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Short communication

Fingerprint analysis of fruiting bodies of cultured *Cordyceps militaris* by high-performance liquid chromatography–photodiode array detection

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Abstract

We have developed and optimized a novel, efficient and accurate fingerprint method using high-performance liquid chromatography–photodiode array detection (HPLC–DAD) for the quality control of cultured *Cordyceps militaris* (L.) Link. The feasibility and advantages of the used chromatographic fingerprint were verified for the evaluation of cultured *C. militaris* by systematically comparing chromatograms with a professional analytical software recommended by State Food and Drug Administration (SFDA) of PR China. The results revealed that the chromatographic fingerprint combining similarity evaluation could efficiently identify and distinguish cultured *C. militaris* from different sources.

Keywords: Quality control; Chromatographic fingerprint; HPLC–DAD; Cultured *Cordyceps militaris*; Traditional Chinese medicines

1. Introduction

The paradigm of traditional Chinese herbal medicine (TCHM) emphasizes the importance of multi-compound, multi-ingredient preparations as being responsible for the activity of the herbal drug, in contrast to modern pharmacology and drug development that often focus on a single chemical entity [1]. The traditional quality control of herbal medicines faces a number of severe challenges in the standardization of TCHM. Chromatographic fingerprint, a comprehensive and quantifiable identification method, is able to reveal chemical information of herbal medicines with chromatogram, spectrograms and other graphs by analytical and chemical techniques [2,3]. State Food and Drug Administration (SFDA) has required that all the injections made from herbal medicines or their raw materials be standardized by chromatographic fingerprinting [4]. Moreover, SFDA has also suggested that all of herbal chromatograms should be evaluated by their similarities, a commonly employed approach based on calculating the correlative coefficient and/or cosine value of vectorial angle of original data [5–7].

The *Cordyceps* species of the traditional Chinese medicinal mushrooms are entomopathogenic fungi. *Cordyceps militaris*, also known as the Chinese caterpillar fungus, possesses pharmacological activities similar to, and according to some studies, more potent than *C. sinensis* (also known as Dong Chong Xia Cao) that is used in certain health food products in Asia [8–10]. Recently, several studies have demonstrated that the extracts and some components of *C. militaris* can exert multiple pharmacological actions, such as anti-inflammatory and humoral-immunity suppressive activities [11–13], ameliorative effects on insulin resistance and insulin secretion [14] and anti-oxidant activity stronger than those of *C. sinensis* and *C. kyushuensis* [15]. Because of the rarity and expensiveness of natural *C. militaris*, scientists have conducted extensive studies on its life cycle with the aim of developing techniques for isolating and therefore harvesting fermentable strains of *C. militaris*. Several strains have been isolated from natural *C. militaris* and produced in high quality by fungus-cultivation technology [16–18]. Our group has undertaken studies on comparing the chemical composition of cultured *C. militaris* with that of the wild *C. militaris* [19]. The products from cultured *C. militaris* have shown to possess similar pharmacological efficacy to that of wild *C. militaris* [20], and cultivated fruiting bodies of *C. militaris* are commonly sold as drug materials and

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health food products in China and South East Asia [21,22]. As a result, it is currently of much interest and importance to develop an effective method for the quality control of cultured *C. militaris*.

In this study, a novel fingerprinting method was first developed using high-performance liquid chromatography–photodiode array detection (HPLC–DAD) for the quality control of cultured *C. militaris*, and similarity evaluation system has also been used to establish the chromatographic fingerprint of the herb.

2. Experimental

2.1. Instrumentation and reagents

HPLC analysis was carried out on a Agilent Series 1100 liquid chromatograph, equipped with a vacuum degasser, a quaternary pump, an auto sampler and a diode array detection (DAD) system, connected to a reversed-phase column (Zorbax SB-Aq RP18e, 5 μm, 250 mm × 4.0 mm i.d., Agilent, USA). Data collection was performed using ChemStation software (Agilent). The water used for all the solutions and dilutions was prepared with a Millipore water purification system. An ultrasonic cleaner (TDL80-2B, Feige, China) was used for extraction. The vacuum concentrator system consisted of a rotary evaporator, a cool ice and a digital bath (EYELA, Japan). Acetonitrile and methanol were of HPLC grade.

