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Fingerprint profiling of acid hydrolyzates of polysaccharides extracted from the fruiting bodies and spores of Lingzhi by high-performance thin-layer chromatography

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Abstract

Modern extraction and planar chromatographic instrumentation were employed for the fingerprint profiling of carbohydrates from an important and popular medicinal mushroom commonly known as Lingzhi. For the first time, the feasibility of employing the high-performance thin-layer chromatography (HPTLC) peak profiles (fingerprints) of carbohydrates for the screening of various Lingzhi species/products was demonstrated. An analytical procedure was developed such that upon acid hydrolysis of the 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 plates (Si 50 000) for separation and 4-aminobenzoic acid as the post-chromatographic derivatization reagent for detection. Also, using automated multiple development (AMD), the acid hydrolyzates from Lingzhi, consisting of simple and more complex sugars, can be separated simultaneously with high degree of automation. An important finding was that unique fingerprint patterns were observed in the monosaccharide profiles between two highly valued Lingzhi species, *Ganoderma applanatum* and *Ganoderma lucidum*, under total or partial acid hydrolysis conditions. Additionally, the HPTLC fingerprint profiles of carbohydrates were obtained from the extracts of the spores and fruiting bodies of Lingzhi and compared.

Keywords: Lingzhi; Fingerprint profiling; Polysaccharides

1. Introduction

In addition to Ginseng, which is well known for its tonic and anti-aging/stress effects, another ancient Chinese medicine which is highly revered in Asian countries as well as the West for its abilities to protect humans against biological, emotional and environmental stresses is Lingzhi [1,2]. Lingzhi was deemed as an elixir of life for thousand of years and, at present, commercial Lingzhi products in the forms of raw herbs, tea, powders and capsules, are commonly found in the market place as remedies for the treatment of more than 20 different illnesses, including migraine and headache, hypertension, arthritis, hepatitis, cardiovascular problems and cancer. Modern

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research has revealed that Lingzhi contains a variety of chemical ingredients, including simple and complex carbohydrates, organic germanium, triterpenes, adenosine, alkaloids, numerous mineral elements and an array of amino acids [3–6]. Among these various phytochemicals, triterpenes and polysaccharides have attracted much attention by the international scientific community, as triterpenes were shown to possess the abilities of lowering blood pressure as well as blood lipids [1,7] and, perhaps more interesting, polysaccharides present in Lingzhi were found to possess potent anti-tumor effect, which was attributed to the immuno-modulation property of these long-chain carbohydrates [8,9].

In recent years, the profiling of the relative amounts of various active ingredients (i.e. fingerprint profiling) has been shown to be a convenient and effective method for the standardization and quality control of various herbal materials, especially when there is a lack of authentic standards for the identification of all the active components present in these complex natural products [10–13]. In fact, the use of high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) for the fingerprint profiling of triterpenes present in the fruiting bodies of Lingzhi has been demonstrated [14,15]. However, to the best our knowledge, the feasibility of using HPLC or TLC for the standardization and quality control of various Lingzhi products based on the fingerprint profiling of carbohydrates from Lingzhi extracts has not been reported. This is perhaps not surprising since the separation and detection of carbohydrates, especially long-chain polysaccharides, is well known to be a highly challenging and difficult analytical problem [16,17].

Although TLC was first demonstrated to be a simple and effective tool for the determination of mono-, di-, oligo- and polysaccharides present in real samples, the rapid instrumental development of HPLC in the 1980s has essentially taken over as the method of choice for the analysis of various carbohydrates [18,19]. In recent years, however, there seems to be a resurgence of interest in TLC, as the introduction of high-performance plates and modern, highly automated TLC instrumentation, starting from the application of samples onto the plate, to their elution and their qualitative or quantitative analysis, have opened up new avenues for the rapid and reliable measurements of various analytes, especially for samples that normally require cumbersome clean-up procedures [20,21]. In particular, the advent of the technique of automated multiple development (AMD) method allows for gradient elution to be achieved in TLC with relative ease and, thus, complex samples such as sugars present in beet or cane molasses, can be analyzed (or screened) on high-performance thin-layer chromatography (HPTLC) plates using modern TLC scanners with relatively high resolution and sensitivity [20].

A key factor in the widespread acceptance of natural remedies, such as Ginseng and Lingzhi, by the international community involves the “modernization” of herbal medicines. In other words, the standardization and quality control of herbal materials by the use of modern science and technology is crucial [10–13]. Currently, however, quality-related problems (e.g. lack of consistency in terms of chemical compositions) seem to be overshadowing the potential benefits of various herbal products, and a major cause of these problems is the lack of simple and reliable analytical techniques/methodologies in the quality assurance/quality control of complex herbal materials, such as various Lingzhi products.

In the present work, the feasibility and advantages of employing a modern extraction technique (pressurized liquid extraction, PLE) and advanced planar chromatographic tools (HPTLC plates, AMD apparatus, automated sampler and scanner) for the fingerprint profiling of carbohydrates obtained from the fruiting bodies of various Lingzhi species were investigated and demonstrated. In particular, an analytical procedure based on the acid hydrolysis of polysaccharides extracted from the samples, followed by the use of AMD for separation and post-chromatographic derivatization with 4-aminobenzoic acid for detection on HPTLC Si 50 000 plates, was developed for the fingerprint profiling of the acid degradation products of polysaccharides extracted from various Lingzhi samples. The acid hydrolysis conditions were optimized so as to obtain unique/distinctive fingerprint profiles for the screening and differentiation of various Lingzhi species/products based on the evaluation of the relative amounts of sugars that appeared on the densitograms. Also, for the first time, the HPTLC fingerprint profiles of carbohydrates obtained from the extracts of the spores of Lingzhi were obtained and compared to those obtained from the fruiting bodies.

