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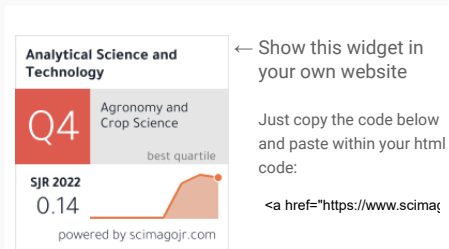
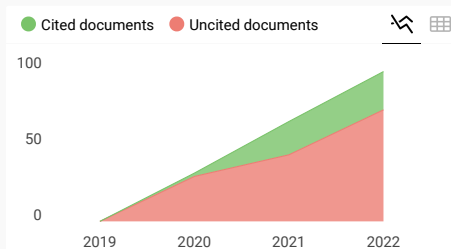
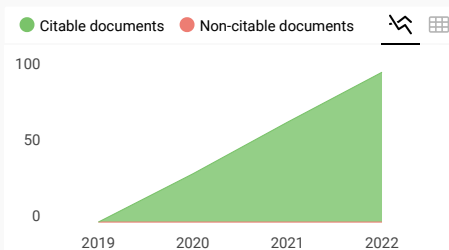
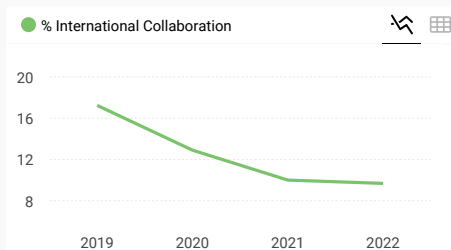
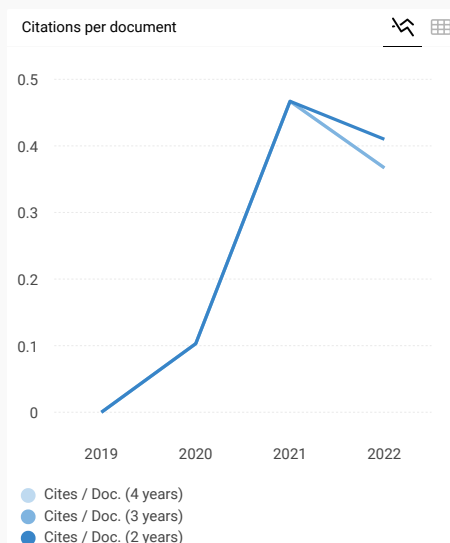
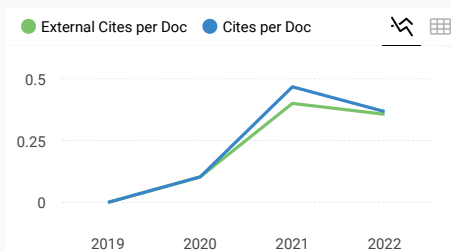
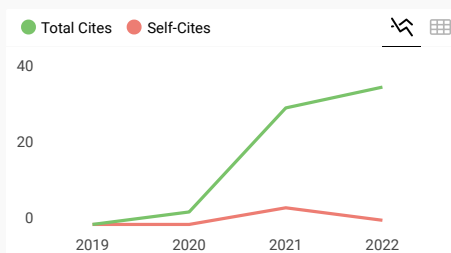
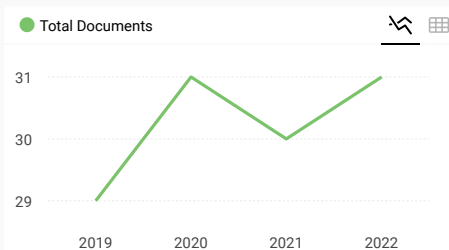
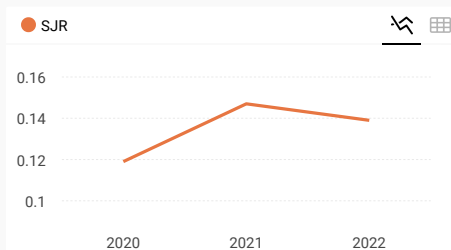
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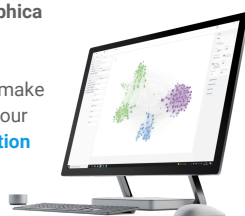
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- 논문제목 : Optimization and Validation of HPLC/DAD Method for the Determination of Adenosine and Cordycepin in Cordyceps Products