2.2. Materials

Eleven fruiting bodies of cultured *C. militaris* collected and cultured by different companies from six provinces of China were examined. All of them were identified by Professor R.M. Yu at Jinan University, China. Six pure nucleosides (guanosine, hypoxanthine, adenine, adenosine, uridine and cordycepin) were used as standards and all of them were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Table 1
The examined mobile phases in optimization of HPLC conditions

Systems	Gradients	Finish time (min)	Peaks
Acetonitrile (A) and water (W)	4–60% A, 96–40% W	10	17
Acetonitrile (A) and buffer solution (B, water–KH_2PO_4, pH 6.86)	4–20% A, 96–80% B	15	23
Methanol (M) and water (W)	5–60% M, 95–40% W	25	22
Methanol (M) and buffer solution (B, water–KH_2PO_4, pH 6.86)	5–50% M, 95–50% B	25	24

Table 2
Optimization standard of the whole chromatographic fingerprint of methanol system

System A: MeOH–H_2O					System B: MeOH–KH_2PO_4				
Peaks	k	S	R	Result	Peaks	k	S	R	Result
1	1				1	1	0.0244	1.81	
2	1.06	0.0148	1.64		2	1.11	0.0255	2.26	
3	1.5	0.0965	11.32		3	1.2	0.0090	0.99	
4	1.76	0.0494	7.64		4	1.24	0.0261	2.42	
5	1.82	0.0108	1.64		5	1.36	0.0268	2.92	
6	2.16	0.0569	6.10		6	1.49	0.0099	1.44	
7	2.28	0.0186	2.19		7	1.54	0.0136	1.90	
8	2.4	0.0180	2.62	0–15 min	8	1.61	0.0151	2.35	0–15 min
9	2.91	0.0698	10.71	$r = 0.00249$	9	1.69	0.0092	1.46	$r = 0.00065$
10	2.99	0.0101	1.39		10	1.74	0.0335	3.53	
11	3.47	0.0567	7.87	15–25 min	11	1.93	0.0200	1.96	15–25 min
12	3.53	0.0067	1.14	$r = 0.52486$	12	2.05	0.0113	1.39	$r = 2.95373$
13	3.72	0.0205	3.53		13	2.12	0.0545	6.01	
14	3.88	0.0167	2.76	0–25 min	14	2.48	0.0400	4.89	0–25 min
15	4.25	0.0365	5.50	$r = 0.001307$	15	2.77	0.0636	7.49	$r = 0.001928$
16	4.44	0.0178	2.27		16	3.35	0.0715	8.91	
17	4.64	0.0181	2.25	R_{min}[a] = 1.14	17	4.02	0.1382	17.70	R_{min}[a] = 0.99
18	4.97	0.0284	3.89		18	5.63	0.0112	1.34	
19	6.97	0.1435	28.50		19	5.78	0.0477	7.49	
20	7.23	0.0160	3.70		20	6.46	0.0224	3.21	
21	8.1	0.0502	9.31		21	6.72	0.0434	5.73	
22	8.53	0.0231	4.40		22	7.42	0.0152	1.86	
					23	7.68	0.0280	4.87	
$\sum$		0.7791	120.37				0.7601	93.93	
$\prod$		1.83×10^{-33}	5.00×10^{12}				1.68×10^{-37}	1.5×10^{11}	

[a] R_{min} represents the lowest resolution in the chromatograms.

2.3. Sample preparation

The samples were prepared as follows: 1.0 g powder of dried materials was precisely weighed and put into a 50 ml volumetric flask. Approximately 20 ml of water was added to the flask, which was subsequently sonicated for 30 min for extraction at 25 °C. The extraction step was repeated twice, and the extracted solution was mixed and filtrated through analytical filter paper. The filtered solution was vacuum-dried at 55 °C and the dried extract was dissolved in 10.0 ml methanol–water (50:50, v/v). The sample was finally filtrated through a 0.45-μm-membrane filter prior to injection into HPLC.

2.4. HPLC conditions

Chromatography was performed on a reversed-phase column with a flow rate of 1.0 ml/min. Separation was carried out by linear gradient elution with methanol (5–60% in 30 min) and water (95–40% in 30 min). The detection wavelength was set at 260 nm, and the column temperature was kept at 25 °C. The loading volume was 1 μl.

2.5. Data analysis

Data analysis was performed using a professional software named Analysis System for Chromatographic Fingerprint and Data of Traditional Chinese Medicine (Version 2004). In this paper, all of the results were calculated by the correlative coefficient [5–7].

