	OAc H	OH	OH H	H_2
18	OAc H	OAc	OH H	H_2
19	H OAc	OAc	OH H	H_2
20	H OH	OH	H OAc	H_2
21	OH H	OH	H OAc	H_2
22	H OH	OH	H OH	H_2
23	OH H	OH	OH H	H_2
24	OH H	H	H_2	H_2
25	OAc H	H	H_2	H_2

Fig. 1. Structures of 25 oxygenated triterpenoids, including eight pairs of stereoisomers and five pairs of positional isomers, isolated from *G. lucidum*. Reprinted from [65].

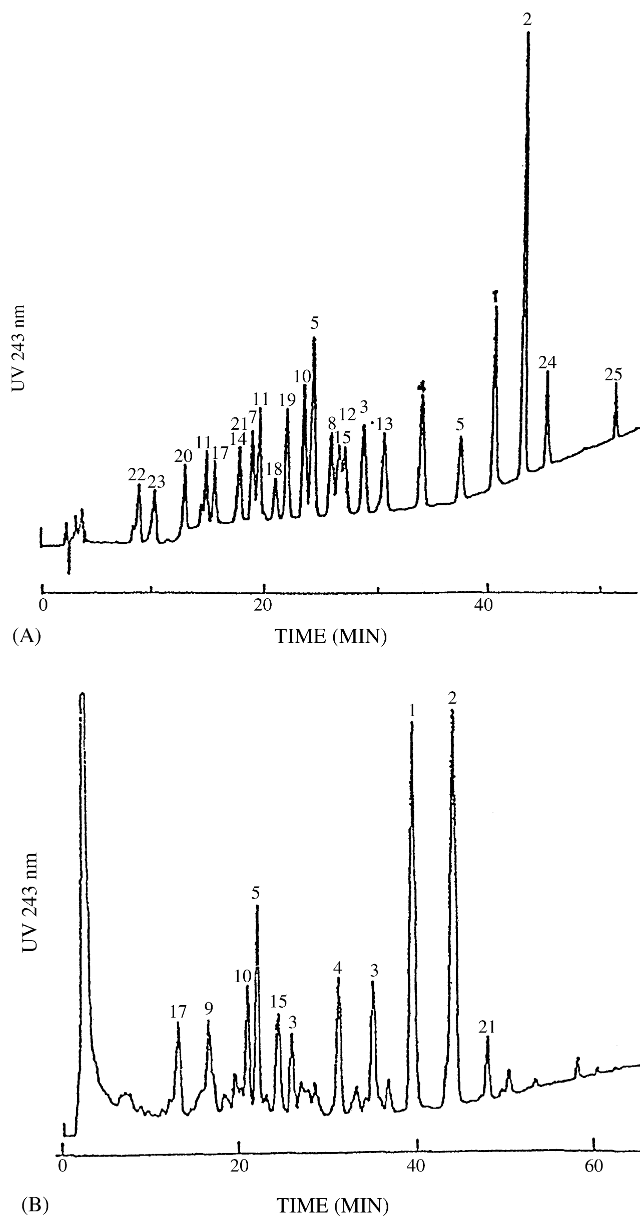


Fig. 2. RP-HPLC separation of triterpenoids: (A) standard solution containing compounds 1–6 and 8–25 as shown in Fig. 1 and (B) methanolic extract of *G. lucidum*. Column, Cosmosil 5 C_{18}, 25 cm × 0.46 cm i.d. Eluent A, methanol–acetic acid (100:0.5, v/v); eluent B, methanol–water–acetic acid (80:20:0.5, v/v/v); flow rate, 1.0 ml/min; UV detection at 243 nm. Gradient elution was started with 80% methanol, increased linearly to 84% in 15 min, to 86% min in another 15 min, to 88% in 10 min, to 94% in another 10 min and finally to 100% in 20 min. Reprinted from [65].

In a study by Chen et al. [54], various strains of *Ganoderma* belonging to *G. tsugae* and *G. lucidum* were cultivated and the triterpenoid patterns of their fruiting bodies were obtained by RP-HPLC using a gradient of acetonitrile and 2% acetic acid. In another study, Chen et al. [66] reported the use of spectral analysis based on simple Fourier transform infrared absorption as well as RP-HPLC, with isocratic elution using acetonitrile–2% acetic acid (1:2), for the classification/identification of various commercial *Ganoderma* products (*G. lucidum and G. tsugae*) into three different types: cultured mycelia, fruiting bodies and mixed mycelia/fruiting bodies. More recently, Chen and co-workers [67] isolated nine triterpenoids from the fruiting body of *G. tsugae* and, among these compounds, ganoderic acids E, C5, C6, G and ganoderenic acid D were analyzed for the first time by RP-HPLC. The separation was carried out on a C_{18} column with a solvent gradient of acetonitrile and 2% acetic acid (1:4 and 1:2, v/v).

An HPLC method for determining ganoderic acid B in different body parts of *G. lucidum* has been developed and validated by Ding et al. [68]. The pilei (caps) were found to be the best source of ganoderic acid B, followed by the stipes (stems), and the spores were the poorest source.

Similarly, Cai et al. [69] reported the effective use of RP-HPLC (YWG-C_{18} column) for the determination of ganoderic acids contents, i.e., ganoderic acid A, ganoderic acid C, ganoderic acid D and ganoderic acid E, in cultured and wild *G. lucidum*, and this method was claimed to be highly suitable for the quality control of herbal formulations containing Lingzhi. Most recently, a validated HPLC method has been reported for the simultaneous analysis of ganosporeric acid A, lucidenic acid A, ganoderic acids B and C from *G. lucidum* by Ma et al. [70]. Interestingly, it was found that the contents of four triterpenes in the fruiting bodies were higher than that in the spores, and the triterpene contents in the spores with a sporoderm-breaking rate of 85% were found to be higher than those obtained from spores having complete sporoderms.

For the comparison of triterpene contents between fruiting bodies and spores, Min et al. [26] have reported an HPLC method for the quantitative analysis of the ganoderic alcohols and acids in the spores and five fruiting bodies of *G. lucidum* collected from different countries. Six ganoderic alcohols, namely, lucidumols A and B, ganoderiols A and F, ganodermanontriol A, ganodermanondiol, were well separated on TSK gel ODS 80 TS column (150 mm × 4.6 mm, 5 μm) with 2% ethanol–acetonitrile (4:6) as the mobile phase. Seven ganoderic acids, namely, ganoderic acids A, B, C1, H, α, β, and ganolucidic acid A, were well separated except for ganoderic acids C1 and H on the same column using 2% ethanol–acetonitrile (7:3) as the mobile phase. A chromatogram of a standard mixture containing the ganoderic alcohols and acids isolated from *G. lucidum* is shown in Fig. 3A and B. The results indicated that the total triterpene content of the spores was 5–20 times higher that of the fruiting bodies. In contrast, other researchers have shown that the spores were the poorest source of triterpenes [32,68,70]. These differing results indicate that the quality and content of triterpenes vary greatly from sample to sample, despite the same species of Lingzhi.

