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Identification of chemical markers in *Cordyceps sinensis* by HPLC-MS/MS

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Abstract

Authentication and quality assessment of *Cordyceps sinensis*, a precious and pricey natural product that offers a variety of health benefits is highly significant. To identify effective chemical markers, authentic *C. sinensis* was thoroughly screened by using HPLC-MS/MS. In addition to many previously reported ingredients, two glycosides, i.e. cyclo-Ala-Leu-rhamnose and Phe-o-glucose were detected for the first time in this material. Six ingredients detected, including cordycepin, D-mannitol, Phe, Phe-o-glucose, cyclo-Gly-Pro, and cyclo-Ala-Leu-rhamnose, were selected as a collection of chemical markers. An HPLC-MS/MS method was developed to simultaneously quantify them with sensitivity and specificity. The method had limits of detection ranging from 0.008 μg mL^{-1} for cordycepin to 0.75 μg mL^{-1} for cyclo-Gly-Pro. Recovery was found between 96% and 103% in all tests. To evaluate the effectiveness of the marker collection proposed, 5 authentic *C. sinensis* samples and 5 samples of its substitutes were analyzed. Cordycepin, D-mannitol, and Phe were found present in all samples. The contents ranged from 0.0076 to 0.029% (w/w) for cordycepin, 0.33 to 18.9 % for mannitol, and 0.0013 to 0.642% for Phe. Interestingly, the two glycosides, Phe-o-glucose and cyclo-Ala-Leu-rhamnose were detected only in authentic *C. sinensis* samples. These results indicated that the proposed protocol based on HPLC-MS/MS quantification of the markers might have a great potential in authentication and quality assessment of *C. sinensis*.

Graphical abstract

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Conflict of interest

The authors declare that they have no conflict of interest.

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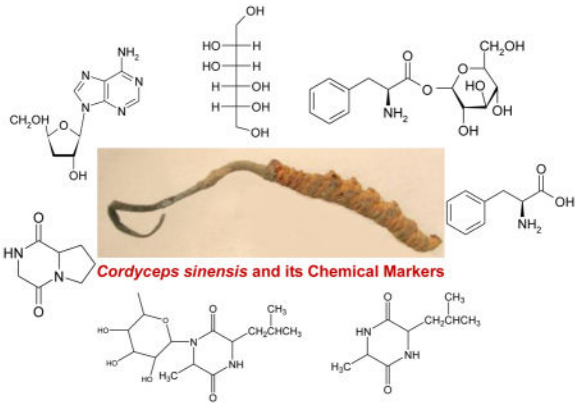

Keywords

Chemical markers; Cordyceps sinensis; Cordycepin; Glycosides; Authentication; HPLC-MS

Introduction

Cordyceps sinensis is a precious and very pricey natural material that offers many health benefits and has been used for a long time in traditional Oriental medicine in order to treat fatigue, respiratory diseases, renal dysfunction, arrhythmias and other heart diseases, etc.[1–5]. Lab studies have shown that extracts of *Cordyceps* exhibit pharmacological effects, including antifungal, antibacterial, anticancer, anti-inflammatory, and antioxidant [6–11]. In addition to its therapeutic use, *C. sinensis* is widely used as a folk tonic food or an invigorant in Asia. Due to a very limited availability of *C. sinensis*, various cultivated or cultured substitutes are produced [3–5, 12–13]. Studies comparing *C. sinensis* with its substitutes in regards to their chemical compositions and medicinal effects have been receiving a great amount of research interest [14–17]. Several active ingredients such as cordycepin (i.e. 3′-deoxyadenosine), nucleosides, and polysaccharides were suggested as markers of *C. sinensis* for quality control purposes [3, 18–20].

