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Quality control of *Cordyceps sinensis*, a valued traditional Chinese medicine

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Abstract

Cordyceps sinensis, a well-known and valued traditional Chinese medicine, is also called DongChongXiaCao (winter worm summer grass) in Chinese. It is commonly used to replenish the kidney and soothe the lung for the treatment of fatigue, night sweating, hyposexualities, hyperglycemia, hyperlipidemia, asthemia after severe illness, respiratory disease, renal dysfunction and renal failure, arrhythmias and other heart disease, and liver disease. As the rarity and upstanding curative effects of natural *Cordyceps*, several mycelial strains have been isolated from natural *Cordyceps* and manufactured in large quantities by fermentation technology, and they are commonly sold as health food products in Asia. In addition, some substitutes such as *Cordyceps militaris* also have been used and adulterants also confused the market. Therefore, quality control of *C. sinensis* and its products is very important to ensure their safety and efficacy. Herein, markers and analytical methods for quality control of *Cordyceps* were reviewed and discussed.

Keywords: *Cordyceps*; Quality control; Quality marker; Analytical methods; HPLC; CE

1. Introduction

Cordyceps, one of the well-known 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 is also known as "winter worm summer grass" because of its appearance during different seasons (Fig. 1). The parasitic complex of the fungus and the caterpillar is found in the soil of a prairie at an elevation of 3500–5000 m. It mostly distributed in Tibet, Qinghai, Sichuan, Yunnan and Gansu province.

Cordyceps has been known and used in China for medication over 300 years. It was first recorded in "Ben Cao Bei Yao" by Wang Ang in 1694 AD. And was described as: "*Cordyceps* derived from Jiading of Sichuan, shows the highest quality. In winter, it appears as an old silk worm in soil, and moves with hair. In summer, hairs grow out of soil, and turn into grass. They have to be collected in summer, if not they will turn into worm again". *Cordyceps* became known to the Western society during 17th century. In 1878 AD, Italian scholar Saccardo named *Cordyceps* derived from China officially as *Cordyceps sinensis* (Berk.) Sacc., and this nomenclature was adopted until today.

Cordyceps is commonly used in China to replenish the kidney and soothe the lung for the treatment of fatigue, night sweating, hyposexualities, hyperglycemia, hyperlipidemia, asthemia after severe illness, respiratory disease, renal dysfunction and renal failure, arrhythmias and other heart disease, and liver disease [1,2]. Modern pharmacological studies showed that *Cordyceps* was beneficial to several systems, including the circulatory, immune, hematogenic, cardiovascular, respiratory and glandular systems in human body [3]. However, its usage has been limited during the past decades due to the high price and the difficulty of its supply. The growth of *C. sinensis* has a very restricted habitat, and the yield is decreasing every year. In 2001, a total of only a few thousand kg of natural *Cordyceps* were collected with a decrease of over 70% as compared to 1978 in China. Therefore, the isolation of mycelial strain from *Cordyceps* is a trend of many scientists to achieve a large-scale production of

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Fig. 1. The habitat of *Cordyceps* in China: (A) a prairie at an elevation of 3500–5000 m, mainly in the provinces of Qinghai, Tibet, Sichuan, Yunnan and Gansu; (B and C) the parasitic complex of the fungus and the caterpillar is found in the soil; (D) freshly collected *Cordyceps*. Arrowheads in (B and C) indicate the *Cordyceps*.

Cordyceps by fermentation. Besides cultivation of *Cordyceps* by fermentation technology, much effort has also been focus on discovering the alternative species. There are more than 350 types of so called *Cordyceps* or its substitutes have been found worldwide today, such as *Cordyceps militaris* (L.) Link (the most commonly used substitute), *C. martialis* Speg., *C. hawkesii* Gray, *C. liangshanensis* Zang, Liu et Hu, sp. nov., *C. barnesii* Thwaites, *C. cicadicola, C. gracilis* (Grav.) Dur. et Mont., *C. ramose* Teng, *C. ophioglossoides* (Ehrh. Fr) Link and *C. gunnii* (Berk.) Berk etc. In addition, there are counterfeits and mimics such as *Stachys geobombycis* C.Y. Wu, *Stachys sieboldii* Miq. and *Lycopus lucidus* Turcz., etc also emerge on the market [4–8]. Thus, it is a serious problem for authentication and quality control of *Cordyceps* on the market.

Reviews of the clinical usage of *Cordyceps* [1,2], biological and pharmacological properties [9] and its effects on apoptotic homeostasis [10] have been described. Herein, markers and analytical methods for quality control of *Cordyceps* were reviewed and discussed.

2. Significant markers for quality control of *Cordyceps*

2.1. Nucleosides—authentication of Cordyceps

Nucleosides are one of the major components in *Cordyceps*. In 1964, 3′-deoxyadenosine, namely cordycepin, was isolated from cultured *Cordyceps militaris* [11], a related species of *C. sinensis* commonly used as a substitute. Since then, nucleosides in *Cordyceps* have been a focus because cordycepin was shown to have anti-tumor activity. More than 10 nucleosides and its related compounds have been isolated from *Cordyceps* including adenine, adenosine, uracil, uridine, guanidine, guanosine, hypoxanthine, inosine, thymine, thymidine, deoxyuridine [12–17]. However, existence of cordycepin in natural *C. sinensis* is controversial in the past decades. Recently, cordycepin has been identified in natural *C. sinensis* with a very low content in some reports [14,15]. In addition, N^6-(2-hydroxyethyl)-adenosine (Fig. 2), which behaves as a Ca^{2+} antagonist and an iontropic agent, was isolated from cultured mycelia of *Cordyceps* [16]. To date, nucleosides are believed to be the active components in *Cordyceps*, and adenosine has been used as marker for quality control of *C. sinensis* [18]. Indeed, nucleosides are involved in the regulation and modulation of various physiological processes in the central nervous system (CNS). Adenosine is known to depress the excitability of CNS neurons and to inhibit release of various neurotransmitters presynaptically [19,20]. There is growing pharmacological evidence from several animal models of seizure disorder that adenosine possesses anticonvulsant activity [21]. However, fresh natural *C. sinensis* contains very little amount of nucleosides, as compared to dry and processed one [13], and more interestingly cultured *Cordyceps* mycelium contains high level of

