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Analysis of Nucleosides (Adenosine and Cordycepin) in the Mushroom Samples by Liquid-Chromatography and Mass Spectrometry; HPLC-MS-MS

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Abstract Cordyceps, a kind of precious natural crude drugs and edible mushrooms, were used as tonic food in East Asia area and enjoyed an extensive praise for its medicinal functions. Cordycepin exhibits various bio-activities, including anticancer, antibacterial, antiviral and immune regulation activities, and it's been a significant focus of research. However, the preparation of high‑purity cordycepin remains challenging. Also, the molecular target with which cordycepin interacts to cause an antibacterial effect remains unknown. A simple and rapid isocratic chromatographic method (HPLC), optimum separation for (adenosine and cordycepin) analytes was achieved using the mixture of water and methanol as a mobile phase (85:15, v/v). The Photo-Diode Array Detector (DAD) WR, an auto injector, and a reverse phase column, Agilent Shield RP C18/4.6 × 150 mm, 4‑micron and confirmation by LC/MSMS coupled with electrospray ionization (ESI) method for simultaneous separation and determination of adenosine and cordycepin in Cordyceps sinensis (Cs) and its substitutes was developed. Selective ion monitoring (SIM) mode ($[M+ H]^-$ at m/z 136, 267 and 252) was used for quantitative analysis of above components. The linearities for the 6 substances were studied in the range between 0.001 to 0.2 mg/L and the coefficients of determination (R^2) were always > 0.999. Matrix effects were also assessed by comparing the slopes obtained in solvent and matrix. The recoveries for all the substances at 3 different spike levels (0.05, 0.10 and 0.20 mg/L) were in the range 70.50-108% with RSDs < 5%. The instrumental limits of quantification were in the range 0.013-0.016 mg/L, while the reporting level of the method was 0.004 mg/L for all the aforementioned compounds. The nucleoside contents of types of natural Cs and its substitutes were determined and compared with this developed method.

Keywords: *adenosine, cordycepin, HPLC, LC-MS-MS, nucleoside*

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1. Introduction

Cordyceps includes several *Cordyceps* species, which are widely used for medicinal purpose or food additives. It a high value medicinal mushroom, naturally distributed in China, India, Nepal, Tibet and Bhutan [1]. *C. sinensis* is a parasitic fungus found at altitude of more than 3,200 meters. It has a characteristic life cycle on the larva of a moth; belongs to *Clavipitaceae* family and the genus *Ascomycetes* [2]. The wild fungus along with the cultivated varieties as well as cultured mycelia, fruiting body and extracts reportedly possesses diverse medicinal properties [3].

Owing to these properties, it has been employed to treat various rehabilitation disorders such as arrhythmias, asthenia after severe illness, bronchitis, cancer, hyperglycaemia, hyperlipidaemia, hyposexuality, liver disease, lungs disorders, night sweating, renal dysfunction and renal failure etc. [4-10]. The Indian isolates of *C. sinensis* possess medicinal properties like anti-bacterial activity, anti-oxidant activities, anti-stress effect, anti-inflammatory, antimicrobial, muscular endurance, immune modulating effects, enhancement of neuromuscular activity, endurance enhancing activity [11-16] and tolerance activity to hypoxia at high altitude [15]. There are various HPLC methods that had been widely used in the determination of adenosine and cordycepin from *C. sinensis* or *C. militaris* [17,18,19]. Considering, its wide medicinal properties, the fungus is regarded as "soft gold" in China and is extremely high in price; approximately USD $20,000 to 40,000 per kg [2]. The Indian variety of *C. sinensis* is found at high altitude between 12,000 and 16,000 ft. at Himalayan plateau of Kumaon and Garhwal hills, Arunachal Pradesh, Uttarakhand and Sikkim [20,21]. In India, it is known by the name of *Ghas Ka Kira* or *Kira Jhar* or *Kira ghas* or *Yartsa Gumba* in hilly regions of Darma and Johaar Valleys in Pithoragarh [16]. The fungus

contains several idiosyncratic secondary metabolites such as cordycepic acid, cordycepin, ergosterol, fatty acids, nucleobases, polyphenols, polysaccharides, steroids, terpenes and vitamins [12,15,22,24,25]. The current study based on validation of Adenosine and Cordycipin by HPLC-MS-MS is one of the main means in qualitative and quantitative monitoring of food and medicine products. (Figure 1)

