Supercritical fluid extraction effectively removes phthalate plasticizers in spores of *Ganoderma lucidum*

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Abstract Phthalate plasticizers residue in food is a serious threat to public health. Spores of *Ganoderma lucidum* are easy to be contaminated with phthalates during collection and processing. In this study, supercritical fluid extraction (SFE) was performed to remove phthalates in spores of *G. lucidum*, and the effects on acid and peroxide values of spores' oil were also evaluated. The results showed SFE removed 100% of the residual di-iso-butyl phthalate, di-*n*-butyl phthalate and di-2-ethylhexyl phthalate in the spores of *G. lucidum*. No significant differences in polysaccharides content and fatty acid composition were observed between SFE and control spores. However, the triterpenoid extracts of SFE spores had a 7.45% increase, significantly higher than that in control spores. Accelerated oxidation tests further implied that SFE could improve the stability of spores' oil. Our results suggested SFE is a potential approach to remove phthalate from food related products.

Keywords Supercritical fluid extraction · *Ganoderma lucidum* · Spore · Phthalate plasticizer · Fatty acid composition

Abbreviations

SFE Supercritical fluid extraction
UE Ultrasonic-assisted extraction (UE)
MAE Microwave-assisted extraction

Introduction

Ganoderma, locally called *Lingzhi* in China or *Reishi* in Japan, a kind of large fungus belonging to polyporaceae plant, is widely used in the traditional Chinese medicine for prevention and treatment of various kinds of diseases in China (Enshasy et al., 2013). So far, a total of 98 *Ganoderma* species have been recorded in China, in which *Ganoderma lucidum* is officially listed in the Chinese Pharmacopoeia (2010) as immunomodulation (Yu et al., 2015), antitumor/anticancer (Gao et al., 2012), antiviral, antioxidative/anti-aging properties (Trebušak et al., 2014; Wan et al., 2017) because of its varieties of bioactive ingredients, such as polysaccharides, triterpenoids, sterols, and fatty acids (Paterson, 2006). The fruit body of *G. lucidum* has been widely used, however, the spores of *G. lucidum*, kind of oval germ cells (Φ 6–8 μ m) ejected from the *G. lucidum* lamella during mature growth period, were only realized and utilized in late 20th century (Liu et al., 2002). The spores of *G. lucidum* contains higher level of bioactive substances than the fruit body, moreover, the active components in spores can be better absorbed after the spores is broken. Therefore, the spores of *G. lucidum* have much higher of medicinal value and health benefits.

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Phthalates are a group of additives widely used as plasticizers in plastics. The plasticizers in plastic films and bags could migrate into in spores of *G. lucidum* during spores' collection and processing (Coltro et al., 2014; Fang et al., 2017). Therefore, spores of *G. lucidum* are easily contaminated with phthalates. These residual toxic compounds contaminated in spores can hinder its commercial application and value. However, residual toxic compounds contaminated in spores of *G. lucidum* were ignored. Actually, phthalates were also detected at high levels in various miscellaneous foods including milk and milk products (Sørensen, 2006), oil and oily food (McCombie et al., 2015), fruit and vegetables (Holadová et al., 2007). Toxicology studies in animals have shown that phthalates such as di-2-ethylhexyl phthalate (DEHP), di-iso-butyl phthalate (DIBP), di-*n*-butyl phthalate (DBP) are teratogens and carcinogens for liver (Silva et al., 2004). In view of the potential toxicity of phthalates to human, especially for the long-term consumers, it is essential and critical to remove phthalates in spores of *G. lucidum*. So far, however, there has been no appropriate method can be used to remove the phthalates in food.

Supercritical fluid extraction (SFE) was used for phthalates extraction from polymers at late 20th century. Due to the specific increased solubility of supercritical CO₂ and its unique advantages of high extraction efficiency, good selectivity, low extraction temperature, nontoxic and no residual (Li et al., 2010), SFE has been widely used in the extraction and analysis of natural active ingredients from food, plant and herbal medicine (Wei et al., 2009). However, few research has been focused on the removal of phthalate plasticizers in food including the spores of *G. lucidum* (Chen et al., 2014). In this study, we quantitatively determined the effect of SFE on removal of phthalate plasticizers in the spores of *G. lucidum*. Also, the effects of SFE on acid and peroxide values of the spores' oil were evaluated.

