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Determination of Ergosterol in Ganoderma Spore Lipid from the Germinating Spores of *Ganoderma lucidum* by High-Performance Liquid Chromatography

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A gradient reversed-phase high-performance liquid chromatography (HPLC) method was developed for the separation and determination of free ergosterol in ganoderma spore lipid (GSL) extracted from the sporoderm-broken germinating spores of *Ganoderma lucidum*. Sodium hydroxide in methanol was added for the hydrolysis of ergosteryl esters to determine the total content of ergosterol in GSL by HPLC. A 0.04 M concentration of sodium hydroxide in reaction mixtures was appropriate for the complete hydrolysis of ergosteryl esters without a significant loss of ergosterol during saponification. In addition, the ergosterol content in four commercial GSL softgel supplements from four different firms was determined. The results showed that the ergosterol content in these samples had significant differences. Ergosterol content may be a suitable marker for evaluating the quality of GSL products.

KEYWORDS: Ergosterol; ganoderma spore lipid; *Ganoderma lucidum*; HPLC; saponification

INTRODUCTION

Ganoderma lucidum (Curt.: Fr.) Karst. has been widely used in traditional Chinese medicines for preventing and treating various diseases (*1*). Although the fruiting bodies of *G. lucidum* have been utilized as medicines for several thousand years in China, the spores of *G. lucidum*, whose bioactivities may be much higher than those of the fruiting bodies of *G. lucidum* (*2*, *3*), were realized and utilized only in the late 20th century. The potential medicinal values of the spores of *G. lucidum* have attracted intense interest in the search for pharmacological compounds from the spores. Numerous triterpene derivatives have been isolated from *G. lucidum*, including highly oxygenated lanostane derivatives and common fungal steroids derived from ergosterol (*4*).

Previous studies have demonstrated that ergosterol and its peroxidation products may contribute to potential health benefits and significant pharmacological activities, including reducing pain related to inflammation, reducing the incidence of cardiovascular diseases, and inhibiting cyclooxygenase (COX) enzyme, antioxidant, antimicrobial, anticomplementary, and antitumor activities (*5*−*14*). The antitumor activity of ergosterol may be due to direct inhibition of angiogenesis induced by solid tumors (*9*, *13*). Dietary ergosterol is absorbed in the alimentary tract, accumulates in the adrenals and other organs, and can be metabolized in vivo to generate newer bioactive products, such as 17α,24-dihydroxyergosterol, which has been found to be able to inhibit the proliferation of skin cells in culture, as demonstrated in human keratinocytes and melanoma cell lines (*15*).

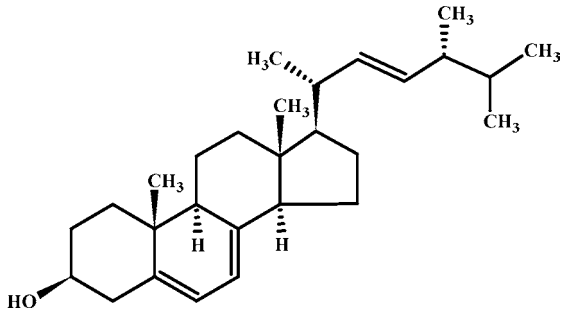

Figure 1. Structure of an ergosterol molecule.

Ergosterol (**Figure 1**) is a principal sterol of the cell membrane to which it is strongly bound in fungi (*16*) and is able to activate expression of a number of defense genes and increase the resistance of plants against the pathogens (*17*). Ergosterol is present in two forms, as free ergosterol and esterified ergosterol, and the relative abundances of free to esterified ergosterol are different among various species (*18*). The ergosterol content has been widely used as an estimate of fungal biomass in various environments because a strong correlation has been found between ergosterol content and fungal dry mass (*19*). A number of methods have been reported for the determination of ergosterol (*20*−*24*). Most of them are based on its specific UV absorption with a maximum at 282 nm, originating from the double bond at positions 5 and 7 (*21*).

Although ergosterol and steroid esters have been isolated from the fruit bodies of *G. lucidum* (*25*, *26*), little is known of the

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level of total ergosterol in the spores of *G. lucidum*. Ganoderma spore lipid (GSL) extracted from the spores of *G. lucidum* has been approved as a health food supplement, which is known as GSL softgel in China. However, rarely has information appeared in the literature to investigate the bioactive constituents in GSL. In this study, a gradient reversed-phase high-performance liquid chromatography (HPLC) method is developed to be suitable for the separation of triterpenoids and quantitation of free ergosterol and total ergosterol in GSL.