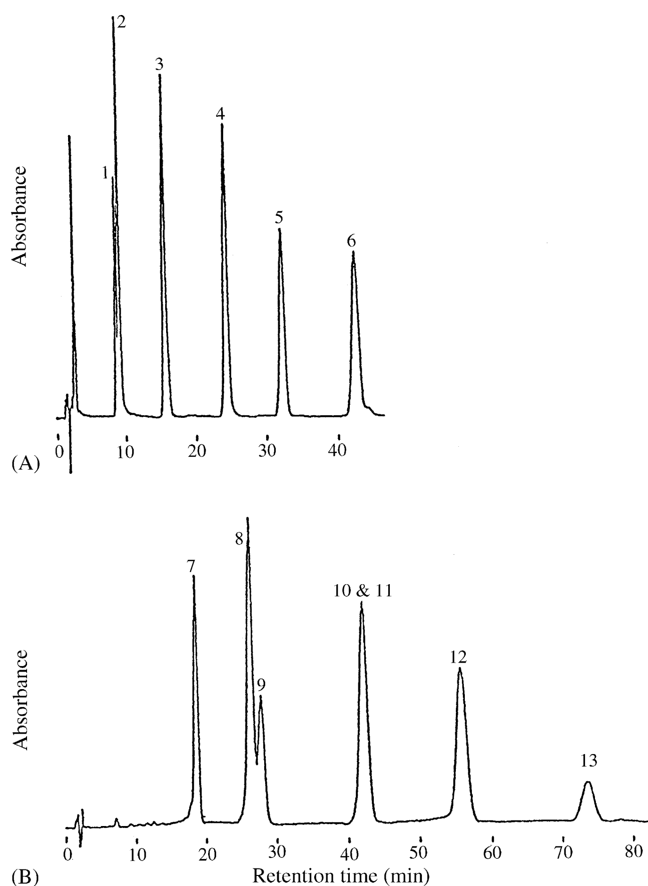


Fig. 3. RP-HPLC profile of a mixture of triterpenes isolated from the spores and fruiting bodies of *G. lucidum*. (A) Ganoderic alcohols: (1) lucidumol A, (2) ganoderiol A, (3) ganodermanontriol, (4) lucidumol B, (5) ganoderiol F, (6) ganodermandiol and (B) Ganoderic acids: (7) ganoderic acid B, (8) ganoderic acid A, (9) ganoderic acid α, (10) ganoderic acid H, (11) ganoderic acid C1, (12) ganolucidic acid A, (13) ganoderic acid β. Reprinted from [26].

3. Polysaccharides

Polysaccharides represent a structurally diverse class of biological marcromolecules with a wide range of physicochemical properties. The importance of polysaccharides (including protein/peptide bound polysaccharides) as pharmaceuticals has a long history and is receiving considerable attention in recent years [71]. Scientific investigations on the anti-tumor and immuno-modulating activities of medicinal mushrooms, including *G. lucidum*, was reported as early as in 1957 [72] and, more recently, extensive studies on the anti-tumor ingredient(s) contained in these higher fungi, especially polysaccharides and protein/peptide bound polysaccharides, have been carried out. For example, several β-glucans (which serve to hold the cell walls of the fungus intact) that were isolated from water and alkali extracts were found to be bioactive by Miyazaki and Nishijima in the early 1980s [73]. Importantly, these Japanese researchers discovered that water soluble polysaccharides of *G. lucidum* strongly inhibited the growth of Sarcoma 180 solid-type tumor, with inhibition ratio above 95%. Most of the anti-tumor β-glucans were reported to contain a branched glucan core with (1 → 3)-β-, (1 → 4)-β- and/or (1 → 6)-β-linkages and an average molecular weight of 1,050,000 Da [73,74].

Polysaccharides are believed to exert anti-tumor activities through an enhancement of the host-mediated immunity rather than a direct cytotoxicity to the tumor cells, such as via an increase in interleukin [75], interferon [76] and antibody production [77], as well as the stimulation of cytotoxic T lymphocytes [78]. It should be noted that the amount of bioactive water insoluble polysaccharides was found to be higher than that of water-soluble polysaccharides, as reported by Mizuno et al. [74,79]. They found that the water-soluble polysaccharides consisted of β-glucan and glucurono-β-glucan, whereas the water-insoluble polysaccharides consisted of hetero β-glucan, xylo-β-glucan, xylomanno-β-glucan and manno-β-glucan. At present, more than 200 polysaccharides have been isolated from the fruiting bodies, spores, mycelia and cultivation broth of Lingzhi.

Recent research into the bioactivities of polysaccharides include the following work: (1) Kim and Kim [80] found that water soluble polysaccharides extracted from the fruiting body of *G. lucidum* was effective in preventing DNA from strand breakage caused by hydroxyl radical and UV irradiation, indicating the anti-tumor and immuno-modulating activities of Lingzhi are strongly related to its anti-oxidative property; (2) You and Lin [81] studied the anti-oxidant property of *Ganoderma* polysaccharide peptide (GLPP) and found that GLPP decreased the oxidation of low density lipoprotein and exhibited anti-oxidant effect by scavenging reactive oxygen species in mice; (3) A bioactive fraction (GLIS) – a proteoglycan having a carbohydrate/protein ratio of 11.5 to 1 – isolated from the fruiting body of *G. lucidum* was found by Zhang et al. [82] to stimulate the proliferation of mouse spleen lymphocytes, resulting in a three to four-fold increase in the percentage of B cells and an increase in the secretion of immunoglobulin, as well as increased production of interleukin 2 and enhanced expression protein kinase C α and β; (4) Lee et al. [83] found that the polysaccharide extracts from the mycelium of *G. lucidum* (GLP) exhibited anti-tumor effect against fibrosarcoma in male and female C3H mice and inhibited the metastasis of the tumor to the lung. The bioactive polysaccharides could stimulate blood mononuclear cells to increase cytokines, tumor necrosis factor, interferon and interleukin production, and the lifespan of tumor-implanted mice was found to increase significantly due to the administration of GLP; and (5) Two protein bound polysaccharides, a neutral protein bound polysaccharide (NPBP) and an acidic protein bound polysaccharide (APBP), were isolated from water soluble substances of *G. lucidum* by Eo et al. [10] and APBP was found to be more potent than NPBP as an antiviral agent against herpes simplex viruses (HSV). The antiviral activity of APBP appeared to be related to its binding with HSV-specific glycoprotein at the cell membrane.

