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Validation and application of HPLC method for determination of cordycepin and adenosine in dietary supplements

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Abstract

A simple method based on High Performance Liquid Chromatography (HPLC)-UV was developed in this study for determining the existence of cordycepin and adenosine in Cordyceps and its products. Chromatographic separation was carried out using a Kinetex® C18 column with a mobile phase of water and acetonitrile (95:5, v/v), a flow rate of 1.0 mL/min, and wavelengths of ultraviolet detection set at 260 nm. The calibration curve was linear over the range of 0.05–20 and 0.02–20 μg/mL for cordycepin and adenosine, respectively, and the limit of detection was 0.005 μg/mL for both compounds. The method was validated with excellent specificity, accuracy, precision, and recovery. The assay has been applied successfully to a quality control study on commercial products containing Cordyceps in the Thai market such as dried cultured materials and pharmaceutical formulations.

Keywords: Adenosine, Cordycepin, Cordyceps, Dietary supplements, HPLC-UV

1. Introduction

Cordyceps is a group of ascomycete fungi that includes two well-known species called *Cordyceps sinensis* and *C. militaris*. Cordyceps is widely used in tonic food, traditional Chinese medicine, and dietary supplements [1]. Cordyceps fungi are difficult to collect in their natural origin, making them very expensive, but now they can be easily grown and harvested in a laboratory [2]. Different bioactive compounds from *Cordyceps sp*. contain nucleosides, ergosterol, polysaccharides, glycoproteins, and peptides, and their biological activities have been reported to include anti-tumor, anti-metastatic, immunomodulatory, anti-oxidant, anti-inflammatory, insecticidal, anti-microbial, hypolipidaemic, hypoglycaemic, anti-aging, as well as neuroprotective and renoprotective benefits [3]. Nucleosides, major active components of Cordyceps, are used as valuable chemical markers for quality control. Cordycepin (3′-deoxyadenosine) (Figure 1) is one of the active nucleosides first extracted from *C. militaris* while adenosine (Figure 1) is a major nucleoside found in *Cordyceps sp*. These compounds exhibit several significant therapeutic properties [4-6]. The increasing prevalence of chronic diseases is expected to drive the adoption of preventive healthcare products made from Cordyceps, potentially further augmenting the growth of the Cordyceps extract market over the forecast period.

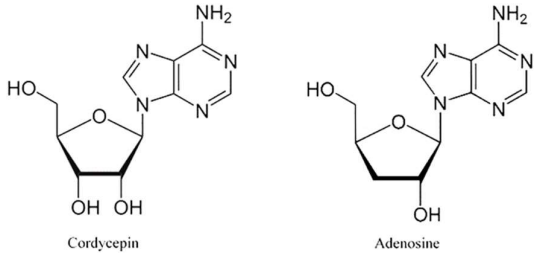

Figure 1 Chemical structures of cordycepin and adenosine.

Different kinds of instrumental methods have been developed for the analysis of cordycepin, but chromatographic assays generally seem to be the most favored. Liquid chromatographic methods have been studied such as High Performance Liquid Chromatography ultra-violet (HPLC-UV) [7-11], High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) [12-15], Ultra-performance Liquid Chromatography-photodiode Array Detection (UPLC-PDA) [16], and liquid chromatography-mass spectrometry (LC-MS) [17,18]. These listing techniques can be applied to analyze either cordycepin only or in conjunction with nucleoside derivatives such as adenosine. Most analytical methods have focused on the dried materials of different species from both wild sources and cultivation. Numerous dietary supplement products have recently appeared on the global market, containing not only Cordyceps extract but also mixtures of active ingredients from other natural sources as well as vitamins and minerals. Therefore, it is necessary to control the quality of existing products. However, few publications mention the need to determine the cordycepin content in these commercial products [10].

In the present study, an HPLC-UV method for the simultaneous determination of cordycepin and adenosine from Cordyceps has been validated according to The International Council for Harmonisation (ICH) guidelines [19]. This analytical method can be applied to samples obtained from both dried materials and dietary supplement products containing Cordyceps.