- 저자 : Sasikarn Panpraneecharoen; Tisorn Chatrakoon; Sompong Sansenya; Saowapa Chumanee

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Thank you for your contribution to the Journal. If you have any questions, feel free to contact us referring to the last contact address.

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Dear Saowapa Chumanee,

This is Prof. Jong-Ho Park, the deputy EIC of the Analytical Science and Technology (AST).

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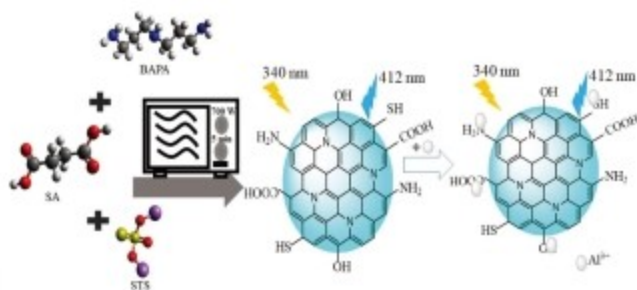
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Optimization and validation of HPLC/DAD method for the determination of adenosine and cordycepin in cordyceps products

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Abstract: Adenosine and cordycepin are bioactive compounds with health benefits. Therefore, both substances are often used to assess the quality of Cordyceps products. Optimization and validation of the HPLC/DAD method for determining two nucleosides were studied. The samples were prepared using an ultrasound-assisted extraction (ultrasonic bath). The result was optimal conditions for aqueous extraction, an extraction time of 35 min, and an extraction temperature of 40 °C. The Chromatographic separation was achieved using a reverse phase column (InfinityLab Poroshell 120 EC-C18, 4.6 × 250 mm, 2.7 μm) at 30 °C with a mobile phase gradient elution of water and methanol at a flow rate of 0.7 mL/min. The eluents were monitored via a diode array detector at 260 nm. Two nucleosides were separated by less than 12 min after injection. The developed method was found to be excellent linear ($r^2 > 0.9999$), accurate (% recovery 95.34-98.51), and precise (% relative standard deviation < 2.0). The limit of detection (LOD) and quantification (LOQ) were 0.45 and 1.38 mg/mL for adenosine and 0.47 and 1.43 mg/mL for cordycepin, respectively. This method was satisfactory for simultaneously quantitating two nucleoside contents, which were used to evaluate Cordyceps products.

Key words: cordyceps, adenosine, cordycepin, ultrasonic bath; HPLC/DAD

1. Introduction

Cordyceps militaris (*C. militaris*) is a herbal mushroom and an entomopathogenic fungus belonging to Clavicipitaceae Ascomycetes. It is a functional food and also has great potential for medicinal use, which contains many bioactive compounds and

nutrients such as adenosine, cordyceps, d-mannitol, polysaccharides, g-aminobutyric acid, protein, vitamins, biominerals, etc.¹⁻³ *C. militaris* has similar pharmacological activities to *Cordyceps Sinensis* (*C. Sinensis*). However, *C. militaris* is much more readily available and less expensive. Furthermore, it revealed that biological activities are an antitumor, immunomo-

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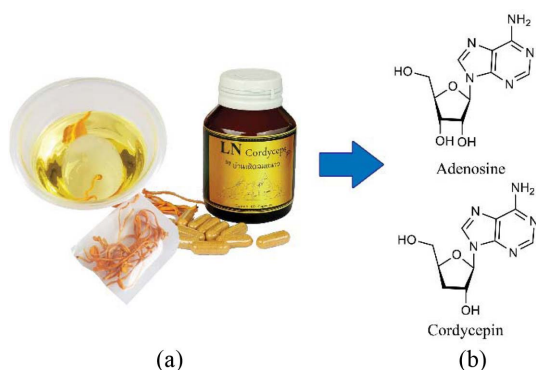