3.1. Extraction and clean-up

To isolate water-soluble polysaccharides from Lingzhi, samples were mostly extracted with hot water (95–100 °C). Afterward, ethanol was added to the concentrated aqueous extract and the resulting precipitate (polysaccharide fraction) was collected by centrifugation [73,74,84–102]. Systematic investigations into basic factors which affected the extraction of polysaccharides from *G. lucidum* have been carried out [97,103]. For the extraction of alkaline-soluble polysaccharides from Lingzhi, sodium hydroxide (0.1–1.0 M) solutions were frequently used instead of hot water [104–110]. Prior to the extraction with hot water or sodium hydroxide solutions, Lingzhi samples can be firstly extracted with ethanol under reflux [93,94,97,98,104,111–114], or successively washed with hot dichloromethane and hot methanol [115]. The advantages of such a sample pretreatment are that lipids present in Lingzhi can be removed and, also, enzymes that could lead to the hydrolysis of polysaccharides can be deactivated. Following ethanol precipitation, the resulting precipitate can be washed with ethanol, acetone, and/or ethyl ether [73,85,88,90–92,95,97,103,114]. In a study conducted by Cheong et al. [116], the chemical compositions of alkali-extracted peptidogylcans from Korean *G. lucidum* was found to be different from those of water-extracted fractions, and the authors noted that the biological activity of peptidoglycan was expected to differ according to the types of extraction solvents and fractionation methods employed.

In addition to the solvent extraction procedures, a clean-up of the extracted crude polysaccharides is often performed, since the extracts not only contained polysaccharides but also proteins and many small molecules which can interfere with the ensuring separation step if they are not properly removed. For example, interfering small molecules can be removed by dialyzing against running water [73,88,91,92,95,110–115,117,118]. For the removal of free proteins, however, Sevag method is often the method of choice [90–92,95,117–119]. This method is based on the denaturation of proteins with the addition of chloroform–*n*-butanol (5:1, v/v) to the extracts, such as crude polysaccharides from the mycelia and culture of Lingzhi. As an alternative to the Sevag method, which is time-consuming (i.e., the deproteinizing steps usually have to be repeated more than 10 times) and may not be very effective for the removal of extracts with higher protein content, the use of trichloroacetic acid for deproteination in Lingzhi extracts have been reported [111–114,120].

For the extraction and subsequent chromatographic analysis of both water-insoluble and water-soluble polysaccharide–protein complexes present in various Lingzhi species, an appropriate enzyme is often employed to effect hydrolysis of the protein-bound polysaccharides. For example, Park et al. [121] reported the use of chymotrypsin for the hydrolysis of water-insoluble protein-bound polysaccharides present in *G. lucidum*, leading to an enhancement of their water solubilities (possibly due to the enzymatic cleavage of the attached hydrophobic polypeptide chains). Also, Peng and Zhang [118] reported the use of trypsin to hydrolyze a water-soluble polysaccharide–protein complex extracted from the mycelium of *G. tsugae* and determined the protein and carbohydrate contents using size exclusion chromatography. Addition of the enzyme pronase to the polysaccharide extracts, followed by the use of Sevag method for deproteination prior to chromatographic analysis has also been reported [73,96,103,104].

It should be noted that the extraction of certain water-insoluble polysaccharides from Lingzhi can be carried out by cellulase hydrolysis reaction [122–124]. This method is based on the attack of the enzyme on the polysaccharide substrates, composed of cellulose and lignin. Compared to conventional extraction methods such as hot water extraction, the enzyme-based method has the advantages of reduced extraction time and increased extraction efficiency. Also, it has been reported that enzyme hydrolysis reaction can be enhanced by ultrasonic waves [124].

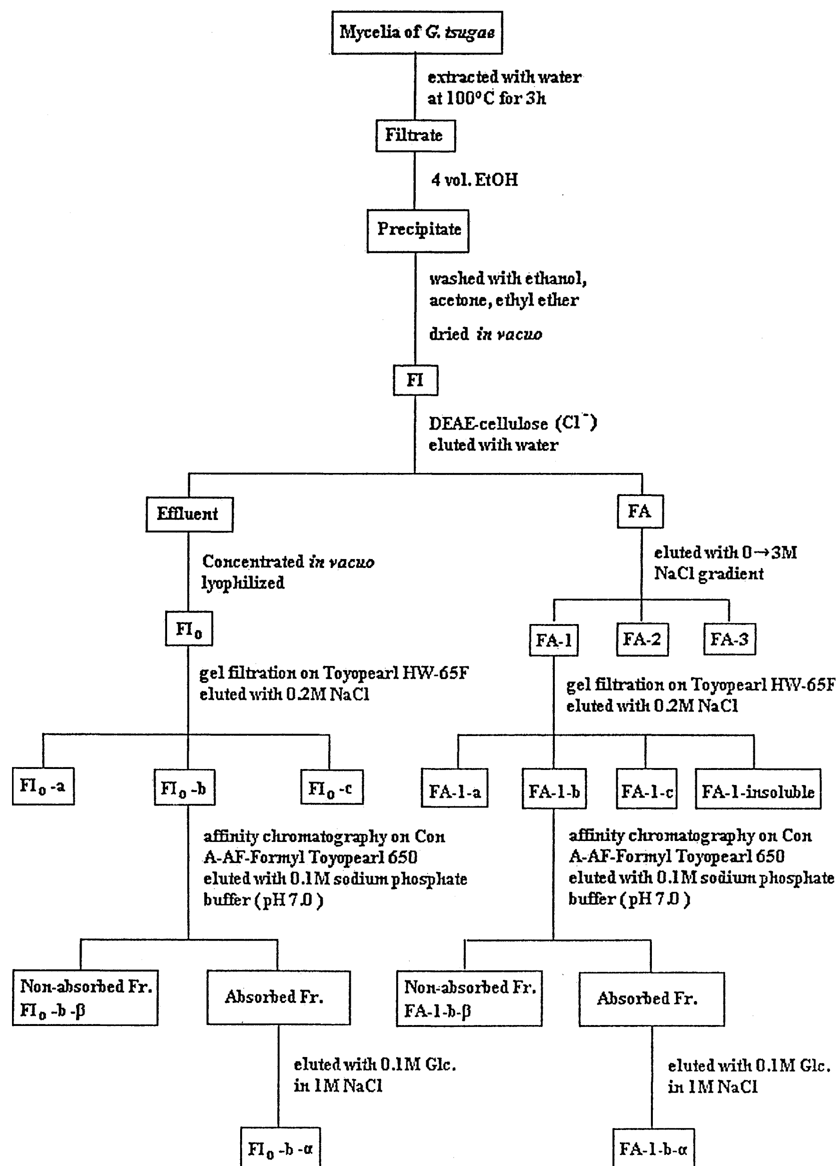


Fig. 4. Scheme for the isolation and purification of 16 polysaccharides from the mycelia of *G. tsugae*. Reprinted from [85].