Materials and methods

SFE process of *G. lucidum* spores

About 1000 g of *G. lucidum* spores, cultivated and collected from Longquan, Zhejiang, China at June 2016, were provided by Infinitus (Chinese) Co., Ltd. The sporoderm of the spores had already been broken with the broken rate of 98.5%. The spores (7 kg) was subjected to a large scale SFE instrument (SF24000, Masson New Separation Technology Co., Ltd., Guangzhou, China) and the phthalates extraction was performed under the following conditions: pressure 200 bar, temperature 32 °C, extraction time 180 min and a CO₂ flow rate of 60 ml/min. The collected

spore oil (about 50 g) was discarded. The spores did not treated by SFE were used as control.

Phthalates detection and analysis

Spores oil was extracted from 0.5 g spores of *G. lucidum* with petroleum ether, then removed water with anhydrous sodium sulfate, concentrated and dried under reduced pressure. The concentrate was dissolved with acetic ether and cyclohexane (volume ratio 1:1), then set the volume to 10.0 ml, vortex mixed for 2 min. The solution was filtered by 0.45 µm filter before gas chromatography/mass spectrometry (GC–MS, 7890A GC, 975C VL MSD, Agilent, USA). Phthalates were detected by GC–MS with a capillary column (30 m × 0.25 mm i.d.) coated with 0.25 µm film 5% phenyl methyl siloxane. High purity helium was used as carrier gas with flowrate at 1 ml/min. The column temperature was set at 60 °C and held for 1 min for injection, then programmed at 20 °C/min to 220 °C and held for 1 min, and finally, at 5 °C/min to 280 °C and held for 4 min. Split injection (1 µl) with a split ratio of 10:1 was used, and injection temperature was set at 250 °C, and detector temperature was 280 °C. The mass spectrometer was operated in electron-impact (EI) mode, the scan range was 40–550 amu, the ionization energy was 70 eV and the scan rate was 0.34 s per scan. The quadrupole, ionization source temperature were 150 and 280 °C, respectively.

Chemical composition analysis of spores' oil

The content of polysaccharides was examined using phenol–sulfuric acid colorimetric method (DuBois et al., 1956). The total content of triterpenoids was determined as previously described (Chen et al., 2007).

Determination of fatty acid composition

Spores oil extraction

The spores of *G. lucidum* and petroleum ether (1:2 based on weight) were mixed and sealed up in a bottle. The mixture was shaken at room temperature with the shaking speed of 150 r/min and extracted for 4 h. Then, it was filtered at vacuum and the petroleum ether was removed by vacuum rotary evaporation at 50 °C to obtain spore oil of *G. lucidum*.

GC–MS

After removing water by anhydrous sodium sulfate, the spore oil of *G. lucidum* was reacted through methyl esterification to obtain fatty acid methyl esters. 60 mg spore oil, 4 ml chromatogram class isooctane and 200 µl 2 mol/l

methanol solution of potassium hydroxide, were mixed in a test tube with plug, shook violently for 30 s, then stood until clarify. 1 g sodium bisulfate was added into the mixture, shook violently again. After the salt precipitated, the upper solution was filtered through a 0.45 μm filter membrane for GC–MS (7890A GC, 975C VL MSD, Agilent, USA) as previously described (Lv et al., 2012).

Morphological characterization

The morphological characterization of the spores of *G. lucidum* was performed using a scanning electron microscope (JSM-5610LV-SEM, Tai Si Ken Trading Co., Ltd., Shanghai, China). The accelerated voltage was set at 10.0 kV and the working distance was about 10 mm. The spores were coated with platinum of 10 nm thicknesses to make the spores conductive.

Color analysis

Chromatic values of spores of *G. lucidum*, including luminance, red and yellow values, were determined by a color difference meter (DC-P3, Xingguang Color Instrument Co., Ltd., Beijing, China).