Fig. 1. (a) Cordyceps products from Khao Kho, Phetchabun, Thailand and (b) structures of adenosine and cordycepin.

dulating, antioxidant, and pro-sexual agent.⁴ Since *C. militaris* contains many bioactive compounds and health benefits. Nowadays, *C. militaris* tissue is cultivated to make products such as beverages,⁵ nutritional supplements, and cosmeceuticals.⁶ Fig. 1 shows *C. Militaris* products from Khao Kho, Phetchabun, Thailand and the structure of adenosine and cordycepin. The quality of *C. militaris* is usually evaluated by the contents of adenosine and cordycepin. Adenosine has many pharmacological effects, such as treating chronic heart failure,⁷ the release of neurotransmitters in the central nervous system,⁸ sleep-wake regulation,⁹ and the regulator of blood vessel tone.¹⁰ Cordycepin, also known as 3'-deoxyadenosine, is a nucleoside analogue proven to possess an array of biological activities, including antibacterial, antifungal, antitumor, antileukemia, and antiviral activities, as well as an immunoregulatory effect.^{3,11,12}

High-performance liquid chromatography (HPLC) is a commonly used technique for the analysis of adenosine and cordycepin with detectors such as ultraviolet (UV) or diode array detector (DAD),¹³⁻¹⁶ mass spectrometry (MS)¹⁷ and tandem mass spectrometry (MS/MS).^{18,19} There are two types of ultrasound-assisted extraction (UEA): ultrasonic baths and probe-type devices. It is often used for sample preparation to extract bioactive compounds from plants.²⁰ Ultrasonic bath is more economical and easier to handle, but its low reproducibility and power.²¹ Nevertheless, several researchers successfully used

UEA (ultrasonic bath) to extract adenosine and cordycepin from the Cordyceps.²²⁻²⁵

The aim of this work was to optimize and validate by HPLC/DAD method for the quantitation of adenosine and cordycepin in various Cordyceps products obtained from Small and Medium Enterprises trader (SME), Khaokho, Phetchabun province, Thailand. Moreover, a rapid and simple method for sample preparation was achieved by ultrasound extraction system (bath-type) for routine analysis in the Science Center laboratory, Phetchabun Rajabhat University.

2. Experimental

2.1. Reagents and standard preparation

The standards of adenosine and cordycepin were purchased from ACROS Organics™ (New Jersey, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively, and stored at -20 °C until use. Methanol of HPLC grade was obtained from Carlo Erba (Milan, Italy). Purified water was produced from the arium® pro ultrapure water system (18.2 MΩcm, Sartorius, Germany).

The standard stock solutions (1.0 mg/mL) of adenosine and cordycepin were prepared by dissolving an accurately weighed reference standard in methanol as diluents and stored at 4 °C. Using the stock solution, a series of mixed working standard solutions were prepared (6 levels each), which the concentration ranges were 1.07-50.34 mg/mL of adenosine and 1.06-50.16 mg/mL of cordycepin for calibration curves and filter through a 0.22 mm nylon membrane filter before injection to chromatographic analysis.

2.2. Instrumentation

Analysis by HPLC/DAD was performed with the Agilent 1260 Infinity system (USA) with a quaternary pump VL (G1311C), degasser (G1322A), an auto-sampler (G1329B), thermostatted column compartment (G1316A), and a diode array detector (G1315D). The data processing and evaluation were performed using the OpenLab CDS ChemStation-Edition Software (Rev.C.01.07). An ultrasonic cleaner model WUC-D03H (DAIHAN Scientific, Korea) and the bench-

top centrifuges model V18R (Dynamica Scientific, UK) were used for sample preparation.

