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Short communication

Simultaneous determination of ergosterol, nucleosides and their bases from natural and cultured *Cordyceps* by pressurised liquid extraction and high-performance liquid chromatography

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Abstract

A simple method is described for the simultaneous determination of ergosterol, nucleosides and their bases in *Cordyceps*. The samples were extracted by using pressurised liquid extraction (PLE). The effects of experimental variables, such as solvent, temperature, static extraction time and cycles, on PLE efficiency have been studied. The results showed a strong influence of the solvent and temperature on extraction efficiency of PLE. The determination was achieved by high-performance liquid chromatography (HPLC) using a Zorbax NH_2 analytical column (250 × 4.6 mm i.d., 5 μm) with diode-array detector (DAD). The automated preparation of the sample permits a very fast analysis which is an important goal for routine purpose.

Keywords: *Cordyceps* spp.; Pharmaceutical analysis; Ergosterol; Sterols; Nucleosides

1. Introduction

The extraction step has often proved to be the bottleneck of most analytical procedures, as it is one of the least evolved parts of the whole method. During the past few years, one of the most promising and recent sample preparation techniques is pressurised liquid extraction (PLE; Dionex trade name ASE for accelerated solvent extraction), which offers the advantages of reducing solvent consumption and allowing for automated sample handling [1]. More recently, the distinct advantages of PLE, such as significantly reduced extraction time and low solvent volume requirement, are being exploited in diverse areas, including biology, pharmaceuticals and foodstuffs [2]. An interesting and important new application area of PLE is in the extraction of chemical constituents from plants or herbal materials [2–10].

Cordyceps, one of the well-known traditional Chinese medicines, is a composite consisting of the stroma of the fungus, *Cordyceps sinensis* (Berk.) Sacc. (Fam. Hypocreaceae), parasitized on the larva of some species of insects (Fam. Hepialidae), and the dead caterpillar. Recent studies have demonstrated its multiple pharmacological actions [11,12]. The natural *Cordyceps* (wild *C. sinensis*) is rare and expensive on the local market. Several mycelial strains, e.g. *Pacilomyces hepiali* and *Hirsutella hepialid*, isolated from natural *C. sinensis* have been manufactured in large quantity by fermentation technology [11]. At present, cultured *Cordyceps* mycelia are commonly sold as health food products in South East Asia.

Nucleosides and sterols are believed to be the active components in *Cordyceps* [11,13]. Several methods have been developed for the separation of nucleosides. The vast majority of these separations have been performed by using either reversed-phase high-performance liquid chromatography (HPLC) with gradient elution [14–16] or using reversed-phase ion-pair chromatography [17]. Capillary electrophoresis [18] and capillary electrochromatography

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[19] methods have also been developed for quantification of nucleosides.

The aim of this work was to develop a simple method for simultaneous determination of ergosterol, nucleosides and their bases in *Cordycedps* based on PLE and HPLC.

2. Experimental

2.1. Chemicals

Ergosterol, nucleosides such as adenosine, cordycepin, cytidine, guanosine, thymidine, uridine, 2′-deoxyuridine and their bases adenine, cytosine, guanine, thymine and uracil (Fig. 1) were purchased from Sigma (St. Louis, MO, USA). Ammonium acetate was purchased from Riedel-de Haën (Seelze, Germany). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA).

2.2. Samples

Natural *C. sinensis* was obtained from different provinces of China: One from Qinghai and two from Tibet. The identities of these natural *Cordyceps* were confirmed by Professor Ping Li, China Pharmaceutical University, Nanjing, China.