2. Experimental

2.1. Apparatus

An ASE 200 pressurized liquid extraction system (Dionex trade name ASE for accelerated solvent extraction; Dionex, Sunnyvale, CA, USA) was used to for all extractions. Sample solutions were applied onto the plates with an Automated TLC Sampler III (Camag, Muttenz, Switzerland) and was controlled by WinCATS software. Plates were developed in an AMD system (Camag, Muttenz, Switzerland) or a horizontal developing chamber (Camag, Muttenz, Switzerland). A TLC Scanner III with WinCATS software (Camag, Muttenz, Switzerland) were used for scanning the TLC plates. A ReproStar 3 with VideoStore 2 documentation software (Camag, Muttenz, Switzerland) was used for the imaging and archiving the TLC chromatograms. The HPTLC silica gel 60 plates (10 cm × 10 cm) and HPTLC Si 50 000 plates (10 cm × 10 cm) were purchased from E. Merck (Darmstadt, Germany).

2.2. Herbal materials and chemicals

The fruiting bodies of *Ganoderma lucidum* (Curtis: Fr.) P. Karst, *Ganoderma nigrolucidum* (Lloyd) Reid, *Ganoderma applanatum* (Pers.) Pat, *Ganoderma tropicum* (Jungh.) Bres, *Trametes versicolor* (L.: Fr.) Pilat and the spores of *G. lucidum* were gifts from ParaPharm Corporation (Hong Kong, China). D-Galacturonic acid (99%), D-glucuronic acid (99%), D-(+)-xylose (99%), L-(+)-arabinose (99%), and L-rhamnose (99%), and 4-aminobenzoic acid were purchased from Aldrich–Sigma. D-(+)-Galactose (99.5%), D-(+)-glucose monohydrate, and D-(+)-fucose (99%) were obtained from Fluka (Buchs, Switzerland). D-(+)-Mannose (99%) was obtained from Acros (Glee, Belgium). All other reagents were of analytical-reagent grade. Water was obtained from a Milli-Q (Millipore, Bedford, MA, USA) water purification system.

2.3. Preparation of reference solution

Standard sugar mixtures containing 0.2 mg/ml of galactose, glucose, mannose, xylose, rhamnose, galacturonic acid, 0.4 mg/ml of arabinose, 0.6 mg/ml of glucuronic acid, 1.2 mg/ml of fucose, were prepared in methanol–water (1:1, v/v).

2.4. Preparation and pressurized liquid extraction (PLE) of samples

The fruiting bodies of *G. lucidum*, *G. nigrolucidum*, *G. applanatum*, *G. tropicum*, *T. versicolor* were dried at 55 °C for 4 h, then ground to fine powders before use. A 0.5 g of each sample (including the spores of *G. lucidum*) was loaded into a 11 ml stainless steel cell and batches of six samples were extracted by PLE in an automatic extraction sequence as follows: the extraction was performed with water at 120 °C and 1500 psi with 7 min preheating time, 5 min static time and 90 s purge time for a total of two cycles (1 psi = 6894.76 Pa). The water extract was concentrated to ca. 5 ml and used for the preparation of crude polysaccharides.

2.5. Preparation of crude polysaccharides

The concentrated water extract was mixed with ca. 25 ml (five times the volume of the concentrated water extract) of 95% ethanol and placed at room temperature for 1 h. The suspension was centrifuged at 3000 rpm for 10 min and the supernatant was discarded. The precipitate was washed with 10 ml of 95% ethanol and then dried under a stream of nitrogen. The crude polysaccharides obtained were used for acid hydrolysis without further purification.

2.6. Hydrolysis procedures for polysaccharides

The crude polysaccharides were mixed with 1 ml of 0.75 M H_2SO_4 in a screw-cap vial and hydrolyzed in a water-bath set at 85 °C for 1 h. After cooling, the solution that contained the hydrolyzates was diluted by adding 1 ml of water and then neutralized by addition of $BaCO_3$. The precipitate was removed after a centrifugation step. The supernatant was then transferred and filtered through a 0.45 μm nylon filter.

2.7. HPTLC procedures

For the separation and detection of carbohydrates, an aliquot of the acid hydrolyzates from the fruiting bodies (4–6 μl) and spores (12 μl) of the Lingzhi samples, the fruiting body of *T. versicolor* (1.5 μl), as well

Table 1
Gradient program used for AMD

Step number[a]	Development time (min)		Addressed bottle number[b,c]
	A[b]	B[c]	
1	4.2	5.8	1
2	10.0	12.2	2
3	16.3	18.6	2
4	23.9	27.0	2
5	32.4	35.7	2
6	44.7	48.0	2
7	56.0	59.5	2

[a] Drying time between each step: 7 min; preconditioning solution in wash bottle: 4.7% NH_3.