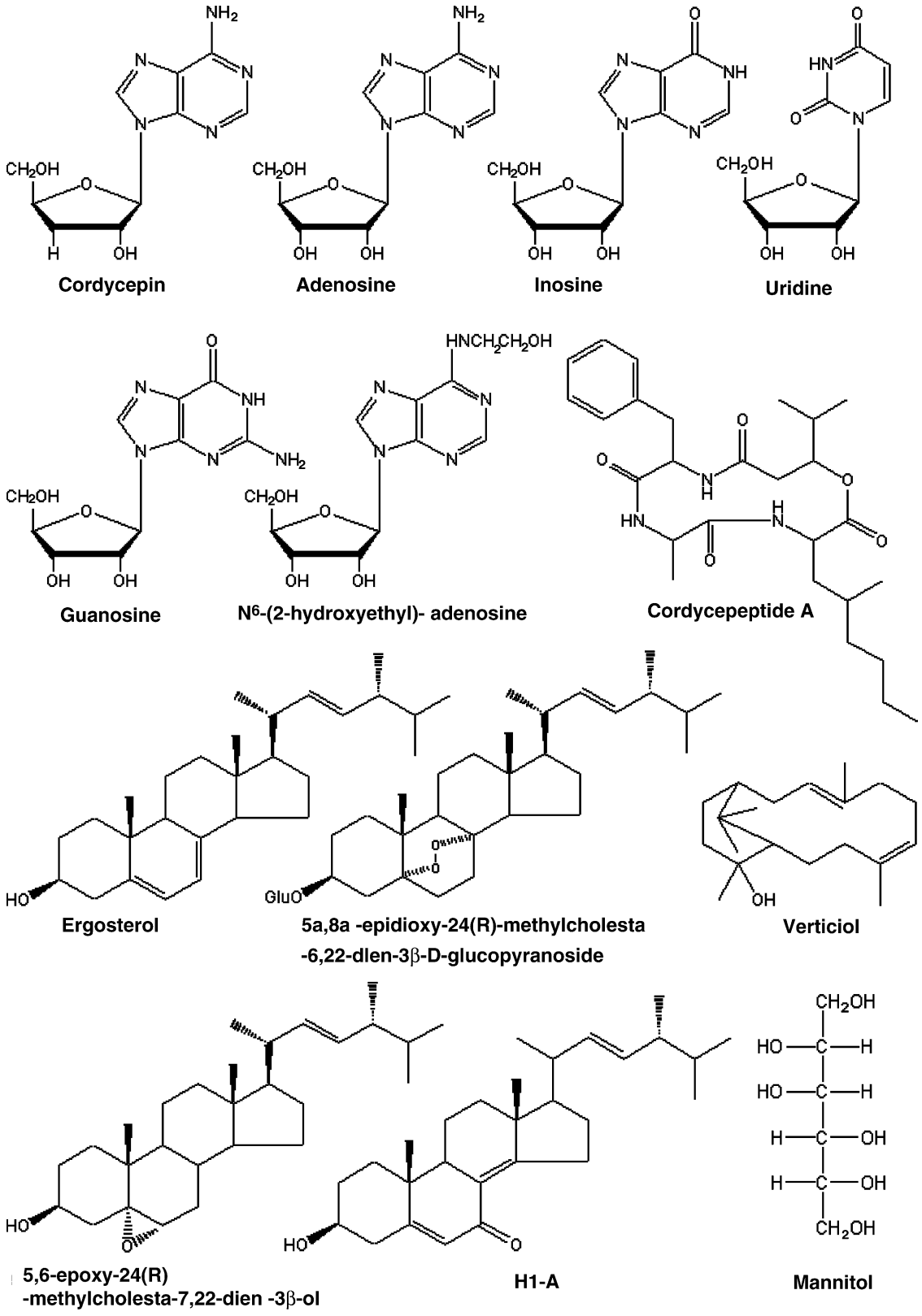

Fig. 2. Structures of some chemical compounds isolated from *Cordyceps*.

nucleosides (Table 1). Furthermore, humidity and heat significantly increased the amount of nucleosides in natural *Cordyceps*. Storage of *Cordyceps* at 75% relative humidity and 40 °C for 10 days, the nucleosides content in natural *Cordyceps* markedly increased to about four-folds. However, the effect of humidity and heat in altering the content of nucleotides could not be revealed in cultured *Cordyceps* mycelia [25]. Therefore, it is believed that the sources of nucleosides in natural *Cordyceps*

Table 1
The contents (mg/g) of main chemical components in *Cordyceps*

Marker	Natural *C. sinensis*		Cultured *C. sinensis*				Cultured *C. militaris*	
	Qinghai	Tibet	Jiangxi	Huadong	Wanfong	Boding	Jining	Oli
Ergosterol	3.65[a]	10.34	1.31	1.10	0.38	0.95	6.33	ND[b]
Adenosine	0.31	0.25	3.23	2.31	5.09	2.16	0.86	0.22
Cordycepin	0.04	0.06	–[c]	–	–	+[d]	9.22	5.71
Guanosine	0.20	0.18	2.80	1.82	4.45	2.55	0.69	0.17
Inosine	0.33	0.20	0.12	0.01	0.03	0.19	0.03	0.02
Uridine	0.66	0.83	3.11	1.54	8.14	1.93	1.96	0.51
Mannitol	38.64	35.42	10.24	12.83	13.41	11.21	ND	ND
Polysaccharides	4.75	8.22	5.83	7.51	5.96	3.84	ND	ND

Source: Data are adapted from Refs. [14,22–24].
[a] The mean values of three determinations are presented. The S.E.M. is less than 5% of the mean, which is not shown for clarity.
[b] No data.
[c] Undetectable.
[d] Beyond lower limit of linear range of detection.

may be different from that of cultured one. In addition, the hypolipidemic activity of adenosine has never been reported. Therefore, having adenosine as a marker for good quality of *Cordyceps* may not be indicative. Moreover, inosine, the major biochemical metabolite of adenosine due to oxidative deamination, can stimulate axon growth in vitro and in the adult central nerve system [26]. It is interesting that natural *Cordyceps* contain much higher amount of inosine than the cultured ones, including *C. sinensis* and *C. militaris* (Table 1). Though the efficacy of *Cordyceps* may be not mainly derived from nucleosides, its profile of nucleosides, especially adenosine, inosine and cordycepin, could be used for discrimination of natural and cultured *C. sinensis* and *C. militaris* (Fig. 3), which is helpful to control the quality of *Cordyceps*.

