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Full Length Research Paper

Simultaneous determination of five fatty acids in *Phellinus* sp. by high-performance liquid chromatography with photodiode-array detection

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Fatty acids are important nutrition and physiology component in fungus. A method has been developed to determine the five fatty acid components—palmitoleic acid (I), linoleic acid(II), oleic acid (III), hexadecanoic acid (IV) and stearic acid(V) in fungus *Phellinus sp*. using high-performance liquid chromatography with a photodiode-array (HPLC–PDA) ultraviolet detection system. The information about the fatty acid constituents of *Phellinus sp*. is necessary for further research and development of this resource. This analysis was carried on a column of Kromasil C18 (250 mm × 4.6 mm, 5 µm) with a mobile phase of acetonitrile and 5% CTAB water (95:5 v/v). The flow rate was 1.5 ml/min and the detector wavelength was set at 242 nm. Calibration curve showed good linearity with correlation coefficients (*r*) more than 0.9994. Average recoveries of the four compounds were more than 97% and relative standard deviations were less than 1.6%. This method appeared to be stable, sensitive and reproducible for determination of the five fatty acid compounds in fungus *Phellinus sp*..

Key words: Fatty acids, high-performance liquid chromatography (HPLC), phellinus.

INTRODUCTION

Phellinus sp. is a species of mushrooms belonging to the *Hymenochaetaceae Basidiomycetes* which is indigenous mainly to tropic America, Africa and East Asia. It is among a number of medicinal mushrooms that have been widely used in East Asia, especially Korea, China and Japan as health booster and ancient herbal medicine (Zhu T et al., 2008). In recent years, the main chemical constitutes of mushrooms have been identified as polysaccharide, flavonoid, protein, amino acid and fatty acid (Tao et al., 2005; Sun et al., 2006; Lee et al., 2010). As we know, fatty acids are natural substances which elicit a number of physiological effects in living organisms and also have an important dietary constituent in human nutrition. Therefore, it is of great practical significance to establish an analytical method for determining them.

Analysis of common fatty acids is usually carried out by gas chromatography (GC) and high-performance liquid chromatographic (HPLC). GC is used widely for fatty acid analysis and has the advantage of providing high resolution, while HPLC techniques with less resolution but higher sensitivity (compared to GC) have been developed for fatty acid analysis (Chen et al., 1992; Tan et al., 2006). Efficient purification and analysis procedures of phenacyl esters have been described in mammalian tissues and plant using reversed-phase HPLC and UV spectrophotometer detection (Chen et al., 1992; Tomas et al., 1998; Nicholas et al., 1988; Golden et al., 2001), but not found in fungi. We report the conversion of a published gradient elution HPLC-PDA method to a more efficient isocratic HPLC system, and describe its application to the measurement of fatty acids. In common with other methods, we used phenacyl esters to produce UV absorbing derivatives of the fatty acids. The method is not recommended for complete compositional analysis, but is useful for determinations of the content of fatty acid during studies on nutrition and metabolic conversions in *Phellinus sp*.

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Table 1. Results of validation of analytical method.

Analyte	Calibration curves Correlation coefficient (*r*2)	Limit of detection (ng)	Precision RSD (%)	Repeatability RSD (%)	Stability RSD (%)		Recoveries (%)	RSD (%)
					inter-day	intra-day		
Palmitoleic acid	y = 11410x + 2443.6(0.9992)	6.8	1.19	2.50	2.62	3.17	101.2	4.22
Linoleic acid	y = 9006.4x + 2466.5(0.9985)	5.7	2.76	3.75	3.33	3.23	99.2	2.81
Oleic acid	y = 9860.5x + 249.9(0.9999)	9.2	1.97	3.21	2.84	3.21	98.6	3.02
Hexadecanoic acid	y = 10814x + 2002.1(0.9999)	10.5	1.65	3.55	3.29	1.92	102.3	1.36
Stearic acid	y = 10278x - 2438.3(0.9988)	5.9	1.86	2.44	1.27	1.03	99.4	3.01

EXPERIMENTAL

Plant materials

The fungal materials (Table 2) were collected in China and conserved Microbiological Research Center of Sichuan Academy of Agricultural Science.