MATERIALS AND METHODS

GSL. *G. lucidum* (Curt.: Fr.) Karst. was cultivated at a base located in a 1000 m high forested area in the Fujian Province, China, which was established by the Food Engineering Research Center of State Education Ministry. The spores of *G. lucidum* collected might enter a resting state, and subsequently, the dormant spores were activated by germinating. The sporoderm of germinating spores was then broken (*27*), and the lipid in the sporoderm-broken germinating spores of *G. lucidum* was extracted on an industrial scale by using supercritical carbon dioxide; ethanol as a polar enhancer was added to elevate the effectiveness of extraction (*28*).

For the purpose of comparison, four commercial GSL softgel supplements (XZ, ZK, HF, and HK) from four different firms in Shenzhen, Nanjing, Guangzhou, and Hongkong, China, respectively, were purchased for the determination of ergosterol.

Chemicals and Reagents. HPLC-grade methanol was obtained from Merck KGaA (Darmstadt, Germany). Ergosterol (75% purity) was obtained from Sigma-Aldrich Co. (St. Louis, MO). Water was purified using a Millipore Simplicity system.

Preparation of GSL Solution. The GSL solution for the determination of free ergosterol in GSL was prepared by dissolving GSL (0.5 g) in 10 mL of diethylether. For each commercial GSL softgel supplement, ten softgels from every sample were opened and the inclusions were exhaustively mixed. A 0.5 g amount of a sample was accurately weighed into a 10 mL volumetric flask and made up to volume with diethylether.

Saponification of GSL. The saponified GSL solution for the determination of total ergosterol in GSL was prepared by dissolving GSL (0.5 g) in 4 mL of diethylether and 5 mL of ethanol, and then, 1 mL of freshly prepared methanolic NaOH at three different concentrations (0.4, 0.7, or 1.0 M) was added to hydrolyze the esterified ergosterol in GSL. The concentration of NaOH in the reaction mixture was 0.04, 0.07, or 0.1 M, respectively. The hydrolysis reaction of ergosteryl esters was carried out at two different temperatures (25 and 50 °C). The reaction mixtures were sampled to HPLC for monitoring the progress of hydrolysis of ergosteryl esters and possible losses of ergosterol during saponification.

HPLC. HPLC was conducted on a Waters liquid chromatograph equipped with a 1525 binary pump and a 2996 photodiode array detector from Waters Corp. (Milford, MA). The GSL solution and saponified GSL solution were separated and analyzed by using a Kromasil 100-5C18 column (250 mm × 4.6 mm, 5 μm) from Eka Chemicals (Bohus, Sweden) at ambient temperature. The mobile phase consisted of solvent A (methanol/0.05% acetic acid, 10:90, v/v) and solvent B (methanol). A gradient procedure was used as follows: starting at sample injection, 10% of B for 10 min; a linear gradient from 10 to 90% of B for 10 min; a linear gradient from 90 to 100% of B for 10 min; 100% of B for 30 min. The flow rate was 1.0 mL/min. Chromatographic peaks were identified by comparing the retention times and spectra against the known standard. The detecting wavelength was set between 220 and 400 nm, and the chromatographic peaks were measured at a wavelength of 280 nm to facilitate the detection of ergosterol. The sample was filtered through a 0.45 μm filter. Aliquots of 20 μL were directly injected into the HPLC for the determination. All injections were repeated three times.

Method Validation. A 20.0 mg amount of ergosterol was placed in one 50 mL volumetric flask and dissolved in ethanol. The stock standard of ergosterol was prepared at 300 μg/mL, and additional calibration levels were prepared by a serial dilution with ethanol. The standard calibration curve was constructed using these ergosterol standard solutions. The linear regression analysis was carried out by plotting the peak areas (A) against the concentrations (C) of ergosterol. The linearity was demonstrated by a correlation coefficient (r^2) greater than 0.999. The limit of detection (LOD) and the limit of quantification (LOQ) were determined based on signal-to-noise ratios (S/N) of 3:1 and 10:1, respectively.

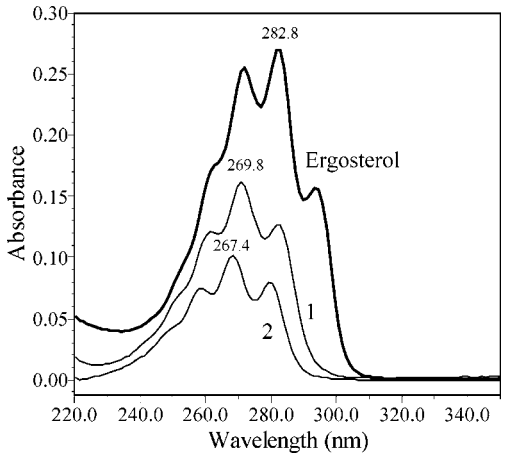

Figure 2. UV spectra of ergosterol, peak 1, and peak 2.