2.2. Polysaccharide represents the most biological properties of Cordyceps

Cordyceps contains high amount of polysaccharide, which could be ranged from 3 to 8% of the total dry weight [27,28]. *Cordyceps* polysaccharide is considered to possess the activities of anti-oxidation [28,29], immuno-potentiation [9,30,31], anti-tumor [32] and hypoglycemic [33] activities.

Based on the activity-guided fractionation, a water-soluble protein-containing galactomannan was isolated from the sodium carbonate extract of *Cordyceps*, and its molecular weight was estimated by gel filtration to be ~23 kDa. The isolated compound composed of D-mannose and D-galactose in a molar ratio of 3:5, and contained a small proportion of protein. It is a highly branched structure and composes of (1 → 6)-and (1 → 2)-linked α-D-mannopyranosyl residues in the main chain [34]. Another polysaccharide with hypoglycemic activity, purified from a hot water extract of the cultured mycelium of *C. sinensis*, was a combination of galactose, glucose and mannose in a molar ratio of 43:33:24; its molecular weight was estimated to be about 15 kDa [33].

In searching for active component(s) of having anti-oxidant activity, a polysaccharide of molecular weight ~210 kDa, named CSP-1, was isolated from cultured *Cordyceps* mycelia by ion-exchange and sizing chromatography [35]. The isolated polysaccharide, having strong anti-oxidant activity, contained glucose, mannose and galactose in a ratio of 1:0.6:0.75. The pre-treatment of isolated polysaccharide on cultured rat pheochromocytoma

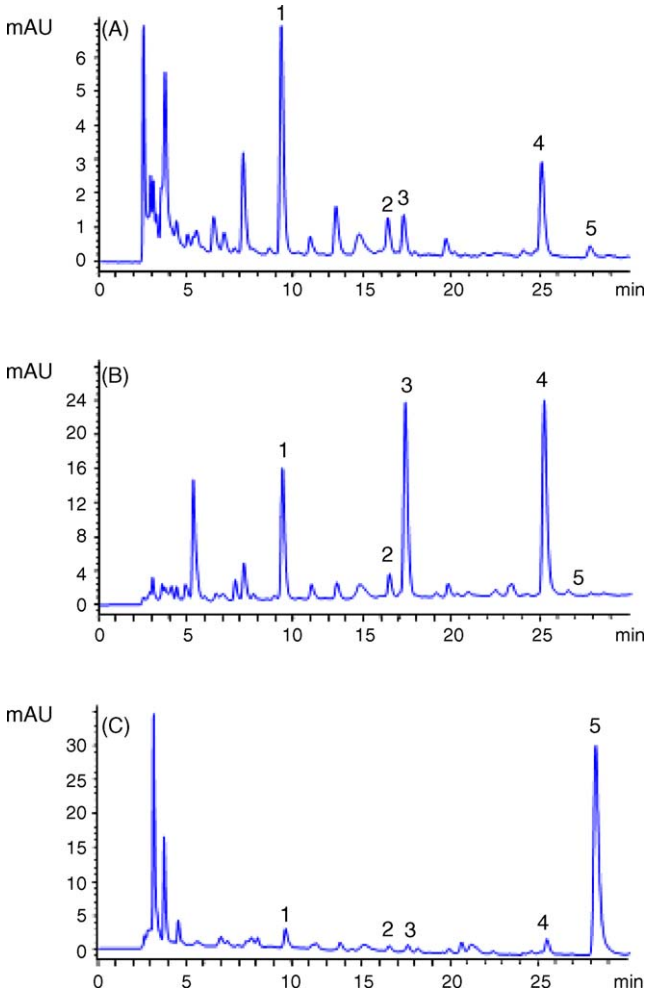

Fig. 3. HPLC profiles of: (A) natural and (B) cultured *Cordyceps sinensis* and (C) cultured *Cordyceps militaris*. 1: Uridine; 2: Inosine; 3: Guanosine; 4: Adenosine; 5: Cordycepin.

Table 2
Summary of markers currently used for quality control of *Cordyceps*

Pharmacological activities	Comments
Nucleosides	
Anti-tumor activities [11]; Ca^{2+} antagonist [16]; depress the excitability of CNS neurons, inhibit release of various neurotransmitters presynaptically and anticonvulsant activity [19–21]; stimulate axon growth in vitro and in the adult central nerve system [26]	Nucleosides, especially adenosine, are usually used as markers, and the profiles can be applied for authentication of *Cordyceps*
Polysaccharides	
Anti-oxidation, immuno-potentiation, anti-tumor, and hypoglycemic activity [9,28–33,35,36]; anti-inflammatory activity and suppress the humoral immunity in mice [37]	Represents the most biological properties of *Cordyceps*, and less used as marker for quality control. Thus, it should be developed
Ergosterol and its analogs	
Cytotoxic activity, anti-viral activity, and anti-arrhythmia effect [44–47]; suppress the activated human mesangial cells and alleviate immunoglobulin A nephropathy (Berger's disease) [48]	Ergosterol shows the characteristics of *Cordyceps* and can be used as marker for quality control
Mannitol	
Diuretic, anti-tussive and anti-free radical activities [49]	It sometimes is used as marker for quality control
Peptides	
Anti-tumor and immuno-potentiation activities [54,55]	A potential marker for quality control of *Cordyceps*?

PC12 cells showed strong protective effect against the free radical-induced neuronal cell toxicity, as well as a significant drop in blood glucose level in both streptozotocin-induced diabetic rats and alloxan-induced diabetic mice [36].