2. Materials and methods

2.1 Chemicals and reagents

Cordycepin (98.0%) for this study was purchased from Chengdu Biopurify Phytochemicals Limited, Sichuan, China, and adenosine (≥99.0%) from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan. Methanol and acetonitrile of HPLC grade were purchased from RCI Labscan Limited, Bangkok, Thailand, and water supplied by A.N.B. Laboratories (Bangkok, Thailand). Three dried materials containing the fruiting bodies of cultured Cordyceps (*C. militaris*) were obtained from different sources: sample A (Bangkok, Thailand); sample B (Tibet, China); and sample C (China). The following three dietary supplement products were used: sample D (Sukhothai, Thailand), each capsule contains 250 mg Cordyceps (*C. militaris*), 75 mg panex ginseng extract, 50 mg black ginger extract, 100 mg long jack extract, 25 mg zinc amino acid; sample E (Bangkok, Thailand), each capsule contains 180 mg ginseng extract, 150 mg Cordyceps (*C. sinensis*) extract, 120 mg oyster extract, 30 mg zinc methionine; sample F (Nonthaburi, Thailand), each 65 mL solution contains 1,000 mg Cordyceps (*C. militaris*) extract, 40 mg hedgehog mushroom, and 0.1 mg astaxanthin.

2.2 Chromatographic conditions

The HPLC system (Shimadzu, Kyoto, Japan) consisted of a solvent delivery system (LC-10ATVP), a system controller (SCL-10AVP), an autosampler (SIL-20ACHT), a column oven (CTO-10ASVP), and a UV-VIS detector (SPD-10AVP). The Kinetex® C18 analytical column was employed (250×4.6 mm i.d.; 5 μm particle diameter, 100 Å average pore size), fitted with a guard cartridge packed with C18 material (Phenomenex, Torrance, CA), and the temperature maintained at 30 °C. The mobile phase comprised water and acetonitrile (95:5, v/v) at a flow rate of 1.0 mL/min. A 10 μL aliquot sample was injected onto the HPLC system for analysis, with the detector set at a wavelength of 260 nm and the run time at 15 min.

2.3 Preparation of calibration standards and quality control

The stock solutions of cordycepin and adenosine were prepared separately in water to yield primary standard solutions with a concentration of 1 mg/mL as the base. The secondary standard solutions were produced from each stock solution by dilution with water to yield concentrations of 50 μg/mL. The working standard solutions of cordycepin and adenosine at 0.02, 0.05, 1, 5, 10, and 20 μg/mL were prepared by serial dilution of the secondary

standard solution with water. Three quality control (QC) samples were set at concentrations of 0.02 and 0.05 μg/mL (low, LQC), 1 μg/mL (medium, MQC), and 20 μg/mL (high, HQC). All stock solutions were stored at 4 °C until analysis.

2.4 Sample preparation

The dried Cordyceps sample was grounded into a powder, with each 1 g accurately weighed in a screw-cap centrifuge tube. The sample was extracted using 10 mL water in an ultrasonic bath for 15 min, centrifuged at 1,000 rpm for 15 min and the supernatant then collected. This process was repeated once again. The collection was adjusted to 20.0 mL and a 10 fold dilution then made with water. The solutions were subsequently filtered through a 0.45 μm VertiClean nylon syringe filter (Vertical Chromatography, Bangkok, Thailand) and transferred to an autosampler vial.

To analyze the Cordyceps capsules, the content of each was removed by a suitable means and blended. Each 1 g of the sample was extracted as described above and diluted to the optimal concentration. In contrast, to analyze the Cordyceps solutions, the sample was prepared by filtering it through a syringe filter without extraction or dilution.

2.5 Validation of the analytical method

2.5.1 Specificity

The specificity of the developed HPLC method for the determination of cordycepin and adenosine was investigated by comparing the chromatogram of the standard solution with those of the Cordyceps samples.

