Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 1172–1178

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Analysis of sterols and fatty acids in natural and cultured *Cordyceps* by one-step derivatization followed with gas chromatography–mass spectrometry

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ARTICLE INFO

Article history:
Received 14 January 2009
Received in revised form 21 February 2009
Accepted 23 February 2009
Available online 9 March 2009

Keywords:
Gas chromatography–mass spectrometry (GC–MS)
Trimethylsilyl (TMS) derivative
Pressurized liquid extraction (PLE)
Fatty acids
Sterols
Cordyceps

ABSTRACT

Ten free fatty acids namely lauric acid, myristic acid, pentadecanoic acid, palmitoleic acid, palmitic acid, linoleic acid, oleic acid, stearic acid, docosanoic acid and lignoceric acid and four free sterols including ergosterol, cholesterol, campesterol and β-sitosterol in natural (wild) *Cordyceps sinensis*, *Cordyceps liangshanensis* and *Cordyceps gunnii*, as well as cultured *C. sinensis* and *Cordyceps militaris* were first determined using pressurized liquid extraction (PLE), trimethylsilyl (TMS) derivatization and GC–MS analysis. The conditions such as the amount of reagent, temperature and time for TMS derivatization of analytes were optimized. Under the optimum conditions, all calibration curves showed good linearity within the tested ranges. The intra- and inter-day variations for 14 investigated compounds were less than 3.4% and 5.2%, respectively. The results showed that palmitic acid, linoleic acid, oleic acid, stearic acid and ergosterol are main components in natural and cultured *Cordyceps* which could be discriminated by hierarchical clustering analysis based on the contents of 14 investigated compounds or the 4 fatty acids, where the contents of palmitic acid and oleic acid in natural *Cordyceps* are significantly higher than those in the cultured ones.

1. Introduction

Cordyceps, one of the valued traditional Chinese medicines, is a composite consisting of the stromata of the fungus, *Cordyceps sinensis* (Berk.) Sacc. (Family: Hypocreaceae) parasitized on the larva of some species of insects (Family: Hepialidae), and the dead caterpillar. It has been used for treatment of several diseases such as hyposexualities, hyperglycemia, hyperlipidemia, asthemia after severe illness, renal dysfunction and renal failure, arrhythmias and other heart disease, and liver disease [1,2]. However, the relationship between the compounds and the pharmacological effects of *Cordyceps* is still, at least partially, unclear. Furthermore, due to the rarity and excellent curative effects of natural *Cordyceps*, several fungus strains have been isolated from natural *Cordyceps* and manufactured in large quantity by fermentation technology, and they are commonly sold as health food products. In addition, some natural substitutes such as *Cordyceps militaris*, *Cordyceps liangshanensis*, *Cordyceps gunnii* and *Cordyceps cicadicola* are also used in the market [3].

To date, several fatty acids and sterols, which are very important bio-functional components in fungi [4,5], were separated or identified from *Cordyceps* [3,6]. Ergosterol is the predominant sterol found in *Cordyceps*, which can be converted by ultraviolet irradiation into vitamin D_2, a nutritional factor that promotes proper bone development in humans and other mammals. Ergosterol analogues also have multiple pharmacological activities, such as cytotoxic, anti-viral and anti-arrhythmic effects [3], as well as suppression of activated human mesangial cells and alleviation of immunoglobulin A nephropathy (Berger's disease) [7]. Actually, phytosterols, mainly β-sitosterol, campesterol and stigmasterol [8], are beneficial to human health [9,10]. On the other hand, free fatty acids (FFAs) are not only essential nutritional components but also modulators of many cellular functions through their receptors of FFAs, which were proposed to be the novel therapeutic targets for diabetes and dyslipidemia, especially type 2 diabetes [11,12]. Generally, FFAs receptors are the G-protein-coupled receptors (GPCR), including G-protein receptor (GPR)41 and GPR43 activated by short-chain FFAs, and GPR40 and GPR120 activated by medium- and long chain FFAs [11–13]. To date, several fatty acids were identified in *Hirsutella sinensis*, anamorph of *C. sinensis* [14], and *C. militaris*, a substitute of *C. sinensis* [15]. Free fatty acids in *Cordyceps* may also contribute to its effects. Therefore, analysis of sterols and fatty acids in *Cordyceps* is not only important for evaluation of their quality but also beneficial to the elucidation of its pharmacological activities. Actually, TMS derivatization followed GC–MS analysis has been used for the analysis of fatty acids and β-sitosterol in soil [16]. In present study, TMS ethers of four free sterols such as ergosterol, cholesterol, campesterol and β-sitosterol, and 10 free fatty acids,

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doi:10.1016/j.jpba.2009.02.025