2.3. Chromatographic Conditions

The chromatographic conditions were performed using the modified methods of Fu *et al.* and Huang *et al.*^{15,16} The chromatographic separation was achieved with an InfinityLab Poroshell 120 EC-C18 column (150 mm × 4.6 mm, 2.7 μm, Agilent Technologies) maintained at 30 °C and water (A) and methanol (B) were mobile phases. Each mobile phase component was filtered through a 0.22 μm nylon membrane filter and degassed by sonication for 20 minutes before use. The gradient elution was followed: 0-5 min, 10 % B; 5-8 min, 10 %-15 % B; 8-10.5 min, 15-25 % B; 10.5-12 min, 25 % B. The column was equilibrated for 15 minutes before each analysis, and the total analysis time per injection was 27 minutes. Standards and samples were injected at a volume of 5 mL with a flow rate of 0.7 mL/min. The diode array detector (DAD) was set to a wavelength of 260 nm.

2.4. Optimization of ultrasound-assisted extraction

The extraction of analytes from samples was carried out using an ultrasonic cleaning bath with a working frequency of 40 kHz. The inner container was a rectangular temperature-controlled water bath (240 mm × 140 mm × 100 mm). Small and medium enterprises (SME) traders provided samples of products ground into a fine powder (approximately 60 mesh) for analysis in this research. An aliquot of 200 mg of each sample was in a 25 mL volumetric flask. In this study, the effects of extraction variables included methanol concentration (0 %-50 %v/v), extraction time (35-240 min), and temperature (40-65 °C). After extraction, the solutions were centrifuged for 15 min and 25 °C at 10,000 rpm. Finally, the supernatant was filtered through a 0.22 μm nylon membrane, and the filtrate was injected into the chromatographic system for analysis.

2.5. Method validation

The HPLC/DAD method for determining adenosine

and cordycepin was based on analytical parameters: selectivity, linearity and range, the limit of detection (LOD), the limit of quantification (LOQ), precision, and accuracy.^{26,27}

2.5.1. Selectivity

The selectivity of an analytical method was its ability to distinguish between the analyte and the other component in the sample matrix. Therefore, the method was assessed by comparing the chromatograms of the standard solution, sample solution, mobile phase and blank solution. The blank sample was prepared by using boiling water to extract the adenosine and cordycepin from the Cordyceps sample.

2.5.2. Linearity, Limit of Detection and Quantification

The linearity was measured by preparing a serial six concentration of standard mixtures. The concentration ranges were 1.07-50.34 μg/mL (1.07, 3.02, 5.03, 10.68, 30.50, and 50.34) for adenosine and 1.06-50.16 μg/mL (1.06, 3.01, 5.02, 10.64, 30.40, and 50.16) for cordycepin. Three injections from each concentration were analyzed under the same condition. Calibration curves for the different concentrations versus peak area plotted for adenosine and cordycepin and obtained data were subjected to regression analysis using the least square method. The correlation coefficient (r) must not be less than 0.999.

The limits of detection (LOD) and limits of quantification (LOQ) of adenosine and cordycepin were calculated by the following equation according to ICH guideline: $LOD = 3.3s/S$ and $LOQ = 10s/S$ respectively, where s is the standard deviation of the response; can be obtained by the standard deviation of the y-intercept of the regression line ($S_{y/x}$) and S is the slope of the linear regression equation.²⁶

2.5.3. Precision

The precision of the method was determined in intraday (repeatability) and interday (intermediate) studies. The intraday precision was evaluated by analyzing the amount of adenosine and cordycepin in Cordyceps products in six replicates (n=6). Similarly,

the interday precision was established on three consecutive days (n=18). The adenosine and cordycepin content were determined, and the relative standard deviations (RSD) were calculated.

2.5.4. Accuracy

A recovery test determined the method accuracy by spiking a known amount of standard solutions to the blank sample matrix before extraction. The solutions at three concentration levels of 25.17, 95.34, and 190.69 mg/100g were determined for adenosine; and 25.08, 95.00, and 190.00 mg/100 g were determined for cordycepin, respectively. Three samples from each concentration were prepared (n=3) and injected for three days (n=9). The percentage recovery and RSD were calculated for each replicate of the sample.