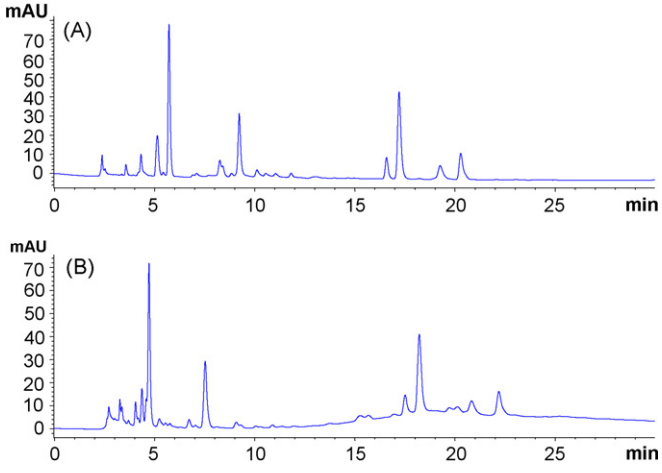

Fig. 1. Chromatogram of the sample in different mobile phases. (A) HPLC conditions—column: Zorbax SB-Aq RP18e, 5 μm, 250 mm × 4.0 mm i.d., Agilent, USA; the mobile phases: methanol (M, 5–60% in 30 min) and water (W, 95–40% in 30 min); flowing rate: 1.0 ml/min; detection wavelength: 260 nm; temperature: 25 °C; injection: 1 μl. (B) HPLC conditions—column: Zorbax SB-Aq RP18e, 5 μm, 250 mm × 4.0 mm i.d., Agilent, USA; the mobile phases: methanol (M, 5–50% in 30 min) and buffer solution (B, water–KH_2PO_4, pH 6.86, 95–50% in 30 min); flowing rate: 1.0 ml/min; detection wavelength: 260 nm; temperature: 25 °C; injection: 1 μl.

3. Results and discussion

3.1. Optimization of HPLC conditions

3.1.1. Optimization of mobile phases

In order to develop a fingerprint for cultured *C. militaris*, an optimized strategy for HPLC conditions was performed on sample no. 1. The various examined mobile phase systems are shown in Table 1 and all the gradients were finished in 30 min. Although, the duration of analysis of both systems with acetonitrile was relatively short, fewer peaks appeared during the elution and the resolution was also unsatisfactory. The methanol–water system had the same analytical time compared with that of the