[b] A: employed for the simultaneous separation of simple and complex sugars; addressed bottle 1 contained *n*-propanol–water (60:40) and bottle 2 contained *n*-propanol–water (83:17).

[c] B: employed for the separation of monosaccharides; addressed bottle 1 contained *n*-propanol–water (60:40) and bottle 2 contained *n*-propanol–water (80:20).

as the 2.0 μl of the sugar standards solution were applied onto the Si 50 000 plate at 8 mm from the lower edge of the plate. The plate was automatically developed in an AMD apparatus with a solvent gradient consisting of seven steps and a development distance increment of about 10 mm per step. The gradient program is shown in Table 1. After AMD, the plate was sprayed with a reagent solution consisting of 0.5 g of 4-aminobenzoic acid, 9 ml of glacial acetic acid, 10 ml of water and 0.5 ml of 85% H_3PO_4, and was then heated at 100 °C for 20 min. The plate was imaged immediately and then scanned at 365 nm using absorption mode with 4.0 mm × 0.1 mm slit dimensions and 20 mm/s scanning speed.

3. Results and discussion

3.1. Selection of extraction, separation and detection methods

PLE has been recently exploited for the extraction of active ingredients from medicinal plants, such as ginsenosides from the roots of Ginseng with improved performances [22–24]. In the present study, it was found that PLE was better than the use of a traditional method (sonication) for the extraction polysaccharides from Lingzhi in terms of speed of extraction and/or recovery. For example, the total amount of polysaccharides extracted using PLE (with water at 120 °C and 1500 psi for 5 min of extraction and a total of two cycles) was found to be higher than those obtained using sonication (with water at 100 °C for 30 min and extracted twice).

Although conventional plates, consisting of silica gel 60 or 70, amino, and diol phases, have been shown to be useful for the resolution of acid or enzymatic hydrolysis products of polysaccharides, the use of a novel synthetic porous silica, Si 50 000, has been demonstrated to be more advantageous for the rapid separation and detection of polar analytes, such as sugars [21]. Compared to regular silica gels, Si 50 000 has an extremely large pore diameter (5000 nm) and a low surface area of about 0.5 m^2/g [25], thus resulting in minimal spot tailing of polar compounds such as carbohydrates. Indeed, when compared to silica gel 60 plate type, we found that Si 50 000 yielded the best resolution and peak shape for the separation of polysaccharide hydrolyzates from Lingzhi (vide infra). In addition to plate type, the availability of modern TLC instrumentation—an AMD apparatus—was found to be important for the rapid fingerprinting, i.e. simultaneous separation/screening of simple and more complex sugars, of various hydrolyzed Lingzhi extracts with relatively high resolution and sensitivity (see below).

The sensitive detection of small amounts of sugars by spectroscopic methods is problematic due to the lack of suitable chromophores among native carbohydrates [19]. For TLC, simple charring of the sugars on the plate surfaces offer a simple and convenient means for the optical detection of these non-absorbing species based on densitometry [21]. However, we found that limits of detection (LODs) using this simple approach was too high for the observation of small amounts of sugars obtained from the acid hydrolyzates of Lingzhi. To achieve lower LODs, derivatization of the sugars with various visualization agents were investigated, and it was found that 4-aminobenzoic acid offered the best LODs (ca. 10 ng) for the visual fluorescent detection of various acid hydrolyzates of polysaccharides separated on Si 50 000 plate.

3.2. Optimization of acid hydrolysis conditions

The major objective of the hydrolysis procedure was to obtain a sufficiently large number of

Table 2
Factors and levels for the optimization of hydrolysis condition

Factor	Level		
	1	2	3
A: H_2SO_4 concentration (M)	0.75	0.5	1.0
B: hydrolysis temperature (°C)	85	70	100
C: hydrolysis time (h)	2	1	3
D: H_2SO_4 volume (ml)	1.5	1.0	2.0

detectable peaks on the TLC chromatogram so that unique/distinctive peak profiles or patterns can be easily observed from different species or parts (spores versus the fruiting body) of Lingzhi. In the present study, crude polysaccharides extracted from the fruiting body of the Lingzhi species, *G. lucidum*, were chosen to determine the optimal hydrolysis conditions. The suitability of employing total and partial acid hydrolysis procedures for the fingerprint profiling of *G. lucidum* was evaluated. For total acid hydrolysis (i.e. complete acid degradation of polysaccharides into individual monosaccharides), commonly used conditions were employed (i.e. 1 M H_2SO_4 at 100 °C) [21] to study the amounts of monosaccharides liberated as a function of hydrolysis time, and it was found that a hydrolysis time of 4 h or more resulted in total hydrolysis of the polysaccharide extracts.

The optimization of conditions for the partial hydrolysis of *G. lucidum* was more complicated, since it involved the study of the effects of four factors: H_2SO_4 concentration, volume, hydrolysis temperature and time. To reduce the number of experiments required to find the optimal conditions involving these four experimental factors, an orthogonal experimental design was applied so that only nine experiments were required (as opposed to 81 experiments required for a full 3^4 design). The experimental factors and corresponding levels were shown in Table 2, and different hydrolysis conditions employed in the nine experiments were presented in Table 3. In the present study, the optimal conditions for partial hydrolysis of *G. lucidum* were considered to be those that resulted in the generation of the most number of peaks and with minimal differences between peak heights. Thus, the sum of the relative peak heights (SRPH) was used as a criterion for the selection of the optimal partial hydrolysis conditions.