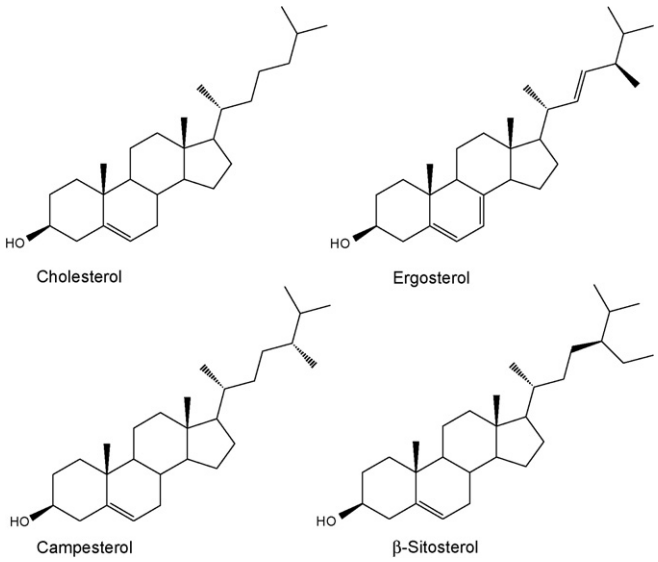

Fig. 1. Chemical structures of four investigated sterols.

including lauric acid, myristic acid, pentadecanoic acid, palmitoleic acid, palmitic acid (PA), linoleic acid (LoA), oleic acid (OA), stearic acid (SA), docosanoic acid and lignoceric acid, extracted from natural and cultured *Cordyceps* by pressurized liquid extraction (PLE) were simultaneous determined by GC–MS. The contents of the free sterols and fatty acids in natural *C. sinensis*, *C. liangshanensis*, *C. gunnii*, and cultured *C. sinensis* and *C. militaris* were also compared.

2. Materials and methods

2.1. Materials and chemicals

Lauric acid (dodecanoic acid, C12), myristic acid (tetradecanoic acid, C14), pentadecanoic acid (C15), palmitoleic acid ((*Z*)-hexadec-9-enoic acid, C16), palmitic acid (hexadecanoic acid, C16), linoleic acid ((9*Z*,12*Z*)-octadeca-9,12-dienoic acid, C18), oleic acid ((*Z*)-octadec-9-enoic acid, C18), stearic acid (octadecanoic acid, C18), docosanoic acid (C22), lignoceric acid (tetracosanoic acid, C24) and ergosterol were purchased from Sigma (St. Louis, MO, USA); cholesterol, campesterol and β-sitosterol were purchased from ChromaDex (Irvine, CA, USA) (Fig. 1). Ethyl acetate, *n*-hexane, petroleum ether (60–90 °C) and dichloromethane were purchased from Merck (Darmstadt, Germany). Derivatization reagent BSTFA (N,O-bis (trimethylsilyl) trifluoroacetamide) containing 1% TMCS (trimethylchlorosilane) was purchased from Supelco (St. Louis, MO, USA). Materials of natural (wild) *C. sinensis* were obtained from three different regions of China, one from Qinghai, two from Tibet and one from Sichuan. Natural *C. liangshanensis* was from Sichuan, and natural *C. gunnii* were from Sichuan and Anhui. The identities of these natural *Cordyceps* were confirmed by the correspondence author. Commercial cultured *C. sinensis* mycelia were obtained from Hebei, Anhui, Jiangxi, Huadong and Wanfeng. Commercial cultured *C. militaris* were obtained from Guobao, Hong Kong, Aoli, Quanxin and Xiankang. The species of the cultured *Cordyceps* were certified by State Food and Drug Administration of China and/or manufacturers. Seven cultured *C. sinensis* mycelia were fermented in our lab. The voucher specimens of *Cordyceps* are deposited at the Institute of Chinese Medical Sciences, University of Macau, Macau, China.

2.2. Sample preparation

Pressurized liquid extractions were performed on a Dionex ASE 200 (Dionex Corp., Sunnyvale, CA, USA) system. In brief, 0.2 g powder of *Cordyceps* were mixed with diatomaceous earth in a proportion (1:1) and placed into an 11 mL stainless steel extraction cells, respectively. The extraction with petroleum ether was performed under optimized conditions: temperature, 160 °C; static extraction time, 10 min; pressure, 1500 psi; flush volume, 40%; static cycle, 1 and one for the number of extraction. The extract (approximate 15 mL) was dried at 40 °C using a rotary evaporator (BÜCHI, Switzerland), then 50 μL derivatization agent (BSTFA) and 200 μL *n*-hexane were added to the residue and reacted at 70 °C for 30 min. The derivative mixture was dried under N_2 (gas) to remove the excess BSTFA, and subsequently re-dissolved in 1000 μL *n*-hexane. The solution was filtered through a 0.45 μm Econofilter (nylon, Agilent Technologies, Palo Alto, CA, USA) before GC–MS analysis.