Moreover, CPS-1, a polysaccharide isolated from cultured *C. militaris*, was shown to possess a significant anti-inflammatory activity and suppression to the humoral immunity in mice. The average molecular weight was 2.3×10^4 and was mainly composed of Rha, Xyl, Man, Glc, and Gla in a molar ratio of 1:6.43:25.6:16.0:13.8. The ^{13}C NMR data showed that CPS-1 might contain mannose bonded by (1 → 2) linkage, xylose bonded by (1 → 4) linkage, and rhamnose bonded with galactose by (1 → 2) or (1 → 3) linkage [37]. Four other polysaccharides named CPS-2, CPS-3, CPS-4 and CPS-5 were also isolated and purified from cultured *C. militaris* [38].

As mentioned above, the pharmacological profile of *Cordyceps* correlates very well with the amount of polysaccharides in the herb. Based on the binding to Mono Q® column, four fractions of polysaccharides were isolated from different types of natural and cultured *Cordyceps*; however, the ratio of these four polysaccharide fractions varied in different cultured products of *Cordyceps* [22]. The molecular weight distribution of polysaccharides isolated from *Cordyceps* was also compared by gel filtration. The polysaccharides in natural *Cordyceps* were predominantly (>50%) those high molecular weight molecules of over 150 kDa, which were rather distinct as compared to the cultured products [39]. Actually, the study showed that the pharmacological activity of polysaccharides was correlated with its characteristics such as molecular weight [40]. Thus, the usage of polysaccharides in determining the quality of *Cordyceps* should be promoted.

2.3. The level of ergosterol and mannitol show the characteristics of Cordyceps

Ergosterol, one of chemical components from mycelium cells, is the predominant sterol found in most fungi. It usually is used as a mould growth indicator [41]. Indeed, during the fermentation of *Cordyceps*, the level of ergosterol changed according to the time of culture; a steady level of ergosterol was revealed when the maturation of *Cordyceps* mycelia was reached [42]. Therefore, ergosterol could indicate the level of mycelia in fermentation products of *Cordyceps*, which is another choice of chemical marker for quality of *Cordyceps*. Other sterols such as Δ^3ergosterol, ergosterol peroxide, ergosteryl-3-*O*-β-D-glucopyranoside, cereisterol, 22,23-dihydroergosteryl-3-*O*-β-D-glucopyranoside, β-sitosterol, daucosterol, cholesterol, cholesteryl palmitate, campesterol and dihydrobrassicasterol were also identified in *Cordyceps* [17,43]. Ergosterol exists as free and combined forms in *Cordyceps*. The content of free ergosterol is high in natural *Cordyceps*, and the level of ergosterol could reflect the amount of *Cordyceps* mycelia [23]. Ergosterol analogues have multiple pharmacological activities, such as cytotoxic activity [44], anti-viral activity [45] and anti-arrhythmia effect [46,47]. H1-A (Fig. 2), which suppresses the activated human mesangial cells and alleviate immunoglobulin A nephropathy (Berger's disease) with clinical and histological improvement, is a purified compound from the fruiting body of *C. sinensis* [48]. These activities are in line with the quality of *Cordyceps* for both natural and cultured products. Therefore, the level of ergosterol is a useful marker for quality of *Cordyceps*, at least which represents part of *Cordyceps*' biological functions.

D-Mannitol is one of the major compounds in natural *Cordyceps*, and which contributes to over 3.4% of the total dry weight (Table 1). D-Mannitol, also called cordycepic acid, was isolated from *C. sinensis* in 1957. It has shown to have diuretic, antitussive and anti-free radical activities [49]. Mannitol is being used to treat many diseases, and the content of mannitol in natural *Cordyceps* was higher than that in the cultured one [22]. Therefore, mannitol shows the characteristic of *Cordyceps*, which has been considered as one of the markers for quality of *Cordyceps* [50].

2.4. Peptides—a potential marker for quality control of Cordyceps?

Over 20% of amino acids can be found in *Cordyceps*, which should be responsible for the tonic and immuno-potentiating activity of *Cordyceps* [51]. The contents of total and individual amino acids in natural and cultured *Cordyceps* had no statistically significant differences [52]. Most peptides show biological activities, and some of which have been developed as drugs. In 1988, a fungus strain, which can produce cyclo-carboxyl peptide, was separated from natural *C. sinensis* [53]. Then, six cyclo-dipeptides were isolated from cultured *Cordyceps*, and one of them, cyclo-(L-glycyl-L-prolyl), showed anti-tumor and immuno-potentiation activities [54]. In addition, a peptide named cordycepeptide A was also isolated from *C. militaris* [17]. The study showed that it could increase phagocytosis of macrophages in mice [55]. Though the study of peptides in *C. sinensis* was not enough, we can suspect that peptides may be a potential marker for quality control of *Cordyceps*.

In a word, although many so called active constituents used as markers for quality control of *Cordyceps* have been identified (Table 2), the exact roles of these compounds for the functions of *Cordyceps* are not known. At present, multiple markers such as nucleosides, ergosterol, mannitol and polysaccharides are helpful to control the quality of *Cordyceps* and its products. However, these markers are far from optimization. Extensive work is still needed to define the pharmacological efficiency of these chemical markers and some other compounds.

3. Analytical methods for quality control of *Cordyceps*

3.1. Titration, colorimetry and thin layer chromatography scanning (TLCs)

Redox titration was commonly used for determination of mannitol in *Cordyceps* [56–67]. However, reductive compounds such as glucose and fructose of monosaccharides in *Cordyceps* interfere with the assay, which results in higher content. In order to avoid the interference, colorimetry, which is more specific, simple and rapid than titration, was developed for determination of mannitol [49,68,69]. Polysaccharides in *Cordyceps* were also determined using colorimetry [70,71]. Actually, reductants still have influence on the determination [68]. For increasing specificity of the assay, TLCs, which could remove the interferents from the analytes, was used for separation and determination of mannitol in *Cordyceps*, and organic solvent such as absolute ethanol was used to increase the accuracy [72]. However, extraction efficiency of mannitol was higher using water as solvent than 95% ethanol [73], which should be considered during the analysis of mannitol in *Cordyceps*. TLCs was also applied for determination of ergosterol [74], nucleosides including adenosine, guanosine and uridine [75] in natural and cultured *Cordyceps*, as well as adenosine in final product of *Cordyceps* [76]. For TLCs, determinations of nucleosides were performed by fluorescence quenching analysis [75] or dual-wavelength scanning [76].