2.5.2 Sensitivity

The sensitivity of each analyte was evaluated by the limit of detection (LOD, the concentration of analytes giving a signal-to-noise ratio of 3:1) and the limit of quantification (LOQ, the concentration of analytes giving a signal-to-noise ratio of 10:1). The LOQ was the lowest measurable concentration of the standard with a relative standard deviation (RSD) of less than 20%.

2.5.3 Linearity and working range

The five-point calibration curves were constructed by plotting the peak area of analytes (y) versus the concentration of analytes (x) on three separate batches. The calibration curves require a correlation coefficient (r^2) of 0.999 or better.

2.5.4 Accuracy and precision

The accuracy and precision were estimated by analyzing the replicates at three different QC levels. Intra-day precision (repeatability) and inter-day precision (intermediate precision) analyses were determined by replicating three samples within a day and three on consecutive days, respectively. The accuracy was calculated as %accuracy based on the given formula (concentration found/concentration taken) ×100. The precision was evaluated by calculating the percentage of relative standard deviation (% RSD). To meet the acceptance criteria for accuracy and precision, the mean value should be within 15% of the actual value and the %RSD should be ≤15%, respectively [20].

2.5.5 Recovery

The recovery of analytes from the dried material samples was determined after extraction by comparing the concentrations obtained from three spiked samples with a standard solution of three unspiked samples. The percentage recovery was calculated from the given formula [(concentration of the spiked sample-concentration of the unspiked sample)/concentration of standard added] ×100.

3. Results and discussion

3.1 Development of the HPLC method and specificity

The published HPLC methods for the determination of bioactive nucleosides in Cordyceps are summarized and compared to the report presented in Table 1. From the previous reports, the compositions of the mobile phase

for HPLC analysis of nucleosides in Cordyceps were used as mixtures of water with either methanol [8,11,13-15] or acetonitrile [7,9,10] operated by isocratic [7-9,13] and gradient elution [10,14,15]. Figure 2 shows the comparison of typical chromatograms for a standard stock solution in the mobile phases referred to. The condition with water:acetonitrile at the ratio of 95:5 provided more resolution than the other mobile phase. Adenosine and cordycepin were well resolved with good symmetry and retention times of 7.9 and 10.5 min, respectively. The water/acetonitrile isocratic system was used for further study. The total run time for determining either cordycepin only or cordycepin and adenosine was equal or less than that reported by Kumar and Spandana [8], Wang et al. [9], and Huang et al [13]. Furthermore, this simple method provides the most versatile HPLC column and the standard HPLC-UV instrument is widely accessible and does not require the internal standard. The specificity of an analytical method is its unequivocal assessment of the analyte in the presence of components in the dried samples as well as other active ingredients and excipients in the products. The method demonstrated excellent chromatographic specificity with free interferences at the retention times of cordycepin and adenosine (Figure 3).

Table 1 Comparison of published HPLC methods for the determination of cordycepin and adenosine in the various Cordyceps products.

