High-Performance Liquid Chromatographic Analysis for the Characterization of Triterpenoids from *Ganoderma*

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Abstract

A high-performance liquid chromatographic (HPLC) analysis of triterpenoids from *Ganoderma* is developed and validated in an attempt to explore a way to differentiate a number of species of the genus *Ganoderma*. Results show that 64 samples examined in this study could be divided into 18 groups based on characteristics of the HPLC pattern of triterpenoids. This result also conforms with those of the morphological examination and the interfertility test by di-monokaryotic mating. The HPLC analysis of triterpenoids further reveals that differentiation among samples from three different regions each of the two species *G. lucidum* and *G. tsugae* is workable. Even then, an incorrect designation is found for two of the groups of samples that were originally classified as *G. resinaceum* but showed different morphological characteristics and mating incompatibility. In conclusion, an HPLC analysis of triterpenoids is a simple and easy way to differentiate among different species of the genus *Ganoderma*.

Introduction

Members of the *Ganoderma* fungus genus have attracted considerable attention because they produce many biologically active triterpenoids (1–3). Among them, *G. lucidum* and *G. tsugae* have most frequently been used as folk medicine in China and Japan. More than 100 new triterpenoids (including ganoderic acid derivatives) have been isolated from the fruiting bodies and the cultured mycelia of these *Ganoderma* species. Some of these compounds have activities inhibiting both histamine release in rat mast cells (2) and the angiotensin-converting enzyme (3), cytotoxicity activity against hepatoma cells in vitro (1), anti-HIV-1 and anti-HIV-1-protease activities (4), and hepatoprotective effects as a β-glucuronidase inhibitor (5). The therapeutic effects of substances occurring in other basidiomycetous mushrooms (including *G. lucidum*) have been reviewed (6).

There are numerous species of *Ganoderma*, and the information gathered has usually been inadequate for identification and classification. Traditionally, classification was based on the morphological characteristics of the fruiting body. However, variations in the morphological characteristics of the fruiting body caused by differences in the growing origin and position, geological distribution, climate, and development of individual species make identification and confirmation difficult. Furthermore, different amounts of emphasis on the importance of the morphological characteristics of the fruiting body can result in the same species being given different names. Uncertainty in the defining of the *Ganoderma* species has possibly caused the misinterpretation or differences in explanation of the experimental results of in vivo studies. Therefore, several methods have been proposed to identify or confirm the genus and species of *Ganoderma*, especially for species with close relations. These methods include the scanning electron microscopy of the outline of basidiospores (7), an interfertility test (8), DNA restriction enzyme fragment length polymorphism (9), DNA sequence (10), isozyme patterns (11), secondary metabolites, API-ZYM analysis (Analytab Products Inc., Plainview, NY) (11), and growing conditions (7). However, most of these methods are time-consuming and not very cost effective.

Previously, it has been found that different species of *Ganoderma* have shown quite varied patterns in the thin-layer chromatography (TLC) chromatograms of triterpenoids. In the literature, a pattern was disclosed that was peculiar to the strain for several terpenoids isolated from the fruiting bodies of *G. lucidum* and *G. tsugae* (12). A chromatographic method has been developed to not only resolve each pair of stereoisomers but also identify certain triterpenoids present in the crude extract of *G. lucidum* (13). Changes in the triterpenoid patterns during formation of the fruiting body in *G. lucidum* have been reported as well (14). It was thought that those secondary metabolites of triterpenoids from *Ganoderma* could

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possibly be used as a way of identification. In this report, a high-performance liquid chromatographic (HPLC) separation of triterpenoids was developed and validated, and the possibility of using this to identify species of the *Ganoderma* genus was explored.

Experimental

Sample extraction and preparation

Two external standards of the ganoderic acids B and C_2 were purified from *G. tsugae* following a procedure previously developed in our laboratory (15). Sixty-four fruiting bodies either collected by our laboratory, supplied by Taiwan Agriculture Research Institute (TARI) of R.O.C. (Taipei, Taiwan), or purchased from the deposit of the Culture Collection and Research Center, (CCRC, Hsinchu, Taiwan, R.O.C.), were included in this study. Detailed information of these samples is included in Table I. All samples were dried and ground into fine powder. Two grams of powdered samples were mixed with 100 mL alcohol and then placed on a rotating shaker for 24 h. Filtrate was collected, and alcohol was evaporated to dryness under reduced pressure. A final volume of 1.5 mL for each sample was prepared as a stock solution with the addition of methanol. For quantitation, a sample solution for each sample was prepared by mixing two external standards at a respective concentration of 6.8 µg/mL (ganoderic acid B) and 0.27 µg/mL (ganoderic acid C_2) with an equal volume of the respective stock solution.