As a biological hybrid of larva and parasitic fungus, *C. sinensis* contains a complex enzymatic system and many ingredients of medicinal value. Nucleosides are believed to be an important group of bioactive components in *Cordyceps* [3]. Cordycepin, was first isolated from cultured *Cordyceps militaris*, a related species of *C. sinensis* commonly used as a substitute. It has been shown to exhibit potent antitumor and antimicrobial activities [8, 11, 21]. Polysaccharides were detected at high levels in *Cordyceps*. Their anticancer effects were found to come from an enhancement of body's immune system instead of direct cytocidal action [4]. In addition, amino acids, sterols, fatty acids and cyclic peptides were also detected in *Cordyceps* [1–5]. To date, various methods have been developed for analysis of bioactive components in *C. sinensis* [18, 20]. Most of them were based on thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and capillary electrophoresis (CE) [22–26]. While easy to assess and to perform, these methods lack the capability of chemical structure identification, which in some cases may produce false results, particular when analyzing such a complex sample as *C. sinensis*. LC [27–28]

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and CE [29] methods with mass spectrometric detection for determination of nucleosides and their bases in *Cordyceps* samples were also reported.

The aim of the present study was to develop a facile and effective protocol based on HPLC-MS/MS quantification for authentication and quality assessment of *C. sinensis*. The authentic material was thoroughly screened in order to identify the ingredients that might serve as effective markers. Special attention has been given to cyclic peptides and glycosides since *C. sinensis* contains a very complex enzymatic system. Peptide cyclization and glycosylation are mainly enzymatic processes in a form of co-translational and post-translational modification. Based on the medicinal significance and detectability of the ingredients identified in *C. sinensis*, a set of compounds were chosen as a collection of chemical markers. An HPLC-MS/MS method was then developed and validated for simultaneous quantification of the target markers. The effectiveness of the HPLC-MS/MS based protocol for authentication and quality assessment of *C. sinensis* was demonstrated by analyzing 5 samples of the authentic natural product and 5 samples of its substitutes.

Experimental section

Reagents and Materials

Cordycepin, D-mannitol, Cyclo-Gly-Pro, Cyclo-Ala-Leu, phenylalanine, HPLC grade methanol, and formic acid were purchased from Sigma-Aldrich (St. Louise, MO, USA). Other chemicals used were of analytical grade. Milli-Q water (Millipore, Bedford, MA) was used throughout the work.

Cordyceps samples

A total of 10 samples were analyzed in this study. These included 5 authentic *C. sinensis* samples, classified as caterpillar host and mycelium of *C. sinensis* which were collected in different regions of China. Five samples of Cordyceps extract products (in the form of tablets) were purchased from local health supplements stores in the US. The sample information is summarized in Table 1.

Sample preparation

C. sinensis samples were cryogenically ground and homogenized to obtain a uniform matrix. The dietary supplements samples were in the form of capsules or tablets. Ten capsules or tablets were weighed for each sample, and the average was taken as the weight of each capsule or tablet of the sample. About 500 mg of *C. sinensis* samples or equivalent amount for the dietary samples were weighed out and transferred into a centrifuge tube. Methanol/water (8:2, 20 mL) was added to the sample. The mixture was placed on a shaker for 12 hours, and then in an ultrasonic bath for 30 min. After extraction, the mixture was centrifuged at 6000 rpm for 10 min to obtain extract. The extract was diluted with the mobile phase (1:1) and filtered through a 0.22 μm nylon syringe filter before being injected into the HPLC-MS/MS system for analysis.

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Standard solutions

A portion of each compound was weighed out and added to 5.00 mL methanol/water (8:2) to make a stock solution at a concentration of 1.20 mg mL^{-1} (for cordycepin and D-mannitol), 3.1 mg mL^{-1} (for Cyclo-Gly-Pro), 2.0 mg mL^{-1} (for Cyclo-Ala-Leu), and 1.0 mg mL^{-1} (for phenylalanine). Stock solutions were diluted appropriately to obtain working standard solution with the mobile phase. The compounds involved in the study are shown in Fig. 1.