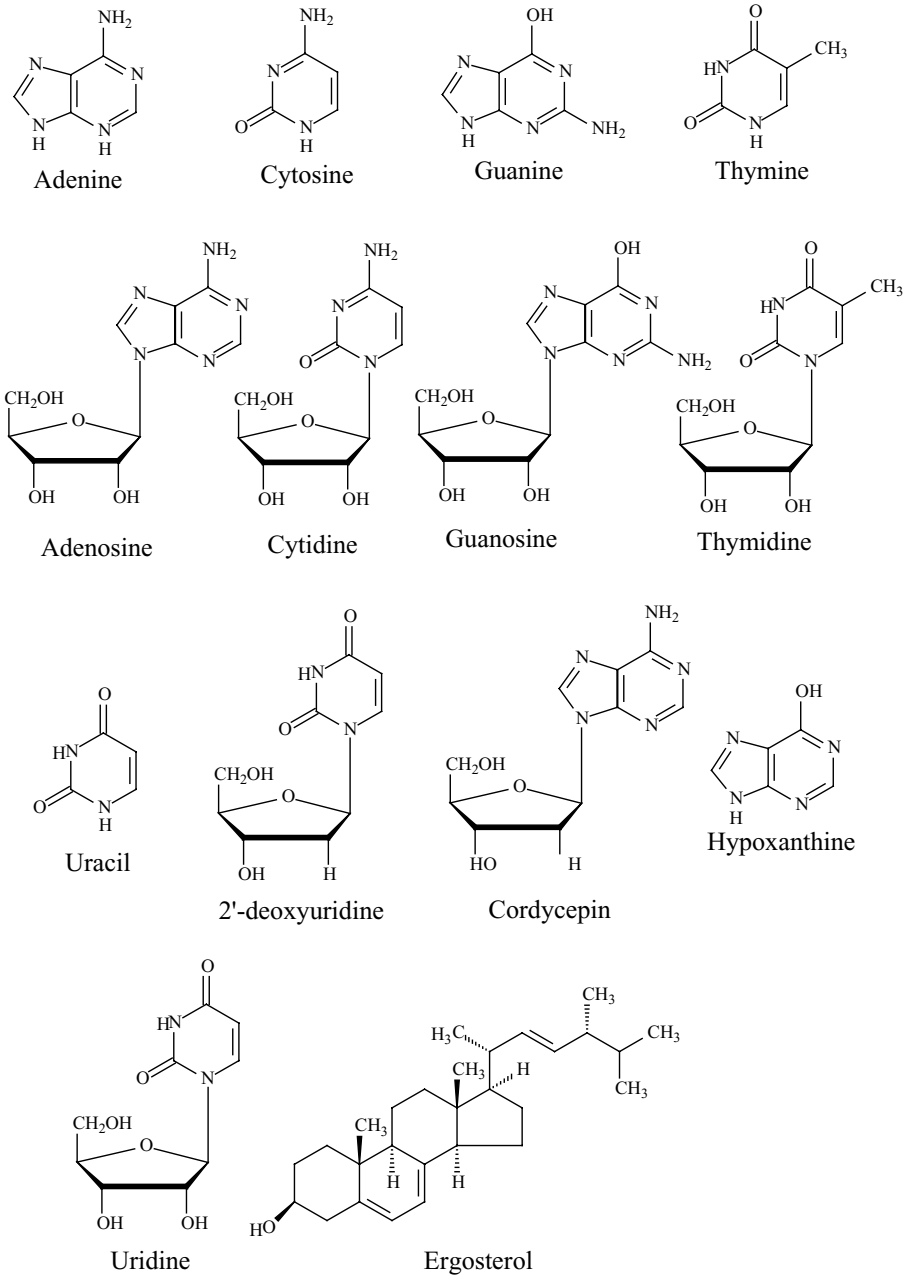

Fig. 1. Structure of ergosterol, nucleosides and their bases.

Cultured *C. sinensis* mycelia were obtained from Jianxi, Huadong, Wanfong and Boding. Cultured *Cordyceps militaris*, one of the substitutes for *C. sinensis*, were obtained from Jining and Oli. The species identities of these cultured *Cordyceps* were guaranteed by State Food and Drug Administration of China. The voucher specimens of *Cordyceps* were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macau, China. Dried samples were ground into powder (0.13–0.15 mm i.d.).