2.3. GC–MS analysis

GC–MS was performed with an Agilent 6890 gas chromatography instrument coupled to an Agilent 5973 mass spectrometer and an Agilent ChemStation software (Version 1701DA, Agilent Technologies, Palo Alto, CA). A HP-5MS capillary column (30 m × 0.25 mm i.d.) coated with 0.25 μm film 5% phenyl methyl siloxane was used for separation. The column temperature was set at 100 °C and held for 5 min for injection, then programmed at 20 °C min^{-1} to 200 °C and held for 10 min, then at 10 °C min^{-1} to 230 °C, and finally, at 5 °C min^{-1} to 320 °C, and held for 5 min. Split injection (2 μL) with a split ratio of 1:10 (or 1:100 if the content of

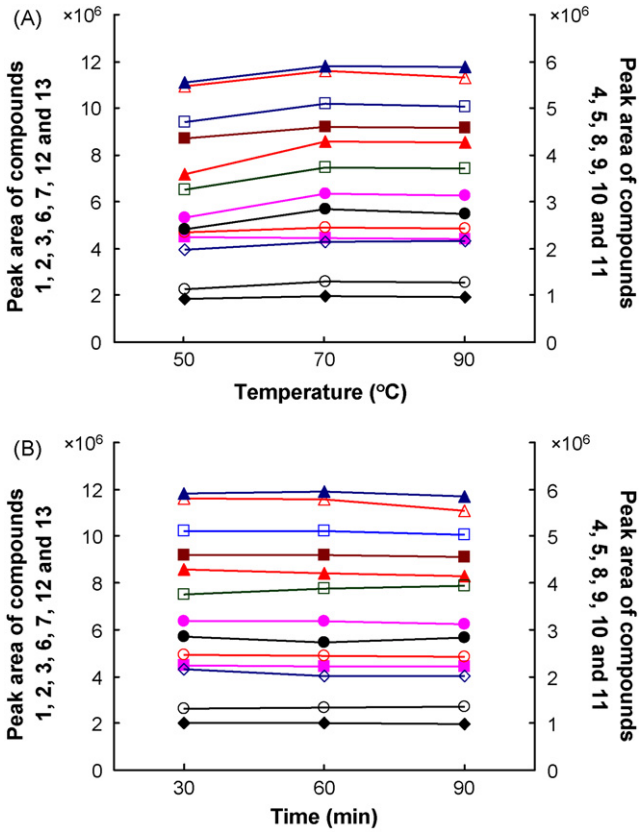

Fig. 2. Effects of temperature (A) and time (B) on derivatization efficiency of the investigated compounds. For optimization of the temperature, amount of BSTFA and reaction time were 50 μL and 30 min, while during time changed, amount of BSTFA and temperature were 50 μL and 70 °C, respectively. (■) lauric acid, (○) myristic acid, (♦) pentadecanoic acid, (■) palmitoleic acid, (▲) palmitic acid, (●) linoleic acid, (□) oleic acid, (▲) stearic acid, (●) docosanoic acid, (◇) lignoceric acid, (□) cholesterol, (▲) ergosterol and (○) β-sitosterol. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
Mass data of TMS derivative of 14 investigated compounds.

Peak	Compound	Mass data[a]
1	Lauric acid	272(M+, 3), 258(20), 257(100), 145(15), 132(24), 129(30), 117(62), 75(54), 73(59), 43(14), 41(14)
2	Myristic acid	300(M+, 6), 286(23), 285(100), 145(19), 132(30), 129(34), 117(68), 75(55), 73(64), 43(20), 41(18)
3	Pentadecanoic acid	314(M+, 7), 300(25), 299(100), 145(19), 132(30), 129(34), 117(67), 75(51), 73(61), 43(21), 41(18)
4	Palmitoleic acid	326(M+, 8), 312(25), 311(100), 145(25), 129(61), 117(67), 75(83), 73(82), 55(43), 43(23), 41(32)
5	Palmitic acid	328(M+, 8), 314(26), 313(100), 145(21), 132(29), 129(33), 117(61), 75(47), 73(56), 43(22), 41(16)
6	Linoleic acid	352(M+, 5), 337(79), 129(46), 95(35), 81(51), 79(34), 75(99), 73(100), 67(62), 55(47), 41(38)
7	Oleic acid	354(M+, 8), 340(27), 339(100), 145(26), 129(60), 117(62), 75(78), 73(82), 55(43), 43(26), 41(34)
8	Stearic acid	356(M+, 11), 342(27), 341(100), 145(23), 132(30), 129(31), 117(60), 75(42), 73(54), 55(16), 43(24)
9	Docosanoic acid	412(M+, 21), 398(32), 397(100), 145(30), 132(34), 129(32), 117(59), 75(37), 73(51), 43(26)
10	Lignoceric acid	440(M+, 27), 426(33), 425(100), 145(30), 132(32), 129(29), 117(53), 75(34), 73(49), 43(27)
11	Cholesterol	458(M+, 47), 368(81), 353(41), 330(26), 329(100), 129(74), 95(27), 75(30), 73(44), 43(28)
12	Ergosterol	468(M+, 29), 364(31), 363(100), 338(19), 337(64), 143(19), 131(20), 73(27), 69(28), 55(21)
13	Campesterol	472(M+, 51), 383(28), 382(91), 367(42), 344(26), 343(100), 129(78), 75(30), 73(46), 43(48)
14	β-Sitosterol	486(M+, 53), 397(31), 396(96), 381(42), 357(100), 129(82), 75(30), 73(45), 57(30), 43(43)

[a] *m*/*z*, relative intensity shown in parenthesis.