Chromatographic conditions

The HPLC equipment consisted of a Shimadzu (Kyoto,

Table I. Status Information for the Analyzed Samples and Grouping

Species	CCRC*	TARI*	Collection area	Grouping
G. australe	36128†		India	C‡
G. formosanum	37048	88-1-49†	Eastern Taiwan	B‡
G. lobatum	36245†		Canada	Q‡
G. lucidum	36021	87-1-1†	Central Taiwan	K
	36041	87-1-2†	Southern Taiwan	K
	37026	87-1-6†	Northern Taiwan	K‡
	37029	87-1-9†	Northern Taiwan	K
	37033	88-1-30†	Central Taiwan	K
	37043	88-1-44†	Eastern Taiwan	I
	37053	88-1-54†	Eastern Taiwan	I‡
	36124†		India	H‡
	36125†		Argentina	I
	36144†		North America	I
G. mirabile	36152†			R
G. neo-japonicum	36049	87-1-4†	Northern Taiwan	A
	37042	88-1-43†	Eastern Taiwan	A
	37051	88-1-52†	Northern Taiwan	A‡
G. oerstedii	36291†			R
G. pfeifferi	36159†			G‡
G. resinaceum	36146†			G
	36147†			R
	36149†			R
G. subamboinense var. laevisporum	36087†		Argentina	H
G. tsugae	36042†	87-1-3†	Central Taiwan	O
	36065†	87-1-14†	Central Taiwan	P
	36090†	87-1-19	Central Taiwan	O‡
		88-1-56†	Northern Taiwan	O
	36203	88-1-59†	Central Taiwan	O
	36204	88-1-60†	Northern Taiwan	P
–§	37063†			P‡
G. weberianum	36145†			F
–	37049†	88-1-50†	Eastern Taiwan	F
	37081	88-1-58†	Central Taiwan	F‡
–		88-1-77†		F
G. boniense	37040	88-1-39†	Central Taiwan	L‡
	37068	88-1-73†		L
G. calidophilum	36205	88-1-61†	Central Taiwan	D‡
G. curtisii		87-1-13†	Central Taiwan	P
		88-1-34†	Central Taiwan	P
		88-1-37†	Central Taiwan	P
		88-1-64†	Central Taiwan	P
		88-1-67†	Central Taiwan	P
	37064	88-1-68†	Central Taiwan	P
G. fornicatum	37067	88-1-71†	Northern Taiwan	N‡
G. mastoporum	37044	88-1-45†	Eastern Taiwan	E
		88-1-62†		E‡
G. tropicum		87-1-8†		M
		87-1-28†	Northern Taiwan	M
		88-1-40†	Northern Taiwan	M‡
		88-1-57†	Central Taiwan	M
		88-1-65†	Northern Taiwan	M
		88-1-69†	Northern Taiwan	M

* Company that assigned accession number.
† Analyzed samples.
‡ Representative of each group.
§ –, Undetermined species.

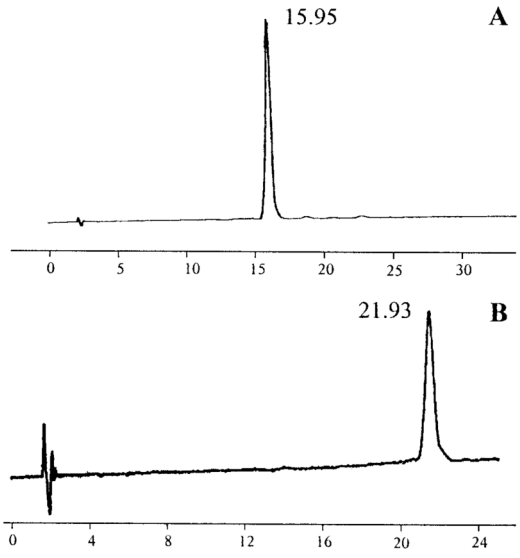

Figure 1. HPLC chromatograms for the ganoderic acids C_2 (A) and B (B).

Japan) LC-4A pump, an SPD-6AV UV detector (Shimadzu) with the wavelength set at 254 nm, and an SP4270 recorder (Integrator, Spectra-Physics, Mountain View, CA). A model CTO-2AS heater module (Shimadzu) was used to keep the column temperature constant at 42°C ± 0.5°C. Separation was carried out on a C-18 column (Lichrosphere 100, 300 × 4.6 mm, 5 µm) (Merck, Darmstadt, Germany). Five microliters of the stock or sample solution was introduced with a Rheodyne valve equipped with a 50-µL external loop. The mobile-phase flow rate was 1 mL/min and consisted of acetonitrile, glacial acetic acid, and distilled water (69.3:0.01:30.69). For normal-phase TLC examination, precoated silica-gel TLC plates (Kieselgel 60 F254, DC-Alufolien, Merck) were used. The developing reagent was composed of chloroform, methanol, and water in a volumetric ratio of 30:4:1. Visualization proceeded by spraying 10% sulfuric acid in alcohol and then heating the sample to 100°C in order to evaporate the solvents. The sample was initially examined under a UV lamp with a wavelength of 254 nm, and plates were further heated to 105°C for visualization.