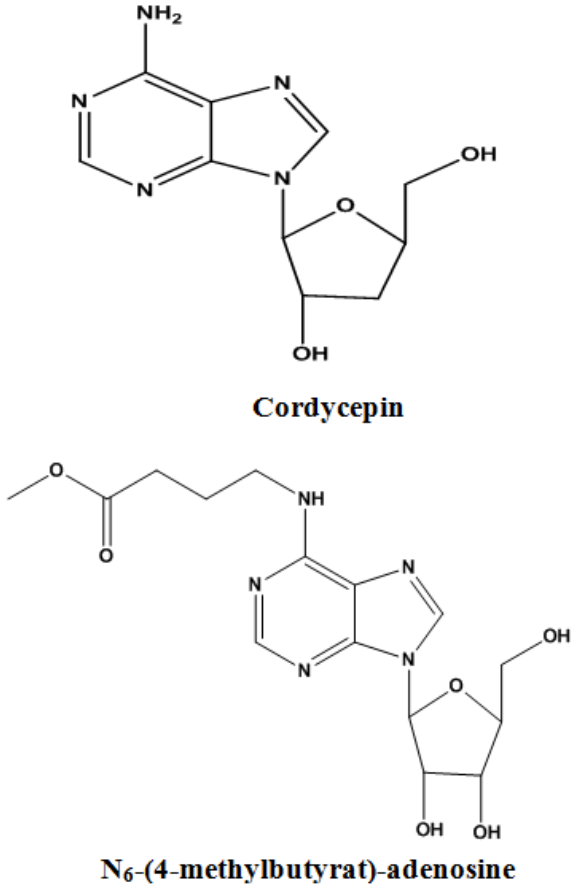

Figure 1. Structure of Nucleosides of active compound of mushroom (Adenosine and Cordicepin)

2. Materials and Methods

2.1. Materials and Standards

Adenosine and cordycepin were purchased from Sigma (St. louis, MO, USA). A stock solution of each standard (0.2 mg ml^{-1}) was prepared in methanol and stored in a refrigerator. All solutions were filtered through a 0.45 mm membrane and degassed. Working standard solution (20 mg ml^{-1}) was prepared by diluting the stock solution with methanol. Formic acid used was analytical grade. HPLC-grade methanol and Milli-Q quality water was used in the preparation of the mobile phase. Samples of Cordyceps sinensis and Cordyceps militarris from different sources were obtained from local drug stores.

2.2. Extraction of Cordycepin and Adenosine from Different Samples

Various samples, under similar method, were prepared to extract of mushroom for the analysis of cordycepin and adenosine, respectively. Take contains whole parts, corpse and capillary, which were grinded into powder (diameter at approximate 50 meshes) together in liquid nitrogen. Then, approximate 1.0 g of sample was precisely weighed and added into 10 ml water: methanol (85:15, V/V) in a 50 ml centrifuge tube which was subsequently placed in an ultrasonic machine for extracting cordycipin and adenosine at a power of 75 watt. After the centrifugation at @ 4500 rpm, the sample extraction procedure was repeated another twice. Supernatant obtained from the three times centrifugation was mixed and exactly measured of its volume. The sample was filtrated through a 0.45 µm filter prior to HPLC analysis. The extraction of cordycepin and adenosine was also carried out in a procedure [25,26].

2.3. Determination of the Bioactive Components by HPLC

All HPLC analysis work was carried out on an Agilent (Agilent, 1260 Infinity II, USA, which consists of an Agilent 1260 Photo-Diode Array Detector (DAD) WR, an auto injector, and a reverse phase column (Agilent Shield RP C18/4.6 × 150 mm, 4 micron). Standards of cordycepin and adenosine were purchased from Sigma Chemical Corporation (St. Louis, MO, USA). The standard adenosine and cordycepin solvent was consecutively injected six times of draw calibration curves. The injection volume was 1, 10, 25, 50,100 and 200 mg/L, respectively. The determination condition of the samples was set as follows: the mobile phase adopted in the analysis consists of water and methanol were in the ratio 85:15, (V/V). The separation was conducted in isocratic elution with a flow rate of 0.7 ml/min. The detection wavelength of photo-diode array was set at 260 nm and the column temperature was 30°C. The injection volume was 20 µl. Data collection and analysis was performed using Open Lab CDS 2.X, DAD software (Agilent Technology). The results were shown as the means of three replicates.