Sample preparation was performed by using pressurised liquid extraction under the pre-set default condition or the optimised conditions. The extract was transferred to a 50 ml volumetric flask which was brought up to its volume with extraction solvent and filtered through a 0.45 μm

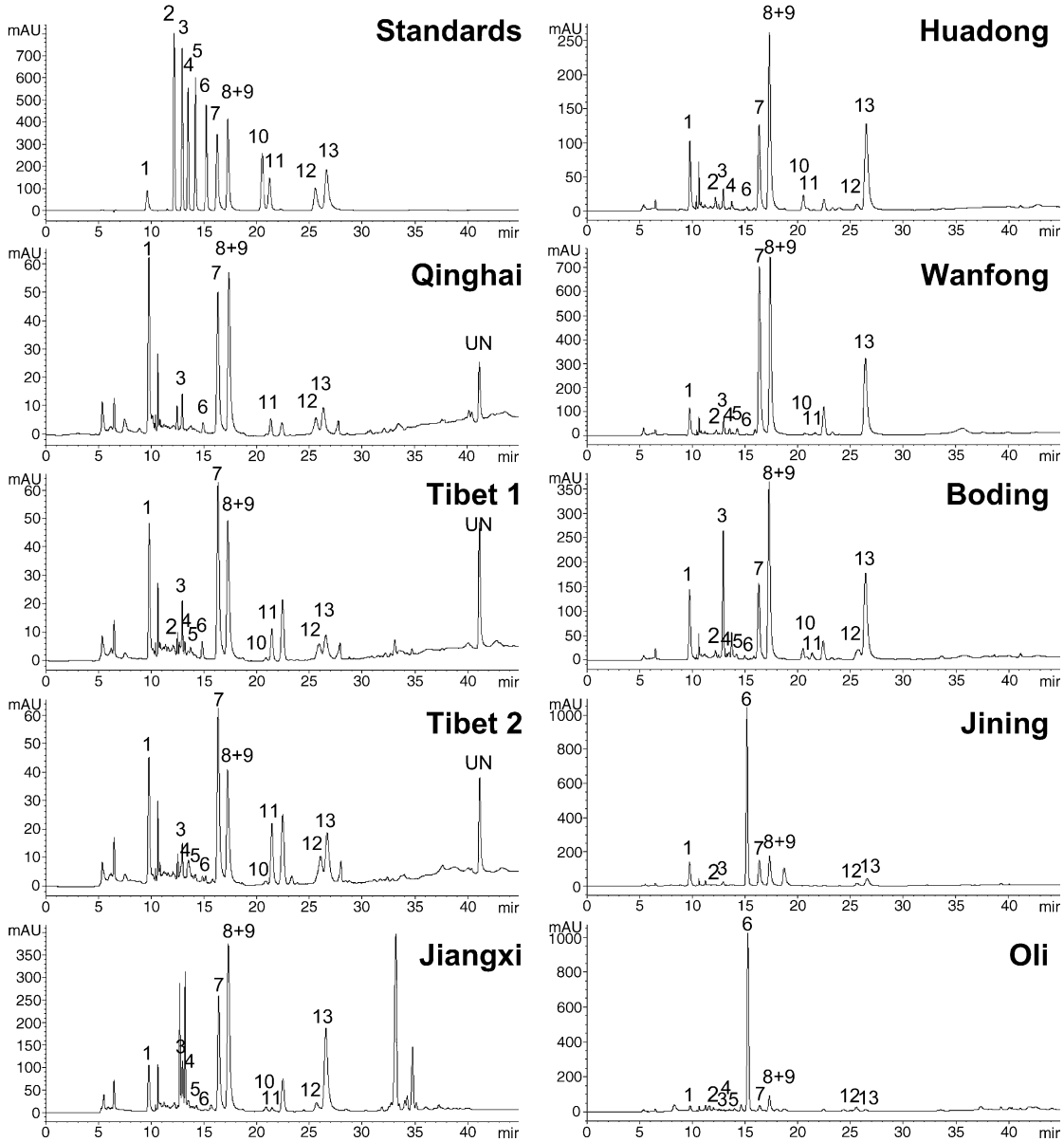

Fig. 2. HPLC chromatograms of PLE extract of natural and cultured *C. sinensis* and cultured *C. militaris*. PLE Condition: solvent, methanol; temperature, 160 °C; static extraction time, 5 min; pressure, 1500 psi; static cycle, 1 and extraction times, 1. Analysis were performed on a Agilent Series 1100 liquid chromatograph, equipped with a vacuum degasser, a quaternary pump, an autosampler and a DAD system, connected to a Agilent ChemStation software. A Zorbax NH_2 column (250 × 4.6 mm i.d., 5 μm) and a Zorbax NH_2 guard column (12.5 × 4.6 mm i.d., 5 μm) were used. Flow rate was 0.6 ml/min. Detection wavelength was at 275 nm for ergosterol and at 254 nm for nucleosides and their bases. Solvents that constituted the mobile phase were (A) acetonitrile and (B) 10 mmol/l ammonium acetate in water. The elution conditions applied were: 0–5 min, linear gradient 0–15% B; 5–25 min, linear gradient 15–20% B; 25–35 min, linear gradient 20–40% B; 35–45 min, linear gradient 40–80% B; 45–50 min, 80% B isocratic. Natural *C. sinensis*: Qinghai, Tibet 1 and Tibet 2; Cultured *C. sinensis*: Jiangxi, Huadong, Wanfong and Boding; Cultured *C. militaris*: Jining and Oli. (1) Ergosterol; (2) thymine; (3) uracil; (4) thymidine; (5) 2′-deoxyuridine; (6) cordycepin (3′-deoxyadenosine); (7) uridine; (8 + 9) adenosine + adenine; (10) cytosine; (11) hypoxanthine; (12) cytidine; (13) guanosine; UN, unknown.

Econofilter (Agilent Technologies) prior to injection into the HPLC system.

2.3. Pressurised liquid extraction

Pressurised liquid extractions were performed on a Dionex ASE 200 (Dionex, Sunnyvale, CA, USA) system. Powder of *Cordyceps* (1.0 g) were mixed with diatomaceous earth in a proportion (1:1) and placed into an 11 ml stainless steel extraction cell, respectively. The extraction cell was extracted under the extraction conditions.

2.4. HPLC analysis

Analysis were performed on a Agilent Series 1100 liquid chromatograph, equipped with a vacuum degasser, a quaternary pump, an autosampler and a diode-array detection (DAD) system, connected to a Agilent ChemStation software. A Zorbax NH_2 column (250 × 4.6 mm i.d., 5 μm) and a Zorbax NH_2 guard column (12.5 × 4.6 mm i.d., 5 μm) were used. Solvents that constituted the mobile phase were (A) acetonitrile and (B) 10 mmol/l ammonium acetate in water. The elution conditions applied were: 0–5 min, linear gradient 0–15% B; 5–25 min, linear gradient 15–20% B; 25–35 min, linear gradient 20–40% B; 35–45 min, linear gradient 40–80% B; 45–50 min, 80% B isocratic; and finally, reconditioning steps of the column was 0% B isocratic for 15 min. The flow-rate was 0.6 ml/min and the injection volume was 20 μl. The system operated at 25 °C. Ergosterol was monitored and quantified at 275 nm, nucleosides and their bases at 254 nm.

3. Results and discussion

3.1. Optimisation of PLE procedure

The optimisation of the PLE procedure was performed using cultured *Cordyceps* mycelia from Wanfong and the optimal conditions found for cultured *Cordyceps* were also applied to natural *Cordyceps*. The parameters include the type of solvent (A), temperature (B), static extraction time (C) and static cycles (D), were studied by the orthogonal test. The area of six peaks including ergosterol, adenosine (adenine), cytidine, guanosine, thymidine and uridine were considered for estimating the results of orthogonal test. The result showed that the order of influence of each variables on the extraction efficiency was A > B > D > C. Moreover, the higher temperature, the higher extraction efficiency during the investigated temperature range (80–140 °C). Therefore, the further observation for temperature (140–180 °C) was performed and 160 °C was selected as the optimum.

The recovery efficiency for the PLE procedure was determined by performing consecutive pressurised liquid extractions on the same sample under the optimised PLE conditions, until no investigated compounds were detected by HPLC analysis. The recovery was calculated based on the total amount of individual investigated components. Taking into account the results of orthogonal test and recovery experiment, the conditions of the PLE method proposed were: solvent, methanol; temperature, 160 °C; static extraction time, 5 min; pressure, 1500 psi and one static cycle and one extraction times (1 psi = 68 × 4.76 Pa).

3.2. Identification and quantitation of investigated compounds

Chromatograms of a PLE extract from natural and cultured *Cordyceps* were shown in Fig. 2. The identification of investigated compounds was carried out by comparison of their retention time and their UV spectra with those obtained injecting standards in the same conditions or by spiking *Cordyceps* samples with stock standard solutions. The linearity ranges and recovery for the investigated compounds were reported in Table 1.