Chemicals and reagents

Palmitoleic acid (1), linoleic acid (2), oleic acid (3), hexadecanoic acid(4), stearic acid(5) were purchased from Sigma. HPLC grade acetonitrile (Merck, Germany) was used for the HPLC analysis. Deionized water was purified by Milli-Q system (Millipore, USA). The acetic acid and CTAB were of AR grade, purchased from Chengdu Changzheng Chemical Corporation (Chengdu, China).

Apparatus and chromatographic conditions

A Shimadzu 20 AT HPLC instrument (Japan) equipped with a quaternary pump, a photo diode-array detector and a column compartment was used. The sample was separated on a Chromasil C18 column (5μm, 4.6×250 mm).

The mobile phase consisted of acetonitrile and 5%CTAB water (95:5,v/v) . HPLC separation was performed at 20℃ and a flow rate of 1.5 ml/min. PAD detector was set to scan from 200 to 800 nm, and 242 nm was used as detection wavelength for analysis.

Sample preparation

Total lipids were extracted using the method described by Dai (2000). The fresh *Phellinus* (1 g) were ground in liquid nitrogen, and 2 ml chloroform, 1 ml 1mol/L sodium hydroxide-methanol were added and saponified at room temperature for 45 min. The extract was suspended in 1 *ml* 2 *mol/L* hydrochloric acid-methanol and vibrated for 2 min. After 10 min, 1.8 ml water was added and subnatant was dehydrated by anhydrous sodium sulfate.

Phenacyl esters were prepared as described by Chen (1992). An aliquot (400 μl) of the extracted lipids was dried under nitrogen and resuspended in 100 μl 2-bromocacetophenone (10 mg/ml in acetone). After vortex-mixing, 100 μl trithylamine (10 *mg/ml* in acetone) was added. The mixture was sealed immediately in a screw-capped glass tube and heated for 5 min in a boiling water bath. After cooling, 160 μl acetic acid (2 mg/ml in acetone) was added and the tube was heated for an additional 5 min. The resulting FAPEs were filtered through a 0.22 μm microfilter membrane and redissolved in 500 μl methanol for HPLC injection

Method validation

Calibration curves of the tested compounds were linear over the studied concentration ranges with $r2 > 0.9985$ for all five analytes. Under the optimized experimental conditions, calibration curves of palmitoleic acid, linoleic acid, oleic acid, hexadecanoic acid and stearic acid showed good linearity in 0.04 to 1.60 μg/mL. The detection limits were all no less than 10.5 ng. Injection precision, repeatability and stability test were evaluated. Recoveries of the quantified constituents were determined using samples of SH-05 in which the contents of corresponding constituents had been predetermined. In each case a mixture of standards with 80, 100 or 120% of the quantified levels of the constituents was spiked into the sample, which was subjected to the extraction procedure described above and then analysed by HPLC in triplicate.

RESULTS AND DISCUSSION

Identification of fatty acid compounds was carried out by comparing HPLC retention times and UV absorptions. Under the current experimental conditions, the retention times for palmitoleic acid, linoleic acid, oleic acid, hexadecanoic acid and stearic acid were at 11.1, 11.9, 19.0, 20.0 and 36.2 min, respectively. Chromatograms were shown in Figure 1a and b. Calibration curves were obtained using standard solutions at six different concentration levels. As shown in Table 1, all calibration curves were linear over the concentration ranges tested, with good correlations. The detection limits (S/N = 3) of the compounds 1–5 ranged from 5.7 to 10.5 ng. The injection precision was evaluated by replicated injection of the same sample solution six times in a day. The RSD of peak areas of five peaks were found in the range of 1.19–2.76%, respectively

Table 2. Contents of five fatty acids in *Phellinus* sp. (mg g-1).