Method (Wavelength)	Analytes	Samples (Cordyceps species)	Stationary phase column	Mobile phase (v/v); Run time	LOD / LOQ (mg/mL)	Range (mg/mL)	References
HPLC-UV (260 nm)	C and A	Culture and product (CS and CM)	Kinetex C_{18} (250 mm×4.6 mm)	Water: acetonitrile (95:5); 15 min	C: 0.005 / 0.05 A: 0.005 / 0.02	C: 0.05-20 A: 0.02-20	Presented report
HPLC-UV (260 nm)	C, A, U, G, 2′-deoxyA and Hypoxanthine	Natural, culture and extract (CS and CM)	Daisopak 120-5ODS-BP (250 mm×4.6 mm)	Water: acetonitrile (98:2); 14 min	C: 0.0160/0.030 A: 0.0126/0.030	C: 0.030-150 A: 0.030-150	[7]
HPLC-UV (254 nm)	C and A	Nutural (CS)	protoSIL C_{18} (250 mm×4.6 mm)	Water: methanol (80:20); 25 min	C: None A: None	C: None A: None	[8]
HPLC-UV (260 nm)	C	Cordyceps recombinant rice (CM)	C_{18} (250 mm×4.6 mm)	Water: acetonitrile (95:5); 25 min	C: None	C: 1-50.0	[9]
HPLC-UV (254 nm)	C, A, U and G	Culture, extract and products (CS and CM)	ACE Generix 5 C_8 (250 mm×4.6 mm)	Water: acetonitrile (gradient elution); 30 min	C: 0.01 / 0.279 A: 0.01 / 1.13	C: 0.279-2.793 A: 1.13-11.30	[10]
HPLC-UV (260 nm)	C and A	Cooking sample (CM)	Zorbax SB-C18 (150 mm×4.6 mm)	Water: methanol (84:16); 25 min	C: None A: None	C: 10-80 A: 10-80	[11]
HPLC-DAD (n/a)	Nucleosides	Culture (CS)	Shimadzu VP-ODS (150 mm×2.0 mm)	Ammonium acetate (40 mM, pH 5.2): methanol (gradient elution); n/a	All analytes: 0.1-0.6 / 0.5-2.0	All: n/a	[12]
HPLC-DAD (254 nm)	C and A	Culture (CS and CM)	Waters Symmetry Shield RP 18 (150 mm×4.6 mm)	Water: methanol (92:8); 15 min	C: 0.25 / 2.85 A: 0.30 / 2.50	C: 2.85-130 A: 2.50-120	[13]
HPLC-DAD (260 nm)	C, A, U, G, Adenine, Inosine and Uracil	Culture (CJ)	Shimudzu VP-ODS (250 mm×4.6 mm)	Water: methanol (gradient elution); 30 min	C: 0.050 / 0.01 A: 0.001 / 0.01	C: 1.13-91.0 A: 1.00-100.00	[14]
HPLC-DAD (260 nm)	A, U, G, Adenine, Uracil	Fermented Cordyceps (CS)	Zorbax-RP (250 mm×4.6 mm)	Water: methanol (gradient elution); 30 min	A: 0.024 / 0.073	A: 0.936-93.6	[15]
UPLC-PDA (254 nm)	14 nucleosides and nucleobases	Culture (CS and CM)	Acquity UPLC BEH C_{18} (50 mm×2.1 mm)	0.5 mM acetic acid: acetonitrile (Gradient elution); 30 min	C: 0.0209 / 0.0725 A: 0.0204 / 0.0787	C: 5.52-486.00 A: 0.32-120.82	[16]
LC/MS	C, A, Adenine and Hypoxanthine	Natural (CS and CM)	Shimadzu VP-ODS (150 mm×2.0 mm)	Water: methanol: formic acid (85:14:1); n/a	C: 0.1 / 0.5 A: 0.1 / 0.5	C: 0.5–131.5 A: 0.5-128.5	[17]
LC/ESI-MS	C, A, Adenine and Thymine	Natural (CS)	Shimadzu VP-ODS (150 mm×2.0 mm)	Ammonium acetate (40 mM, pH 5.2): methanol (gradient elution); 17.5 min	C: 0.1 / 0.5 A: 0.1 / 0.6	C: 0.5-107.5 A: 0.6-114.0	[18]

HPL = High-performance liquid chromatography; UPLC = Ultra-performance liquid chromatography; LC = Liquid chromatography; UV = Ultraviolet detector; DAD = Diode-array detector; PDA = photodiode array detector; MS = Mass spectrometer; C = Cordycepin; A = Adenosine; U = Uridine; G = Guanosine; CS = *C. sinensis*; CM = *C. militarris*; CJ = *C. jiangxiensis;* n/a = Not available; LOQ = Limit of quantification; LOD = Limit of detection