The sum of the three SRPH values at levels 1, 2 and 3 (denoted as I, II and III, respectively) for each of the four experimental factors shown in Table 2 were calculated and the *R* values (which represent the difference between the maximal and minimal SRPH values among I, II and III at each of the four experimental factors) were determined. The experimental factor which has the largest *R* value is the one that influenced the hydrolysis process the most. In the present study, hydrolysis temperature (with $R = 6.28$) was determined to be more important than the other three factors (i.e. hydrolysis time, acid concentration and volume, which yielded *R* values in the range of 1.5–2.0) in governing the partial hydrolysis conditions, and the optimal conditions (according to SRPH values) was found to occur in run 1 (H_2SO_4 concentration = 0.75 M; volume = 1.5 ml; hydrolysis temperature = 85 °C; time = 2 h). Since the hydrolysis time and volume of the acid were found to be less critical factors according to their *R* values, an hydrolysis time = 1 h and volume of H_2SO_4 = 1 ml

Table 3
Orthogonal experimental design $L_9(3^4)$

Run number	Concentration, A (mol/l)	Temperature, B (°C)	Time, C (h)	Volume, D (ml)	SRPH
1	1 (0.75)[a]	1 (85)	1 (2)	1 (1.5)	7.772
2	1 (0.75)	2 (70)	2 (1)	2 (1.0)	4.805
3	1 (0.75)	3 (100)	3 (3)	3 (2.0)	5.604
4	2 (0.5)	1 (85)	2 (1)	3 (2.0)	5.805
5	2 (0.5)	2 (70)	3 (3)	1 (1.5)	4.765
6	2 (0.5)	3 (100)	1 (2)	2 (1.0)	6.007
7	3 (1.0)	1 (85)	3 (3)	2 (1.0)	7.318
8	3 (1.0)	2 (70)	1 (2)	3 (2.0)	4.852
9	3 (1.0)	3 (100)	2 (1)	1 (1.5)	5.989

[a] Level (experimental value).

were employed to reduce extraction time and reagent consumption.

3.3. Fingerprint profiling of water extracts from different Lingzhi species

Lingzhi is available in different varieties and medicinal activities may vary significantly from one species to another, mainly due to differences in the chemical composition/concentration of the active ingredients. The most well-known Lingzhi variety is *G. lucidum*, which is being cultivated in many countries for its high commercial value. However, many still preferred Lingzhi that is grown in the wild, such as *G. applanatum*: an ancient Lingzhi of legend and was highly revered as the "mushroom of longevity" [1,2]. In recent years, polysaccharides extracted from various Lingzhi species are being actively promoted in the market place as "new and effective" natural remedies for a variety of aliments and diseases. Acid degradation products of polysaccharides derived from natural products, such as gums and seaweed, have been found to contain various simple and complex sugars, including uronic acids [21]. Fig. 1 shows the HPTLC chromatogram of a mixture of nine monosaccharide standards, including galacturonic acid and glucuronic acid. It can be seen that using the present chromatographic conditions, baseline resolution of almost all of the nine sugar standards on Si 50 000 plate can be achieved, without showing any significant tailing for the separated peaks.

Fig. 2A shows the HPLTC chromatogram (fingerprint profile) due to the total acid hydrolysis of polysaccharides obtained from the water extract of the fruiting body of *G. applanatum*. It is important to note that all of the nine monosaacharide standards that appear in Fig. 1 can be matched with corresponding peaks shown in Fig. 2A according to their retention values (R_f). The peaks with the highest intensity was due to peak 4 (glucose), followed by peak 2 (glucuronic acid), peak 3 (galactose) and peak 5 (mannose), whereas peak 6 (arabinose), peak 7 (xylose), peak 8 (fucose) and peak 9 (rhamnose) exhibited lower peak intensities, which can be attributed in part

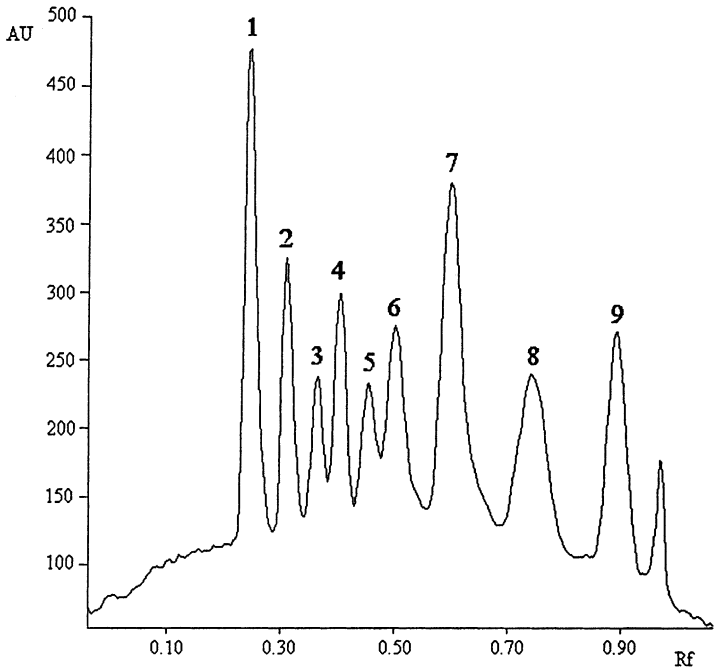

Fig. 1. HPTLC chromatogram of nine monosaccharide standards. Peaks: 1: galacturonic acid; 2: glucuronic acid; 3: galactose; 4: glucose; 5: mannose; 6: arabinose; 7: xylose; 8: fucose; 9: rhamnose. The standard sample solution contained 0.2 mg/ml each of galactose, glucose, mannose, xylose, rhamnose, galacturonic acid, 0.4 mg/ml of arabinose, 0.6 mg/ml of glucuronic acid, and 1.2 mg/ml of fucose.