2.6. Adenosine and Cordycepin quantification in Cordyceps products

Ten samples of Cordyceps products were obtained from Small and medium enterprises (SME), Khaokho, Phetchabun province, Thailand. Physical characteristics were obtained from community enterprise operators for 1-3 as capsule samples and 4-10 as fruiting bodies of *C. militaris* samples. All samples were homogenized into a power and sieve (60 mesh) before analysis. Briefly, it was precisely weighed (about 200 mg) and added to 20 mL water in a 25-volumetric flask. The solution was directly sonicated in the ultrasonic bath for 35 min at 40 °C and a frequency of 40 kHz, and then the mixture was diluted to mark with water. After centrifugation at 25 °C for 10 min at 10,000 rpm, the supernatant was filtered through a 0.22 nylon membrane filter before injection to HPLC. Triplicate analytical samples were prepared for each sample. The quantities of target compounds were calculated by comparing their peak area to those of the standards.

The concentration of analyte in the test sample (mg/100 g) = (concentration of analyte in solution (mg/mL) × volume of sample solution (mL)) / (10 × sample weight (g)). This analytical method was applied to quantify the content of adenosine and cordycepin in samples.

2.7. Statistical analysis

The statistical analysis was done using Microsoft Excel and SPSS software (version 22.0) for windows. All data were analyzed in triplicate, and results were expressed as mean ± standard deviation (SD). Analysis of variance was performed by ANOVA. A significant difference between means was determined by Duncan's multiple range test at a level of $p < 0.05$.

3. Results and Discussion

3.1. Optimize separation condition for adenosine and cordycepin

The present work was to optimize chromatographic conditions for simultaneous separation and quantitation of two nucleosides (adenosine and cordycepin) in Cordyceps products by HPLC/DAD. Initially, this study examined the types of reversed-phase columns suitable for the separation of these two nucleosides: ZORBAX Eclipse Plus C8 (250 mm × 4.6 mm, 5 μm), YMC-Triart C18 (250 mm × 4.6 mm, 5 μm), Inertsil ODS-3 (250 mm × 4.6 mm, 5 μm), ZORBAX Eclipse Plus C18 (150 mm × 4.6 mm, 3.5 μm), ZORBAX Eclipse Plus C18 (100 mm × 4.6 mm, 3.5 μm), and InfinityLab Poroshell 120 EC-C18 (150 mm × 4.6 mm, 2.7 μm). InfinityLab Poroshell 120 EC-C18 (4.6 × 150 mm, 2.7 μm) had the best separation efficiency in our experiments considering the system suitability parameter (Table S1 and Fig. S1-S6). Furthermore, this study used mobile phase systems such as methanol-water and acetonitrile-water. The results showed that the separation was best using methanol and water, according to research by Fu *et al.* and Huang *et al.*^{15,16} The HPLC system in this work required a gradient elution, detailed in section 2.3. Under the optimized chromatographic conditions, elution of the analyte was completed in 12 min, and adenosine and cordycepin retention times were 8.40 and 10.05 min, respectively. The chromatogram of the mixed standard solution of adenosine and cordycepin was shown in Fig. 2(a).