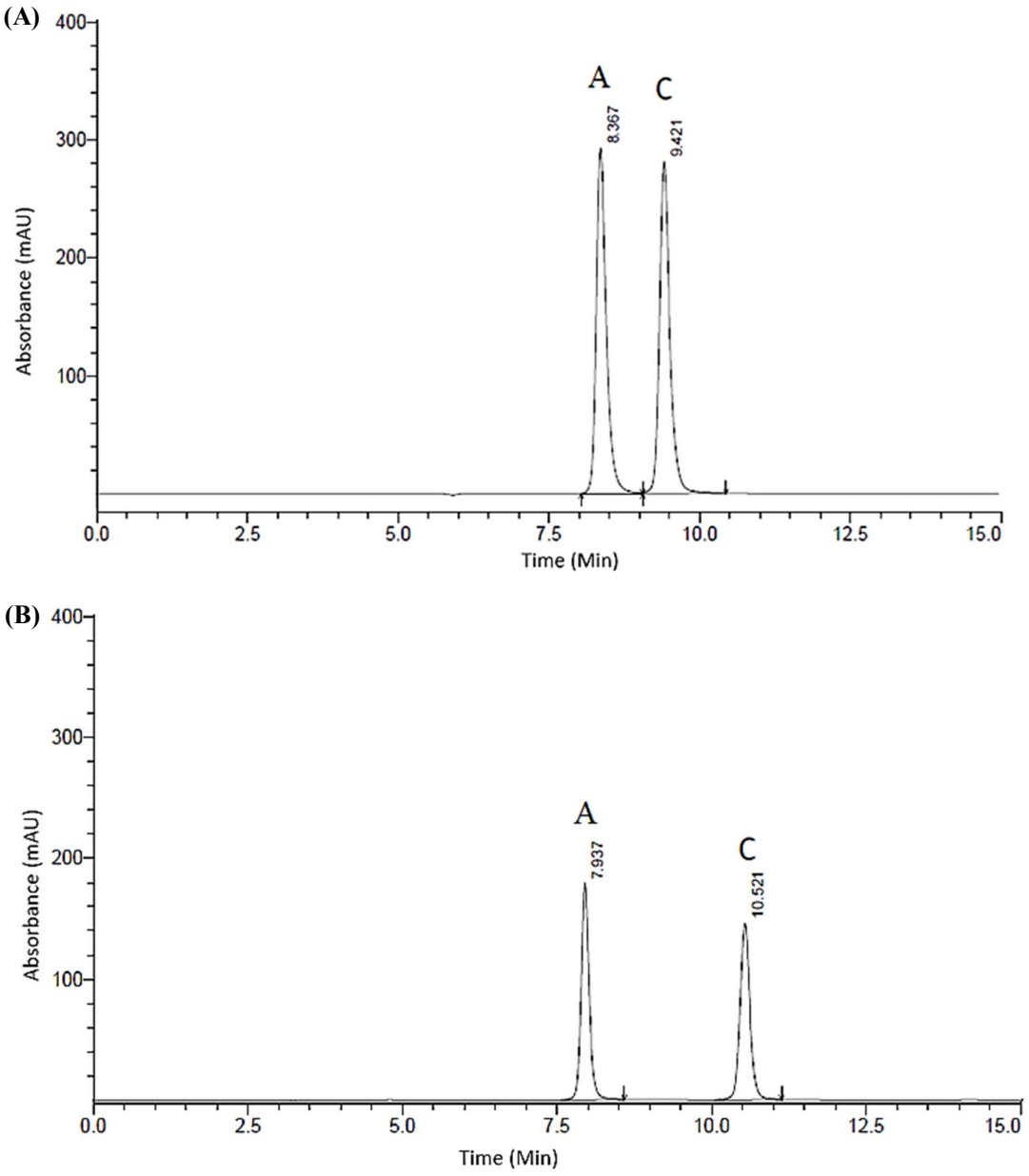

Figure 2 HPLC chromatogram for standard stock solutions of cordycepin (C) and adenosine (A) in different mobile phase conditions: (A) water: methanol (80:20); (B) water: acetonitrile (95:5).