HPLC-MS/MS analysis

The system consisted of two pumps (LC-10ADvp, Shimadzu, Toyoto, Japan), an on-line degasser (DGU-12A, Shimadzu), and a triple quadrupole mass spectrometer equipped with a heated ESI source (TSQ Quantum, Thermo Scientific, San Jose, CA, USA). The mass spectrometer was controlled by Xcalibur software (Thermo Finnigan). A C_{18} reversed-phase column (Ascentis® 3 μm particle size, 10 cm × 2.1 mm, Sigma-Aldrich Chemicals, St. Louise, USA) was used for separation. MeOH/Water mixture (5/95, v/v) with 0.2% formic acid was used as the mobile phase at a flow rate of 0.20 mL/min. Sample injection volume was 5 μL. Data was acquired in full scan and SRM mode. The MS detector was operated in positive ion mode with the following settings: spray voltage 3 KV, vaporization temperature 250 °C, capillary temperature 300 °C, sheath gas pressure 35 (arb), auxiliary gas pressure 10 (arb), tube lens voltage of 150 V, and capillary voltage of 35 V, respectively. SRM parameters for MS detection of the test compounds are summarized in Table 2.

Method validation

Validation of method linearity, selectivity, sensitivity, and recovery was performed according to the US Food and Drugs Administration (FDA) guidelines analytical assay [30].

Results and Discussion

Detection of bioactive ingredients in C. sinensis

Detection of various bioactive compounds in *C. sinensis* has been reported [1–5]. These compounds included nucleosides and the bases, polysaccharides, amino acids, cyclic peptides, and sterols, etc. To identify effective biomarkers for authentication and quality control purposes, the present study was focused on detection of cordycepin, mannitol, amino acids, cyclic peptides and glycosides. These compounds were selected either because they were known as the active ingredients for pharmacological effects of C. sinensis or because they specifically occurred to *C. sinensis*. Authentic *C. sinensis* samples were carefully screened by HPLC-MS/MS. Chemical structures of the ingredients detected were verified by comparing HPLC retention times and MS^2 spectra with authentic chemicals. Cordycepin, D-mannitol, and phenylalanine were detected. D-Mannitol, once being mistaken as “cordycepic acid”, is one of the major bioactive compounds in natural Cordyceps. Studies have shown that it exhibits diuretic, antitussive, and anti-free radical activities. Therefore, mannitol was considered as one of the markers for quality control of *Cordyceps* [31]. It’s well documented that *C. sinensis* contains many amino acids at high levels. In this work phenylalanine was included because during the screening its glycoside with a molecule of glucose, i.e. phenylalanine-o-glu, was detected. To our knowledge, this is the first report on the detection of glycosylated amino acids in *C. sinensis*. Two cyclic peptides, cyclo-Gly-Pro

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and cyclo-Ala-Leu-rhamnose were detected. Little study has been so far carried out to investigate small cyclic peptides in *C. sinensis*. Detection of cyclo-Gly-Pro was reported in only one study [32]. There has been no report on the occurrence of cyclo-Ala-Leu-rhamnose in *C. sinensis*. It's worth noting that the cyclodipeptide, cyclo-Ala-Leu, was found not detectable. Its concentration in the extract sample was below the detection limit of the assay which was estimated to be at the sub-μg/mL level. Interestingly, several cyclic peptides isolated from cultures of *C. sinensis* and from *C. militaris*, i.e. cycloaspeptides A, C, F, and G [33–35] were not detected in *C. sinensis*. The MS spectra of the compounds detected are shown in Fig. 2. The six compounds, i.e. cordycepin, D-mannitol, phenylalanine, phenylalaine-o-glu, cyclo-Gly-Pro, and cyclo-Ala-Leu-rha were selected as a collection of chemical markers for authentication and quality assessment of *C. sinensis*. Although they are not necessarily the most abundant components in *C. sinensis*, they either specifically occur to this natural material or make significant contribution to its pharmacological activity.