Table 2
SIM, regression data, LOD, LOQ, repeatability and recovery of 14 investigated compounds analyzed by GC–MS.

Analytes	SIM (*m*/*z*)	Linear regression data			LOD (μg/mL)	LOQ (μg/mL)	Repeatability (%)		Recovery (%, *n* = 3)
		Regressive equation (μg/mL)	Test range	R^2			Intra-day	Inter-day	
Lauric acid	257	$y = 16{,}679x - 17{,}690$	2.7–32.4	0.9995	0.1	0.4	1.3	2.2	105.6
Myristic acid	285	$y = 18{,}122x - 13{,}710$	2.1–24.7	0.9996	0.2	0.6	0.7	1.9	99.2
Pentadecanoic acid	299	$y = 18{,}164x - 11{,}927$	1.7–20.0	0.9993	0.2	0.8	0.4	1.9	95.3
Palmitoleic acid	311	$y = 9096.3x - 31{,}693$	4.4–88.5	0.9964	0.4	1.5	1.1	2.3	99.8
Palmitic acid	313	$y = 24{,}183x - 97{,}771$	5.0–201.2	0.9983	0.2	0.6	1.3	2.5	103.5
Linoleic acid	337	$y = 4869x - 29{,}509$	11.9–237.6	0.9987	1.1	2.1	1.2	2.6	100.2
Oleic acid	339	$y = 11{,}838x - 43{,}558$	4.9–196.4	0.9984	0.4	0.6	1.0	1.0	98.9
Stearic acid	341	$y = 7866.7x - 131{,}117$	16.5–164.9	0.9976	0.2	0.8	0.8	2.1	104.5
Docosanoic acid	397	$y = 19{,}424x - 94{,}692$	5.1–50.9	0.9937	0.6	2.1	1.3	4.9	95.3
Lignoceric acid	425	$y = 16{,}392x - 141{,}556$	10.0–80.0	0.9986	1.2	3.3	3.4	5.2	107.3
Cholesterol	329	$y = 10{,}131x - 25{,}465$	3.3–130.0	0.9991	0.3	1.2	0.7	1.9	101.1
Ergosterol	363	$y = 12{,}277x - 197{,}676$	16.5–660.0	0.9943	0.9	3.2	2.1	1.3	93.0
Campesterol	343	$y = 25{,}000x - 6868$	1.1–21.7	0.9974	0.2	0.4	0.5	2.8	106.0
β-Sitosterol	396	$y = 3960x - 22{,}174$	7.6–303.3	0.9979	0.7	2.5	0.6	1.9	102.5

analytes beyond the upper limit of linearity ranges) was used, and injection temperature was set at 260 °C. High purity helium was used as carrier gas of 1.0 mL min^{-1} flow-rate.

The spectrometers were operated in electron-impact (EI) mode, full scan of 40–550 amu or selected ion monitoring (SIM) was used, the ionization energy was 70 eV and the scan rate was 0.34 s per scan. The quadrupole and ionization source temperatures were 150 and 280 °C, respectively.

2.4. Calibration curves

n-Hexane stock solutions containing 14 reference compounds were prepared and diluted into appropriate concentrations (refer to Table 2) for the construction of calibration curves using SIM method of GC–MS, where *m*/*z* 257, 285, 299, 311, 313, 337, 339, 341, 397, 425, 329, 363, 343 and 396 were used for lauric acid, myristic acid, pentadecanoic acid, palmitoleic acid, palmitic acid, linoleic acid, oleic acid, stearic acid, docosanoic acid, lignoceric acid, ergosterol, cholesterol, campesterol and β-sitosterol, respectively. At least six concentrations of the solution were analyzed in duplicates, and then the calibration curves were constructed by plotting the peak area versus the concentration of each analyte.

2.5. LOD and LOQ

n-Hexane stock solution containing 14 reference compounds was diluted to a series of appropriate concentrations with the same solvent, and an aliquot of the diluted solutions was injected into GC–MS for analysis. The limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined at the ratio of signal to noise (*S*/*N*) equal to 3 and 10, respectively.

2.6. Precision, repeatability and accuracy

Intra- and inter-day variations were chosen to determine the precision of the developed GC–MS assay. A certain concentration (about at the middle of linear range) solution of 14 reference compounds was tested. For intra-day variability, the samples were analyzed for six times within 1 day, while for inter-day variability, the samples were examined in duplicate for consecutive 3 days. Variations were expressed by the relative standard deviations (R.S.D.).