3.2. Gas chromatography (GC)

GC is a unique and versatile technique, which is conventional method for analysis of volatile compounds. If the sample to be analyzed is nonvolatile, the techniques of derivatization or pyrolysis GC can be utilized. The chemical compositions of the essential oil of *C. sinensis* were analyzed by GC–MS [77]. The result showed that 72 peaks were separated and 41 of them were identified. It was noteworthy that verticiol and some analogs were found in *C. sinensis* [77,78]. The former was a substance strongly resembling with verticine, a medication for respiratory disease, in structure. Verticiol and its analogs may contribute to the anti-tussive and expectorant effects of *C. sinensis*, though further studies are needed. Mannitol is a carbohydrate with six hydroxyl groups and with no volatile property. For analysis of mannitol by GC, a derivatizing procedure is essential. Wang et al. [79] developed a GC method for quantitative determination of mannitol in *C. militaris*. The derivatization of sample powder (75 mg) was performed adding 2.5 mL pyridine and 5.0 mL acetic anhydride, allowing reaction for 1 h at 90 °C. Other derivatization procedures such as trimethyl-silylation (TMS) [80] and *n*-butyldiboronation [81] have also been used. As mannitol has bifunctional hydroxyl groups, organic boronic acids are most useful reagents to use for derivatization, as compared with TMS or acetate. An *n*-butyldiboronate derivative of mannitol gave a better separation from other polyols and only 10 min at room temperature was required for derivatization [82].

GC–MS has been used for determination of ergosterol in organic dust [83] and mouldy building materials [84], though there is no report for determination of ergosterol in *Cordyceps* using GC. Other free and esterified sterols in edible oil [85,86] and various food matrices [87] were also determined by GC with flame ionization detector or mass spectrometric detector. Since sterols can efficiently decrease serum cholesterol concentration [88], the analysis of sterols may be helpful to elucidate the hypocholesterolemic effects of *Cordyceps*, which may be used as marker for quality control of *Cordyceps*.

3.3. High performance liquid chromatography (HPLC)

HPLC is a conventional method for analysis of non-volatile compounds. For most cases, HPLC with UV–vis detection is the prevailing technique, which has been widely used for determination of components in Chinese medicine. Using HPLC coupled with UV detector, ergosterol [14,23,89], adenosine [90–92], cordycepin [93] and other nucleosides [94,95] in *Cordyceps*

Table 3
HPLC of chemical components in *Cordyceps*

Samples	Sample preparation	Column	Mobile phase	Detection	Ref.
Ergosterol					
Natural *C. sinensis*, cultured *Cordyceps* mycelia	1. Soxhlet extraction with petroleum ether; 2. Removing the solvent; 3. Dissolving the residue with ethanol	Allsphere ODS (250 mm × 4.6 mm, i.d.), 2.5 μm	Methanol: water (95:5)	UV 275 nm	[23]
Mannitol					
Natural *C. sinensis*, cultured *Cordyceps* mycelia	1. Ultrasonic extraction with water; 2. Filtrate passed through Sep-Pak C18 column; 3. Eluent used for analysis.	HC-75 (305 mm × 7.8 mm, i.d.) calcium type cation ion exchange column	Water	Refractive index (RI)	[96]
Adenosine					
Natural *C. sinensis*	1. Reflux extraction with 90% methanol; 2. Removing the solvent; 3. Dissolving the residue with 90% methanol	ECONOSPHERE C_{18} (250 mm × 4.6 mm, i.d.), 5 μm	Phosphate buffer (pH 6.5)–methanol (17:3)	UV 260 nm	[90]
Cultured *Cordyceps* mycelia	Reflux or ultrasonic extraction with 50% ethanol	Dupont C_{18} (250 mm × 4.6 mm, i.d.)	Methanol–0.06 mol/L monopotassium phosphate–THF (10:150:1.5)	UV 260 nm	[91]
C. hawkesii	1. Ultrasonic extraction with 30% ethanol; 2. removing the solvent; 3. dissolving the residue with 30% ethanol	YMC–ODS (150 mm × 6 mm, i.d.)	0.3% Aqueous acetic acid–methanol (93:7)	UV 260 nm	[97]
Ergosterol, nucleosides (uridine, adenosine)					
Natural *C. sinensis*	For ergosterol: 1. Reflux with saturated potassium hydroxide and absolute ethanol; 2. The extract was extracted with cyclohexane; 3. Vaccum drying the extract; 4. Dissolving the residue with absolute ethanol. For nucleosides: 1. Ultrasonic extraction with distilled water; 2. Vacuum drying the extract; 3. Dissolving the residue with methanol.	Zorbax–ODS (250 mm × 4.6 mm, i.d.), 5 μm	Methanol	UV 260 nm	[98]
Nucleosides (adenosine, cordycepin)					
Natural *C. sinensis*, cultured *C. militaris*	Soxhlet extraction with methanol	Shimadzu VP-ODS (150 mm × 2.0 mm, i.d.), 5 μm	Water–methanol–formic acid (94:5:1)	ESI-MS	[99]
Nucleosides (uridine and adenosine)					
Natural *C. sinensis*, cultured *Cordyceps* mycelia, Cultured *C. militaris*	1. Soxhlet extraction with methanol; 2. removing the solvent; 3. dissolving the residue in Milli-Q water.	Shimadzu VP-ODS column	Water-methanol-formic acid (90:9:1)	ESI-MS	[100]

Table 3 (*Continued*)