Number	Source	Palmitoleic acid	linoleic acid	Oleic acid	Hexadecanoic acid	Stearic acid
SH-01 *Phellinus linteus*	Zhouyulin Edible Mushroom Institute of Wuhan	0.05296±0.0010	1.3486±0.0869	0.09857±0.0093	1.0798±0.0598	0.2774±0.0233
SH-02 *Phellinus linteus*	Culture Center of Huazhong Agricultural University	0.02619±0.0018	0.4449±0.0269	0.04101±0.0026	0.6283±0.0997	0.3040±0.0048
SH-03 *Phellinus linteus*	Xinyu Edible Mushroom Institute of Wuhan	0.03127±0.0058	0.9397±0.0317	0.07742±0.0033	0.8191±0.0748	0.2256±0.0095
SH-04 *Phellinus linteus*	Agricultural Culture Collection of China	0.01194±0.0008	0.4136±0.0194	0.03369±0.0010	0.657±0.0091	0.3391±0.0104
SH-05 *Phellinus linteus*	Microbiological Research Center of Sichuan Academy of Agricultural Science	0.04008±0.0014	0.8667±0.0232	0.04813±0.0038	0.8459±0.0185	0.2562±0.0419
SH-06 *Phellinus linteus*	Microbiological Research Center of Sichuan Academy of Agricultural Science	0.03932±0.0022	0.5941±0.0174	0.06317±0.0109	0.9894±0.0443	0.2998±0.0274
SH-07 *Phellinus linteus*	Agricultural Culture Collection of China	0.10032±0.0089	1.6483±0.0852	0.10889±0.0078	1.0196±0.1066	0.3336±0.0584
SH-08 *Phellinus baumii*	China General Microbiological Culture Collection Center	0.05205±0.0025	0.7721±0.0019	0.0762±0.0018	0.4871±0.0298	0.1464±0.0029
SH-09 *Phellinus baumii*	Guangda Edible Mushroom Center of Jining in Shandong	0.06016±0.0028	1.144±0.0889	0.06089±0.0071	1.1034±0.0553	0.2345±0.0285
SH-10 *Phellinus baumii*	Xinyu Edible Mushroom Institute of Wuhan	0.00461±0.0003	0.7316±0.0185	0.05094±0.0139	0.6312±0.0549	0.2910±0.0003
SH-11 *Phellinus baumii*	Fungi Institute of Jinxiang in Shandong	0.00854±0.0002	0.5181±0.0205	0.03801±0.0057	0.9778±0.0606	0.2839±0.0002
SH-12 *Phellinus baumii*	Edible Mushroom Institute of Yichun in Heilongjiang	0.09963±0.0122	1.0969±0.0911	0.09553±0.0002	1.2403±0.0617	0.3821±0.0600

Table 2. Contd.

SH-13 *Phellinus pomaceus*	Agricultural Culture Collection of China	0.02215±0.0045	0.2982±0.0078	0.02074±0.0045	0.3952±0.0333	0.2292±0.0303
SH-14 *Phellinus pini*	Agricultural Culture Collection of China	0.02344±0.0024	0.1869±0.0043	0.03654±0.0023	0.4405±0.0171	0.2984±0.0257
SH-15 *Phellinus robustus*	Agricultural Culture Collection of China	0.02834±0.0060	0.1629±0.0067	0.02732±0.0019	0.5223±0.0771	0.2591±0.0465
SH-16 *Phellinus sp.*	Guangda Edible Mushroom Center of Jining in Shandong	0.02592±0.0003	0.4638±0.0053	0.01644±0.0025	0.7253±0.0064	0.3242±0.0051