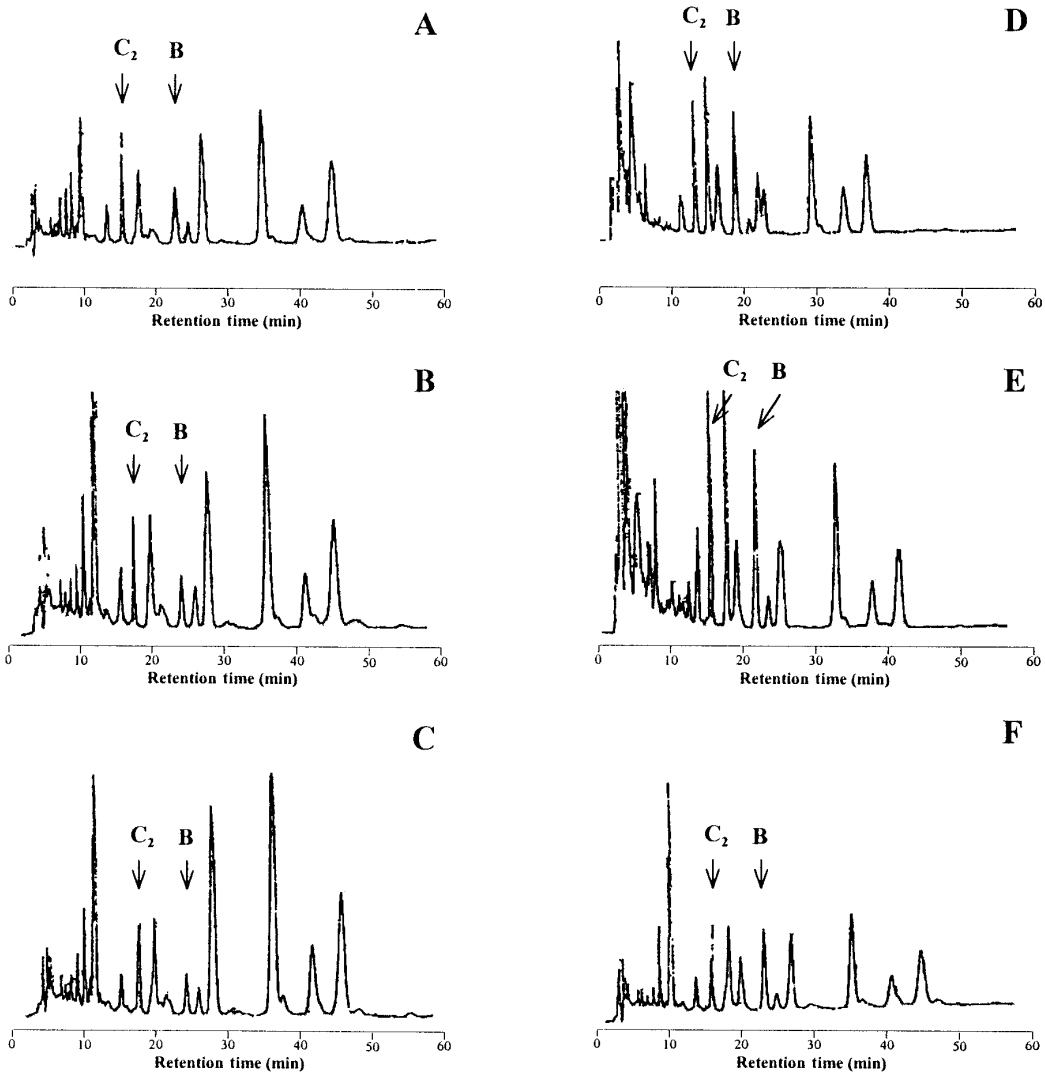

Figure 2. HPLC chromatograms of the triterpenoids of *G. tsugae* from six different regions.

Results and Discussion

Typical HPLC chromatograms for the ganoderic acids B and C_2 are shown in Figure 1. This figure demonstrates the applicability of these chromatographic conditions to the separation of triterpenoids in the crude extract of these *Ganoderma* species. The suitability was further confirmed in an analysis of *G. tsugae* from six different origins, and their HPLC chromatograms are illustrated in Figure 2.