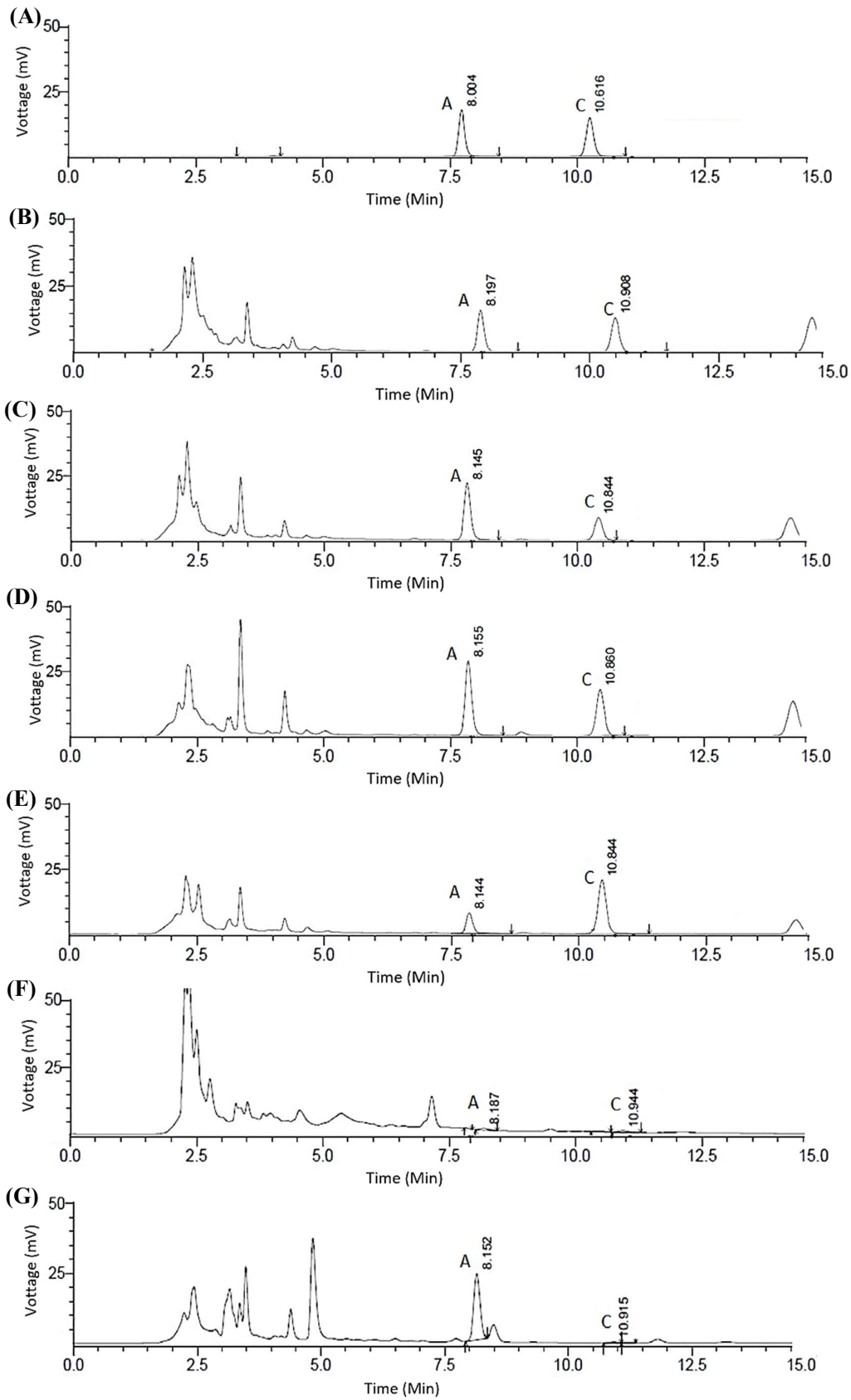

Figure 3 HPLC chromatogram of the mixed standard solutions of cordycepin (C) and adenosine (A); (A) standard, (B-D) dried samples of Cordyceps, and (E-G) dietary supplement products containing Cordyceps.