2.4. LC-MS-MS Confirmation

We carried out LC-MS-MS using an Agilent 1260 Infinity II (Ultivo LC/TQ), QQQ-pole mass spectrometer with an electrospray ionization performed in positive and negative mode, full-scan spectra were recorded from m/z 100 to 500 at a scan time of 500 ms and 1 min delay for post run (to remove the carryover effect of previous run). The main other instrumental settings were capillary voltage 4 kV, ion energy 0.1, source temperature 250 C, shealth temperature 400 C, cone gas (N2) flow rate 720L/h, and desolvation gas (N2) flow rate 660 L/h. Collision energy Adinocine 30 Volt and Cordicipine 20 Volt, Fermenter 80 simultaneously, dwell time 150 and 50. Selected-ion monitoring of the most abundant ion was used for quantification. The MS-MS detector was tuned for the maximum sensitivity of the parent ion for Adenosine at m/z 267 and for Cordesepin at m/z 252 respectively for confirmation. Analysis and Confirmations was performed using Mass Hunter ver 10.0 software (Agilent Technology).

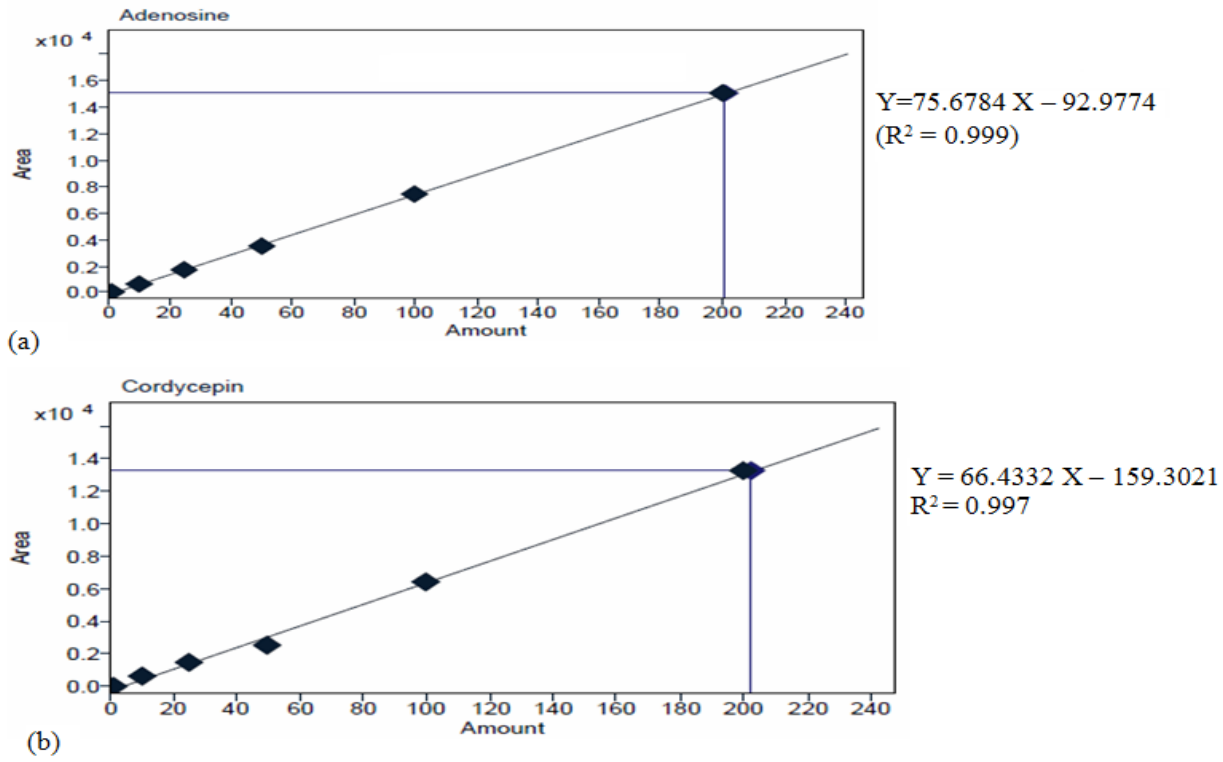

Figure 2. Calibration curve of Nucleosides Standards (a): Adenosine (b): Cordycepin