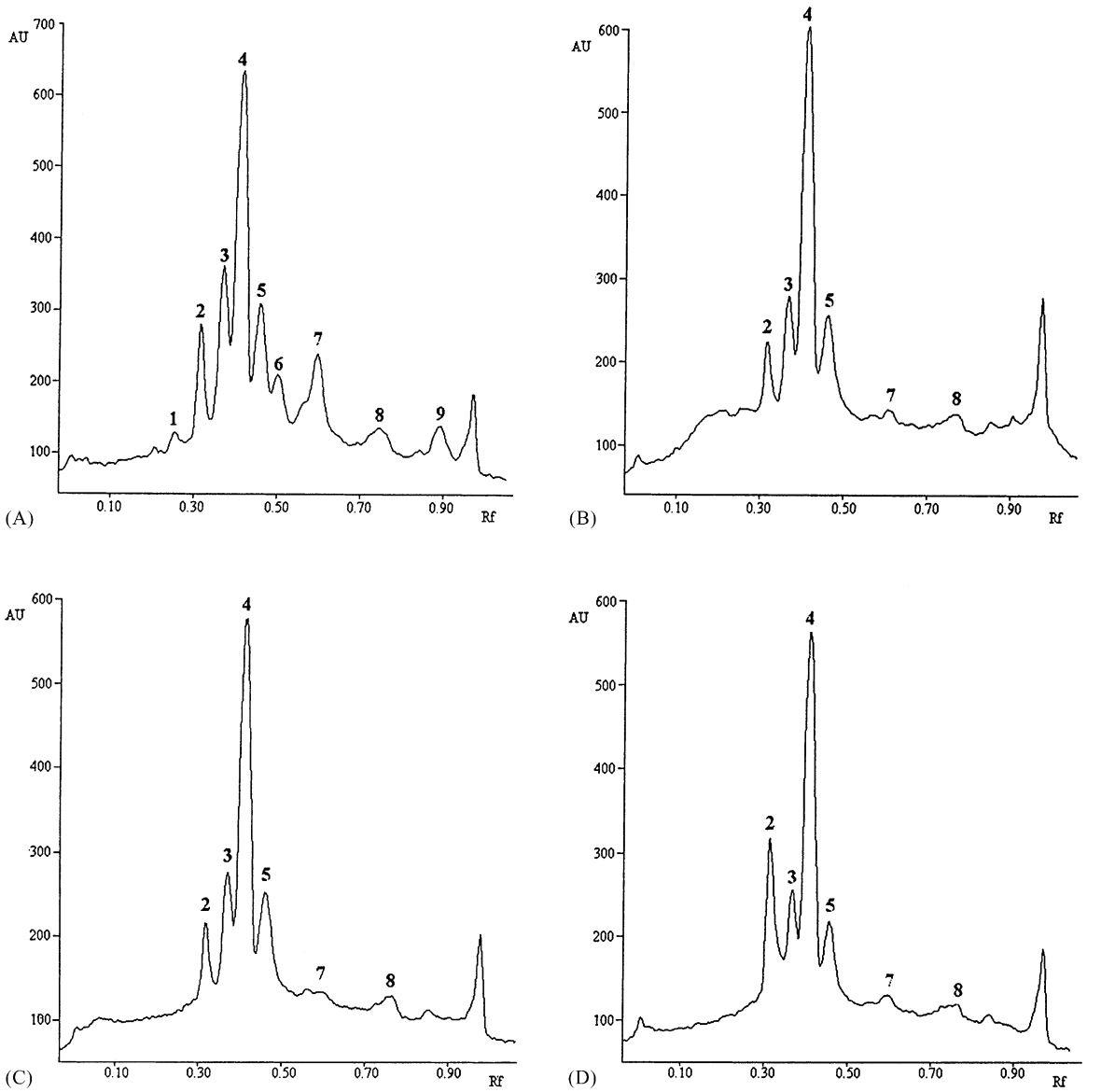

Fig. 2. HPTLC chromatogram of acid hydrolyzates of polysaccharides from water extracts of four different Lingzhi species obtained under total hydrolysis conditions. Fruiting bodies of: (A) *G. applanatum*; (B) *G. lucidum*; (C) *G. nigrolucidum*; (D) *G. tropicum*. Peak assignments from (A) to (D) are the same as those in Fig. 1.

to the possibility that 6-deoxy sugars (e.g. rhamnose and fucose) and furanose (e.g. arabinose and xylose) are more vulnerable to acid/thermal degradation [26].