By using the calibrated curve of each investigated compound, natural and cultured *Cordyceps* were analysed.

Table 1
Linear regression data and PLE recoveries (%) of investigated compounds from *Cordyceps*

Analyte	Linear regression data				Recovery (%)	
	Linear range (μg/ml)	Slope	Intercept	r^2 ($n = 8$)	Mean	R.S.D.
Ergosterol	13.3–266.0	19.5	109.7	0.9999	100.2	0.7
Adenine	1.2–98.4	175.5	26.1	1	–	–
Adenosine	0.9–71.4	108.9	−60.6	0.9998	99.1	0.8
Cytosine	1.3–50.4	84.7	−5.3	1	98.8	1.3
Cytidine	2.1–83.5	46.9	−38.5	0.9999	97.5	1.5
Cordycepin	1.6–64.5	110.7	2.5	1	99.3	0.8
Guanosine	0.4–70.4	84.7	−79.1	0.9997	99.5	0.6
Thymine	0.9–73.4	97.7	4.4	1	99.1	1.2
Thytidine	2.7–85.6	55.1	0.6	1	97.7	1.1
Uracil	0.9–75.6	148.6	13.4	1	98.4	2.3
Uridine	1.5–60.3	64.9	−55.0	0.9996	99.3	0.4
2′-Deoxyuridine	1.4–55.8	75.2	−0.3	1	98.4	2.2
Hypoxanthine	0.3–84.8	137.4	−6.6	1	97.6	2.5

r^2, Squares of correlation coefficients for the standard curves. Mean and percentage of relative standard deviation (R.S.D.) for three replicates.

Table 2
The contents (mg/g) of ergosterol, nucleosides and their bases in *Cordyceps*

Analyte	Natural *C. sinensis*			Cultured *C. sinensis*				Cultured *C. militaris*	
	Qinghai	Tibet 1	Tibet 2	Jiangxi	Huadong	Wanfong	Boding	Jining	Oli
Ergosterol	1.43	1.00	0.97	2.71	2.68	3.23	4.14	4.19	1.00
Adenosine (+Adenine)	0.45	0.42	0.36	3.07	1.97	5.13	2.66	1.42	0.64
Cytosine	–[a]	+[b]	+	0.10	0.24	0.09	+	–	+
Cytidine	0.29	0.19	0.32	0.58	0.25	0.04	0.71	0.47	0.59
Cordycepin	+	+	+	+	+	+	+	4.96	4.79
Guanosine	0.20	0.18	0.32	2.80	1.82	4.45	2.55	0.69	0.17
Thymine	–	+	–	–	0.11	0.15	0.19	0.05	0.13
Thymidine	–	+	+	0.29	+	0.28	0.15	–	+
Uracil	+	0.05	0.05	0.36	0.10	0.26	0.08	0.11	+
Uridine	0.66	0.83	0.83	3.11	1.54	8.14	1.93	1.96	0.51
2′-Deoxyuridine	–	+	+	0.16	–	0.24	0.09	–	0.17
Hypoxanthine	0.03	0.06	0.13	0.06	–	0.07	0.10	–	+

[a] Undetectable.
[b] Beyond lower limit of linear range.

Table 2 shows the summary results. Cordycepin, one of the metabolites, is first isolated from *C. militaris* [20]. However, it is rare detected in *C. sinensis* [16]. Indeed, cultured *C. militaris* showed a much higher concentration of cordycepin, though cordycepin contained in natural and cultured *C. sinensis* was confirmed in this report. The amount of nucleosides, especially uridine, adenosine (adenine) and guanosine, in cultured *C. sinensis* was higher than that in natural *C. sinensis*. In addition, a unique unknown peak was found in natural *C. sinensis*. Therefore, It may be possible that identifying natural and cultured *C. sinensis* or *C. militaris* based on their HPLC chromatograms (Fig. 2).

4. Conclusion

The method described provides efficient simultaneous determination of ergosterol, nucleosides and their bases coupled with pressurised liquid extraction, which has good precision and recovery and less time-consuming.

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