2.5. Recovery Studies and Quality Control

Adenosine and cordicepin was identified by matching the retention time of the sample with its external standard and confirm by LC-MS-MS. Procedural blanks consisting of all reagents and glassware's used during the analysis were periodically determined to check for cross-contamination. Since no compounds that interfere with the sample were detected, values were not corrected for procedural blanks. Absolute recovery of Adenosine and Cordycepin was measured by analyzing three samples of mushroom fortified at 1, 10, 25, 50, 100 and 200 mg/L, (Figure 2) which indicated that overall recovery ranged from 70 to 108% the relative standard deviation from (%RSD) 5.22. An Adenosine and cordicipin calibration curve was generated, and the linear relationship was evaluated across the range of expected sample concentrations. Linearity was obtained by a linearregression plot of known concentration versus response using a minimum of six different concentrations of Adenosine and Cordycipin. Residues were evaluated across at least three separate runs from different concentrations of Adenosine and Cordycipin. Under an optimum chromatographic conditions, to the standards of adenosine, the retention time (RT) was 1.96, limit of detection (based on a signal to noise ratio of 3:1) 0.2 and processed channel (UVʎ) 260.0, Linear range 1 - 200 mg/L. To the standards of cordycepin, the RT 7.72, limit of detection (based on a signal to noise ratio of 3:1) 0.1 and processed channel (UVʎ) were 260.0, Linear range 1 - 200 µg/ml. Based on the chromatographic conditions, we established the standard curves cordycepin and adenosine standards. The regression equations of calibration curves and their coefficients were calculated as follows: for cordycepin, Y = 66.4332 X – 159.3021 (R = 0.9970); for adenosine, Y=75.6784 X – 92.9774 (R = 0.9997) was respectively. The separation was conducted in isocratic elution with a flow rate of 1.0 ml/min. The detection wavelength of photo-diode array was set at 190 - 400 nm, and the column temperature was 30°C. The injection volume was 20 µl.

3. Results and Discussion

3.1. Optimization of Chromatographic Conditions

The aim of the present work is to develop a method for simultaneous separation and determination of nucleosides in mushroom sample by HPLC and LC/MS-MS. Although the mixture of adenosine and cordycepin standards can be separated at the optimal chromatographic conditions and monitored at 260 nm with a photo-diode array detector**.** The method has been applied to simultaneous separation and determination of active components in Cordyceps sinensis. The results shown the best separation was obtained under a specific concentrations of methanol and water. Based on the flow rate of mobile phase, the elution time was determined. It was found that 1.0 ml/min was a proper flow rate. As cordycepin and adenosine are polarity organic matters, with the increase of percentage of methanol in mobile phase, the difference of retention time of cordycepin and adenosine become small which resulted in a poor separation [24]. When using water-mathanol (85: 15, V/V) as mobile phase, the retention time was less than two minutes. Considering the elution time and retention times, flow rate of 1 ml/min and mobile phase of water-methanol (85:15, V/V) was the optimized chromatographic condition used for all following analysis (Figure 3a, Figure 3b, and Figure 3c).