Fig. 2B shows the fingerprint profile of the acid degradation products of polysaccharides extracted from the fruiting body of *G. lucidum*, obtained under the same total acid hydrolysis conditions as in Fig. 2A. It is interesting to note that when compared to the case of *G. applanatum* (Fig. 2A), the fingerprint profile of *G. lucidum* (Fig. 2B) was found to contain much lesser number of peaks, i.e. peak 1 (galacturonic acid), peak 6 (arabinose) and peak 9

(rhamnose) were undetectable, and peak 7 (xylose) and peak 8 (fucose) showed a marked decreased in intensity and were barely distinguishable from the baseline. Therefore, the apparent differences in the peak intensity profiles as observed between Fig. 2A and B can be conveniently used for the differentiation between *G. applanatum* and *G. lucidum*. It should be noted that some minor differences occurred in the fingerprint profiles between different samples of the same Lingzhi species. However, these experimental uncertainties were found to be insignificant relative to the differences observed from the acid hydrolysis of polysaccharides from samples of different Lingzhi species, such as *G. applanatum* and *G. lucidum*, as shown in Fig. 2A and B.

The fingerprint profile of a Lingzhi species related to *G. lucidum*, namely, *G. nigrolucidum*, is shown in Fig. 2C. It can been seen that the fingerprint profiles of these two related Lingzhi species obtained under total hydrolysis conditions are almost identical by comparing Fig. 2B and C and, thus, cannot be used for differentiation of these two Lingzhi species. On the other hand, the fingerprint profile of another Lingzhi species, *G. tropicum*, is shown in Fig. 2D. It is clear that the fingerprint profile of this particular Lingzhi variety is very different than that of *G. applanatum* as shown in Fig. 2A, e.g. peak 1 (galacturonic acid), peak 6 (arabinose) and peak 9 (rhamnose) were not detectable and the intensity of peak 2 (glucuronic acid) was higher than peak 3 (galactose) in the peak profile of *G. tropicum* (Fig. 2D). Similarly, some minor differences can be observed in the fingerprint profiles between *G. tropicum* (Fig. 2D) and those of *G. lucidum* (Fig. 2B) and *G. nigrolucidum* (Fig. 2C), i.e. the peak intensity ratio of peak 2 (glucuronic acid) and peak 3 (galactose) in Fig. 2D was found to be much higher when compared to that in Fig. 2B and C.

Using partial acid hydrolysis conditions for the degradation of polysaccharides from the fruiting body of *G. applanatum*, Fig. 3A shows that this wildly grown Lingzhi species yielded a fingerprint profile that contained a larger number of peaks and yielded a somewhat different peak intensity profile when compared to the same sample extract obtained under total hydrolysis conditions. For instance, in comparison to Fig. 2A, it can be seen that the intensity of peak 6 (arabinose) and peak 7 (xylose) is much higher relative to peak 4 (glucose) as shown in Fig. 3A, as well as the intensity of peak 3 (galactose) relative to peak 4 (glucose). On the other hand, the signal intensity of the two uronic acids, peak 1 (undetectable) and peak 2, showed a marked decreased in relative intensity when compared to those in Fig. 2A, especially for glucuronic acid (peak 2). Additionally, under partial hydrolysis conditions, a series of minor peaks appeared in a region between the sample origin and the location of glucuronic acid ($R_f = 0.32$) on the plate, and this series of minor peaks can be observed for other Lingzhi species as well under partial hydrolysis conditions, as shown in Fig. 3B–D.

The HPTLC chromatogram of *G. lucidum* obtained under partial hydrolysis conditions is shown in Fig. 3B. As in the case of total hydrolysis, it can be seen that the fingerprint profile of *G. applanatum* (Fig. 3A), can be differentiated from *G. lucidum* with relative ease. For instance, by comparing Fig. 3A and B, it is clear that peak 6 (arabinose), peak 7 (xylose) and peak 9 (rhamnose) are clearly visible in the case of *G. applanatum* (Fig. 3A), which can be used as distinctive markers for this highly valued Lingzhi species. On the other hand, for the differentiation of the two related Lingzhi species—*G. lucidum* (Fig. 3B) and *G. nigrolucidum* (Fig. 3C)—under partial hydrolysis conditions, their respective fingerprint profiles were found to be very similar except that an extra peak appeared to occur adjacent to peak 7 (xylose) in the fingerprint profile of *G. nigrolucidum* when comparing Fig. 3B and C. However, under further investigation, it was found that this unidentified peak (peak u), which appeared to be missing in the profile of *G. lucidum* (Fig. 3B) was observable in the photographic recordings due to higher resolution that can be obtained (data not shown). As in the case of total hydrolysis, the fingerprint profile of *G. tropicum* (Fig. 3D) can be easily differentiated from that of *G. applanatum* (Fig. 3A) under partial hydrolysis conditions, e.g. peak 6 (arabinose) and peak 9 (rhamnose) were not detectable in the chromatogram of *G. tropicum* (Fig. 3D).