HPLC-MS/MS quantification of C. sinensis markers selected

No study on simultaneous determination of the six compounds above selected has been reported. Since this group of compounds represented a broad variation of structural features, HPLC-MS/MS, one of the most powerful analytical techniques, was deployed for their quantification in this work. Several mobile phases, including MeOH/H_2O (60:40) containing 5mM ammonium acetate, MeOH/H_2O (60:40) containing 0.1% formic acid, ACN/H_2O (60:40) containing 5 mM ammonium acetate, MeOH/ACN/H_2O (60:20:20) were tested for the separation on a C_{18} column. It was found that isocratic elution with MeOH/H_2O (5:95) containing 0.2% formic acid resulted in the best analytical results in terms of separation efficiency and detection sensitivity. To obtain the analytical figures of merit for the HPLC-MS/MS method, analysis of mixture solutions prepared from authentic cordycepin, D-mannitol, phenylalanine, cyclo-Gly-Pro, and cyclo-Ala-Leu was performed. Under the selected HPLC-MS/MS analytical conditions the six compounds were well separated from each other within 6 min as shown in Figure 3A. Moreover, a characteristic product ion was obtained for each of the compounds involved (see Table 2), which ensured sensitive assays. Following the US Food and Drugs Administration (FDA) guideline for analytical assay [30], validation of linearity, selectivity, sensitivity, and recovery was performed. Peak areas were used for the calculation. The results are summarized in Table 3. Five-point calibration curves for all the compounds involved showed good linearity. In the tested concentration ranges (0.05 – 35 μg mL^{-1}), regression coefficients (R^2) were ≥ 0.997. Limits of detection (LODs, signal-to-noise ratio = 3) were in the range from 0.008 μg mL^{-1} for cordycepin to 0.75 μg mL^{-1} for cyclo-Gly-Pro. The corresponding limits of quantification (LOQs, signal-to-noise ratio = 10) ranged from 0.0181 μg mL^{--1} to 1.55 μg mL^{-1}, respectively. Selectivity and accuracy were evaluated by spiking a pooled sample of *C. sinensis* extracts with authentic test compounds at three concentrations. Recoveries were found in the range from 96% and 103% in all tests. Relative standard deviation values (RSD) were ≤ 2.0%. Therefore, all compounds were within the acceptable limits for bioanalytical method validation. The data indicated that the proposed HPLC-MS/MS method for simultaneous quantification of the target compounds in *C. sinensis* and its bio-derivatives products was accurate and selective. It should be pointed out that in the present work good method

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validation results were obtained without using an internal standard for the quantification. It was because the methanol extract of C. sinensis was relatively clean and showed no matrix effects on the HPLC-MS determination. However, in routine analysis of samples from a variety of sources, use of an internal standard, e.g. stable isotope labeled analogs of mannitol and phenylalanine, will certainly improve the repeatability and reliability of the analytical results.

Analysis of C. sinensis samples

Authentication and quality control of precious natural products are highly significant.[36–37] In order to evaluate the effectiveness of the proposed marker collection for authentication and quality assessment of *C. sinensis*, 5 authentic *C. sinensis* samples and 5 samples of its substitutes were analyzed by the validated HPLC-MS/MS method. Typical chromatograms obtained from analysis of authentic *C. sinensis* and its substitute samples are shown in Figure 3B and 3C. Peaks corresponding to the target compounds were well identified. No unknown peaks appeared in the chromatogram, indicating the method was specific for the determination of the markers selected. The analytical results are summarized in Table 5. Cordycepin, D-mannitol, and Phe were detected in all the samples tested. The contents ranged from 0.0076 to 0.0290% (w/w) for cordycepin, from 0.33 to 18.9 % for mannitol, and from 0.0013 to 0.642% for Phe. These results are in consistence with those reported previously [1–3, 18–20, 38–39]. It should be pointed out that samples #6 ~#10 were tablets of *C. sinensis* extracts sold at local healthy supplements stores and the %(w/w) content values may not be comparable with those for authentic *C. sinensis* since information on the composition of these tablet samples was not available. Very interestingly, the two glycosides, i.e. cyclo-Ala-Leu-rha and Phe-o-glu, were detected only in the samples of authentic *C. sinensis*. These results indicated a high level of glycosidases activity in *C. sinensis*, but not in the cultured or cultivated substitutes. Further study is needed to confirm this point of view. From the results of sample analysis, the collection of *C. sinensis* markers proposed herein has a good potential in authentication and quality assessment of this precious natural product.