3.2. Isolation and purification

3.2.1. Ion-exchange chromatography (IEC)

Following solvent extraction, the polysaccharides or protein/peptide bound polysaccharides fractions are often further fractionated by anion-exchange chromatography using different DEAE materials like DEAE-cellulose (Cl^-, OH^-, or $BO_4{}^-$ form) [73,74,88–95,98,99,104–106,111–114,125–129], DEAE-Sepharose 4B or 6B [126], DEAE-Sephadex G-75 [73], DEAE-Toyopearl 650M [86,87] and DEAE-Sephacel [82], and/or by cation-exchange chromatography using columns such as SP-Sepharose [82]. Elution was carried out with water, followed stepwise by NaCl, $NaHCO_3$ or borate solution if necessary. The carbohydrate contents in the eluted fractions were pooled, dialyzed, concentrated and lyophilized, with the use of anthrone–sulfate reagent [130] or phenol–sulfuric acid reaction [131] for total carbohydrate determination.

3.2.2. Gel filtration chromatography (GFC)

In addition to IEC, GFC is often a method of choice for the purification of crude polysaccharide fractions. Common gel filtration columns that have been employed included Sephadex G-100, G-75, G-50, G-25 [74,86,87,90,91,99,104,132], Sepahrose CL-4B, CL-6B [73,87,89,105,116,117,121,125,127,129,133], Toyopearl HW-65F [85,98], Sephacryl S-200, S-300 [82,86,87,111–114] and Fractogel HW-65 [134]. The columns were usually eluted with water or 0.1–0.2 M NaCl. Recently, Lu et al. [135] reported the coupling of the phenol–sulfuric acid method with GFC for the analysis of crude polysaccharides contents in Lingzhi, and this combined

method was shown to be more accurate than the traditional phenol–sulfuric acid method [131].

3.2.3. Affinity chromatography (AC)

AC carried out on a column of concanavalin (Con A)-Sepharose 4B has been demonstrated to be effective for the purification of heterogalactans from the fruiting bodies of *G. applanatum* and *G. lucidum* [74,99,101,102]. Con A, a lectin (phytohemagglutinin) obtained from a jack bean, is known to interact specifically with polysaccharides having terminal D-mannopyranosyl residues and related structures [136]. AC is also known to be a highly useful method for the fractionation and purification of polysaccharide–protein complexes, e.g., by employing Con A-AF-Formyl Toyopearl 650M column and eluting with 0–3 M NaCl solution [85,98]. A fractionation scheme which involved the combined use of ion-exchange chromatography, gel filtration and affinity chromatography for the purification of three active anti-tumor active protein-containing glycans from the mycelium of Songshan Lingzhi (*G. tsugae*) as reported by Zhang et al. [85] is illustrated in Fig. 4.

3.3. Determination of homogeneity and molecular weight

3.3.1. Electrophoresis

Electrophoresis is a method of choice for the examination of the homogeneity of the isolated carbohydrate polymers. The electrophoretic supports include cellulose acetate [89,93], glass-fibre paper [73,86,89,93,94,96,115,133] and polyacrylamide gel [86,95,125,137,138]. NaOH–$Na_2B_4O_7$ (pH 9) or Tris–HCl (pH 9) is often used. After finishing the electrophoresis, the spot can be detected with 1-naphthnol–sulfuric acid reagent [115].

3.3.2. Gel filtration chromatography (GFC)

GFC is often employed for the examination of the homogeneity and molecular weight determination of the isolated polysaccharides. Conventional GFC is usually conducted on Sephadex G-50, G-100, G-200 [88,90,91,95,139], Sephacryl S-200 [86], or Sepharose CL-4B [90,91,93,94,96,115,133] with aqueous solutions containing NaCl, Tris–HCl or NaOH as the mobile phase. For the determination of molecular weight, a series of dextrans [88,139] or pullulans [86] are often used as standards. To increase the separation performance, high-performance size exclusion chromatography (HP-SEC) is a method of choice for determining homogeneity and molecular weight, using ultrahydrogel 500, 1000 or 2000 column and elution with 0.001 M NaOH or 0.003 M NaOAc at a flow rate of 0.5 ml/min [111–114], or Shodex Ionpak S-80 column and elution with 0.2 M Na_2SO_4 at a flow rate of 0.8 ml/min [90]. Also, TSK-5000 PW and TSK-GEL G6000PWXL column have been used [110,117]. The eluate was monitored by UV, differential refractive index (DRI) and laser light scattering (LLS). Recently, HP-SEC combined with LLS as a method for determination of the absolute molecular weight has received increasing attention [107,117,118,140,141]. Table 2 shows a number of bioactive polysaccharides extracted/isolated from various Lingzhi species and their corresponding weights.

Table 2
Bioactive polysaccharides isolated from various Lingzhi species

Polysaccharide	Molecular weight	Source	Reference
SP	1.0×10^4	Spores of *G. lucidum*	[111]
PL-1	8.3×10^3	Fruiting body of *G. lucidum*	[112]
PL-3	6.3×10^4	Fruiting body of *G. lucidum*	[112]
PL-4	2.0×10^5	Fruiting body of *G. lucidum*	[112]
PSGL-I-1A	7.18×10^5	Spores of *G. lucidum*	[113]
Ganoderan A	2.3×10^4	Fruiting body of *G. lucidum*	[87]
Ganoderan B	7.4×10^3	Fruiting body of *G. lucidum*	[86,87]
Ganoderan C	5.8×10^3	Fruiting body of *G. lucidum*	[86]
GL-1	4.0×10^4	Fruiting body of *G. lucidum*	[115]
G-A	8.2×10^4	Fruiting body of *G. sinense*	[115]
FI_0-b-α	1.0×10^4	Mycelia of *G. tsugae*	[85]
FA-1-b-α	1.6×10^4	Mycelia of *G. tsugae*	[85]
TGLP-2	2.1×10^5	Fruiting body of *G. tsugae*	[88]
TGLP-3	4.5×10^4	Fruiting body of *G. tsugae*	[88]
TGLP-6	3.2×10^4	Fruiting body of *G. tsugae*	[88]
TGLP-7	1.0×10^5	Fruiting body of *G. tsugae*	[88]

3.4. Compositional analysis and linkage analysis

3.4.1. Paper chromatography (PC) and TLC

Polysaccharides of Lingzhi and related species have been reported to compose of a variety of monosaccharides, including glucose, galactose, mannose, arabinose, xylose, fucose, rhamnose, glucuronic acid and galacuronic acid [142]. Carbohydrate analysis of complex polysaccharides in Lingzhi can be carried out by the determination of their constituent sugar residues obtained after chemical hydrolysis of the native polymers, followed by the use of PC or TLC (e.g., silica gel or cellulose plates) for separation and *p*-anisidine hydrochloride or silver nitrate reagent for detection [86,114,122]. The polysaccharides were usually subjected to either total hydrolysis with 1–2 M H_2SO_4 at 100 °C for 4–8 h [86,99], or partial hydrolysis with 0.1N sulfuric acid [73] or 0.1 M trifluoroacetic acid for