Samples	Sample preparation	Column	Mobile phase	Detection	Ref.
Nucleosides (uracil, uridine, adenine, adenosine)					
Cultured *Cordyceps* mycelia	1. Ultrasonic extraction with methanol; 2. Removing the solvent; 3. Dissolving the residue with methanol.	Polaris C_{18}-A (250 mm × 4.6 mm, i.d.), 5 μm	Gradient elution with acetonitrile and water	UV 260 nm	[95]
Nucleosides (adenine, hypoxanthine, adenosine, cordycepin)					
Natural *C. sinensis*, cultured *C. militaris*	1. Ultrasonic extraction with distilled water; 2. Vacuum drying; 3. Dissolving the residue with methanol.	Shimadzu VP-ODS (150 mm × 2.0 mm, i.d.), 5 μm	Water–methanol–formic acid (85:14:1, v/v/v)	DAD-ESI-MS	[101]
Nucleosides (uracil, hypoxanthine, uridine, thymine, guanine, adenine, adenosine, cordycepin)					
Natural *C. sinensis*, cultured *C. sinensis* mycelia	1. Ultrasonic extraction with distilled water; 2. Vacuum drying; 3. Dissolving the residue with methanol.	Shimadzu VP-ODS (150 × 2.0 mm, i.d.), 5 μm	Gradient elution with ammonium acetate (40 mM, pH 5.2) and methanol	DAD-ESI-MS	[15]
Ergosterol, nucleosides and bases (adenosine, adenine, cordycepin, cytosine, cytidine, guanine, guanosine, thymidine, uridine, 2′-deoxyuridine, thymine, uracil)					
Natural *C. sinensis*, cultured *Cordyceps* mycelia, cultured *C. militaris*	Pressurized liquid extraction	Zorbax NH_2 (250 mm × 4.6 mm, i.d.), 5 μm	Gradient elution with acetonitrile and 10 mmol/L ammonium acetate in water	Ergosterol: UV 275 nm; Nucleosides and bases: UV 254 nm.	[14]
Nucleosides and bases (Cytosine, cytidine, uracil, pseudouridine, uric acid, hypoxanthine, guanine, 2′-deoxycytidine, xanthine, uridine, adenine, thymine, 2′-deoxyuridine, adenosine, inosine, cordycepin, guanosine, thymidine, 2′-deoxyguanosine, 2′-deoxyadenosine)					
Natural *C. sinensis*, cultured *Cordyceps* mycelia	1. Sample extracted with distilled water (20, w/v); 2. The aqueous extracts were passed though cartridge columns (reversed phase); 3. After eluting with distilled water, the cartridge column was eluted with a mixture of methanol-water (1:1, v/v) to recover samples.	Cosmosil $5C_{18}$ (250 mm × 4.6 mm, i.d.), 5 μm	Gradient elution with Solvent A (2.5% MeOH in 0.01 mmol/L $(NH_4)H_2PO_4$) and solvent B (20% MeOH in 0.01 mmol/L $(NH_4)H_2PO_4$)	UV 260 nm	[12]
Polysaccharides					
Natural *C. sinensis*, cultured *Cordyceps* mycelia	1. Sample extracted with boiling water; 2. Centrifugation and the supernatant was condensed; 3. Adding three folds absolute alcohol; 4. Centrifugation; 5. Dissolving the residue with mobile phase	TSK-Gel G3000 SW_{XL} (300 mm × 7.8 mm, i.d.), 5 μm	0.1 M Sodium sulfate buffer (pH 6.8)	RI	[39]

Table 3 (*Continued*)

Samples	Sample preparation	Column	Mobile phase	Detection	Ref.
Water-soluble protein					
Natural *C. sinensis*, cultured *Cordyceps* mycelia	1. Ultrasonic extraction with water; 2. Centrifugation; 3. The supernatants for analysis	TSK-Gel G2000 SW (300 mm × 7.5 mm, i.d.), 5 μm	Water	UV 280 nm	[102]
Amino acids					
Cultured *Cordyceps* mycelia	1. Sample hydrolyzed with 6 mol/L HCl; 2. Drying and dissolving the residue in water; 3. Filtration and the filtrate derivatized in 0.5 mol/L sodium bicarbonate (pH 9.0), 1% 2,4-dinitrofluorobenzene acetonitrile solution; 4. Then adding phosphoric acid buffer.	Shim-pak CLC-ODS (150 mm × 6 mm, i.d.)	Gradient elution with solvent A (CH_3CN–H_2O 1:1) and B (0.05 mol/L NaAc–HAc buffer, pH 6.1)	UV 360 nm	[103]
Soluble sugar, free amino acid, 5′-nucleotides					
Cultured *C. militaris* mycelia	For soluble sugar: 1. Sample extracted with 80% aqueous ethanol (95% pure); 2. Filtration; 3. Drying the filtrate and dissolving the residue with deionized water. For free amino acid: 1. Sample extracted with 100 mmol/L HCl; 2. Filtration; 3. The filtrate mixed with *o*-phthalaldehyde for derivatization. For 5′-nucleotides: 1. Sample extracted with deionized water; 2. Centrifugation; 3. Supernatant condensation.	For soluble sugar: Phase Sep-NH_2 (250 mm × 4.6 mm, i.d.), 5 μm. For free amino acid and 5′-nucleotides: Prodigy 5 ODS-2 (250 mm × 4.6 mm, i.d.), 5 μm.	For soluble sugar: Acetonitrile–deionized water (75:25, v/v). For 5′-nucleotides: 500 mmol/L KH_2PO_4–H_3PO_4 buffer (pH 4.3)	For soluble sugar: UV 190 nm. For free amino acid: Fluorescence detector, λ_{ex}: 340 nm λ_{em}: 450 nm. For 5′-nucleotide: UV 254 nm.	[104]
Sugar, polyol, free amino acid, 5′-nucleotides					
Cultured *C. militaris*	For sugar and polyol: Sample extracted with 80% aqueous ethanol (95% pure); 2. Filtration; 3. Drying the filtrate and dissolving the residue with deionized water. For free amino acid: 1. Sample extracted with 100 mmol/L HCl; 2. Filtration; 3. The filtrate mixed with *o*-phthalaldehyde for derivatization. For 5′-nucleotides: 1. Sample extracted with deionized water; 2. Centrifugation; 3. Supernatant condensation.	For sugar and polyol: Phase Sep-NH_2 (250 mm × 4.6 mm, i.d.), 5 μm. For free amino acid and 5′-nucleotides: LiChrospher 100 RP-18 (250 mm × 4.6 mm, i.d.), 5 μm.	For sugar and polyol: Acetonitrile–deionized water (75:25, v/v). For free amino acid: Gradient elution with 50 mmol/L sodium acetate (pH 5.7) containing 50 mL/L tetrahydofuran, deionized water and methanol. For 5′-nucleoside: 500 mmol/L KH_2PO_4–H_3PO_4 buffer (pH 4.3)	For sugar and polyol: RI For free amino acid: Fluorescence detector, λ_{ex}: 340 nm λ_{em}: 450 nm. For 5′-nucleotides: UV 254 nm.	[105]