3.2. System suitability

System suitability test (SST) parameters are one of

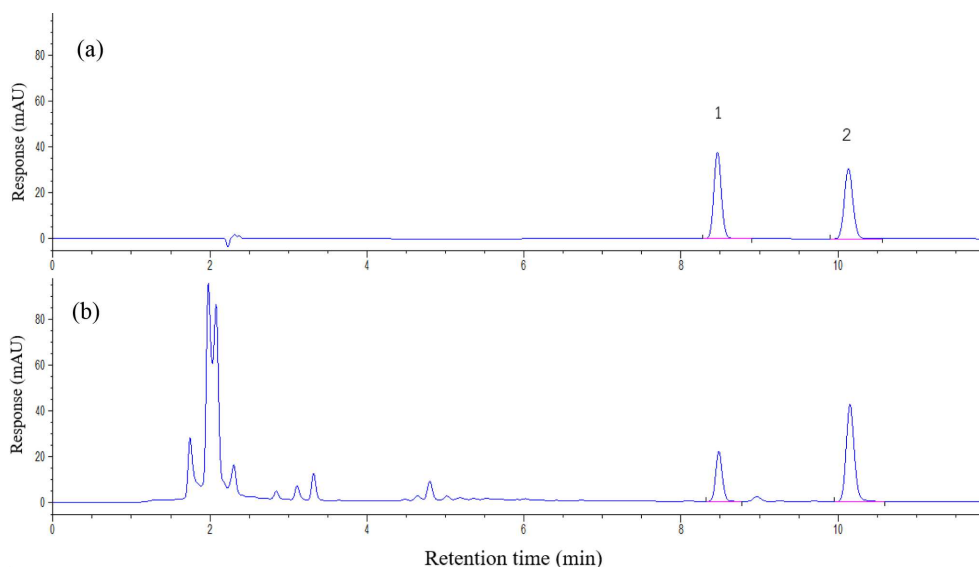


Fig. 2. The chromatograms of (a) mixed standard solution of adenosine (1) and cordycepin (2) at 10 µg/mL, and (b) Cordyceps product solution

Table 1. The suitability parameters of the developed method for adenosine and cordycepin determination (n=10)

Compounds	$R_t \pm SD$ (min)	%RSD of R_t	%RSD of peak area	k'	N	R_s	T
Adenosine	8.40 ± 0.01	0.07	0.54	2.66	114007		1.07
Cordycepin	10.05 ± 0.01	0.06	0.57	3.38	127237	15.59	1.06
Suitability criteria		$\leq 1\%$	$\leq 1\%$	≥ 2	≥ 2000	≥ 2	≤ 2

the most critical and integral parts of HPLC method development and calibration.²⁸ The parameter used in the system suitability testing included retention time (R_t), peak area, capacity factor (k'), number of the theoretical plate (N), resolution (R_s), and tailing factor (T). SST was achieved by injecting ten replicates of mixed standard solutions at 10 µg/mL in ten replicates. Table 1 showed the values of system suitability parameters of adenosine and cordycepin. The results were satisfactory with reasonable specificity. The % relative standard deviation of retention time and peak area ($\leq 1\%$), capacity factor and resolution (≥ 2), theoretical plate (≥ 2000) and tailing factor (≤ 2) were within suitability criteria.

3.3. Obtaining optimal extraction conditions

The determination of optimal conditions for adenosine and cordycepin extraction in Cordyceps

products was modified or adapted from the research of Song *et al.*, Wang *et al.*, and Zhang *et al.*^{22,23,29} The study included three factors: solvent, extraction time and extraction temperature. The initial (pre-experiment) in the solvent study (data not shown) contained water, methanol, and ethanol. Although the extraction of ethanol showed the best of value²² but the peaks were asymmetrical or not sharp peaks when the extracts were analyzed by HPLC/DAD chromatogram technique. In the methanol concentration test (0-50 %) in the extraction found that the percentage of methanol increased, the adenosine content was decreased, but the cordycepin content had no effect, as shown in Fig. 3(a). In this research, water was chosen as a good solvent for extraction, according to the result of Zhang *et al.*²⁹ The effect of ultrasonic time (35-240 min) showed that the longer the extraction time. The adenosine content decreased, while