For recovery studies on added ergosterol, known volumes of ergosterol standard solutions were added to 0.5 g of GSL at three levels. This spiked sample was dissolved in diethylether following the described procedure. Background levels were subtracted in all recovery determinations. The assay of precision and accuracy was carried out with the ergosterol standard. The intraday precision of the procedure was determined by analyzing four standard solutions at 7.5, 37.5, 75.0, and 150.0 μg/mL. The interday precision was determined by analyzing these standards on three different days. Precision was calculated as a relative standard deviation (%RSD) for the repeated measurements. Accuracy is expressed as (assayed concentration)/(standard concentration) × 100.

RESULTS AND DISCUSSION

GSL is a complicated mixture of triglycerides and bioactive substances such as triterpenoids and sterols. In some of GSL samples, α-tocopherol acetate as an antioxidant is added during the manufacturing process. In order to avoid the effect of α-tocopherol acetate on the determination of ergosterol, it is important to well-separate ergosterol, α-tocopherol acetate, and α-tocopherol, the hydrolysis product of α-tocopherol acetate. Therefore, in the present study, a mixed solvent of methanol, water, and acetic acid as the mobile phase and the gradient elution procedure were used to separate the bioactive constituents in GSL. The separation of ergosterol, α-tocopherol acetate, and α-tocopherol could be achieved by adjusting the program of the gradient elution and the proportion of methanol and water. Using a photodiode array detector, peaks were identified by taking the spectra of each peak during elution. The identification of ergosterol was achieved by comparing its retention time and spectrum (**Figure 2**) against the known standard. The external standard method was used for the determination of ergosterol. The content of ergosterol was measured by comparing the peak area with the standard. The peak purities might also be examined by the photodiode array detector.

This HPLC method was validated for linearity, the LOD and the LOQ, precision, accuracy, repeatability, and recovery. The calibration curve ($A = 36324C - 18349$, $r^2 = 0.9997$) of the peak-area (A) ratio against the concentration (C) for ergosterol gave a linear response over a wide range of concentrations (5−300 μg/mL). The LOD and the LOQ with a 20 μL injection

Table 1. Precision and Accuracy of the Determination of Ergosterol in Intraday and Interday Analysis

ergosterol (μg/mL)	mean ± SD (n = 3)	RSD (%)	accuracy (%)
	intraday analysis		
7.5	7.47 ± 0.17	2.28	99.6
37.5	37.47 ± 0.58	1.55	99.9
75.0	74.27 ± 1.42	1.91	99.0
150.0	151.12 ± 3.06	2.02	100.7
	interday analysis		
7.5	7.43 ± 0.21	2.83	99.1
37.5	37.81 ± 0.62	1.64	100.8
75.0	75.23 ± 1.56	2.07	100.3
150.0	151.62 ± 3.23	2.13	101.1

Table 2. Recovery of Ergosterol after Spiking a GSL Sample at the Levels of 0.5, 1.0, and 2.0 mg/g

sample	spiked levels (mg/g)	recovery (%) (n = 3)
GSL	0.5	100.6 ± 3.1
	1.0	99.2 ± 1.8
	2.0	101.2 ± 2.4
XZ	0.5	98.6 ± 2.7
	1.0	99.7 ± 1.3
	2.0	102.4 ± 0.8

were 0.02 and 0.07 μg/mL, respectively, corresponding to 0.4 and 1.4 ng injected on the column. **Table 1** shows intraday and interday precision (%RSD) and accuracy of this HPLC method. As can be seen from **Table 1**, the precision ranges from 1.55 to 2.28% for intraday and from 1.64 to 2.83% for interday while the accuracy ranges from 99 to 101% for both intraday and interday. For recovery, an ergosterol standard was spiked into 0.5 g of GSL samples at the levels of 0.5, 1.0, and 2.0 mg/g. The spiked samples were assayed, and recoveries of ergosterol were found to be between 98.6 and 102.4% (**Table 2**).