It is important to note that the series of minor peaks that appeared under partial acid hydrolysis conditions (Fig. 3A–D) were not detectable when extracts were degraded under total hydrolysis conditions (Fig. 2A–D). Furthermore, it is interesting to note that the peak intensity of a number of monosaccharides, such as galactose (peak 3), glucose (peak 4),

mannose (peak 5), as well as that of the two uronic acids (peaks 1 and 2) obtained under total acid hydrolysis was much higher when compared to the same peaks obtained under partial hydrolysis conditions. Therefore, these observations suggest that the series of minor peaks that occurred between the sample origin and the location of glucuronic acid (peak 2) as shown in Fig. 3A–D were hydrolyzed into various monosaccharides under total hydrolysis conditions, and these minor peaks were likely degradation products from polysaccharides that appeared in the forms of di-, tri- and/or oligosaccharides [21]. Unfortunately, the signal intensities of these minor peaks were relatively small and their fingerprint profiles appeared to be quite similar between different Lingzhi species as shown in Fig. 3A–D, making their

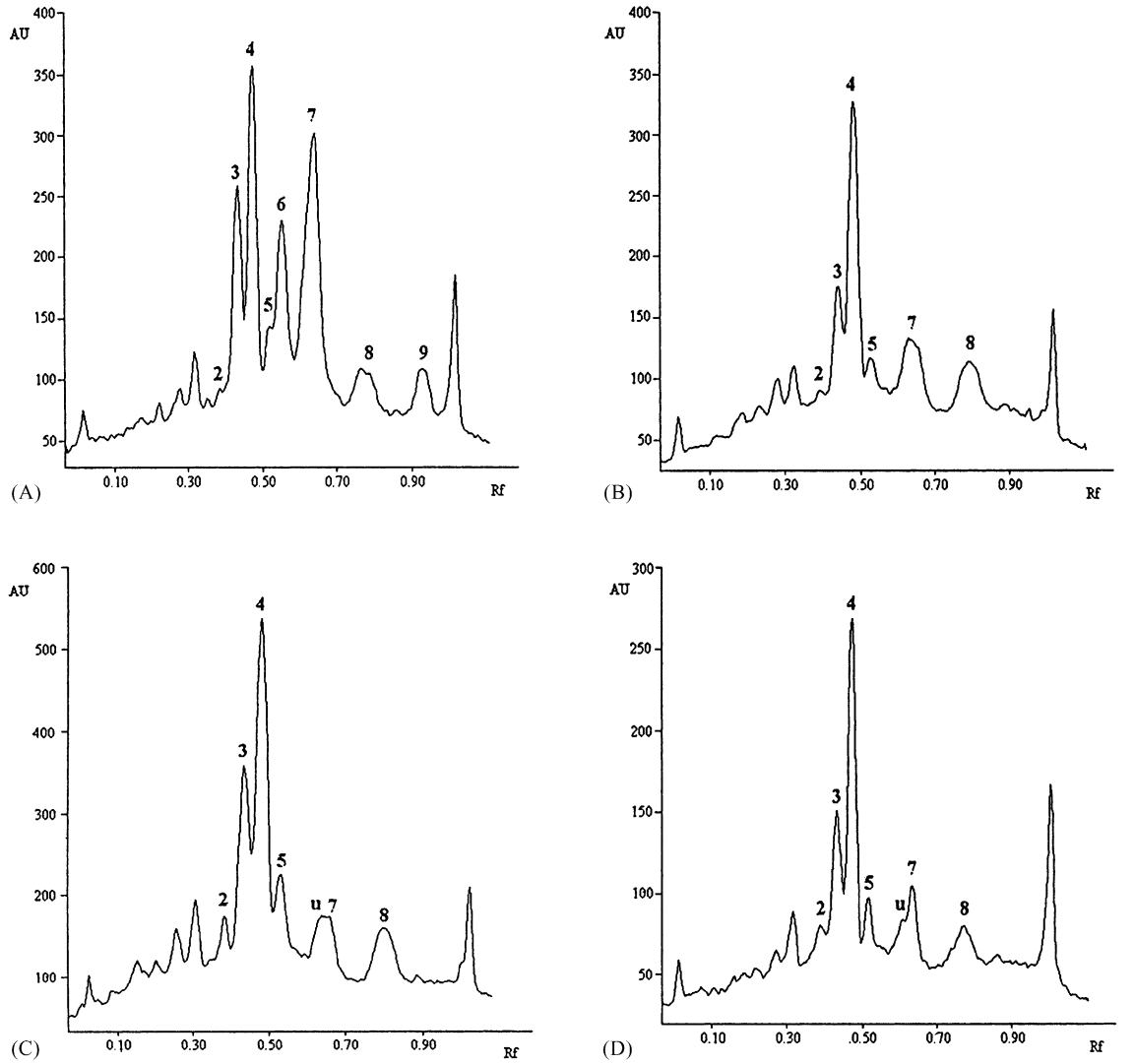

Fig. 3. HPTLC chromatogram of acid hydrolyzates of polysaccharides from water extracts of the fruiting bodies of four different Lingzhi species: (A) *G. applanatum*; (B) *G. lucidum*; (C) *G. nigrolucidum*; (D) *G. tropicum*, of the fruiting body of (E) *T. versicolor*, and of the spores of (F) *G. lucidum*, obtained under partial acid hydrolysis conditions. Peak assignments from (A) to (F) are the same as those in Fig. 1, except for peak u, which was unidentified.