Accelerated oxidation test

Index determination

Acid value, was determined by titration method with 0.05 mol/l potassium hydrate. Peroxide value, was determined by the iodine titration method (Oliveira et al., 2002).

Thermal stabilities of *G. lucidum* spore

The spores of *G. lucidum* were stored under the environment of 40 and 60 °C (Relative humidity (RH) of 75%), respectively. Then the spore oil was regularly extracted from the storage spores to measure its acid value and peroxide value.

Thermal stabilities of *G. lucidum* spore oil

The acid value and peroxide value of the spore oil, which was stored under the environment of 40 °C and 60 °C (75% RH), respectively, were measured at regular intervals.

Light stabilities of *G. lucidum* spore

The spores of *G. lucidum* were stored under light and the spore oil was regularly extracted from the storage spores, in order to measure its acid value and peroxide value.

Statistical analysis

SPSS 16.0 was used for statistical analysis. One way analysis of variance (ANOVA) and student *t* test were used to determine the differences among various treatments. A probability level of 5% ($p < 0.05$) was considered as significant.

Results and discussion

Effect of SFE on polysaccharides, triterpenoid, fatty acids, morpholog and color of *G. lucidum* spores

The contents of main bioactive compounds and the fatty acid composition of *G. lucidum* spores were compared before and after SFE treatment (Table 1). No significant difference in contents of polysaccharides was observed between SFE and control spores. However, the triterpenoid extracts of SFE spores had a 7.45% increase, which was significantly ($p < 0.05$) higher than that in control spores. A total of fourteen fatty acids were identified from the spore oil of *G. lucidum* by GC–MS, among which C_{18:1} (about 60%), C_{16:0} (about 15%), C_{18:2} (about 14%) and C_{18:0} (about 4–5%) are the major constituents, which was consistent with previous report (Liu et al., 2007). Moreover, SFE treatment did not affect the fatty acid composition of the spores, and trans fatty acids were not detected from SFE spores. Although treated by SFE, the spore of *G. lucidum* was still an excellent oil source enriched in oleic acid (C_{18:1}). This indicated that oxidation reaction of spore oil was not existed for SFE treatment.

Under SEM, almost all of the spores were totally broken (Supplementary Fig. S1), and the SFE spores showed no morphological changes compared to the control spores. In addition, no significant differences in the color were observed between SFE spores and control spores, both of which maintained normal color: no caking, just a small amount of deposit (Table 1). In conclusion, SFE did not affect polysaccharides content, fatty acid composition, morphological characterization and color of spores of *G. lucidum*, but significantly enhanced the content of triterpenoid extracts of *G. lucidum* spores.

The polysaccharides, triterpenoids and fatty acids are the main bioactive compounds of spore oil, which has been demonstrated to exhibit immunomodulatory, anti-tumor, anti-oxidation and hepatoprotection effects (Choi et al., 2005; Xu et al., 2011). These compounds potentially represent the quality and efficacy of the spores of *G. lucidum*. Consequently, our results might indicate that SFE could not affect the quality of *G. lucidum* spores.

Table 1 Basic components of *G. lucidum* spores

Ingredient		Group (mean $\pm$ S.D., n = 3)	
		Control	SFE
Polysaccharides (g/100 g)		1.161 $\pm$ 0.027	1.130 $\pm$ 0.018
Triterpenoids (g/100 g)		8.145 $\pm$ 0.248	8.752 $\pm$ 0.437*
Fatty acids (relative content/%)	C _{14:0}	0.247 $\pm$ 0.001	0.245 $\pm$ 0.000
	C _{15:0}	0.474 $\pm$ 0.011	0.470 $\pm$ 0.003
	C _{16:0}	15.101 $\pm$ 0.026	15.089 $\pm$ 0.029
	C _{16:1}	0.313 $\pm$ 0.011	0.310 $\pm$ 0.003
	C _{17:0}	0.250 $\pm$ 0.003	0.251 $\pm$ 0.002
	C _{18:0}	4.469 $\pm$ 0.156	4.520 $\pm$ 0.032
	C _{18:1}	60.441 $\pm$ 0.191	60.346 $\pm$ 0.052
	C _{18:2}	13.792 $\pm$ 0.055	13.784 $\pm$ 0.016
	C _{20:0}	0.391 $\pm$ 0.009	0.404 $\pm$ 0.007
	C _{20:1}	0.404 $\pm$ 0.004	0.394 $\pm$ 0.007
	C _{20:2}	0.055 $\pm$ 0.000	0.057 $\pm$ 0.001
	C _{22:0}	0.499 $\pm$ 0.004	0.499 $\pm$ 0.005
	C _{22:1}	–	0.055 $\pm$ 0.049
	C _{24:0}	0.556 $\pm$ 0.028	0.529 $\pm$ 0.006
Color analysis	Luminance	19.21 $\pm$ 0.39	19.19 $\pm$ 0.10
	Red value	0.32 $\pm$ 0.08	0.42 $\pm$ 0.10
	Yellow value	23.91 $\pm$ 0.54	24.74 $\pm$ 0.28