After careful examination, these 64 samples were divided into 18 patterns (A–R) based on the following criteria: (*a*) the existence of chromatographic peaks for ganoderic acids B and C_2; (*b*) the peak number before ganoderic acid B and after ganoderic acid C_2; (*c*) the peak number between gan-

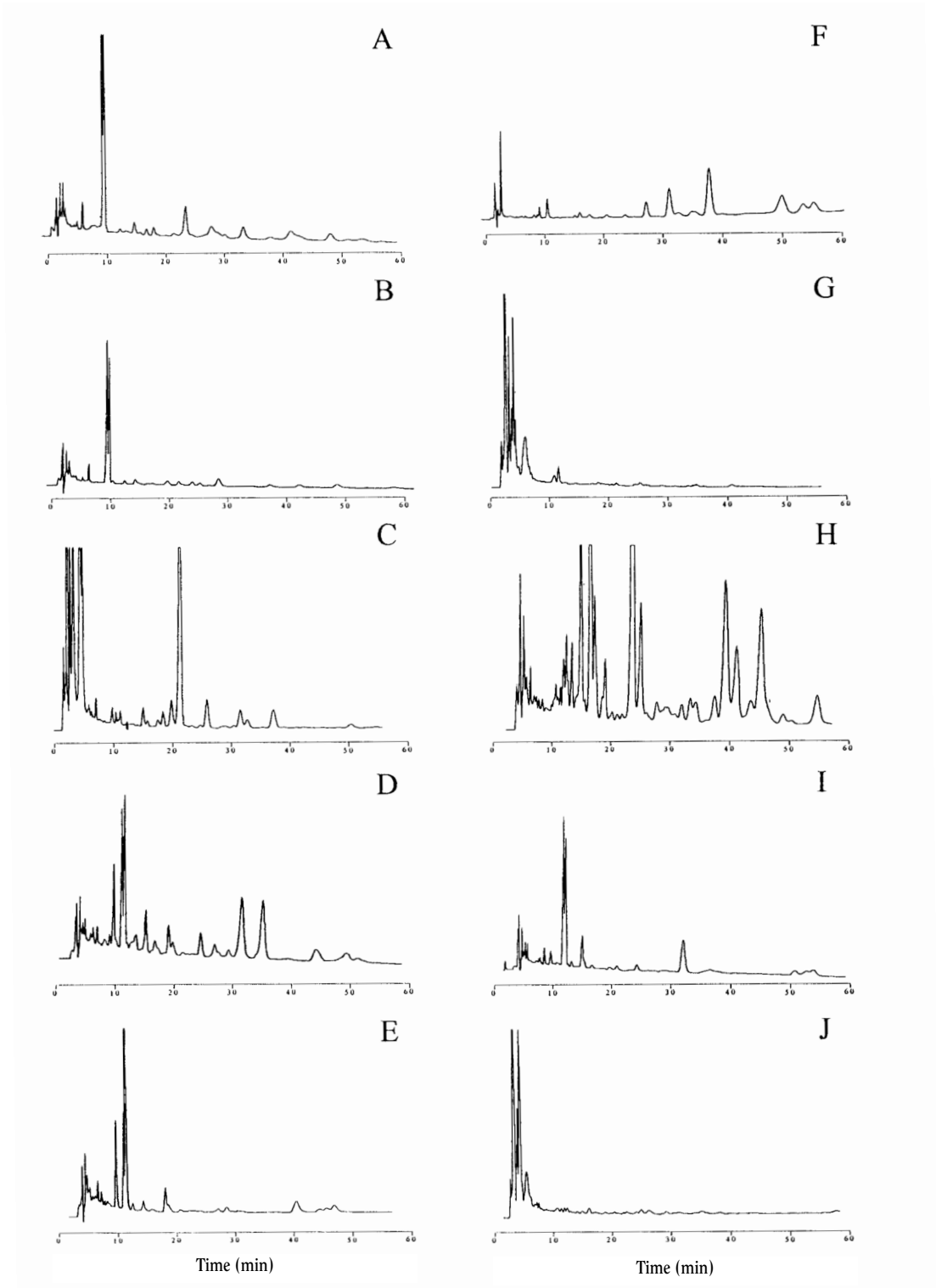

Figure 3. HPLC chromatograms of the triterpenoids for groups A through J.

oderic acid B and C_2; and (*d*) the total peak number in a chromatographic time period of 55 min when there were no peaks for ganoderic acids B or C_2. A representative sample for each pattern is marked in Table I, and typical HPLC chromatograms are displayed in Figures 3 and 4. Furthermore, it was found that the TLC chromatograms for these 64 samples could be grouped into 18 groups as well, as shown in Figure 5. Obviously, the TLC results showed a similar grouping pattern as that for HPLC, as listed in Table I. The comparison of the TLC chromatograms for the representative sample of each pattern is demonstrated in Figure 5.