In the present separation condition, no ergosteryl ester peaks were found. In comparison with free ergosterol, ergosteryl esters were strongly retained on the chosen reversed-phase column and could not be eluted by 100% of solvent B (methanol). For the determination of total ergosterol, the GSL solution was saponified for the hydrolysis of ergosteryl esters. The addition of hydroxides was necessary for the hydrolysis reaction of ergosteryl esters. It was important to add a newly prepared sodium hydroxide solution for effectual hydrolysis of ergosteryl esters. Because saponification might result in the destruction and structural transformation of some compounds, mild saponification for GSL should be achieved (*29*). In the present experiment, the complete hydrolysis of ergosteryl esters was achieved at different concentrations of sodium hydroxide and saponification temperatures. **Table 3** shows the effect of sodium hydroxide concentrations and saponification temperatures on the required least saponification time for almost complete hydrolysis of ergosteryl esters. As can be seen from **Table 3**, a higher concentration of sodium hydroxide and a higher saponification temperature can make the rate of the hydrolysis reaction increase and shorten the required saponification time. However, during saponification of ergosteryl esters, triglyceride in the sample reacts by base-catalyzed hydrolysis to form glycerol and salts of fatty acids (soap). A flocculent precipitate forms at a more concentrated solutions of NaOH and a higher temperature. Therefore, 0.04 M sodium hydroxide in reaction mixtures at ambient temperature was appropriate for hydrolysis of ergosteryl esters without significant production of flocculent precipitates. After the hydrolysis reaction finished (at least 15 h), the reaction mixtures were still sampled to HPLC for monitoring the possible changes of ergosterol. The determination results indicated that no significant loss of ergosterol occurred during saponification. Because the loss of some compounds could be reduced by using antioxidants during saponification, the presence of a large

Table 3. Required Least Saponification Time (h) at Different Concentrations of NaOH and Temperatures

	NaOH concentration (M)		
temperature (°C)	0.04	0.07	0.1
25	15 ± 0.5	7 ± 0.3	3 ± 0.1
50	4 ± 0.2	2 ± 0.1	1 ± 0.1

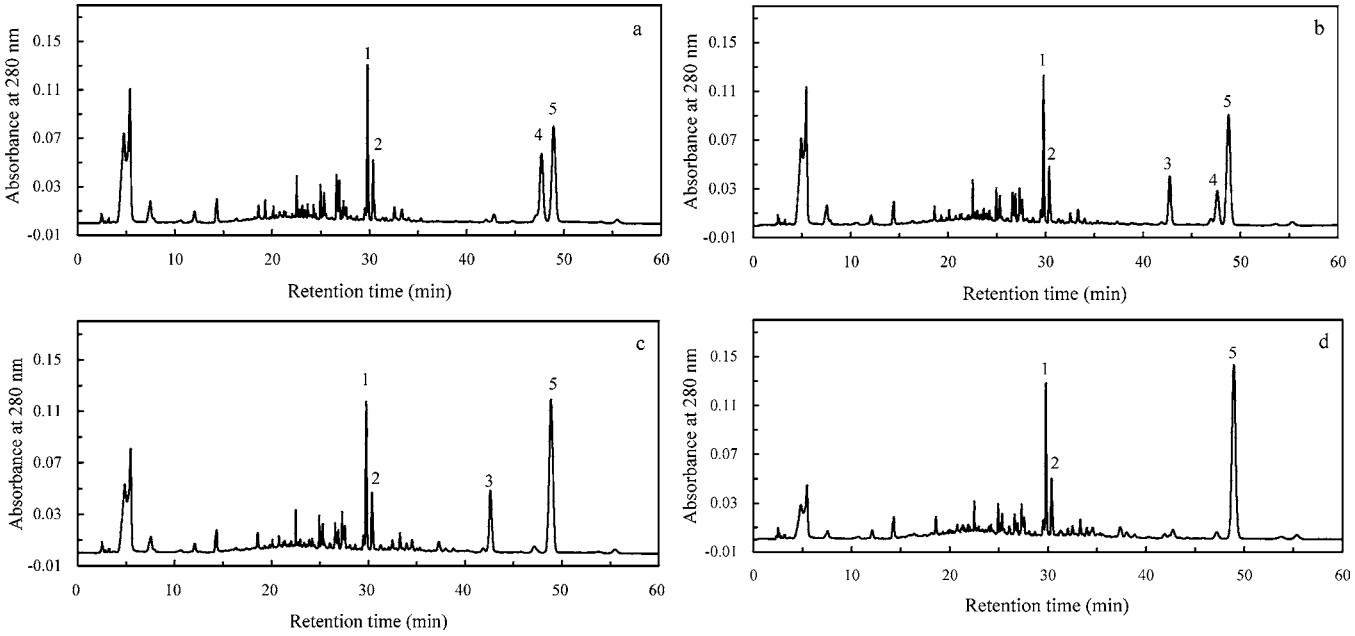

Figure 3. Chromatograms of the GSL solution with 0.04 M sodium hydroxide before saponification (**a**) and after 3.5 (**b**), 6.8 (**c**), and 48.0 h (**d**) of saponification at ambient temperature. Peak identification: 3, α-tocopherol; 4, α-tocopherol acetate; and 5, ergosterol.