To test the repeatability of derivatization, a certain concentration solution of mixture of 14 investigated compounds was derivatized under the optimum conditions triplicates and analyzed by GC–MS as mentioned above. Variations were expressed by R.S.D.

Recovery test was used to evaluate the accuracy of the developed method. Accurate amounts of 14 investigated compounds were added to approximate 0.1 g of cultured *Cordyceps* (from Wanfeng), and then extracted and analyzed as described above:

$$\text{recovery}\,(\%) = \frac{100 \times (\text{amount found} - \text{original amount})}{\text{amount spiked}}$$

3. Result and discussion

3.1. Optimization of derivatization conditions

Conventionally, methyl esters of fatty acids are widely used for GC analysis though butyl ether can be used for the analysis of short-

Table 3
The contents (μg/g) of 10 fatty acids and 4 sterols in natural and cultured *Cordyceps*.

	No.	Samples	LA	MA	PtA	PoA	PA	LoA	OA	SA	DA	LiA	ChS	ErS	CaS	StS
NCS	1	Sichuan	17.6[a]	36.7	35.5	220.7	1469.5	2896.5	3978.0	800.7	35.9	52.6	88.2	134.3	+[b]	103.6
	2	Qinghai	+	21.5	23.8	218.7	1760.0	2854.1	3943.6	609.2	33.1	47.9	69.3	108.0	+	105.1
	3	Tibet1	14.0	47.1	34.3	613.7	3842.4	6012.3	11517.6	1238.6	38.7	52.9	82.5	120.3	+	95.9
	4	Tibet2	+	26.8	30.2	245.9	2050.2	3581.0	5869.4	1269.2	38.9	55.4	68.8	89.7	+	78.1
NCG	5	Anhui-A	+	62.9	15.2	120.9	6385.1	2129.4	4776.8	1943.0	38.3	46.9	60.5	508.6	–[c]	85.0
	6	Anhui-B	+	20.0	28.3	38.5	1736.1	1076.0	1301.4	1254.7	34.7	47.3	151.7	869.7	+	115.3
	7	Anhui-C	13.8	52.4	+	180.7	7010.1	1701.7	4843.5	1850.1	35.3	46.2	69.7	305.8	+	111.6
	8	Sichuan-A	+	24.7	+	363.8	2350.8	1987.6	6553.5	1447.2	37.0	49.4	72.8	451.6	+	52.1
	9	Sichuan-B	+	61.2	+	94.4	10553.5	1868.8	5531.5	1703.6	35.0	46.0	95.9	398.1	+	51.0
NCL	10	Sichuan-A	17.5	24.5	13.2	338.9	2037.3	1014.0	1967.6	1245.3	31.9	48.8	30.0	226.7	+	52.2
	11	Sichuan-B	+	30.1	19.4	402.9	2053.5	1149.8	2082.1	1235.9	32.2	+	22.4	219.1	+	+
CCCS	12	Wanfeng	+	14.7	19.1	98.3	870.5	2155.2	1471.9	1445.1	232.1	145.6	–	642.1	12.6	208.7
	13	Hebei	+	16.4	19.3	36.3	1011.3	2561.2	895.7	2275.6	149.2	99.5	24.1	541.7	15.1	157.2
	14	Huadong	+	14.9	61.5	162.8	1139.7	4549.2	749.3	1297.1	34.6	57.3	37.1	318.6	34.4	75.6
	15	Jiangxi	17.6	13.7	10.5	65.4	843.1	1780.2	909.6	799.2	48.3	59.7	+	212.0	+	164.4
	16	Anhui	21.9	40.3	82.9	968.3	2718.1	13653.7	4182.0	3308.5	177.2	135.7	–	111.2	+	65.8
CCCM	17	Xiankang	31.6	23.8	89.5	77.9	821.6	2508.7	668.1	1237.2	33.3	47.5	+	273.2	+	–
	18	Quanxin	26.8	14.5	36.6	66.4	715.4	2132.0	386.6	621.1	35.7	51.9	–	306.6	+	–
	19	Guobao	+	+	133.6	75.8	710.0	1922.8	317.2	690.2	53.8	+	+	187.0	–	–
	20	HKUST	+	+	133.0	72.8	677.0	2564.4	442.8	803.8	60.4	95.8	+	205.2	–	+
	21	Aoli	18.3	36.4	31.2	233.2	3439.3	1269.3	5148.2	3428.3	69.7	60.4	78.8	93.7	+	73.7
CCS	22	HS	90.9	+	+	99.4	349.1	483.1	201.6	499.1	+	+	+	506.0	25.7	+
	23	CS50542	+	+	–	–	665.2	726.8	442.1	483.9	+	48.8	–	145.2	+	51.6
	24	CS50562	+	+	–	57.5	552.3	1020.3	332.2	295.5	+	48.2	–	397.1	+	49.8
	25	CS50677	20.8	+	+	74.1	557.5	1052.8	604.2	762.6	36.8	60.4	–	514.5	+	–
	26	PH	26.2	+	+	75.4	749.3	1457.0	498.2	465.0	+	+	–	314.5	–	–
	27	GR	16.8	+	+	64.3	929.5	1453.5	1061.3	929.3	45.8	+	–	706.9	–	–
	28	LCT	+	–	–	37.7	166.3	718.0	176.0	208.8	26.8	+	–	259.9	–	–