Conclusions

A facile and reliable protocol for authentication and quality assessment of *C. sinensis*, a precious and pricey medicinal material, was developed. The authentic material was thoroughly screened by HPLC-MS/MS to identify effective markers. Occurrence of cordycepin (i.e. 3′-deoxyadenosine), D-mannitol, phenylalanine, and cyclo-Gly-Pro was confirmed. Two glycosides, i.e. phenylalanine-o-glu and cyclo-Ala-Leu-rha, were detected for the first time. The six compounds were selected in a collection as markers of *C. sinensis*. A fast and reliable HPLC-MS/MS method was developed for simultaneous quantification of these chemical markers. As demonstrated, the proposed protocol based on HPLC-MS/MS quantification of this marker collection has a great potential in authentication and quality assessment of *C. sinensis*.

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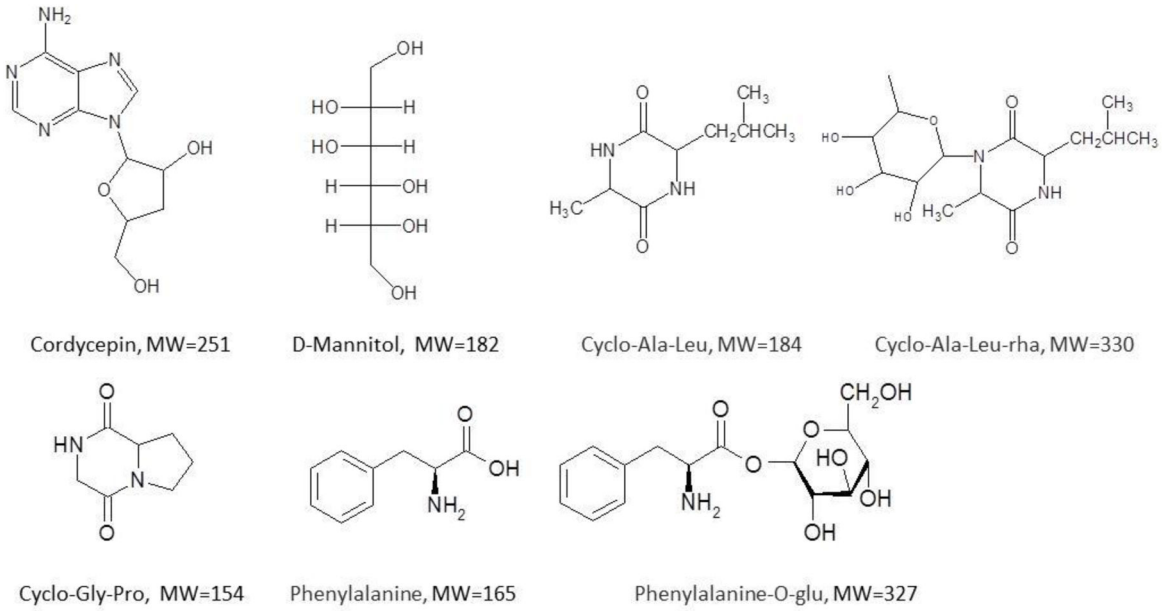

Figure 1.

Chemical structures of the compounds involved in this work: cordycepin, D-mannitol, cyclo-Ala-Leu, cyclo-Ala-Leu-rha, cyclo-Gly-Pro, phenylalanine, and phenylalanine-o-glu.