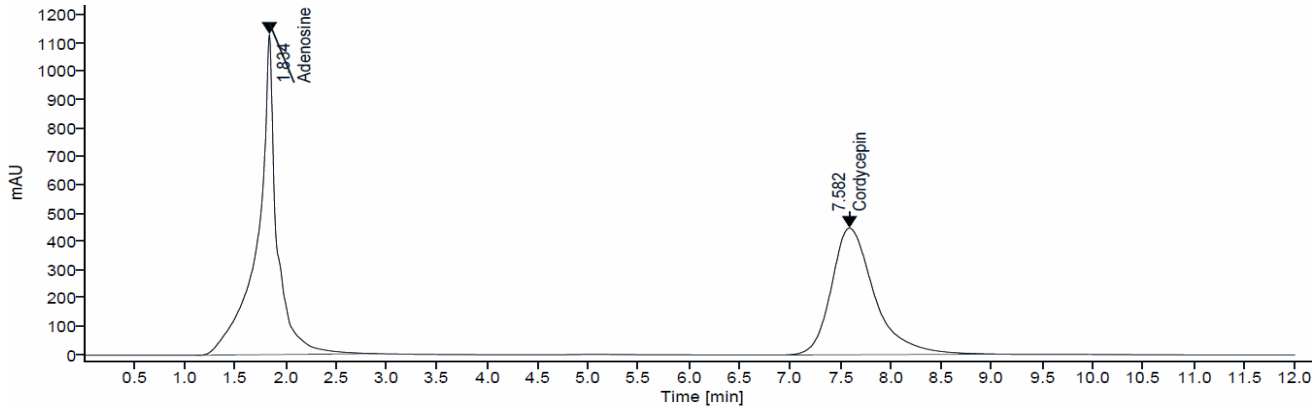

Figure 3a. Chromatogram of adenosine and cordycepin standard under water and methanol in mobile phase; water methanol (85:15,V/V), retention time of adenosine and cordycepin 1.834 and 7.758 min, respectively

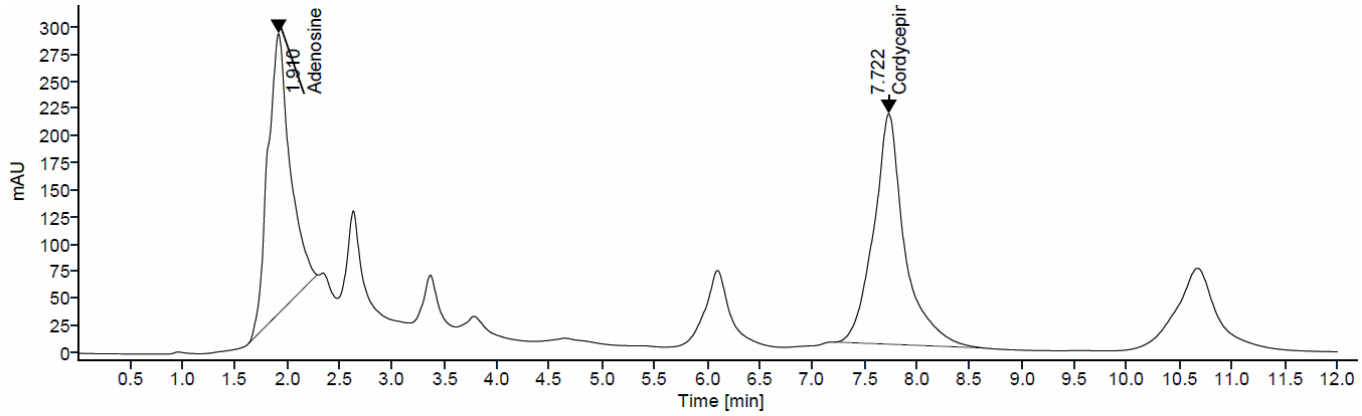

Figure 3b. Recovery study of chromatogram of adenosine and cordycepin under water and methanol in mobile phase; water methanol (85:15,V/V), retention time of adenosine and cordycepin 1.834 and 7.758 min, respectively