* $p < 0.05$ versus control group, – not detected

Effect of SFE on thermal and light stabilities of *G. lucidum* spores

Accelerated oxidation tests were conducted to explore the stabilities of *G. lucidum* spores after SFE treatment. The

thermal stabilities of spores and spore oil, and the light stability of spores were showed in Figs. 1, 2 and 3, respectively. Data in Fig. 2 were fitted and the resulting equations were showed in Supplementary Table S1. For spores of *G. lucidum*, the initial acid value of SFE group

Fig. 1 Thermal stabilities of spores of *G. lucidum* (A, B: 40 °C, 75% RH; C, D: 60 °C, 75% RH)

Fig. 2 Thermal stabilities of spore oil of *G. lucidum* (A, B: 40 °C, 75% RH; C, D: 60 °C, 75% RH)

Fig. 3 Light stabilities of spores of *G. Lucidum*

was slightly higher than that of the control group, which closely related with the higher triterpenoid extracts of SFE spores. Because both triterpenoids and free fatty acids can react with alkaline solution, higher content of triterpenoids and acid might increase acid value. However, no significant differences in acid value and peroxide value were displayed between SFE and control groups during the storage at 40 °C, 75% RH for 3 months [Fig. 1(A, B)]. At 60 °C storage temperature, the acid values of the two groups increased volatility in the initial 6 days, then it occurred a linear increase for the control group and caught up with the SFE group on the eighth day [Fig. 1(C)], which might indicate that the thermal stabilities of control spores had a little bit poorer than the SFE spores at 60 °C storage temperature. Similarly, peroxide values of the two groups showed a volatility increase trends during the storage at 60 °C [Fig. 1(D)].

The initial acid value of control spore oil of *G. lucidum* was 20–60% higher than the SFE spore oil [Fig. 2(A, C)], moreover, the increasing rate of the control group ($0.062 \text{ mg g}^{-1} \text{ d}^{-1}$) was significantly ($p < 0.05$) faster than that of the SFE group ($0.049 \text{ mg g}^{-1} \text{ d}^{-1}$) during the storage at 60 °C (Supplementary Table S1). The peroxide values of spore oil of the two groups increased exponentially at 40 °C storage temperature, but increased linearly at 60 °C storage temperature [Fig. 2(B, D)]. The increasing speed of peroxide values of the control group was significantly ($p < 0.05$) faster than the SFE group at 40 °C storage temperature, however, no obviously difference in increasing rate of peroxide value was found during storage at 60 °C (Supplementary Table S1). This might be due to the high temperature which dramatically accelerated the deterioration speed of spore oil of the two groups. For stability of light, the acid value and peroxide value of the two groups showed similar trends. The acid value of both group first slightly increased and thereafter maintained relatively stable, and the peroxide value curves had a larger fluctuation (Fig. 3).

Overall, these results of accelerated oxidation tests suggested that SFE could improve the thermal stability of spores and spore oil of *G. lucidum* by inhibiting the oil hydrolysis and delaying the oxidation, but showed no effect on the light stability.