Note: LA, lauric acid; MA, myristic acid; PtA, pentadecanoic acid; PoA, palmitoleic acid; PA, palmitic acid; LoA, linoleic acid; OA, oleic acid; SA, stearic acid; DA, docosanoic acid; LiA, lignoceric acid; ChS, cholesterol; ErS, ergosterol; CaS, campesterol; StS, β-sitosterol; NCS, natural *Cordyceps sinensis*; NCG, natural *C. gunnii*; NCL, natural *C. liangshanensis*; CCCS, commercial cultured *C. sinensis*; CCCM, commercial cultured *C. militaris*; CCS, self-cultured *C. sinensis*; HS, *Hirsutella sinensis*; PH, *Paecilomyces hepiali*; GR, *Gliocladium roseum*; CS, *Cordyceps sinensis*; LCT, isolated from the caterpillar of *Cordyceps sinensis* from Qinghai province.

[a] Average of duplicates.

[b] Under the limit of quantification.

[c] Undetectable.

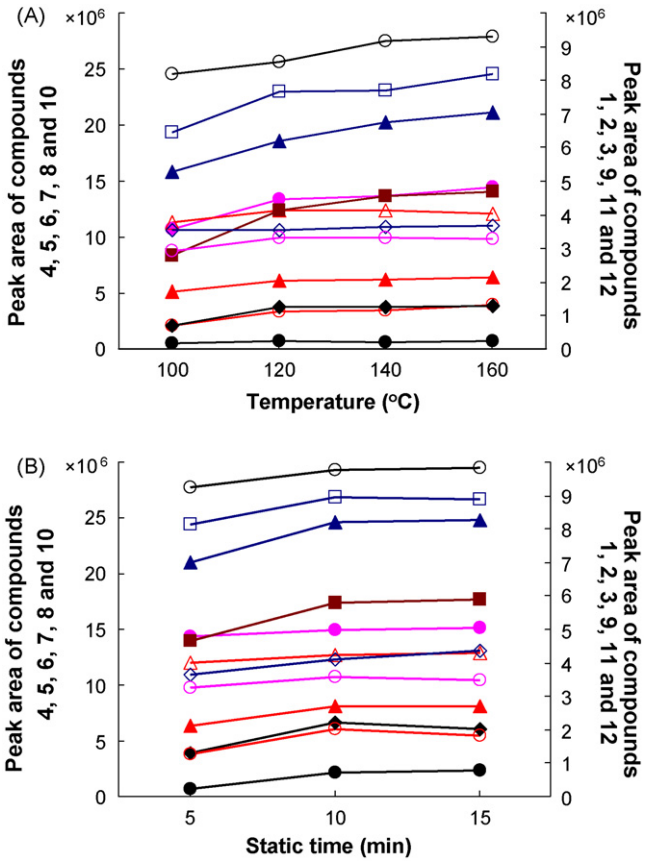

Fig. 3. Effects of temperature (A) and static time (B) on extraction efficiency of the investigated compounds in cultured *C. sinensis* from Wanfeng. To determine the parameters of temperature and static extraction time, the others were set: pressure, 1500 psi; flush volume, 40%; static cycle, 1 and one for the number of extraction. (○) Campesterol, the others are the same as Fig. 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

chain fatty acids to avoid the loss of volatile fatty acids during sample preparation [17]. Sterols are commonly analyzed as their TMS or acetate derivatives by GC [18]. Actually, BSTFA containing 1% TMCS can react with both free hydroxyl (sterols) and carboxyl (fatty acids) groups to form their corresponding trimethylsilyl (TMS) ethers under relative mild conditions [19], which were employed for simultaneous derivatization of fatty acid and sterols in this study.

The derivatization conditions for the investigated compounds (except campesterol due to limit standard amount) were optimized. The sufficient amount of derivatization agent is necessary for good derivatization efficiency. The parameters including the amount of BSTFA (50 and 100 μL), derivatization temperature (50, 70 and 90 °C) and the reaction time (30, 60 and 90 min) were investigated using univariate approach. Peak area of each investigated compound was used as the marker for evaluation of the derivatization efficiency (Fig. 2). Finally, the optimum derivatization conditions, which had highest efficiency in short reaction time under mild temperature, were: 50 μL BSTFA reacted with free sterols and fatty acids (total amount ~ 1.5 mg) in 200 μL of *n*-hexane at 70 °C for 30 min.