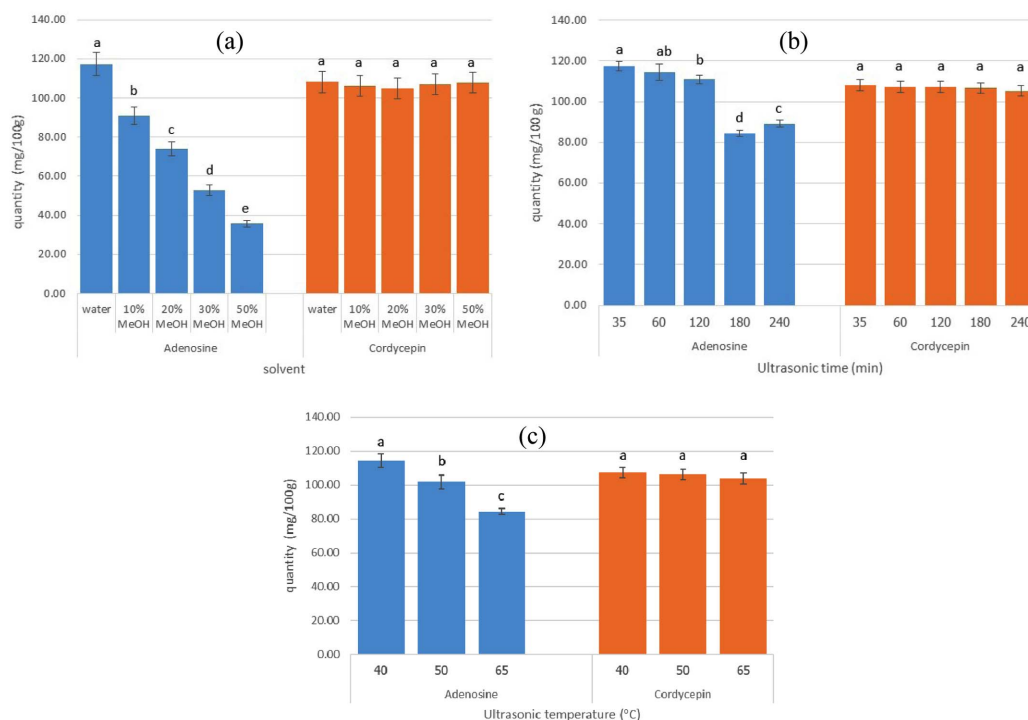


Fig. 3. Effects of three-factor on adenosine and cordycepin contents (mg/100 g) of (a) methanol concentration 0-50 %, (b) ultrasonication time 35-240 min, (c) ultrasonication temperature 40-65 °C. The same letters in the figures indicate no significant differences at the 0.05 level (Duncan, $p > 0.05$)

Table 2. Regression equations, correlation coefficient (r), the limit of detection (LOD), and the limit of quantitation (LOQ)

Compound name	Linear range (µg/mL)	Regression equation	Correlation coefficient (r)	LOD (µg/mL)	LOQ (µg/mL)
Adenosine	1.07-50.34	$y=23.13x+0.57$	1.00	0.45	1.38
Cordycepin	1.06-50.16	$y=21.73x+1.25$	1.00	0.47	1.43

cordycepin had no effect, as shown in Fig. 3(b). So, the optimum extraction time was 35 min and the effect of ultrasonic temperature (40-65 °C). A higher temperature was affected to adenosine content but not affected to cordycepin content, as shown in Fig. 3(c). Therefore, the optimum temperature was 40 °C for extraction.

3.4. Selectivity

Selectivity was evaluated by the peak purity test using a DAD detector. According to the results obtained, peak purity values were higher than 0.9990. Also, no interferences were detected at the retention time of the analyte in the sample solution, which shows that

the developed method was selective (Fig. 2).

3.5. Linearity, the limit of detection and the limit of quantification

The linear relationships were established between concentrations and standard solutions peak areas. As shown in Table 2, correlation coefficient ($r > 0.9999$) values indicated a good correlation between the concentrations and peak areas within the linear ranges. The LOD and LOQ of the method ranged from 0.45 to 0.47 and 1.38 to 1.43 µg/mL, respectively.