The calibration curves that were applied for quantitation based on the HPLC method were constructed respectively for ganoderic acid B and C_2. The linear range for both compounds was found to be between 0.5 and 20 µg/mL. The accuracy and precision for interday runs were 0.53 to 6.54% and –3.11 to 4.86%, respectively, and for intraday runs were 1.25 to 8.94% and 2.55 to 8.65%. The contents of the ganoderic acids B and C_2 in each sample were calculated by interpolation from the corresponding calibration curves based on the peak area (the results are summarized in Table II and Figure 6). These results showed that *G. lucidum*, *G. boniense*, *G. tropicum*, *G. fornicatum*, *G. curtisii*, *G. lobatum, G. resinaceum* (36146), *G. mirabile,* and *G. oerstedii* contain both ganoderic acid B and C_2 in various amounts, but they were not found in *G. neo-japonicum*, *G. formasanum*, *G. australe*, *G. calidophilum*, *G. mastoporum*, *G. weberianum*, *G. pfeifferi*, *G. resinaceum* (36147 and 36149), *G. subamboninese var. laevisporum*, or part of *G. lucidum*. The same results were observed in the TLC

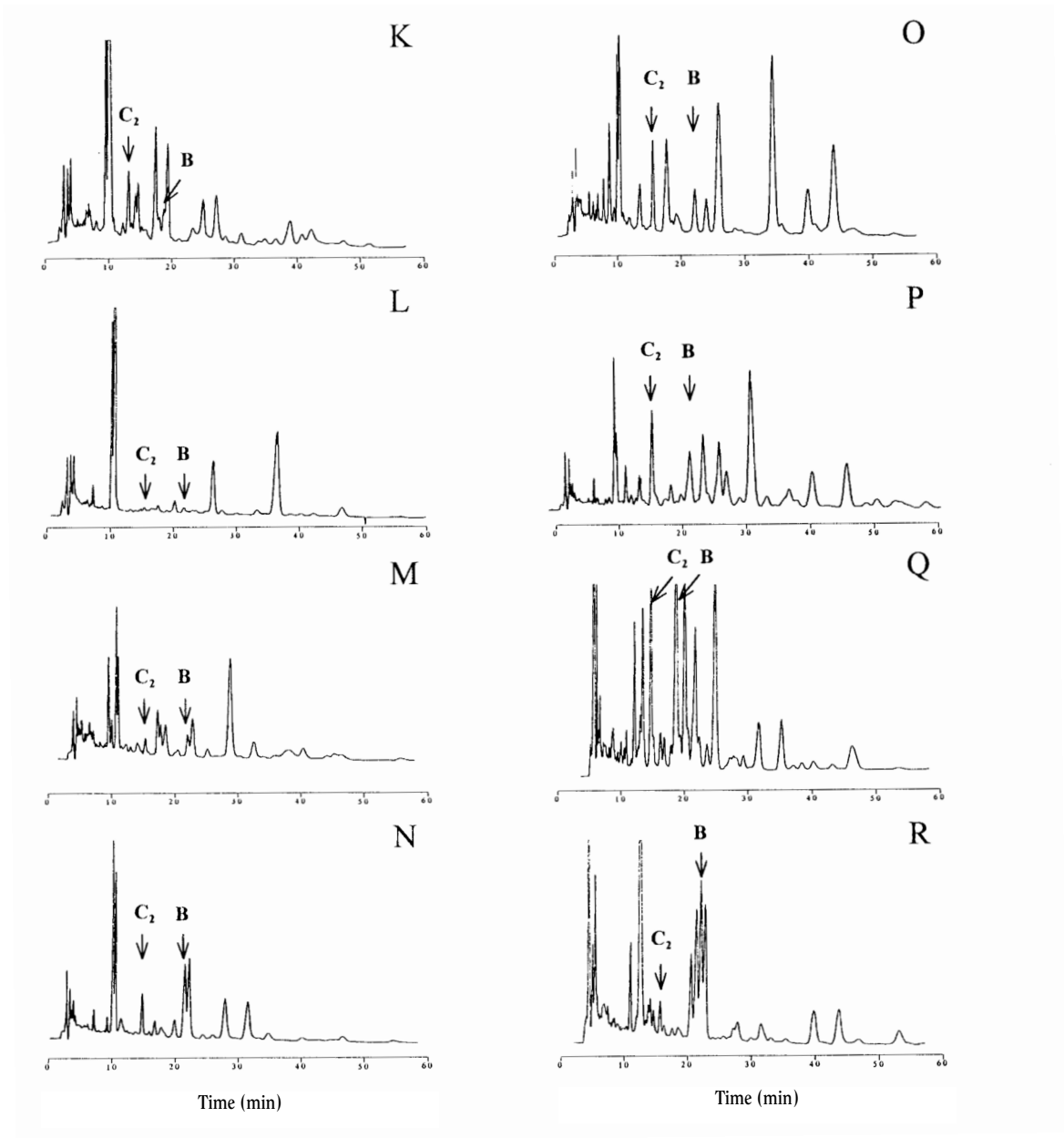

Figure 4. HPLC chromatograms of the triterpenoids for groups K through R.