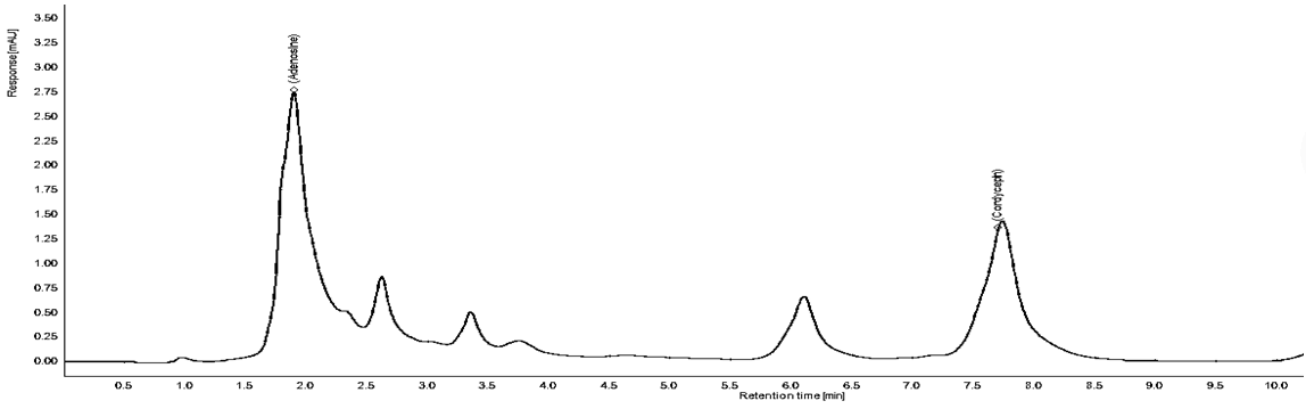

Figure 3c. Chromatogram of adenosine and cordycepin in sample under water and methanol in mobile phase; water methanol (85:15,V/V), retention time of adenosine and cordycepin 1.834 and 7.758 min, respectively

Furthermore, some components with very high contents in real samples cannot be detected with HPLC-DAD due to the low inherent UV absorbance of these components. Here a first combined isocratic LC/MS method based on coupling with the ESI interface was developed. In positive ion mode, LC/ESI-MS mass spectra of adenosine and cordycepin standards were obtained by scanning between m/z 50 and 350 per second (see Figure 4). The mass spectra of adenosine and cordycepin obtained from scan mode were characterized by a protonated molecular ion [M+H] as base peak. However, the mass spectrum of hypoxanthine was characterized by a Na adduct two molecular ion $[2M+Na]^+$as base peak and the protonated molecular ion $[M+H]^+$ was the second peak. In additional to $[M+H]^+$or $[2M+Na]^+$ as a base peak, the MS spectra of adenosine, and cordycepin obtained from scan mode had an $[M+Na]^+$ or $[2M+H]^+/[2M+Na]^+$. The results were summarized in Table 1. SIM mode involved the use of the $[M+ H]^+$ ions at m/z 136, 137, 268, 252 and 302 was chosen for simultaneous determination of the four active components. A mixture containing two standard compounds and IS was injected into the LC/MS system. They were monitored using SIM mode. It was observed that adenosine and cordycepin were LC/ESI-MS mass spectra of mixture of nucleoside standards in positive ion and scan mode between m/z 50 and 350 per second [14,17,18].

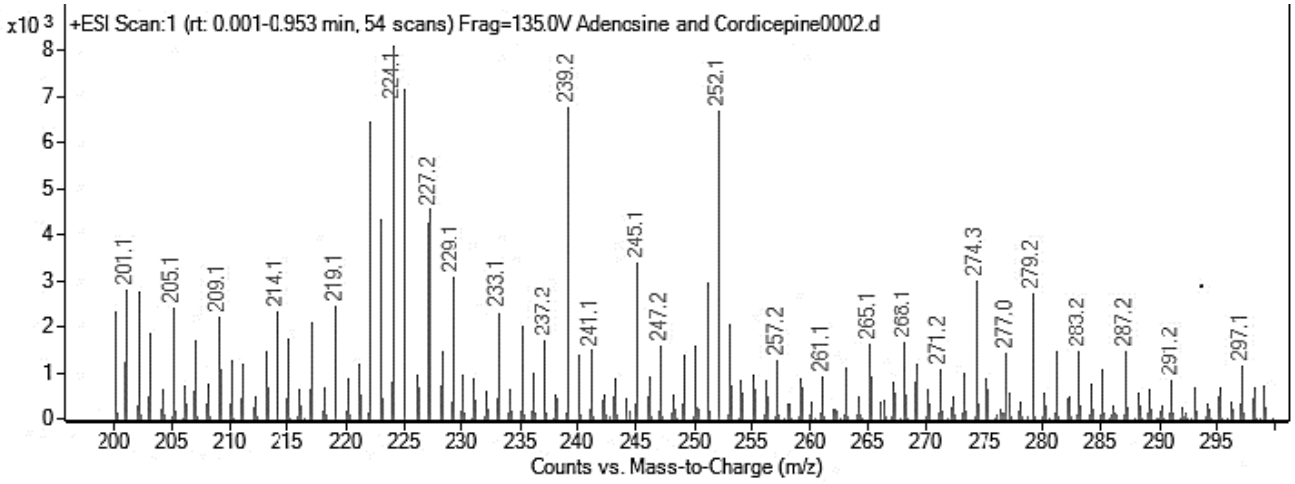

Figure 4. LC/ESI-MS mass spectra of mixture of nucleoside standards in positive ion and scan mode between m/z 50 and 350 per second