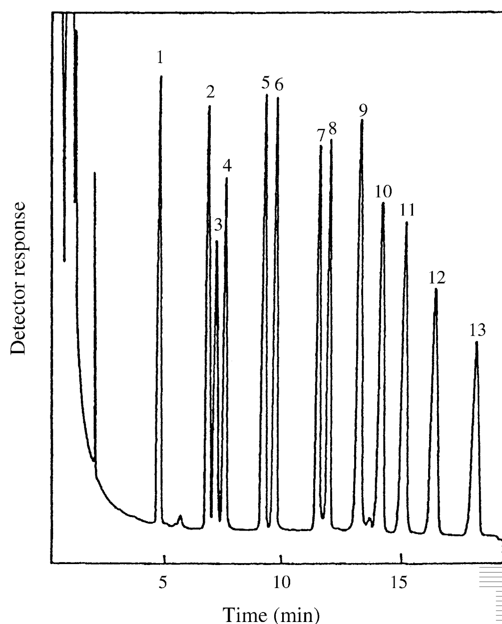


Fig. 5. GC separation of the alditol acetates of 13 monosaccharides on a Silar 10C glass-capillary column. The temperature was kept at 190 °C for 4 min and then increased to 230 °C at 4 °C/min. Peaks: (1) erythritol; (2) 2-deoxy-erthro-pentitol; (3) rhamnitol; (4) fucitol; (5) ribitol; (6) arabinitol; (7) xylitol; (8) 2-deoxy-arabino-hexitol; (9) allitol; (10) mannitol; (11) galactitol; (12) gluctitol; (13) *myo*-inositol. Reprinted from [143].

1–2 h at 100 °C [104]. After neutralization with $BaCO_3$, the acid hydrolyzates were applied on filter paper and developed with ethyl acetate–pyridine–water (10:4:3) [73], *n*-butanol–glacial acetic acid–ethanol–water (4:1:1:2) [88,90–92,95], acetyl acetate–pyridine–ethanol–water (5:2:2:1) [88,90–92,95], *n*-butanol–pyridine–water (6:4:3) [110] or *n*-butanol–benzene–pyridine–water (5:1:3:3) [110].

3.4.2. Gas chromatography (GC), GC–MS and HPLC

For the quantitative analysis of carbohydrate contents in polysaccharides present in Lingzhi, GC coupled with flame ionization detector (FID) or MS detection is the method of choice. Following chemical hydrolysis of the native polysaccharides, GC analysis of the sugars can be performed by converting the acid hydrolyzates into methyl glycosides [87,89]. Alternatively, GC analysis of neutral sugars and their methyl ethers can be carried out after conversion of the acid hydrolyzates into their corresponding alditol acetates [87,91,92,95,98,99,104,111–114,117,139,143]. For example, GC separation of alditol acetates has been performed on a Silar 10C glass-capillary column (28.5 m × 0.5 mm, i.d.) fitted to a Hewllett-Packard 5710 chromatograph equipped with a FID detector [143]. Baseline separation of 13 alditol acetates was achieved as shown in Fig. 5. For the determination of acidic sugar components present in polysaccharides of *G. lucidum*, e.g., galacturonic acid and glucuronic acid, GC analysis can be performed after acid hydrolysis, reduction, lactonization, and trimethylsilation [86].

Methylation analysis and Smith degradation, coupled with GC–MS analysis, are often employed for the structural/compositional analysis of polysaccharides in Lingzhi [102,111,113,133]. For example, for the structural determination of a major polysaccharide from the spores of *G. lucidum* [111], the exact branching of the structure of this complex glucan was elucidated by carrying out methylation of the native glucan, and the fully methylated polysaccharides were hydrolyzed with acid, reduced and then acetylated, followed by GC–MS analysis. The results suggested that the backbone chain of the native glucan was essentially composed of consecutive (1 → 3)-linked glucopranosyl residues with a highly branched structure. Also, structural analysis of water-soluble polysaccharide GFb of fermented mycelia from *G. tsugae* was accomplished by partial acid hydrolysis, Smith degradation and methylation, followed by GC and GC–MS analysis [133]. The main chain of GFb was found to be composed of (1 → 6)-linked galactose and (1 → 6)-linked glucose residues.

HPLC has been accepted as one of the major techniques for the analysis of sugars, especially in the form of anion-exchange chromatography coupled with pulsed amperometric detection of the underivatized sugars [82,144]. For example, after hydrolysis with TFA and determination by high-pH anion-exchange chromatography, a bioactive proteoglycan isolated from *G. lucidum* was found to be composed of eight different monsaccharides, predominately D-glucose, D-galactose and D-mannose in the molar ratio of 3:1:1 [82]. As an alternative to electrochemical detection, Kim et al. [145] have shown that the labeling of acid hydrolyzates (using TFA hydrolysis) of various fungal polysaccharides, including those of *G. lucidum*, with 7-amino-1,3-naphthalenedisulfonic acid (7-AGA) by reductive amination, followed by RP-HPLC separation of the sugar–AGA conjugates using a Spher-5 RP 18 column and fluorescent detection with the excitation wavelength set at 250 nm.

3.5. Fingerprint profiling of polysaccharides and quality control/validation

Although the use of HPLC and TLC for the fingerprint profiling of triterpenes present in Lingzhi has been clearly demonstrated [48,146], the use of HPLC or TLC for the standardization and quality control of various Lingzhi products based on the fingerprint profiling of carbohydrates from Lingzhi extracts is rarely reported [147,148]. High-performance size exclusion chromatography (HP-SEC) has been used for the quality control of a Lingzhi product called Siwei Lingzhi mixture based on the profiling of carbohydrate contents [149]. The TSK-G 4000 PW_{XL} column was used with 0.7% Na_2SO_4 solution as the mobile phase and RI for detection. Recently, the feasibility of employing high-performance thin-layer chromatography (HPTLC) for the fingerprint profiling of polysaccharides extracted from the fruiting bodies and spores of Lingzhi has been demonstrated