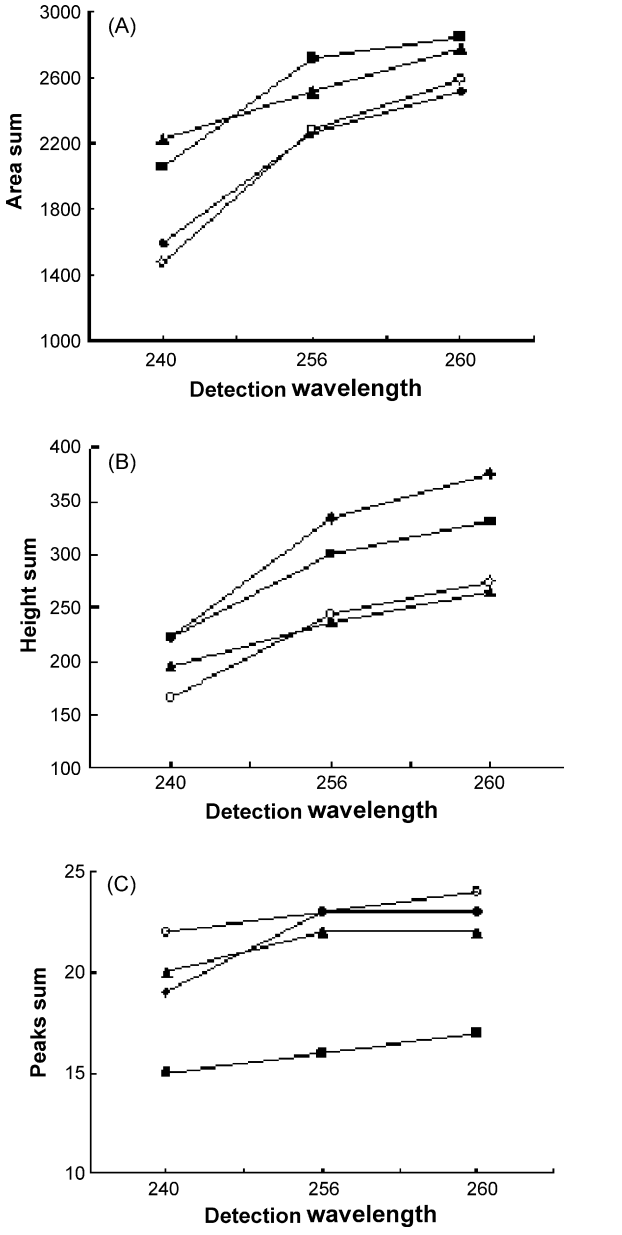

Fig. 2. Influence of detection wavelength on area sum (A), height sum (B) and peak sum (C) of the examined mobile phase system: acetonitrile and water (●), acetonitrile and buffer solution (■), methanol and water (◯) and methanol and buffer solution (▲).

Table 3
Results and analysis of the optimization experiments using an $L_9(3^4)$ orthogonal array design

Run no.	A: extraction temperature (°C)	B: ethanol concentration (%)	C: extraction time (min)	D: solvent volume (ml)	A_{14p}
1	25	0	45	20	2421
2	25	30	15	10	1231
3	25	50	30	30	1595
4	50	0	30	10	2110
5	50	30	45	30	1378
6	50	50	15	20	1651
7	75	0	15	30	1127
8	75	30	30	20	1436
9	75	50	45	10	1164
K_1	5246	5657	4009	4505	
K_2	5139	4045	5140	5508	
K_3	3728	4410	4963	4100	
k_1	1749	1886	1336	1502	
k_2	1713	1348	1713	1836	
k_3	1242	1470	1654	1366	
Rang	506	537	377	470	
Optimized scheme	A1	B1	C2	D2	
Primary and secondary order	2	1	4	3	

A_{14p} represents the area sum of 14 peaks.

methanol–buffer solution system. The parameter of optimization standard was used to determine the resolution of both systems with methanol.

According to the method described in references [23,24], the optimization standard of the whole chromatographic fingerprint of methanol system was computed and shown in Table 2. Out of the given results, the lowest resolution of system A in the chromatograms is 1.14, and the optimization standard is 0.001307. On the other hand, the lowest resolution of system B in the chromatograms is 0.99, and the optimization standard is 0.001928. The optimization standard of system A is much better than system B between 0 and 15 min and in addition, from 15 to 25 min, there is a significant excursion of baseline of system B that is absent in system A (Fig. 1). On the basis of above factors, the methanol (M) and water (W) system was chosen. The linear gradient elution was applied in order to ensure good reproducibility without compromising their resolution.

3.1.2. Optimization of detection wavelength

Three UV wavelengths, 240, 256 and 260 nm, were selected, because most of the nucleosides can be detected by UV in the range of 200–270 nm. The results of pilot experiments on the effects of detection wavelength are shown in Fig. 2. Out of the three detection wavelengths, 260 nm is optimal for establishing the chromatographic fingerprint of cultured *C. militaris*.

3.2. Optimization of extraction conditions

An orthogonal array experimental design was used for optimizing the extraction conditions. Four factors were considered: (A) extraction temperature; (B) proportion of ethanol and water; (C) extraction time and (D) solvent volume. A three-level $L_9(3^4)$ orthogonal array design was displayed in Table 3.

The optimal condition for extraction of cultured *C. militaris* can be obtained by range analysis of the experimental results of the orthogonal array design. The relative sum area of the 14 biggest peaks, which was more than 90% of the area of all peaks, was used as a criterion for the selection of the optimal sonication conditions. The biggest range of the four factors was 537.33 of factor B; the smallest was 377.23 of factor C. Therefore, the factor B was the most important in the extract conditions of cultured *C. militaris*. Optimized factors' ordering was obtained according to range analysis (Table 3). The optimal condition was presented in detail in Section 2.3.