3.6. Precision

The % relative standard deviation of intraday and

Table 3. Results for intraday and interday precision data

Compound	Intraday (n=6),						Interday %RSD ^b (n=18)
	Day1 ^a	%RSD ^b	Day2 ^a	%RSD ^b	Day3 ^a	%RSD ^b	
Adenosine	35.00±0.47	1.33	35.07±0.31	0.89	35.03±0.50	1.42	1.16
Cordycepin	155.40±0.33	0.21	155.91±0.29	0.19	155.11±0.32	0.21	0.29

^aAverage ± standard deviation (n=6), value expresses in mg/100 g of sample. ^b% Relative standard deviation

Table 4. The accuracy studies of adenosine and cordycepin

Compound	Amount added (mg/100 g)	Amount found (mg/100 g) Mean ± SD (n=9)	Mean Recovery (%) ± %RSD
Adenosine	25.17	24.29±0.14	96.51±0.59
	95.34	93.92±0.65	98.51±0.70
	190.69	185.13±0.49	97.09±0.26
Cordycepin	25.08	23.91±0.13	95.34±0.55
	95.00	91.83±0.17	96.66±0.19
	190.00	184.00±0.67	96.84±0.37

intraday accuracy was calculated, and the results were summarized in Table 3. The data obtained were less than 2 % for adenosine and 1 % for cordycepin. The results of the method showed high precision with an acceptable range (%RSD < 2).

3.6. Accuracy

The mean recovery of adenosine and cordycepin at different concentrations ranged from 96.51-98.51 % and 95.34-96.84 %, respectively. The accuracy method was shown as the recovery percentages for all compounds in the acceptable range of 92-105 %.³⁰ % Recovery and RSD data was shown in Table 4. The results revealed that the present method was accurate, reliable, and reproducible.

3.7. Quantification of adenosine and cordycepin in cordyceps products

After optimization and validation, the HPLC/DAD method was successfully applied to determine adenosine and cordycepin in ten Cordyceps products. There was no interference in the separation of two nucleosides in the sample. Furthermore, peak identification for the investigated compounds was performed by comparing their retention time. The chromatogram of the Cordyceps product is shown in Fig. 1(b). The quantitative results of the analysis are summarized

Table 5. Adenosine and cordycepin content in different cordyceps products

No.	Substance content (mg/100g)	
	Adenosine	Cordycepin
Sample1	48.94±0.29 ^g	134.43±0.32 ^e
Sample2	169.41±0.19 ^a	96.46±0.21 ^h
Sample3	61.07±0.21 ^f	125.51±0.11 ^f
Sample4	101.60±2.41 ^d	173.70±0.21 ^b
Sample5	27.77±0.25 ^h	182.21±0.21 ^a
Sample6	18.60±0.83 ⁱ	165.85±0.43 ^c
Sample7	64.64±0.50 ^e	153.75±0.41 ^d
Sample8	59.16±0.45 ^f	110.43±0.28 ^g
Sample9	125.53±1.95 ^c	62.14±0.03 ⁱ
Sample10	133.79±1.76 ^b	48.14±0.16 ^j

Note: Values are means ± standard deviation (n=3). Values in the same column with the same superscript letters are not a significant difference (Duncan, $p > 0.05$)

in Table 5. The results concluded that the ten samples were analyzed the adenosine content in the range of 18.60-169.41 mg/100 g and the adenosine content in samples 3 and 8 was not significantly different. The range of 48.14-182.21 mg/100 g of cordycepin content in every sample significantly differed (Duncan, $p > 0.05$). The adenosine and cordycepin in this study were compared with Guo *et al.*³ The results showed that the adenosine and cordycepin were not different. The period of research found 113.6 mg/100 g of adenosine content and 74.1 mg/100 g of cordycepin

content. However, the adenosine and cordycepin contents depended on food for tissue culture.

4. Conclusions

The proposed HPLC/DAD method to separate and determine adenosine and cordycepin content in Cordyceps products. An ultrasound extraction system (bath-type) was used to extract adenosine and cordycepin from Cordyceps products using water as the solvent. The extraction time was 35 min, and the temperature was 40 °C. The method validation was evaluated and it was found that the proposed method was precise, accurate, sensitive, and selective with good linearity. Furthermore, the results demonstrated that this method could be readily used for routine analysis of Cordyceps products. Finally, qualitative and quantitative analysis was applied for the quality investigation of Cordyceps products.

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