3.2. Optimization of PLE parameters

For the extraction of fatty acids and sterols from *Cordyceps* by PLE, different types of solvents including ethyl acetate, dichloromethane and petroleum ether were tested. There was no significantly different (R.S.D. < 4%) among these three types of solvent, but petroleum ether extract gave the least polar compounds, which may interfere with the derivatization. Therefore petroleum ether was selected as extraction solvent. Furthermore, the parameters for PLE such as extraction temperature (100, 120, 140 and 160 °C) and static time (5, 10, 15 and 20 min) were studied by using univariate approach. Peak area of each investigated compound was used as the marker for evaluation of the extraction efficiency. Taking into account the results of optimization (Fig. 3), the optimum conditions for PLE were: solvent, petroleum ether; temperature, 160 °C; static time, 10 min; flush volume, 40%; static cycle, 1 and the number of extraction, 1.

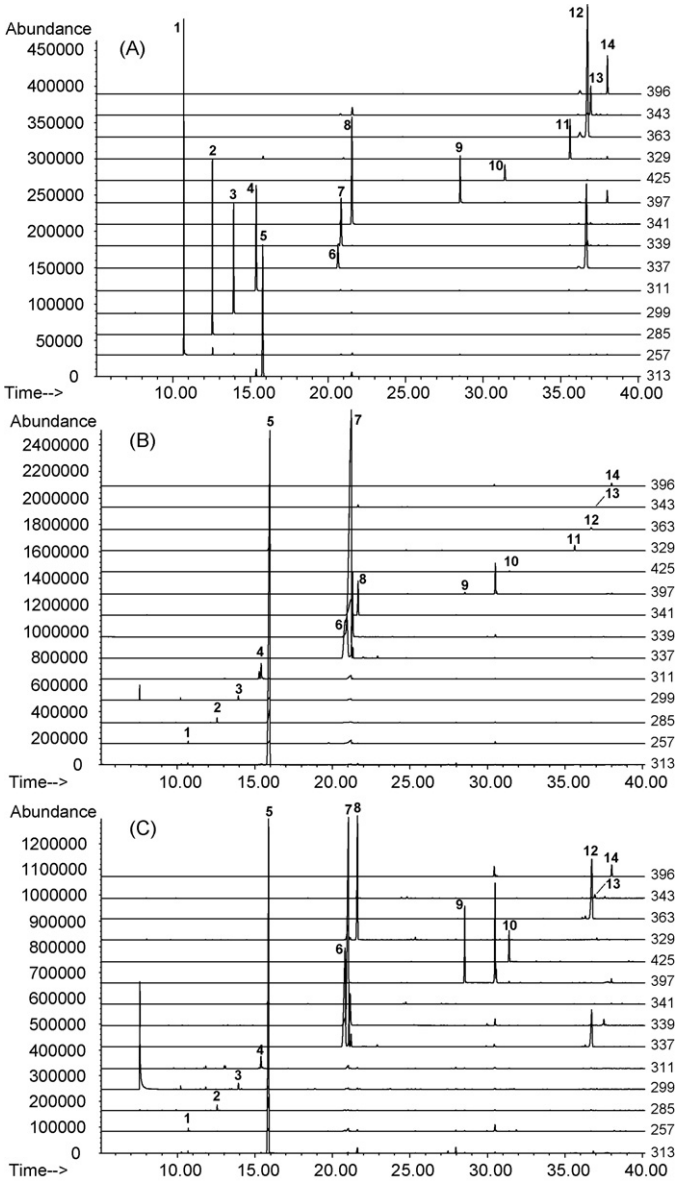

Fig. 4. Typical SIM profiles of GC–MS of mixed standards (A), natural *C. sinensis* (B, from Qinghai), commercial cultured *C. sinensis* (C, from Wanfeng). **1**, lauric acid; **2**, myristic acid; **3**, pentadecanoic acid; **4**, palmitoleic acid; **5**, palmitic acid; **6**, linoleic acid; **7**, oleic acid; **8**, stearic acid; **9**, docosanoic acid; **10**, lignoceric acid; **11**, cholesterol; **12**, ergosterol; **13**, campesterol; **14**, β-sitosterol.

3.3. Validation of method

MS data of 14 investigated compounds were shown in Table 1, while SIM method throughout GC–MS analysis for simultaneous investigation of the analytes was used for the quantification. The