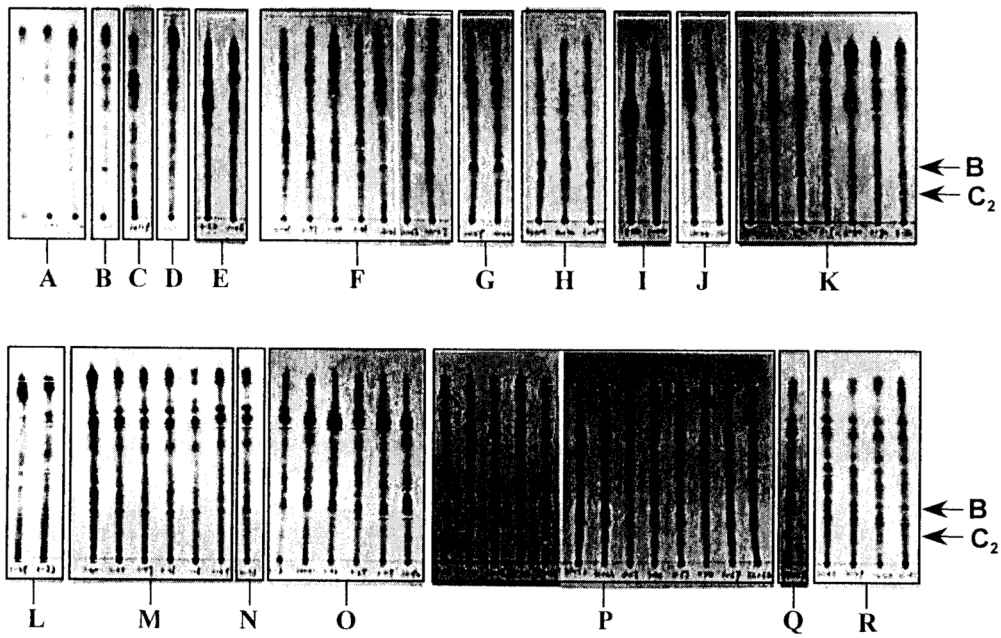

Figure 5. TLC chromatograms of the triterpenoids for 18 groups.

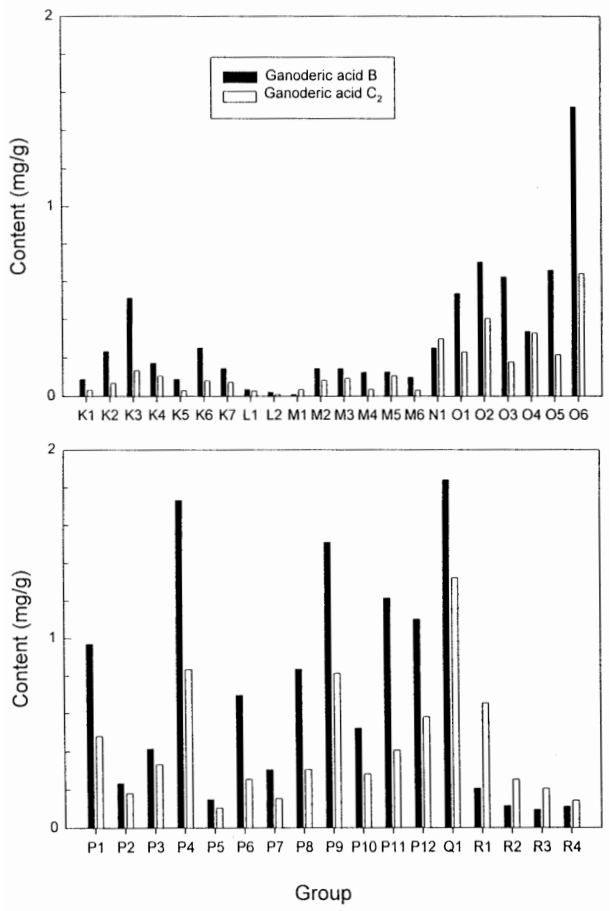

Figure 6. Comparison of the ganoderic acid B and C_2 content for groups K through R.

chromatograms for these samples. Among them, *G. lobatum* had the highest content of both triterpenoids. The contents of these two triterpenoids in *G. tsugae* and *G. curtisii* were also quite high. The results also demonstrated that the amounts of the ganoderic acids B and C_2 in these species are in a comparable level, thus implying a stable status for the production of these two and all other triterpenoids for the species in these samples.