were determined. The applications of HPLC for analysis of chemical components in *Cordyceps* are listed in Table 3.

Mannitol is a carbohydrate, which has no UV absorptivity. To detect intact mannitol, refractive index (RI) detection has been used for analysis by HPLC [24,96]. However, RI detector is one of the least sensitive LC detectors, and it cannot be used for gradient elution. Therefore, enrichment of the analytes or sample clean up may be necessary for improving resolution and faster separation. Solid phase extraction has been used for determination of mannitol in *Cordyceps* [96,106]. For UV detection of mannitol by HPLC, a derivatizing procedure such as *p*-nitrobenzoylation is essential [107], which increases the complexity of sample preparation. The evaporative light scattering detector (ELSD) response does not depend on the samples' optical characteristics, which eliminates the problems associated with RI detector. Therefore, ELSD is increasingly being used in liquid chromatography as a quasi-universal detector, which has been successfully applied to analyze the compounds less volatile than the mobile phase, such as saponins [108,109], carbohydrate [110] and lipids [111,112]. Some main components such as mannitol, polysaccharides and amino acids in *Cordyceps* cannot be detected by UV detection, so it is valuable to develop HPLC–ELSD method for quality control of *Cordyceps*.

Nucleosides are considered to be one of the major active components in *Cordyceps*. Therefore, identification and determination of nucleosides in *Cordyceps* are very important for controlling its quality. LC–MS, which allows more definitive identification and quantitative determination of compounds that are not fully resolved chromatographically, has been applied for identification and determination of nucleosides in *Cordyceps* [15,99–101]. However, at most eight nucleosides and their bases were considered, which excluded guanosine with high content and inosine with significant pharmacological activities in *Cordyceps* [15]. Therefore, qualitative and quantitative determination of nucleosides in natural and cultured *Cordyceps* are necessary for quality control of *Cordcyeps*. There is no report for systematical analysis of nucleosides in *Cordyceps*, though more than 10 nucleosides and their bases were determined using HPLC [14]. Furthermore, cordycepin, a nucleoside first isolated from *C. militaris*, shows multiple pharmacological activities [113–115]. It was not detected in *C. sinensis* [12]. However, using HPLC [14] and LC–MS [13,101], it was confirmed that cordycepin was contained in natural *C. sinensis*. It was noteworthy that high content of cordycepin was found in a sample of cultured *Cordyceps* mycelia, but the strain of the fungus was not mentioned [15]. Actually, our study showed that cordycepin was mainly contained in natural *C. sinensis* and cultured *C. militaris*, while cordycepin isomer, identified as 2′-deoxyadenosine, was contained in cultured *C. sinensis*, as well as natural one (Fig. 4).

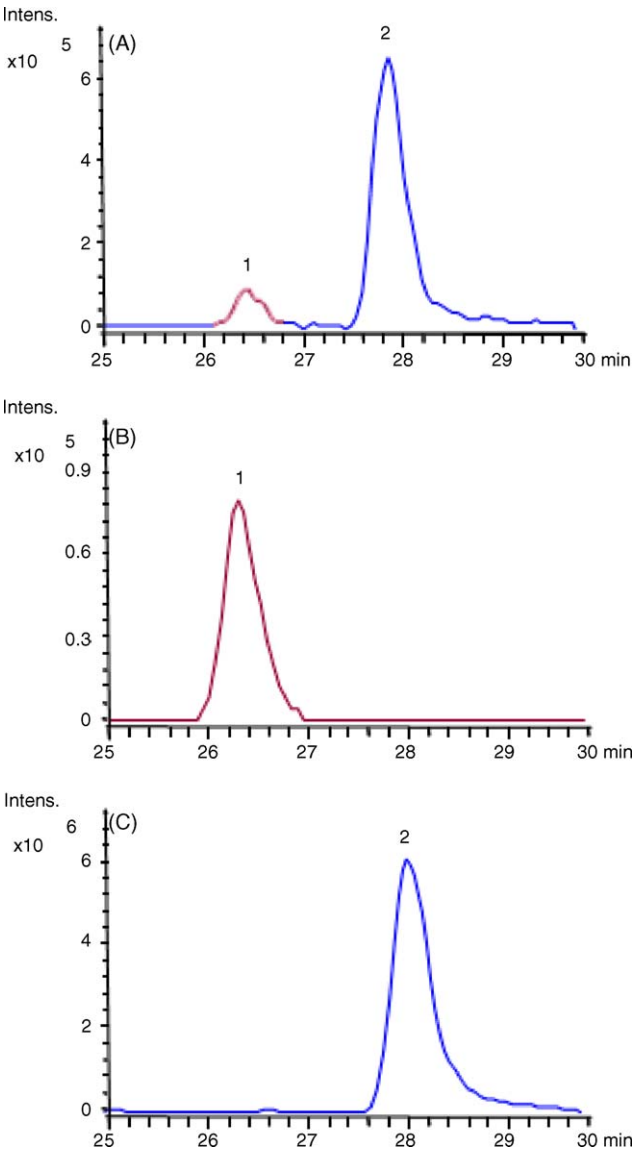

Fig. 4. Extracted ion chromatograms of 2′-deoxyadenosine (1) and 3′-deoxyadenosine (cordycepin, 2) in: (A) natural and (B) cultured *Cordyceps sinensis* and (C) cultured *Cordyceps militaris*.