3.2 Linearity, calibration curves, and sensitivity

The calibration curves for cordycepin and adenosine were linear from 0.05–20 and 0.02–20 μg/mL, respectively. Table 2 shows the individual calibration equations of both analytes from three replicate experiments. The equations of the curves for cordycepin and adenosine, obtained by the least-squares method, were y=34140x+414.03 and y=35089x+47.13, respectively, with r^2 being greater than 0.999. The LOQ of cordycepin and adenosine was deemed to be 0.05 and 0.02 μg/mL, respectively, while the LOD was found to be 0.005 μg/mL for both analytes. Based on the use of HPLC-UV [10], HPLC-DAD [12,13,15], and UPLC-PDA [16], the sensitivity of this developed method was better than the previous reports. Moreover, the LOQ and LOD of the presented analysis were lower than the performance results obtained by Huang et al. [17] and Xie et al. [18] from LC-MS instruments.

Table 2 Linearity of the method for determining the concentrations of cordycepin and adenosine in Cordyceps products (*n*=3).

Analytes	Batch	Slope	Intercept	r^2
Cordycepin	1	33840	-827.43	0.9999
	2	34405	+1982.40	0.9989
	3	34177	+87.15	0.9998
	Mean	34140	+414.03	0.9998
Adenosine	1	34384	-503.05	0.9999
	2	35485	+1602.70	0.9989
	3	35399	-958.26	0.9996
	Mean	35089	+47.14	0.9997

3.3 Accuracy, precision, and recovery

The intra- and inter-day accuracy and precision results for cordycepin and adenosine are summarized in Table 3. In the case of the intra-day assay, the accuracy ranged from 89.67–102.22% while the precision expressed as %RSD for all QC samples was 0.20–4.72%. The inter-day accuracy ranged from 94.67 to 102.90% while the inter-day precision was less than 2.95% for LQC, MQC, and HQC samples analyzed over three consecutive days. These results indicate that the method was reliable and reproducible within the acceptance ranges [20]. The average recoveries of cordycepin and adenosine in three different samples of dried Cordyceps were 99.61 and 101.01%, respectively (Table 4)

Table 3 Accuracy and precision of the method for determining the concentrations of cordycepin and adenosine in Cordyceps products (*n*=3).

Analytes	Known concentration (mg/mL)	Found concentration (Mean±SD; mg/mL)	Accuracy (% Accuracy)	Precision (% RSD)
Cordycepin	Intra-day:			
	0.05 (LQC)	0.045 ± 0.001	89.67	3.15
	1 (MQC)	1.00 ± 0.01	99.99	0.53
	20 (HQC)	20.44 ± 0.05	102.22	0.20
	Inter-day:			
	0.05 (LQC)	0.047 ± 0.001	94.67	2.95
	1 (MQC)	1.02 ± 0.02	102.15	1.63
	20 (HQC)	20.37 ± 0.39	101.86	1.92
Adenosine	Intra-day:			
	0.02 (LQC)	0.019 ± 0.001	95.83	4.72
	1 (MQC)	1.00 ± 0.02	99.92	2.00
	20 (HQC)	20.37 ± 0.11	101.84	0.54
	Inter-day:			
	0.02 (LQC)	0.019 ± 0.001	96.31	2.88
	1 (MQC)	1.03 ± 0.02	102.90	1.78
	20 (HQC)	20.34 ± 0.56	101.71	2.73

Table 4 Recovery of the method for determining cordycepin and adenosine in the different samples of dried Cordyceps (*n*=3).