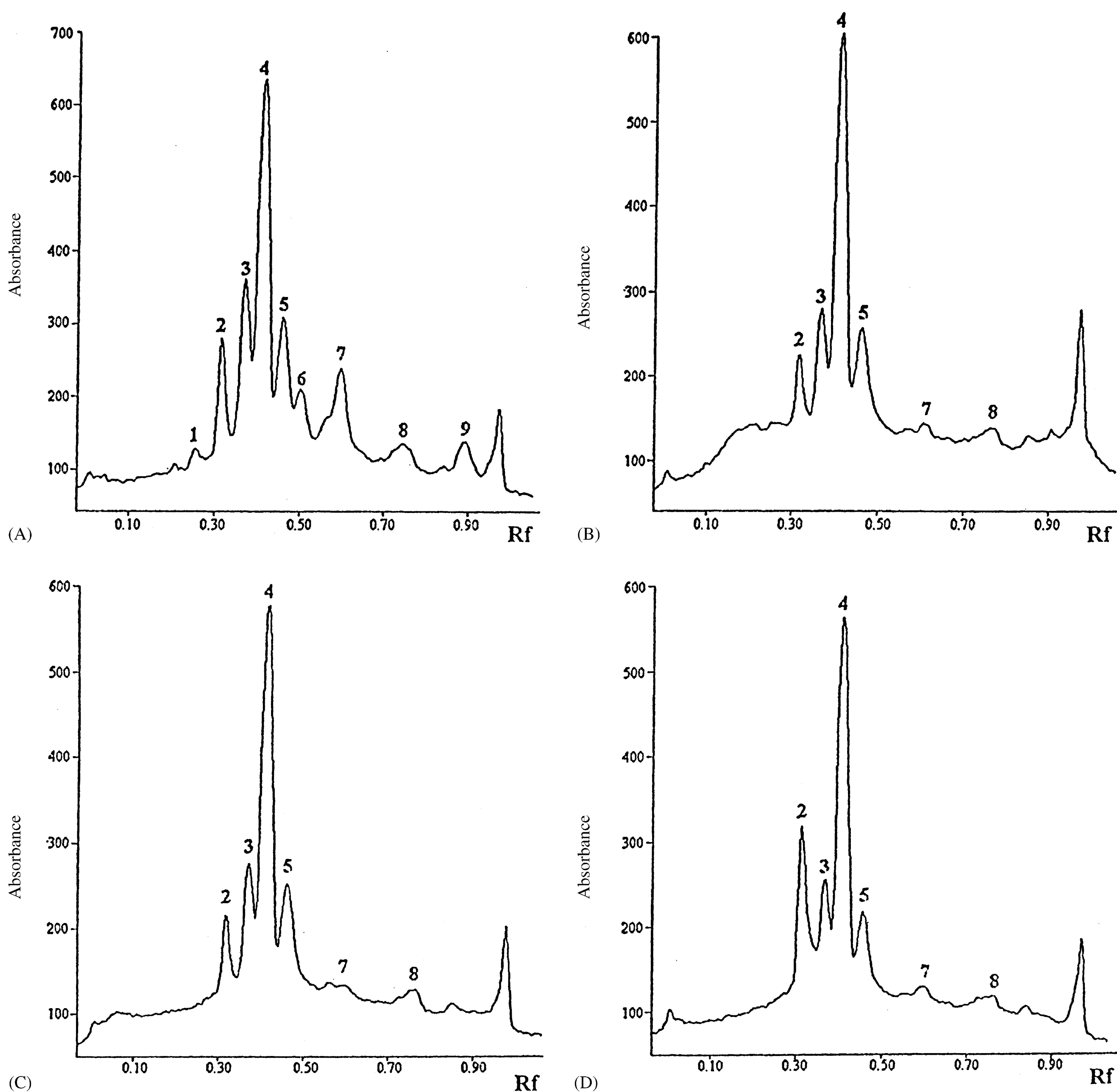


Fig. 6. HPTLC chromatogram of acid hydrolyzates of polysaccharides from water extracts of four different Lingzhi species obtained under total hydrolysis conditions. Fruiting bodies of: (A) *G. applanatum*; (B) *G. lucidum*; (C) *G. nigrolucidum*; and (D) *G. tropicum*. Peaks: (1) galacturonic acid; (2) glucuronic acid; (3) galactose; (4) glucose; (5) mannose; (6) arabinose; (7) xylose; (8) fucose; and (9) rhamnose. Reprinted from [150].

[150]. Based on the acid hydrolysis of polysaccharides extracted from various Lingzhi samples, fingerprint profiles that reveal the relative amounts of the degradation products, such as mono- and oligosaccharides, can be obtained using HPTLC Si 50,000 plates for separation and 4-aminobenzoic acid as the post-chromatographic derivatization reagent for detection. Fig. 6A–D show the HPTLC chromatogram of acid hydrolyzates of polysaccharides from water extracts of four different Lingzhi species obtained under total hydrolysis conditions.

4. Proteins and amino acids

Bioactive proteins have been isolated from various Lingzhi species and characterized by chromatographic/electrophoretic techniques. For example, a new immunomodulatory protein, known as Ling Zhi-8 (LZ-8), was isolated from the mycelia of *G. lucidum* and its biochemical and immunological properties were described by Kino et al. [151]. LZ-8 was purified by two chromatographic systems, i.e., gel filtration (Sephadex G-75 column) followed by

ion-exchange (DEAE-Sephadex A-25 column), using an in vitro bioassay that monitored blast-formation stimulatory activity toward mouse spleen lymphocytes to monitor purification.

Another protein with relatively low sugar content (a lectin) that has been extracted from the mycelia (GLL-M) as well as the fruiting body (GLL-F) of *G. lucidum* was reported by Kawagishi et al. [152]. Both GLL-M and GLL-F were isolated using 80% ammonium sulphate to induce protein precipitation and purified by anion exchange chromatography (Superose 12 HR 10/30 column) and affinity chromatography (BSM-Toyopearl affinity column), followed by the use of SDS–PAGE for molecular weight determination. Using gel filtration chromatography for fractionation and SDS–PAGE for molecular weight determination, Ye et al. [153] have found three kinds of bioactive proteins (LZP-1, LZP-2 and LZP-3) from the fruiting body and spores of Lingzhi which showed obvious mitogenic activity.

Proteins in the form of enzymes have also been isolated and characterized by various types of column chromatography as well as electrophoretic techniques. For example, Sripuan et al. [154] reported the use of DEAE-Sephadex column (gel filtration) and Con A-Sepharose (affinity chromatography for the purification of α-galactosidase from the fruiting body of *G. lucidum*. α-Galactosidase was believed to be involved in the production of D-galactose in bioactive polysaccharides, such as ganoderan C [86], due to its hydrolytic activity as well as galactosyl transfer activity. The electrophoretic separation of isoenzymes of various *Ganoderma* species using cellulose acetate gel electrophoresis (CAGE) was demonstrated by Smith and Sivasithamparam [155] to be a potentially powerful method for the rapid diagnostic tool for identifying different species of *Ganoderma*. Similarly, isoenzyme patterns generated by electrophoresis has also been reported by Kim et al. [156] to be useful for the taxonomy of *Ganoderma* species cultivated in Korea and its enzyme/protein patterns were compared with *G. lucidum* and *G. tsugae*. Furthermore, Hseu et al. [157] has reported the application of laccase isozyme electrophoretic patterns (using PAGE) in the identification of various *Ganoderma* species, and Lan et al. [158] have concluded that esterase isoenzyme analysis based on PAGE separation was an effective way to classify different strains of *Ganoderma*. Table 3 shows a list of various enzymes that have been isolated from Lingzhi and characterized using various chromatographic and electrophoretic techniques [159–169], such as gel filtration, ion-exchange chromatography, and SDS–PAGE.