3.3. Establishment of chromatographic fingerprint of cultured C. militaris

Eleven fruiting bodies materials from various locations, including almost all of the growing sites of *C. militaris* in China, were obtained to ensure that the reference fingerprint thus developed was geographically representative and authentic. The clustering analysis was operated in a SPSS software, and the results are shown in Fig. 3. Using this method, we are able to classify the 11 samples into two broad categories containing 6 and 5 samples, respectively. The average of peaks of samples no. 1, 2, 3, 5, 6 and 9 was selected to be the mutual mode, and the similarities of all the fruiting bodies were shown in Table 4 [25]. Based on the retention time, 11 common peaks were determined and shown in the chromatogram of sample no. 5 (Fig. 4). Peaks of 7, 9 and 11 were identified as uridine, adenosine and cordycepin, respectively. In general, using the reference fingerprint, fruiting bodies of cultured *C. militaris* could be easily identified and assessed.

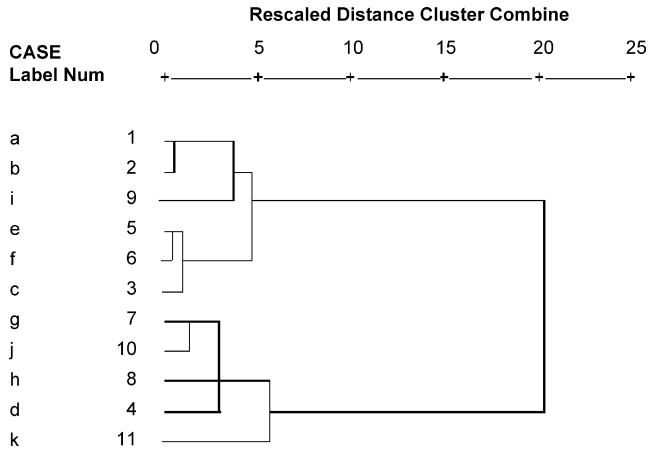

Fig. 3. The clustering analyses of chromatograms of 11 cultured *C. militaris* from different sources. Samples sources: (1) Shenyang, Liaoning 1; (2) Shenyang, Liaoning 2; (3) Beijing 1; (4) Beijing 2; (5) Hohhot, Inner Mongolia; (6) Heze Shandong; (7) Dongtai Jiangsu 1; (8) Dongtai Jiangsu 2; (9) Jiangmen Guangdong; (10) Xinhui Guangdong 1; (11) Xinhui Guangdong 2.

Table 4
The similarities of 11 chromatograms

Sample no.	Correlative coefficient	Cosine value of vectorial angle
1	0.987	0.993
2	0.991	0.995
3	0.992	0.994
4	0.903	0.951
5	0.992	0.996
6	0.987	0.993
7	0.904	0.952
8	0.953	0.975
9	0.976	0.988
10	0.896	0.946
11	0.887	0.943

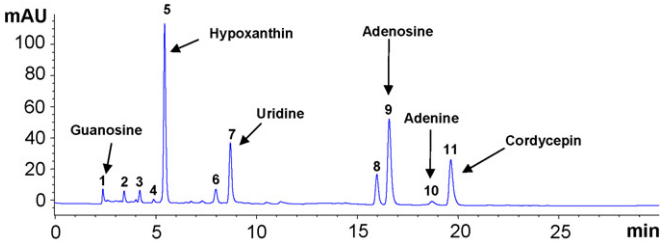

Fig. 4. The reference fingerprint of cultured *C. militaris*. HPLC conditions—column: Zorbax SB-Aq RP18e, 5 μm, 250 mm × 4.0 mm i.d., Agilent, USA; the mobile phases: methanol (M, 5–60% in 30 min) and water (W, 95–40% in 30 min), flowing rate: 1.0 ml/min; detection wavelength: 260 nm; temperature: 25 °C; injection: 1 μl.

4. Conclusion

In the present study, the HPLC fingerprint of cultured *C. militaris* is successfully established for the first time. Eleven fruiting bodies of cultured *C. militaris* from different sources were identified and distinguished by the chromatographic fingerprint in combination with similarity analysis.

Since 1964, when cordycepin was isolated from cultured *C. militaris* and subsequently demonstrated to possess anti-tumor activity, more than 10 nucleosides have been isolated from *Cordyceps* and deemed to be the active components in the genus [9]. The results of our study showed that the chromatographic fingerprint of nucleosides is specific for identifying cultured *C. militaris*.

In summary, the fingerprinting analysis combining similarity evaluation is a novel, valid and rapid method for the quality control of cultured *C. militaris*. The advantage of using chromatographic profiles for quality assessment of herbs or herbal products is that it is often unnecessary to know the individual components that make up the fingerprint [26]. Thus, this presents itself as a highly rapid and efficient process for assessment.

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