3.4. Capillary electrophoresis (CE)

High performance capillary electrophoresis (HPCE) has become a powerful tool in natural product analysis [13,116–120], due to its high resolution, short analysis time, and low solvent and sample consumption. The applications of HPCE for analysis of chemical components in *Cordyceps* are listed in Table 4.

For HPCE analysis of nucleosides in *Cordyceps*, different sample preparation, including reflux [13,122] and ultrasonic extraction [119,124] with different solvent, were used. Usually, ultrasonication using running buffer as solvent is easy for the extraction of nucleosides [119]. Sample buffer [13] or deionized water [124] were also used as solvent for avoiding band broadening induced by extremely high salt concentrations. However, proteins, which can contaminate the capillary and affect selectivity, precision and accuracy, are rich in aqueous extract of *Cordyceps*. Therefore, absolute ethanol was used for reflux extraction of nucleosides in *Cordyceps*. Then ethanol was removed, and the residue was vortexed with sample buffer (10 mmol/L boric acid, pH 8.5) to prepare the sample solution for HPCE determination of nucleosides in *Cordyceps* [13]. In addition, 20% ethanol

Table 4
CE of chemical components in *Cordyceps*

Sample	Sample preparation	Column	Running buffer	Detection	Reference
Nucleosides (cordycepin, adenosine)					
Natural *C. sinensis*, cultured *C. militaris*	Ultrasonic extraction with deionized water	Uncoated silica capillary (41 cm × 45 μm, i.d.), 30 cm effective length	0.025 mol/L Sodium borate (pH 9.4)	UV 258 nm	[121]
Nucleosides and bases (adenosine, guanosine, hypoxanthine, uracil)					
Natural *C. sinensis*	Reflux extraction with 20% ethanol containing 0.01% acetic acid	Fused-silica capillary (60 cm × 75 μm, i.d.), 52 cm effective length	36 mmol/L Borate–15 mmol/L sodium dihydrogen phosphate (pH 8.90)	UV 254 nm	[122]
Nucleosides (adenosine, guanosine, inosine, uridine)					
Natural *C. sinensis*, cultured *Cordyceps* mycelia	Ultrasonic extraction with water	Agilent uncoated silica capillary (50 cm × 50 μm, i.d.), 42 cm effective length	0.025 mol/L Sodium borate (pH 9.5)	UV 260 nm	[123]
Nucleosides (adenosine, guanosine, uridine)					
Natural *C. sinensis*, cultured *Cordyceps* mycelia	1. Soxhlet extraction with absolute ethanol; 2. Removing the ethanol; 3. Dissolving the residue with sample buffer (10 mmmol/L boric acid, pH 8.5); 4. Centrifugation and the supernatant for analysis	Beckman untreated fused-silica capillary (57 cm × 75 μm i.d.), 50 cm effective length	0.2 mol/L Boric acid-sodium hydroxide (pH 8.5)	UV 254 nm	[13]
Nucleosides (cordycepin, adenosine)					
Natural *C. kyushuensis*, cultured *C. kyushuensis*, cultured *C. militaris*	Ultrasonic extraction with deionized water	Uncoated silica capillary (41 cm × 45 μm, i.d.), 30 cm effective length	0.025 mol/L Sodium borate (pH 9.4)	UV 258 nm	[124]
Nucleosides (adenine, adenosine, uracil, inosine, guanosine, uridine)					
Natural *C. sinensis*, cultured *Cordyceps* mycelia	Ultrasonic extraction with running buffer	Fused silica capillary (56 cm × 75 μm, i.d.), 48 cm effective length	0.5 mol/L Boric acid with 12.2% acetonitrile (pH 8.6)	UV 254 nm	[119]
Tris–glycine buffer extract, Basic proteins extract, and acidic proteins extract					
Natural *C. sinensis*	1. Sample extracted with: (1) Tris–glycine buffer (Tris–glycine buffer extract); (2) 0.1 mol/L Tris–HCl buffer (pH 8) with 0.1% ascorbic acid and 10 mmol/L mercaptoethanol (Basic proteins extract); (3) 80 mmol/L citric acid with 32 mmol/L Na_2HPO_4, 5 mmol/L ascorbic acid and 10 mmol/L mercaptoethanol (pH 2.8) for acidic proteins extract. 2. Centrifugation and the supernatant for analysis.	Silica capillary (60 cm × 70 μm, i.d.), effective length was not mentioned	30 mmol/L Borate buffer (pH 8.5)	UV 200 nm	[125]

was also used for reflux extraction of nucleosides in *Cordyceps* to increase the extraction efficiency [122]. To date, nucleosides in *Cordyceps* were determined using CZE only. Actually, capillary electrochromatography (CEC), a rapidly evolving hybrid technique between HPLC and CE, was successively used for separation and determination of nucleosides [126]. The resolution of CEC is much higher than those of HPLC and CE. On the other hand, MS is a more universal detector than UV–vis detectors because of its selectivity and specificity, which also compensates the variation in migration times that frequently occurs in CE. Thus, MS detection for CE has been increasingly used and developed [127–130]. Therefore, it is worthy to develop CEC and CE–MS for determination of nucleosides and/or other components in *Cordyceps* in future.

4. Conclusion

The methodology for quality control is crucial to ensure authenticity and quality of *Cordyceps* and its products. The rational markers, which are related with the safety and efficacy of *Cordyceps*, are essential. At present, multiple markers such as nucleosides, ergosterol, mannitol and polysaccharides are being used for quality control of *Cordyceps* and its products. Unfortunately, these markers are not optimized thoroughly, and extensive work is still needed to define these compounds contribute to the pharmacological efficiency of *Cordyceps*.

Another approach in quality control of the herb is using chemical profile instead of a single compound. By CE, distinct fingerprints could be revealed in water-soluble constituents derived from different sources of *Cordyceps* [118]. This method does not depend on the identities of any chemicals. Thus, the profiles generated from chromatography could serve as fingerprints for the quality control of *Cordyceps.*

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