Table 1. Recovery study of Cordycipin and Adenosine in different spiking levels

Compound	Spiked mg/Kg	R 1	R 2	R 3	Mean	SD	%RSD	LOD	LOQ	m/z
Adenosine	0.05	70.51	72.35	77.18	73.34	2.81	5.22	0.004	0.016	252
	0.1	97.8	100.15	104.54	100.83	2.79	2.77			
	0.2	102	107	105.7	104.9	2.11	2.01			
Cordyecipin	0.05	71.35	73.62	76.58	73.85	2.14	2.89	0.005	0.013	267
	0.1	100.08	105.15	107.54	104.25	3.11	2.98			
	0.2	103	106	108.7	105.9	2.32	2.19			

R-Represented recovery
LOD- Limit of Detection
LOQ- Limit of Quantification.

Table 2. Confirmation of Adenosine and Cordycipin by LC-MSMS

Compound	t_R (min)	UVʎ max	$[M + H]^+$ *m/z*	$[2M + H]^+$ *m/z*	$[M + Na]^+$ *m/z*	$[2M + Na]^+$ *m/z*
Cordyecipin	4.56	206, 260	267	290	-	-
Adenosine	6.12	207,260	252	274	-	-

tR- Retention Time.

UV spectrum and mass spectrum of standard of each component were used for comparison with the chromatograms of extracts of Cordyceps sinensis and its substitute's identification of components. Their retention time (tR), [M+H], [2M+ H], $[M+Na]^+$, [2M+Na]+ and UV ʎ max values were shown in Table 2.

HPLC is one of the main means in quality monitoring of food and medicine products. Under an ideal chromatographic conditions including flow rate, mobile phase, detection wavelength and column temperature, the elution time is short and it has a good separation between analyses. The flow rate had great effect on the elution time. Such that when the rate was too low, the separation between the analyses experience a low-efficiency; when efficiency; when too high, the elution time becomes too short and the peak of adenosine and cordycepin could not be separated from interference peaks caused by other matters in the sample usually appearing mainly in the beginning 5 min. At a flow rate of 1.0 ml/min, the elution time was shorter than 10 min and the retention time of analyses was later than 6 min, which was proper condition for the determination of adenosine and cordycepin. As for the separation of cordycepin and adenosine, mobile phase had been used including methanol and water gradient elution. However, the separation was very good and they have separate retention times because of their similar structure.

In this present research were developed for simultaneously determination of active compounds of Adenosine and cordycepine by HPLC-PDA and LC-MS-MS for quality control.

4. Conclusion

The HPLC-MS-MS method was developed and validated for found to be simple, sensitive, and accurate result in wide linearity range, which can be used for the quantification of adenosine and cordycepin in crude mushroom samples. The mass spectra of nucleosides obtained from the extracts of actual samples were characterized by $[M+H]^+$ or $[2M+Na]^+$as base peak, for quantitative analysis of two active components. It can also be used for quality control and standardization of mushrooms for in which cordycepin and adenosine are present as a major constituent.

The successfully simultaneous separation and determination of the two components in Adenosine and

Cordycepin shows that LC/MS-MS method is a powerful technique for the analysis of bioactive components.

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Highlight

1. The aim of the present work is to develop a method for simultaneous separation and determination of nucleosides in mushroom sample by HPLC and LC/MS-MS
2. The HPLC-MS-MS method was developed and validated for found to be simple, sensitive, and accurate result.
3. The recoveries for all the substances at 3 different spike levels (0.05, 0.10 and 0.20 mg/L) were in the range 70.50-108% with RSDs < 5%. It is good to know that its analyzed in 8 min and good separation

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