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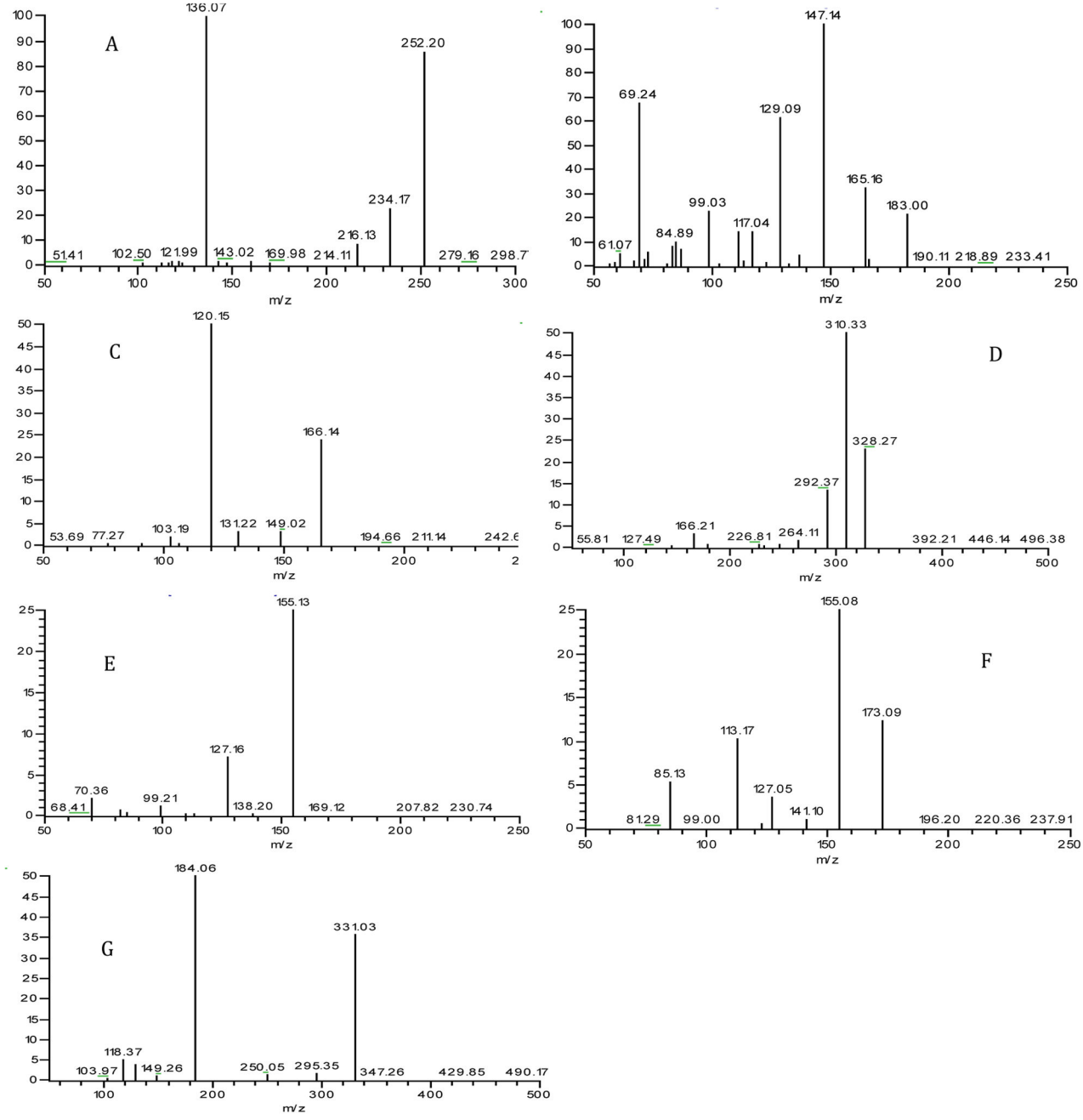

Figure 2.

MS spectra of: A) cordycepin, B) D-mannitol, C)Phe, D) Phe-o-glu, E) cyclo-Gly-Pro, F) cyclo-Ala-Leu, and G) cyclo-Ala-Leu-rha.