A variety of amino acids have been found in Lingzhi [142]. Interestingly, the spores of *G. lucidum* have been reported to contain higher amounts of amino acids compared to the fruiting bodies [170]. It should be noted that the percentage composition of amino acids in the fruiting bodies varied from sample to sample despite the same Lingzhi species [171]. Amino acid composition is usually determined by amino acid analyzer [171–175]. For example, the peptides and amino acids in *G. lucidum* were determined by means of

Table 3
Enzymes isolated from various sources of Lingzhi

Enzyme	Source	Reference
Carboxyl proteinase (II)	Culture of *G. lucidum*	[159]
Endopolygalacturonase and endopectin methyltranseliminase	Culture of *G. lucidum*	[160]
Amylase	*G. lucidum*	[161]
1,4-B-D-Glucan glucanohydrolase	Culture of *G. lucidum*	[162]
Endo- and exo-polygalacturonase	*G. lucidum*	[163]
Cellulases	Culture of *G. lucidum*	[164]
Manganese superoxide dismutase	Mycelium of *G. lucidum*	[165]
Carboxymethyl cellulose	Culture of *G. lucidum*	[166]
Lignin-modifying enzymes	Culture of *G. lucidum*	[167]
Laccase isozymes	Culture of *G. lucidum*	[168]
Chymotrypsin inhibitors isoforms	*G. lucidum*	[169]

Cu–Sephadex G-25 column chromatography and capillary electrophoresis (CE) as reported by He et al. [175]. The CE electropherogram was found to have 13 peptide peaks and the amino acids components of the peptides were measured by an amino acid analyzer. Alternatively, the determination of amino acids in *Ganoderma* has been reported by Shang et al. [176] to be more reliable using RP-HPLC for separation and UV absorbance for detection. To make the primary and secondary amino acids amenable to UV detection, pre-column derivatization with 1-fluoro-2,4-dinitrobenzene was carried out. To increase the detection sensitivity for the RP-HPLC determination of amino acids from polysaccharides of *G. lucidum*, Kim et al. [106] reported the pre-column formation of fluorescent-labeled sugar conjugates, based on the use of 7-amino-1,3-naphthalene disulfonic acid as the label, prior to HPLC separation of the sugar hydrolyzates.

5. Nucleosides, nucleotides and RNAs

Yu and Zhai [177] were the first to report the isolation of adenine, adenosine, uracil and uridine from the mycelia of a Lingzhi species, *G. capense*. Among these nucleosides, uridine and uracil were found to be capable of lowering the serum aldolase level of mice suffering from experimental myotonia. Subsequently, Kim and Nam [178] studied the distribution of RNA contents and mononucleotides distribution from the mycelium and fruiting bodies of *G. lucidum* and found that the levels of RNAs from the young basidiocarp mycelium were much higher than those of mature basidiocarp. Also, Shimizu et al. [179] demonstrated that the water-soluble fraction of *G. lucidum* suppressed platelet aggregation, with the active substance identified as adenosine.

In the method reported by Yu and Zhai [177], the mycelia of *G. lucidum* were extracted with 92–95% ethanol by refluxing. The ethanol extract was suspended in water and extracted with ethyl ether. The resulting water solution was further fractionated by ion-exchange chromatography to give the neutral fraction and basic fraction. The neutral fraction was

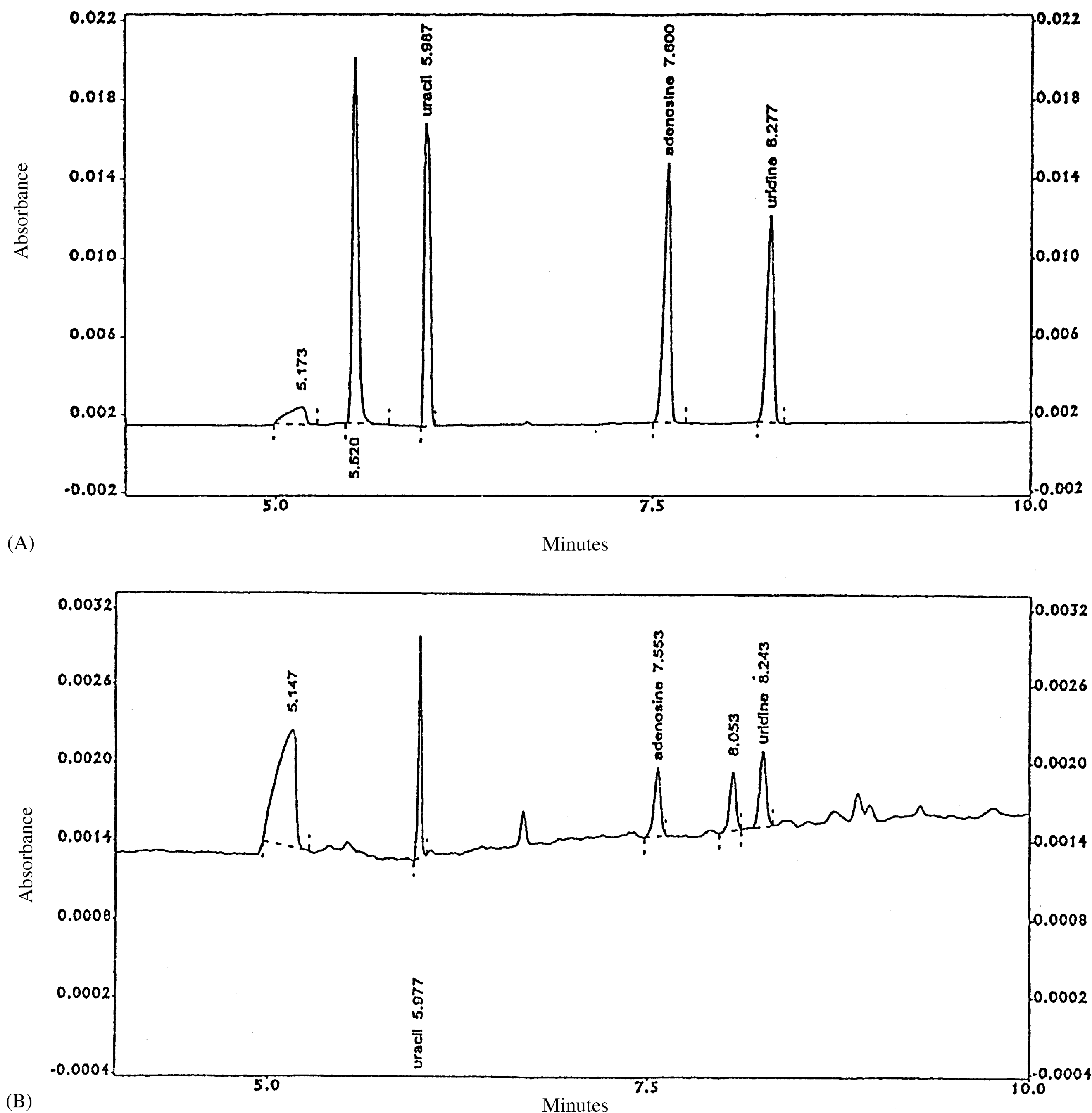


Fig. 7. MEKC chromatogram of nucleoside (adenosine, uridine) and base (uracil) markers in: (A) a standard sample solution and (B) an aqueous extract of *G. lucidum*. Reprinted from [182].