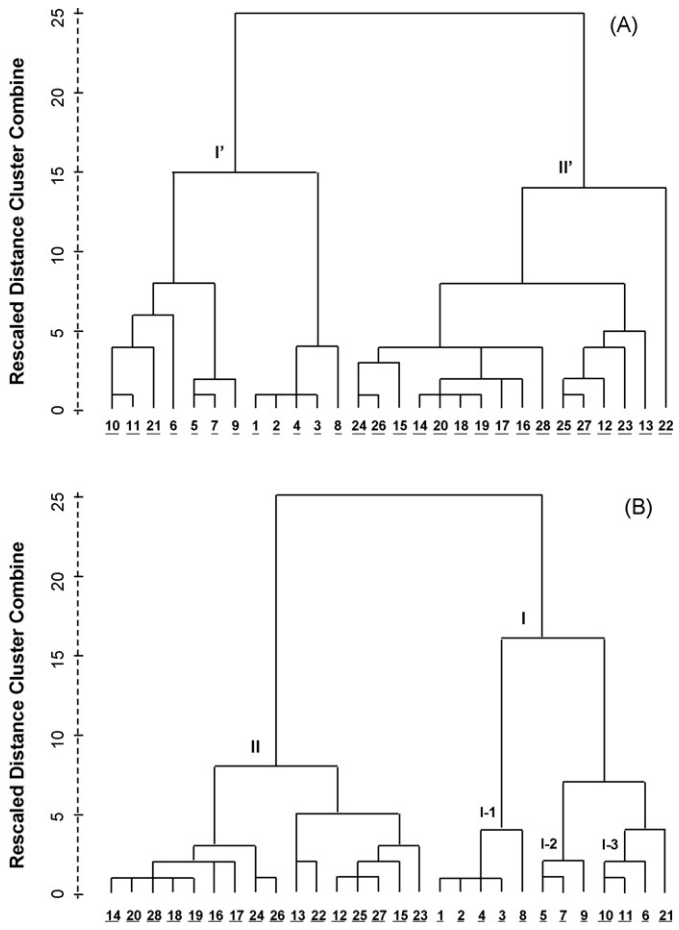

Fig. 5. Dendrogram resulting from average linkage between groups hierarchical cluster analysis. (A) and (B) are dendrograms resulting from the contents of 14 investigated compounds and four fatty acids (palmitic acid, linoleic acid, oleic acid and stearic acid), respectively. The hierarchical clustering was performed using SPSS software. A method named as average linkage between groups was applied, and Cosine distance was selected as measurement. Sample numbers 1–28 are the same as shown in Table 3.

fragment ions for simultaneous monitoring of analytes in extracts were listed in Table 2.

The overall R.S.D. for the repeatability of derivatization process were less than 1.7% for the investigated compounds. LOD and LOQ for all investigated compounds were less than 1.2 and 3.3 μg/mL, respectively, and the overall intra- and inter-day variations (R.S.D.) of 14 analytes were less than 3.4% and 5.2%, respectively. The developed method had good accuracy with overall recovery of 93.0–107.3% for the analytes. The linearity, regression, and linear ranges of 14 analytes were shown in Table 2.

3.4. Quantification and comparison of the investigated compounds in Cordyceps

Typical SIM chromatograms of TMS derivatives of mixture of 14 reference compounds and PLE extracts from *Cordyceps* were shown in Fig. 4. The identification of investigated compounds was carried out by comparison of their retention time and mass spectra with those obtained by injecting standards in the same conditions.

By using the calibration curves of each analyte, the contents (μg/g) of 10 fatty acids and 4 sterols in natural and cultured *Cordyceps* were determined. The results are summarized in Table 3. The data showed that palmitic acid, linoleic acid, oleic acid and stearic acid are the main fatty acids in *Cordyceps*, which are corresponded to previous report for entomopathogenic fungi [4]. Ergosterol, the characteristic compound in fungi, was found at the highest levels of the four sterols investigated. However, it was much lower than that in Ref. [3], which may be derived from the different sample and/or peak overlapping during HPLC analysis.

Hierarchical clustering analysis based on the contents of 14 investigated compounds showed that 28 tested samples can be grouped into two main clusters (Fig. 5A), which are in accordance with the clustering result derived from the contents of four fatty acids, PA, LoA, OA and SA (Fig. 5B). Interestingly, Cluster I are mainly natural *Cordyceps* samples (except one cultured *C. militaris* sample from Aoli), which contained much higher contents of PA and OA than those in Cluster II, cultured *Cordyceps* samples. Furthermore, Cluster I could be divided into three sub-groups, I-1, I-2 and I-3. Among these three sub-groups, OA is the richest in samples of sub-group I-1, PA is the highest in sub-group I-2.

It was reported that OA and LoA could activate GPR40 in breast cancer cell lines while PA and SA had no such effect [20]. Actually, saturated fatty acids such as PA and SA are proapoptotic agents and unsaturated fatty acids including OA and LoA can increase proliferation of breast cancer cells [21]. Therefore, the contents and the variation of PA, LoA, OA, SA as well as other fatty acids in *Cordyceps* may induce their difference of pharmacological activities, but further study is need.

4. Conclusion

Four sterols and ten fatty acids in natural and cultured *Cordyceps* were quantitatively determined using PLE extraction, one-step TMS derivatization and GC–MS analysis. The results show that palmitic acid, linoleic acid, oleic acid, stearic acid and ergosterol are their main fatty acids and sterols, but the contents of palmitic acid and oleic acid are significantly higher in natural *Cordyceps* than those in the cultured ones.

Acknowledgements

We are grateful to Leon Lai and J. Guan from our institute for their expert assistance. The research was supported by grants from Macao Science and Technology Development Fund (077/2005/A and 028/2006/A2), University of Macau (RG086/04-05S) to S.P. Li.

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