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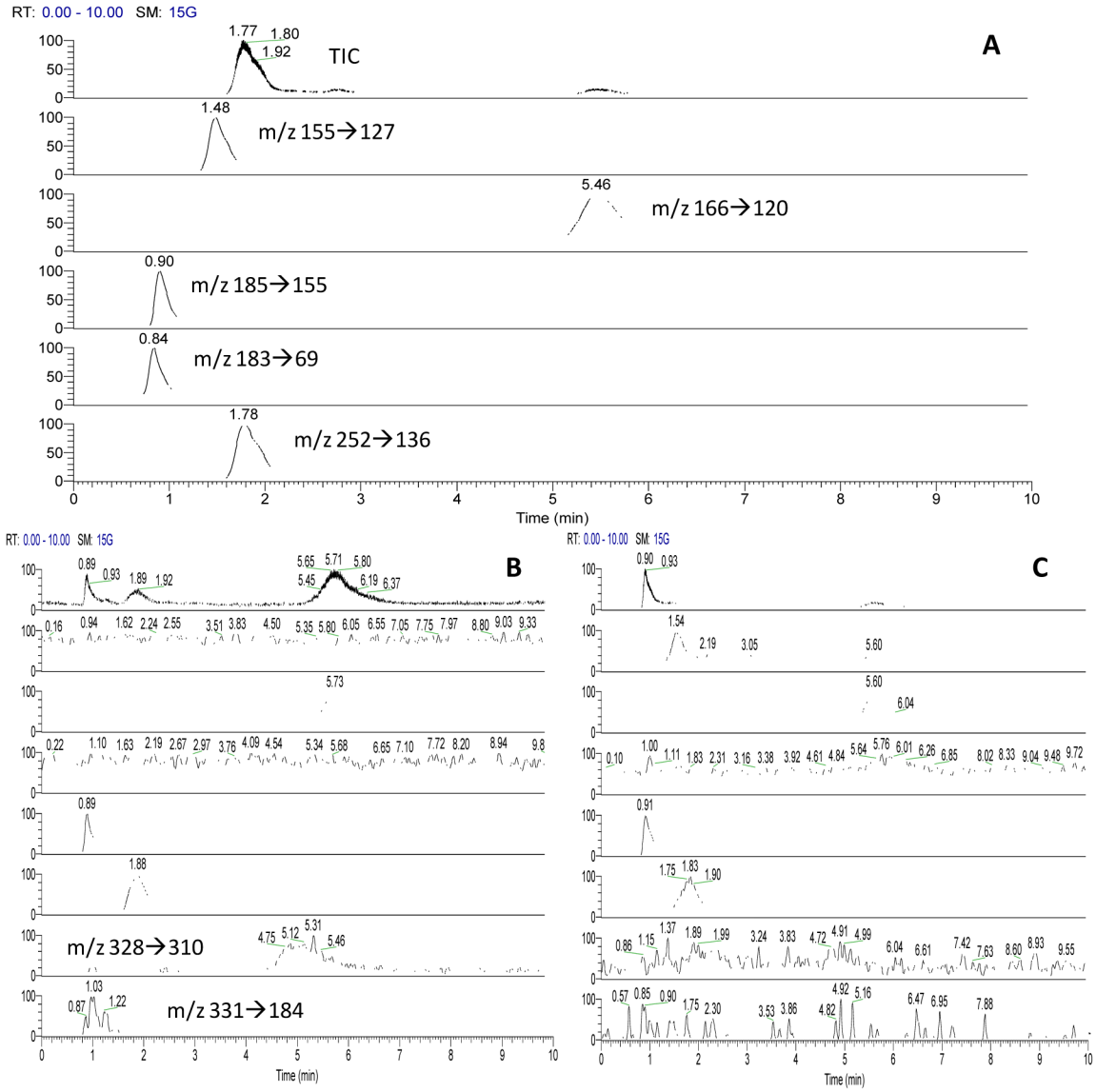

Figure 3.

TIC and extracted ion chromatograms obtained from analysis of: A) a mixture of authentic compounds, including cordycepin, D-mannitol, Phe, cyclo-Gly-Pro, and cyclo-Ala-Leu; B) a sample of authentic *C. sinensis*, and C) a sample of supplement tablets claimed as *C. sinensis* extract. Monitored ion transitions: *m/z* 155→127 for cyclo-Gly-Pro, *m/z* 166→120 for Phe, *m/z* 185→155 for cyclo-Ala-Leu, *m/z* 183→69 for D-mannitol, *m/z* 252→136 for cordycepin, *m/z* 328→310 for Phe-o-glu, and *m/z* 331→184 for cyclo-Ala-Leu-rha.