As spore oil is the volatile component of spores of *G. lucidum*, oxidative rancidity of spore oil directly affects the shelf life and efficacy of spores of *G. lucidum*. However, high pressure during SFE could inactivate the lipase, lipoxygenase, peroxidase and polyphenoloxidase (Lozano et al., 2004). Also, CO₂ could change the microenvironment pH around the enzyme so that to affect the enzyme activity (Knez and Habulin, 2002). Thus, partial inactivation of inherent enzymes in the spores of *G. lucidum* during SFE could hamper the increasing of acid and peroxide

values, so as to infer that SFE spores was more resistant to storage.

On the other hand, temperature is also an important factor affecting the stability of organic compounds (Li et al., 2013). Recently, headspace solid-phase micro-extraction (HS-SPME) was widely used to determine phthalates in food (Cao, 2008), but was not suitable for phthalate removal from spores of *G. lucidum*. Because the reaction temperature of HS-SPME is generally more than 60 °C, or even as high as 90 °C. The thermal sensitive component in food must be destroyed at such high temperature. Contrarily, SFE with lower reaction temperature is much suitable for phthalate removal in thermal sensitive food. Therefore, pressure, media and temperature were three important factors to result in stability improvement of spores of *G. lucidum* after SFE treated.

Effect of SFE on phthalate plasticizers removal in spores of *G. lucidum*

Screening and determination of phthalates in the spores was carried out by GC–MS (Table 2). Three kinds of phthalates, DIBP, DBP and DEHP, with the contents of 0.57, 0.42 and 1.56 mg/kg, respectively, were observed in control spores (Table 2). In 2011, the Office of Ministry of Health of China stated that the maximum residue limit of phthalates in food and food additives were 1.5 mg/kg for DEHP, 0.3 mg/kg for DBP, and 9.0 mg/kg for DINP. Accordingly, the contents of DBP and DEHP in control spore of *G. lucidum* exceeded the national limit value of 40 and 4%, respectively. However, none of phthalate was detected in the SFE group (Table 2).

Supercritical CO₂ was a more effective extraction medium with the maximum amount of dioctyl phthalate (DOP) extraction from multilayer ceramic capacitors (Shende and Lombardo, 2002), and it had been successfully applied in phthalate plasticizers removal from PVC (Shieh et al., 2010). SFE might inhibit the plasticizer migration from packaging to foods under low temperature and acidic environments (Li et al., 2016). Moreover, SFE could prevent oxidation and dissipation of heat-sensitive components and maximize the retention of nutrients, thus especially suitable for the extraction and separation of bioactive components from heat sensitive plant (Ivanovic et al., 2013) and Chinese herbal medicine (Chen et al., 2011). Our results further suggested that SFE is effective in removing phthalates from spores of *G. lucidum*. Such findings will help in effectively improving the quality of spores of *G. lucidum*, and extended the application field of SFE.

Several phthalates with the structures similar to hormone are suspected of possessing endocrine disrupting properties (Petersen and Breindahl, 2000), which may

Table 2 Phthalates contents in spores of *G. lucidum*

Phthalates	Group (mg/kg, mean $\pm$ S.D., n = 3)	
	Control**	SFE
Di-iso-butyl phthalate (DIBP)	0.57 $\pm$ 0.01	–
Di-n-butyl phthalate (DBP)	0.42 $\pm$ 0.02	–
Di-2-ethylhexyl phthalate (DEHP)	1.56 $\pm$ 0.04	–

***p* < 0.01 versus control group, – not detected

cause reproductive system abnormalities or even increased incidence of various cancers and birth defects for the long-term consumption. In particular, phthalates with carbon lengths of C₄–C₆ are well known testicular toxicants, DBP and BBP shown weak oestrogenic properties in vitro (Harris et al., 1997). This result indicated that SFE is effective in removing phthalates and the removal efficiencies, suggesting that SFE could improve the safety of spores of *G. lucidum*.

In conclusion, our results confirmed that SFE could not significant affect the polysaccharides content and fatty acid composition of *G. lucidum* spores, however, SFE could effectively remove phthalates of DIBP, DBP and DEHP and improve the safety of spores of *G. lucidum*. Furthermore, SFE could improve the stability of *G. lucidum* spore oil. Such findings will help in effectively improving the quality of spores of *G. lucidum*. Further studies are required to extend SFE application in other foods.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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