Based on morphological examinations conducted in our laboratory and by experts from TARI, the differentiation among these 18 groups conformed to the results of the HPLC analysis. The use of interfertility testing by homokaryotic mating also found that all were mating incompatible except groups H, I, J, and K. It was determined by mating incompatibility that they were not the same species, which was consistent with the results of the HPLC analysis of the triterpenoids. However, for those groups growing in different regions but assigned to the same species of *G. lucidum* (H, I, J, and K), these showed quite a different distribution pattern for their triterpenoid content. The growing regions for H, I, J, and K are the southern U.S., eastern Taiwan, India, and western Taiwan, respectively. Although the mating compatibility among them revealed that they still belonged to the same species, slight differences in the morphology of the fruiting bodies and a different distribution pattern of the triterpenoid contents by HPLC analysis confirmed that they should be divided into subspecies. This seemed to indicate that the need for segregation into different species was progressing for groups H, I, J, and K, but had not yet reached the level of being recognized as different species.

The morphology of *G. tsugae* (the second most commonly used species of *Ganoderma* next to *G. lucidum*) is easily

confused with that of *G. lucidum*. Based on the results of the HPLC analysis of triterpenoids (Figures 3 and 4), samples of these two species from three different regions displayed quite varied patterns for their distribution of triterpenoid contents. This made it easy to distinguish between these two species based on this HPLC analysis of triterpenoids. Similarly, *G. tropicum* demonstrated its characteristic patterns in the HPLC analysis of triterpenoids as well as its differentiation from other species.

We also found that two samples with the accession numbers CCRC-36147 (CBS 194.76) and CCRC-36149 (CBS352.74) were labeled as *G. resinaceum* and showed a similar HPLC pattern of triterpenoids. However, it was quite different from patterns displayed by the other sample (accession number CCRC-36146, CBS 152.27), which was designated as the same species. This could be the result of incorrect designation based merely on traditional methods and further confirms that an HPLC analysis of the triterpenoid contents of *Ganoderma* is a superior way to differentiate species—even subspecies growing in different regions.

Table II. HPLC Analysis of the Ganoderic Acids B and C_2

Group	Accession number	Species	B (mg/g)	C_2 (mg/g)
K	87-1-1 (K1)	*G. lucidum*	0.0883	0.0334
	87-1-2 (K2)	*G. lucidum*	0.2322	0.0677
	87-1-6 (K3)	*G. lucidum*	0.5104	0.1317
	87-1-9 (K5)	*G. lucidum*	0.1706	0.1027
	88-1-30 (K6)	*G. lucidum*	0.0878	0.0289
	88-1-70 (K7)	*G. lucidum*	0.2516	0.0791
	88-1-76 (K8)	*G. lucidum*	0.1427	0.0722
L	88-1-39 (L1)	*G. boniense*	0.0353	0.0269
	88-1-73 (L2)	*G. boniense*	0.0207	0.0096
M	87-1-8 (M1)	*G. tropicum*	0.0940	0.0343
	87-1-28 (M2)	*G. tropicum*	0.1433	0.0820
	88-1-40 (M3)	*G. tropicum*	0.1416	0.0915
	88-1-57 (M4)	*G. tropicum*	0.1236	0.0356
	88-1-65 (M5)	*G. tropicum*	0.1251	0.1052
	88-1-69 (M6)	*G. tropicum*	0.0974	0.0312
N	88-1-71 (N1)	*G. farnicatum*	0.2504	0.2974
O	87-1-3 (O1)	*G. tsugae*	0.5338	0.2290
	36042 (O2)	*G. tsugae*	0.7030	0.4024
	88-1-56 (O3)	*G. tsugae*	0.6230	0.1764
	88-1-59 (O4)	*G. tsugae*	0.3357	0.3276
	87-1-19 (O5)	*G. tsugae*	0.6585	0.2139
	36090 (O6)	*G. tsugae*	1.5218	0.6401
P	87-1-14 (P1)	*G. tsugae*	0.9697	0.4800
	36065 (P2)	*G. tsugae*	0.2330	0.1797
	88-1-60 (P3)	*G. tsugae*	0.4140	0.3322
	37063 (P4)	*G. tsugae*	1.7319	0.8325
	87-1-13 (P5)	*G. tsugae*	0.1476	0.1051
	36064 (P6)	*G. tsugae*	0.6956	0.2537
	88-1-34 (P7)	*G. curtisii*	0.3039	0.1533
	88-1-37 (P8)	*G. curtisii*	0.8339	0.3058
	88-1-64 (P9)	*G. curtisii*	1.5071	0.8120
	88-1-67 (P10)	*G. curtisii*	0.5221	0.2808
	88-1-68 (P11)	*G. curtisii*	1.2139	0.4062
	Lai-41 (P12)	*G. curtisii*	1.0997	0.5793
Q	36245 (Q1)	*G. lobatum*	1.8409	1.3198
R	36147 (R1)	*G. resinaceum*	0.2050	0.6525
	36149 (R2)	*G. resinaceum*	0.1141	0.2527
	36152 (R3)	*G. mirabile*	0.0944	0.2033
	36291 (R4)	*G. oerstedii*	0.1108	0.1417