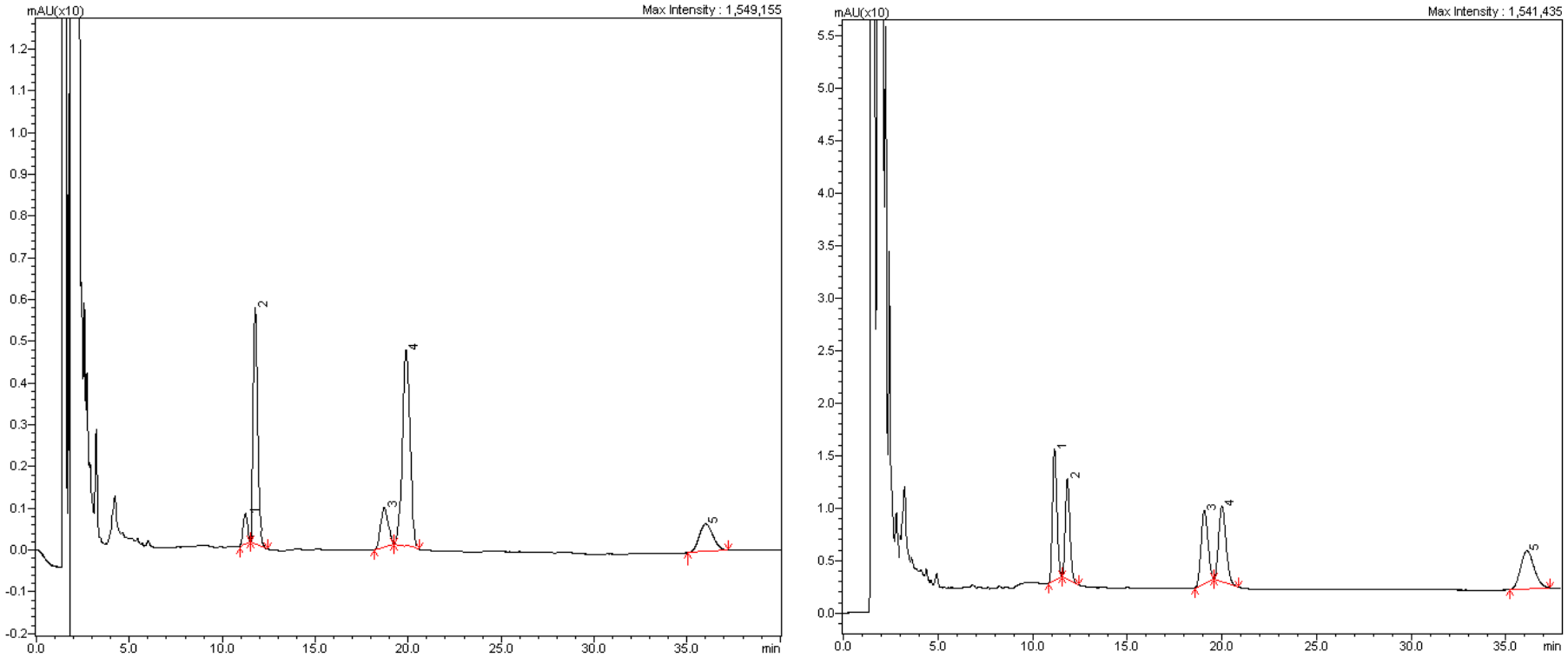

Figure 1. HPLC chromatograms of fatty acid in Phellinus sp. a: the standard; b: SH-05. (1:Palmitoleic acid; 2: linoleic acid; 3: oleic acid; 4: hexadecanoic acid; 5: stearic acid).