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Table 1

Cordyceps Samples analyzed in the study*

Sample Number	Sample name	Origin	Date of collection or Batch number
1	Caterpillar host of *C. sinensis*	Qinghai Province, China	05/11/2014
2	Mycelium of *C. sinensis*	Qinghai Province, China	05/11/2014
3	Caterpillar host of *C. sinensis*	Hubei Province, China	07/08/2014
4	Caterpillar host of *C. sinensis*	AnHui Province, China	09/23/2014
5	Mycelium of *C. sinensis*	AnHui Province, China	09/23/2014
6	Cordyceps 8:1 extract	Local Market	140417 04/16
7	Reishi Standardized	Local Market	20027414
8	Cordyceps Mushroom	Local Market	764402
9	Cordyceps Sinensis	Local Market	04/30/2016
10	Cordyceps	Local Market	H2372 08/15

* Samples #6~10 were supplement tablets prepared from *C. sinensis* or its substitutes.

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Table 2

SRM parameters for the test compounds

Compound	Mol. Weight	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy
Cordycepin	251	252	136, 234	10
Mannitol	182	183	147, 69	10
Cyclo-Gly-Pro	154	155	127, 70	10
Cyclo-Ala-Leu	184	185	173, 155	10
Cyclo-Ala-Leu-rha	330	331	184, 118	10
Phenylalanine	165	166	120, 103	10
Phenylalanine-o-glu	327	328	310, 292	10

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Tab. 3

Linearity and LOD, LOQ of the present HPLC-MS/MS method

Standard	Linear dynamic range (μg mL^{-1})	Slope	Intercept	R^2	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
Cordycepin	0.05~10.0	8556285.9	4148044.3	0.998	0.008	0.018
Mannitol	0.6~12.0	15099.9	59059.7	0.997	0.3	0.6
Cyclo-Gly-Pro	1.55~31.0	64396.1	174127.5	0.998	0.75	1.55
Cyclo-Ala-Leu	1.0~24.0	97875.9	57706.7	0.999	0.3	0.83
Phenylalanine	0.5~10.0	3465243.2	3251173.1	0.998	0.071	0.20

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Table 4

Recovery of target compounds from *C. sinensis* sample matrix

Compound	Content (μg)	Spiked (μg)	Observed (μg)	Recovery (%)	Mean (%)	RSD (%, n=9)
Cordycepin	0.080	0.06	0.1421	103.5%	101.2	1.90
		0.12	0.2008	100.7%		
		0.18	0.2591	99.5%		
Mannitol	37.3	18.0	55.20	99.4%	101.4	1.87
		36.0	73.91	101.7%		
		54.0	92.99	103.1%		
Cyclo-Gly-Pro	0.0	2.40	2.412	100.5%	101.8	1.36
		4.80	4.876	101.6%		
		7.20	7.434	103.3%		
Cyclo-Ala-Leu	0.0	1.00	1.004	100.4%	101.5	1.96
		2.00	2.076	103.8%		
		3.00	3.009	100.3%		
Phenylalanine	2.14	1.00	3.105	96.4%	97.8	1.33
		2.00	4.098	97.9%		
		3.00	5.111	99.0%		

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Table 5

Analytical results of *C. sinensis* samples

Sample #	Cordycepin (%, w/w)	D-Mannitol (%, w/w)	Phe (%, w/w)	Phe-O-glu (%, w/w)	Cyclo-Gly-Pro (%, w/w)	Cyclo-Ala-Leu-rha (%, w/w)
1	0.0182	11.195	0.6423	0.0366	ND	0.1888
2	0.0290	9.782	0.1413	0.0243	ND	0.1105
3	0.0173	18.568	0.4587	0.0138	ND	0.0468
4	0.0167	18.862	0.1884	0.0221	ND	0.0153
5	0.0217	7.291	0.0413	0.0148	ND	0.0063
6	0.0076	9.713	0.0097	ND	0.0016	ND
7	0.0070	0.325	0.0617	ND	ND	ND
8	0.0068	5.372	0.1033	ND	0.0057	ND
9	0.0074	5.212	0.1646	ND	0.0024	ND
10	0.0155	0.967	0.0013	ND	ND	ND

ND: not detected.

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