Conclusion

In conclusion, an HPLC analysis of the triterpenoid contents of *Ganoderma* is a simple and easy method to differentiate the species of *Ganoderma*. This should prove beneficial to the research fields of classification, biological activity, and structural determination.

Acknowledgments

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References

1. J.O. Toth, B. Luu, and G. Ourisson. Les acides Ganoderiques T à Z: Triterpenes cytotoxiques de *Ganoderma Lucidum* (Polyporacée), *Tetrahedron Lett.* **24:** 1081–1084 (1983).
2. H. Kohda, W. Tokumoto, K. Sakamoto, M. Fujii, Y. Hirai, K. Yamasaki, Y. Komoda, H. Nakamura, S. Ishihara, and M. Uchida. The biologically active constitutents of *Ganoderma-lucidum* histamine release-inhibitory triterpenes. *Chem. Pharm. Bull.* **33:** 1367–74 (1985).
3. A. Morigiwa, K. Kitabatake, Y. Fujimoto, and N. Ikekawa. Angiotensin converting enzyme-inhibitory triterpenes from *Ganoderma-Lucidum. Chem. Pharm. Bull.* **34:** 3025–28 (1986).
4. S. El-Mekkawy, M.R. Meselhy, N. Nakamura, Y. Tezuka, M. Hattori, N. Kakiuchi, K. Shimotohno, T. Kawahata, and T. Otake. Anti-HIV-1 and anti-HIV-1-protease substances from *Ganoderma lucidum. Phytochemistry* **49:** 1651–57 (1998).
5. D.H. Kim, S.B. Shim, N.J. Kim, and I.S. Jang.

Beta-glucuronidase-inhibitory activity and hepatoprotective effect of *Ganoderma licidum. Biol. Pharma. Bull.* **22:** 162–64 (1999).
6. S.P. Wasser and A.L. Weis. Therapeutic effects of substances occuring in higher basidomycetes mushrooms: a modern perspective. *Critical Rev. Immunol.* **19:** 65–96 (1999).
7. J.E. Adaskaveg and R.L. Gilbertson. Cultural studies and genetics of sexuality of *Ganoderma-lucidum* and *Ganoderma-tsuage* in relation to the taxonomy of the *Ganoderma-Lucidum* complex. *Mycologia* **78:** 694–705 (1986).
8. Z.Y. Yeh. "Taxonomic study of *Ganoderma australe* complex in Taiwan". Ph.D. Thesis, Institute of Botany National Taiwan University, 1990, p 109.
9. J.E. Adaskaveg and R.L. Gilbertson. Basidiospores, pilocystidia, and other basidiocarp characters in several species of the *Ganoderma lucidum* complex. *Mycologia* **80:** 493–507 (1988).
10. R.S. Hseu, H.H. Wang, H.F. Wang, and J.M. Moncalvo. Differentiation and grouping of isolates of the *Ganoderma lucidum* complex by random amplified polymorphic DNA-PCR compared with grouping on the basis of internal transcribed spacer sequences. *Applied Environ. Microbiol.* **62:**1354–63 (1996).
11. R.S. Hseu. "An identification system for *Ganoderma* isolated in Taiwan". Ph.D. Thesis, Institute of Botany, National Taiwan University, Taipein, Taiwan, 1990, p 105.
12. T. Nishitoba, H. Sato, S. Shirasu, and S. Sakamura. Evidence on the strain-specific terpenoid pattern of *Ganoderma-Lucidum. Agric. Biol. Chem.* **50:** 2151–54 (1986).
13. L.J. Lin and M.S. Shiao. Separation of oxygenated triterpenoids from *Ganoderma lucidum* by high-performance liquid chromatography. *J. Chromatogr.* **410:** 195–200 (1987).
14. M. Hirotani and T. Furuya. Changes of the triterpenoid patterns during formation of the fruit body in *Ganoderma lucidum. Phytochemistry* **29:** 3767–71 (1990).
15. C.H. Su, C.S. Sun, S.W. Juan, H.O. Ho, C.H. Hu, and M.T. Sheu. Development of fungal mycelia as skin substitutes: effects on wound healing and fibroblast. *Biomaterials* **20:** 61–68 (1999).

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