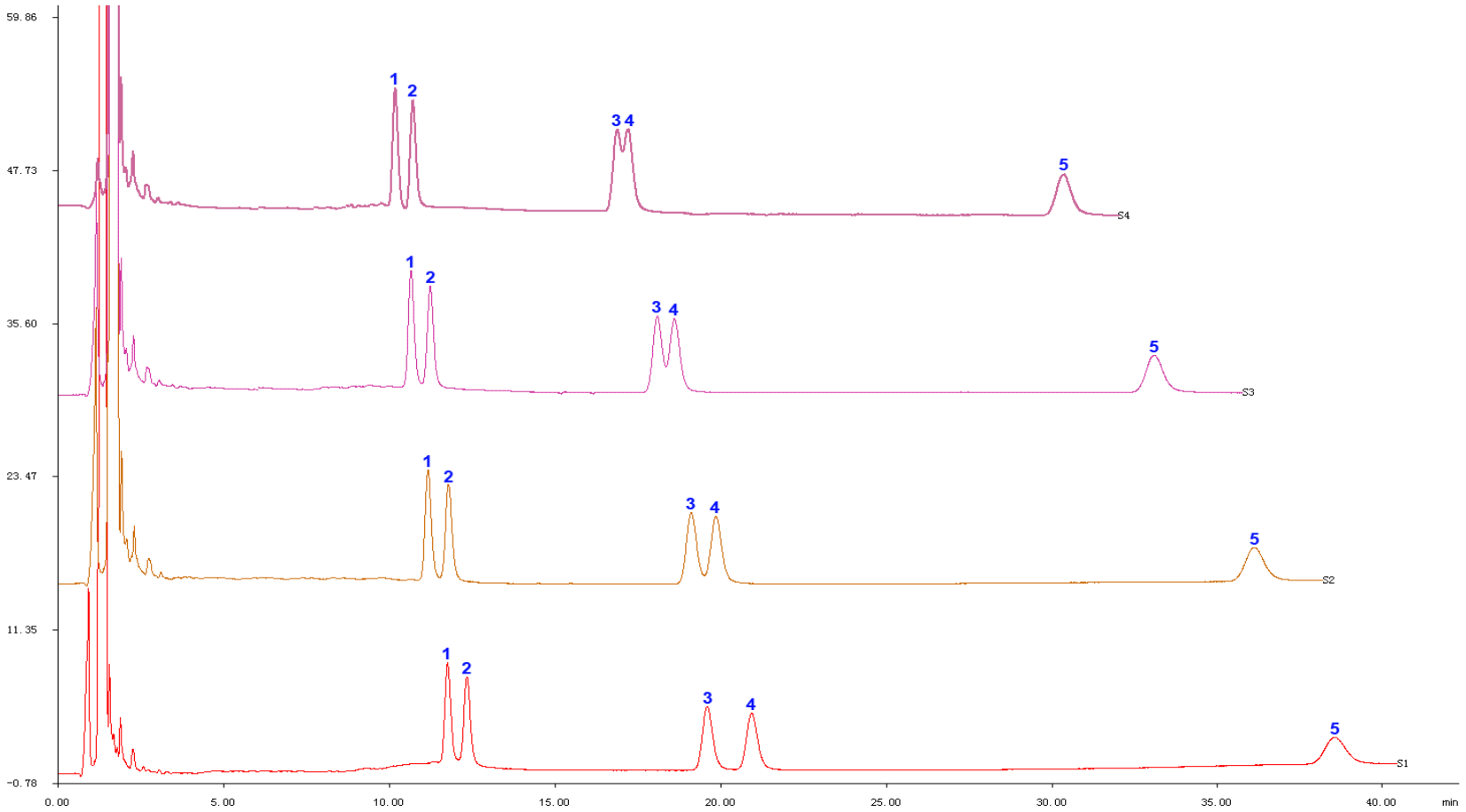

Figure 2. HPLC chromatograms of five fatty acids in Phellinus sp. under different column temperature. (1:Palmitoleic acid; 2: linoleic acid; 3: oleic acid; 4: hexadecanoic acid; 5: stearic acid.

(Table 1). The repeatability was assessed by analyzing six independently prepared samples. The RSD values for method repeatability were found to be within the range of 1.51–5.13% for inter-day assays and 0.85–2.87% for intra-day assays (Table 1). A stability study was conducted to determine storage time for samples. The samples showed good stability up to 7 days. The RSD values for samples stability were found to be within the range of 1.27–3.33% for inter-day assays and 1.03–3.23% for intra-day assays (Table 1) Recoveries of the quantified compounds were within the range of 98.6–102.33%, with RSD less than 4.22% (Table 1). All results of injection precision, repeatability and stability test indicate that this method was adequate, valid and applicable.Selection of column temperature was one of the key factors contributing to reliable and reproducible HPLC condition. Relationships between column temperature and resolution were shown in Figure 2. Therefore, 20℃, at which all compounds could be separated well in 40 min, was chosen. The contents of the five fatty acids in *Phellinus linteus, Phellinus baumii, Phellinus*

pomaceus, Phellinus pini and *Phellinus robustus* were determined, which demonstrated that the varieties exhibited a wide variability (Table 2). The richness in total unsaturated fatty acids and saturated fatty acids content were *P. linteus* from Agricultural Culture Collection of China and *P. baumii* from Edible Mushroom Institute of Yichun in Heilongjiang, respectively. While the poorest in total unsaturated fatty acids and saturated fatty acids contents were *P. robustus* and *P. pomaceus* from Agricultural Culture Collection of China, respectively. In all samples, the content of linoleic acid and hexadecanoic acid were higher than palmitoleic acid, oleic acid and stearic acid; the percentage of linoleic acid in unsaturated fatty acid ranged from 74.5 to 92.9%, and hexadecanoic acid in saturated ranged from 59.6 to 79.6%. That is, the main unsaturated and unsaturated fatty acid constitutes were linoleic and hexadecanoic acid, respectively.

Conclusion

In summary, a stable and sensitive HPLC-PDA method had been developed for determination of five fatty acid components in *Phellinus.sp*.-palmitoleic acid, linoleic acid, oleic acid, hexadecanoic acid, stearic acid. The extraction and derivatization procedures are simple and rapid, characteristic compounds were well separated and had good limits of detection. We believe the described method satisfies the need for controlling the quality and detecting physiological properties of *Phellinus sp*.

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