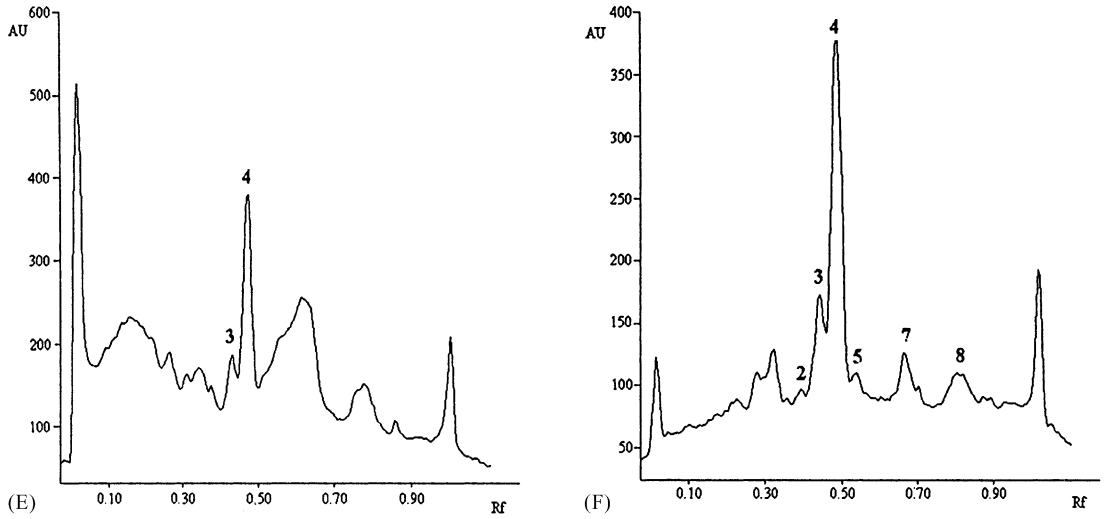

Fig. 3. (*Continued*).

usage for differentiation rather difficult under the present experimental conditions. However, it is interesting to observe in Fig. 3E that the signal intensity of a broad band that appeared in the region near the sample origin and $R_f = 0.32$ due to the acid hydrolysis of a medicinal mushroom that does not belong to the Lingzhi genus, i.e. *T. versicolor*, was significantly higher, albeit the chromatographic resolution was rather poor. These results, however, do suggest that the relative signal intensity in the region of the chromatogram where more complex sugars would be located under the present experimental conditions could be used for the screening and differentiation of mushrooms belonging to different genus.

The fingerprint profiles as shown in Figs. 2A–D and 3A–D were obtained from the extraction of polysaccharides from the fruiting bodies of various Lingzhi species. In the market place, however, Lingzhi products derived from the spores of Lingzhi (rather than the fruiting bodies) are being actively promoted and sold at much higher prices, as recent scientific evidence has demonstrated that polysaccharides extracted from the spores of Lingzhi are more effective or potent in the killing of certain cancer cells in in vitro experiments [27]. Fig. 3F shows the HPTLC chromatogram obtained from acid hydrolysis of polysaccahrides extracted from the spores of *G. lucidum*. It is clear that by comparing with Fig. 3B (from the fruiting body of *G. lucidum*), the carbohydrate fingerprint profiles of the spores and fruiting body of this very common Lingzhi species are quite similar and cannot be easily differentiated, at least for the samples examined in the present study.

4. Concluding remarks

The analytical instrumentation and methodologies described in the present work should be useful in the standardization and quality assurance/control of various Lingzhi products. Based on differences observed in the fingerprint profiles of carbohydrates using HPTLC, the feasibility of screening/differentiating various Lingzhi as a function of its species/varieties as well as different parts of the mushroom (spores versus fruiting bodies) was demonstrated. Importantly, unique fingerprint profiles from the acid degradation of polysaccharides were observed for *G. applanatum* and *G. lucidum* under total or partial hydrolysis conditions, and these distinctive carbohydrate patterns can be readily used for the rapid differentiation of these two important Lingzhi species. In future work, application and further improvement of the present analytical procedures for the screening/quantitative evaluation of product consistency due to, for example, the effects of geographic origins, growing and

manufacturing conditions, etc. on the carbohydrate fingerprint profiles, as well as of safety and efficacy by the correlation of biological activities with these carbohydrate profiles, for a variety of Lingzhi species/products should be highly beneficial.

The use of fingerprint profiles from the degradation products of polysaccharides for the differentiation of related Lingzhi species, such as *G. lucidum* and *G. nigrolucidum*, however, was found to be rather difficult, due to the similarities of their profiles. Although the use of AMD allowed for the simultaneous separation of both simple and more complex sugars on the TLC plate, the series of peaks that appeared in the region of the plate where di-, tri- and oligosaccharides would be located (under partial acid hydrolysis conditions) were found to be too few and minute for the reliable fingerprinting/differentiation of various Lingzhi species, albeit the overall signal intensity within this particular region of the plate was found to be markedly different between mushrooms of different genus. More research is necessary to optimize the hydrolysis procedures (using acids or enzymes), as well as the separation and detection of degradation products of polysaccharides from Lingzhi, especially the di-, tri- and oligosaccharides, since the profiles of these more complex sugars, in combination with those of monosaccharides, may reveal some subtle but distinct differences between closely related Lingzhi species, and these comprehensive carbohydrate profiles should be reflective of the chemical compositions and structures of the various complex polysaccharides present in Lingzhi.

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