sub-fractionated into uracil and uridine by adsorption chromatography on macroporous resin. By extraction with chloroform and *n*-butanol, and crystallization in methanol and hot water, the basic fraction yielded adenine and adenosine. In the method described by Shimizu et al. [179], a water-soluble fraction of *G. lucidum* was applied to a Sephadex G-25 column. The active fraction was obtained by polyacrylamide gel chromatography on a Bio-Gel P-2 column and the purity of this fraction was confirmed by HPLC. The column was Zorbax-ODS and mobile phase was 10 mM potassium phosphate (pH 5.1) containing 20% methanol or 15% methanol, or 10 mM potassium phosphate (pH 8.5) containing 20% methanol or 15% methanol. With these four solvent systems, adenosine can be well separated from the other nucleosides (cystidine, guanosine and uridine).

TLC has been employed for identifying adenine, adenosine, uracil and uridine from the fruiting bodies of *G. lucidum* [180]. On the other hand, Zhang et al. [181] reported a RP-HPLC method for the determination of nucleosides and their bases extracted from the mycelia of *G.*

capense. Adenine, adenosine, uracil, uridine and the internal standard *p*-toluenesulfonic acid were baseline separated on an ODS column, with phosphate buffer solution (pH 6.98)–tetrahydrofuran (100:1) as the mobile phase and the detection wavelength set at 260 nm. More recently, capillary electrophoresis operated in the mode of micellar electrokinetic chromatographic (MEKC) method has been developed for the determination of the selected base (uracil) and nucleoside (adenosine, uridine) markers in the aqueous extracts of *G. lucidum*, *G. japonicum* and Lingzhi capsules as reported by Cheung et al. [182]. A semi-validation of the MEKC method was performed, with each analyte showing a good linear relationship over a concentration range of 0.2–20 ppm. A representative MEKC chromatogram of the nucleosides in a standard solution and a water extract of *G. lucidum* is shown in Fig. 7A and B, respectively. A similar MEKC method for the determination of nucleosides in a commercial Lingzhi preparation (Siwei Lingzhi mixture) was developed and validated by Dai et al. [183].

6. Alkaloids, vitamins, essential minerals, flavors, steroids/sterols and fatty acids

Hou et al. [184] reported the isolation of two alkaloids, choline and betaine, from the spores of *G. lucidum*. By successive extractions with ethanol and water, defatting with ethyl ether, and precipitation with Reinecke's salt, the obtained water-soluble fraction was chromatographed on an alumina column. Elution with chloroform–methanol (9:1) gave fraction I (identified as choline), whereas elution with methanol gave fraction II, which was further separated by preparative TLC on alumina layer for the isolation of betaine. On the other hand, Yu et al. [185] reported the isolation of two novel pyrrole alkaloids (ganoine and ganodine) and a novel purine alkaloid (ganoderpurine) from the mycelia of *G. capense*.

Vitamins and essential elements have been isolated from various Lingzhi species. For example, Zhang and Zhang [186] recently analyzed the vitamin contents in the spores of *G. lucidum* using RP-HPLC and the results indicated the presence of Vitamins C and E, as well as β-carotene. Shang et al. [187] reported the isolation and purification of two kinds of polysaccharides containing selenium in *G. lucidum* using ion-exchange chromatography (DEAE-cellulose column), gel filtration chromatography (Sephadex-100 column) and electrophoresis (PAGE). Aroma compounds produced by shake flask cultured medium of 20 basidiomycete strains, including *G. applanatum*, have been analyzed by gas–liquid chromatography–olfactometry as well as GC–MS techniques for the development of flavors through novel biotechnologies [188]. Over 120 volatile flavor compounds, mostly alcohols, aldehydes, ketones, esters, and phenols, have been identified.

The free sterols of *G. applanatum* and *G. lucidum* has been determined by Kac et al. [189] to contain mainly 24-methylcholesta-5,7,22-trien-3β-ol (ergosterol) and 24-methylcholesta-7,22-trien-3β-ol. The extracts from these fungi were first saponified and then the crude sterol mixtures were fractionated by preparative TLC (silica gel G), followed analysis by GC and GC–MS as free sterols and as their trimethyl-silyl either derivatives. Chiang and Chu [190] also described the use of preparative TLC and GC for the determination of a number of steroidal compounds from *G. lucidum*, such as ergosterol. Importantly, the first isolation of 8,9-epoxyergosta-5,22-dien-3β,15-diol from *G. lucidum* was reported in their work.

Very-long-chain fatty acids (VLCFA) with more than 23 carbon atoms have been reported by Rezanka et al. [191] to exist in higher fungi such as *G. applanatum* at trace levels (1–2%). Hence, the determination of trace amounts of these VLCFA in *Ganoderma* required sample enrichment. It was found that, in contrast to other chromatographic methods such as RP-TLC, semi-preparative RP-HPLC (Separon SGX C_{18} column) was the only suitable method for the enrichment of samples with VLCFA, which provided excellent yields with no sample degradation. Also, fatty acids present in *G. lucidium* have been analyzed by common chromatographic methods, such as GC and TLC, as reported by Casalicchio et al. [192] as well as Murai [193].

7. Concluding remarks

Unlike Western medicine in which therapeutic effects usually derive from a single chemical substance within the drugs, the pharmacologically activities of herbal medicine invariably arise from a mixture of "active" ingredients within the herbal materials [194]. In fact, in many cases, the biological activities of many of the active components administrated or consumed alone were found to be lower than those obtained from the original mixture of active ingredients present in herbs, such as Lingzhi [3]. Thus, although recent scientific studies have begun to provide some important information concerning the chemical identities of bioactive compounds isolated from different Lingzhi species, as well as some insights into the relationships between their chemical structures and pharmacological activities, much more research efforts are needed to gain a better understanding of not just the effect of a single active ingredient, but also the combined or synergistic effects of a mixture of active components that are present in Lingzhi on biological activities. Towards this end, in addition to conventional techniques such as GC, GC–MS and HPLC, the use of modern chromatographic/electrophoretic techniques with higher separation and detection power, such as micro-LC [195], CE [195–197], CE–MS [198] and/or LC–MS–MS [199] for the characterization and determination of a variety of major and minor (both "active" and "non-active" components) within Lingzhi extracts would be necessary. Also for improving the quality assurance and quality control of Lingzhi products, the use of high resolution and sensitive techniques, such as CE-laser induced fluorescence [200,201] and comprehensive

two-dimensional gas chromatography (GC × GC) [202,203], for the fingerprint profiling of active ingredients in Lingzhi should be highly beneficial.

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