Table 4. Contents of Ergosterol in GSL and Four Commercial GSL Softgel Samples ($n = 3$)

sample	free ergosterol (mg/g)	total ergosterol (mg/g)	free ergosterol (%)
GSL	1.202 ± 0.031	2.267 ± 0.048	53.0
XZ	0.995 ± 0.022	1.713 ± 0.039	58.1
ZK	0.090 ± 0.005	unmeasured	
HF	0.032 ± 0.003	unmeasured	
HK	<detection limit	unmeasured	

amount of α-tocopherol acetate might be one of the reasons that no loss of ergosterol and other compounds was observed.

As an application, the bioactive constituents in the GSL solution and saponified GSL solution were analyzed. The chromatograms of the GSL solution and saponified GSL solution during saponification were shown in **Figure 3**. The contents of free ergosterol and total ergosterol in GSL were shown in **Table 4**. On the basis of the above results, the contents of free ergosterol and total ergosterol in four commercial GSL softgel samples were analyzed and the results were also shown in **Table 4**. As can be seen from **Figure 3**, the content of α-tocopherol (peak 3) increased and the content of α-tocopherol acetate (peak 4) decreased during saponification. After 48.0 h of saponification (**Figure 3d**), no α-tocopherol was detected, indicating that α-tocopherol had been completely degraded.

As shown in **Table 4**, the contents of free ergosterol and total ergosterol in GSL were 1.202 and 2.267 mg/g, respectively. The free ergosterol content constituted 53.0% of total ergosterol in GSL. In contrast, only a very small quantity of ergosterol (samples ZK and HF) or no ergosterol (sample HK) was found in the commercial GSL softgel samples with the exception of sample XZ, which was made from the pure GSL extracted from speroderm-broken germinating spores of *G. lucidum*. The results show that the contents of free ergosterol in these commercial GSL softgel samples have significant differences. The reason for this is that some of commercial GSL softgel samples do not consist of pure GSL and may be a mixture of GSL and vegetable oil. Because of rarity, GSL has become a target for adulteration with cheaper vegetable oils, in which no ergosterol is found (*30*−*32*). Ergosterol, a principal sterol in fungi, is either absent or a minor component in most higher plants (*19*) and may be used as an estimate of GSL products.

In addition, ergosterol strongly bound to the cell membrane is difficult to extract when the spore is not fully broken. In fact, it is difficult to completely break the sporoderm of a ganoderma spore due to its prestressed ovoid structure and very hard bilayer sporoderms, especially on a commercial scale. Thus, the bioactive substances stored in the holes between the inner and the outer walls of spores, especially for ergosterol strongly bound to the cell membrane, are difficult to extract. We have succeeded in fully breaking the sporoderms of germinating spores. Large-scale supercritical carbon dioxide extraction of GSL from speroderm-broken germinating spores has been achieved, and up to 37.5 g of GSL has been obtained from 100 g of speroderm-broken germinating spores (*27*, *33*). After germination, the sporoderms soften and become brittle. The mechanical method is further used to break the sporoderms of germinating spores by using a crushing apparatus, whose crushing device is made of yttria-stabilized tetragonal zirconia polycrystalline ceramics because of their excellent mechanical properties, especially the high fracture toughness and flexural strength, instead of stainless steel. Using this apparatus, the contamination of spores by heavy metals such as chromium and nickel from stainless steel can be avoided (*27*). For completely sporoderm-broken spores, it is easy to extract lipids from the spores by either supercritical carbon dioxide extraction or traditional lipid solvent extraction systems.

Many constituents in GLS are still not identified. For example, compounds 1 and 2 (peaks 1 and 2) are two of the main constituents in GSL and their UV spectra are also shown in **Figure 2**. During saponification of the GSL solution, the contents of compounds 1 and 2 did not change, indicating that they did not esterify and were also stable to hydroxide. Unlike ergosterol, these two compounds are not detected in the extracts of the fruit bodies of *G. lucidum*. Therefore, it is interesting and important to further separate and identify these two compounds and investigate their pharmacological activities. Although ergosterol only is a portion of the bioactive constituents in GSL from spores of *G. lucidum*, the ergosterol content may be a suitable marker for evaluating the quality of GSL products.

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