Analytes	Standard added (mg/mL)	Unspiked samples (mg/mL)	Spiked samples (mg/mL)	Recovery (%)	RSD (%)
Cordycepin	4.00	4.52 ± 0.10	8.50 ± 0.08	99.61 ± 2.75	2.76
Adenosine	4.00	5.19 ± 0.18	9.23 ± 0.24	101.01 ± 4.79	4.74

3.5 Application for the analysis of Cordyceps products

The developed method was used to determine cordycepin and adenosine in Cordyceps products from different commercial preparations and the reports are summarized in Table 5. Based on three representations of dried cultured *C. militarris*, the results show deviations between the nucleoside content in the samples from different sources, corresponding to the results from wild Cordyceps samples reported by Xie et al. [18]. Both concentrations of nucleoside in the samples were uncorrelated. The cordycepin content ranges from the present study were remarkably lower than in previous publications [7,13,16]. However, the concentration of cordycepin in *C. militaris* was around 2–3 μg/g [21]. In contrast, the amounts of adenosine were analogous to those revealed by Ikeda et al. [7], Huang et al. [13], and Yang and Li [16]. Interestingly, among the different dried products, Cordyceps with large heads of stroma (sample C) exhibited higher cordycepin and adenosine content compared to those with long fruiting bodies (samples A and B).

Many HPLC methods have been used to determine the main bioactive nucleosides from *C. sinensis*, *C. militarris*, and *C. jiangxiensis*. Many papers publish similar research results for the determination of adenosine and cordycepin, but collected different sample types for analysis, including natural Cordyceps [8,17,18], cultured Cordyceps [7,10,12–14,16], Cordyceps extracts [7,10], Cordyceps recombinant rice [9], fermented Cordyceps [15], and cooked Cordyceps [11]. In the case of commercial products containing Cordyceps, Chutvirasakul et al. [10] used tablets and capsules as samples for the specificity test, but these were not used in the quantitative analysis. Therefore, no study has yet focused on the analysis of dietary supplement products containing Cordyceps plus other active ingredients by the HPLC method. As a result, other active compounds included in the supplements were not affected by the chromatograms. However, the method developed in this study can be applied to samples containing either *C. sinensis* or *C. militarris*. Although the main compounds in Cordyceps were found at very low concentrations and varied among different sources, the exact dosage of crude Cordyceps used for supplement products is unavailable in the scientific literature. Whereas the administration of pharmaceutical preparations containing cordycepin ranging from 0.01–100 μg/kg body weight per day produces the desired effect in humans [22]. According to the results, the presented content of the main active ingredients was less than the requirements or expectations. Since extraction techniques were used to concentrate the chemicals of interest, there should be more active compounds in the extract than would be the case from dried powder. However, the analyzed samples containing the extracts showed very low cordycepin and adenosine contents. Consequently, formulations with either extract or dried powder influenced the decision to choose the product containing Cordyceps.

Table 5 Cordycepin and adenosine contents in the dried cultured materials and dietary supplement products containing Cordyceps (n=3).

Cordyceps samples	Concentration (mg/mL)		Content (mg/g sample)	
	Cordycepin	Adenosine	Cordycepin	Adenosine
Sample A: dried *C. militaris*	4.16 ± 0.42	4.13 ± 0.34	832	827
Sample B: dried *C. militaris*	3.02 ± 0.14	6.22 ± 0.29	605	1,245
Sample C: dried *C. militaris*	6.50 ± 0.31	8.09 ± 0.34	1,299	1,618
Sample D: Cordyceps capsule (1 capsule contains 250 mg *C. militaris* powder)	7.27 ± 0.04	2.26 ± 0.04	1,454 (727)	452 (226)
Sample E: Cordyceps capsule (1 capsule contains 150 mg *C. sinesis* extract)	0.27 ± 0.03	0.65 ± 0.01	5 (2)	13 (6)
Sample F: Cordyceps solution (65 mL contains 1,000 mg *C. militaris* extract)	0.09 ± 0.01	6.02 ± 0.18	6 (6)	39 (39)

The values in square brackets represent the content in units of mg/dose (mg per 1 capsule).

4. Conclusion

Dietary supplement products containing Cordyceps are not required to undergo the same rigorous testing as pharmaceutical products. However, to ensure quality or when selecting supplements, the content of active ingredients should be analyzed. A simple, accurate, and reliable HPLC-UV method for the quantitation of cordycepin and adenosine in the samples of Cordyceps has been developed in this study and validated according to international guidelines. This method could be applied for quality control as well as the identification of various products containing Cordyceps.

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