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ENHANCED ADENOSINE AND CORDYCEPIN EXTRACTION FROM *CORDYCEPS MILITARIS*: OPTIMIZATION VIA ULTRASOUND-ASSISTED EXTRACTION AND RESPONSE SURFACE METHODOLOGY

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Abstract: This article describes a comprehensive and detailed study on the simultaneous extraction process of two important compounds, Adenosine and Cordycepin, from *Cordyceps militaris* powder with the aid of ultrasound. The Plackett-Burman model was used to screen for univariate factors affecting the analyte content in the extract. Subsequently, the optimization process using Response Surface Methodology (RSM) with the Box-Behnken design was employed to establish an optimal extraction procedure. Accordingly, the best extraction conditions for both Adenosine and Cordycepin were determined to be a water ratio of 100:0 as solvent, an extraction temperature of 45 °C, ultrasound power of 300W, material ratio of 1:20, and ultrasound duration of 30 min. The contents of Adenosine and Cordycepin determined by HPLC-DAD under optimal conditions were 2508 ± 65 mg/kg and 2454 ± 37 mg/kg, respectively.

***Keywords*:** *Cordyceps millitaris*, extraction, Adenosine, Cordycepin

Introduction

Cordyceps militaris (CM), also known as caterpillar fungus or "Dong chong xia cao," is a parasitic fungus (*Ophiocordyceps sinensis* (Berk.)) that infects the larvae of Thitarodes moths.[1] Wild CM (primarily *C. sinensis*) is highly valued in traditional medicine for its pharmacological properties and is considered one of the most expensive medicinal fungi due to the challenges in harvesting.[2] However, over-harvesting and illegal exploitation have led to environmental damage and threaten the species with extinction.[3] To address these issues, researchers have focused on cultivating CM under controlled conditions, employing sustainable cultivation and resource management techniques. Cultivated CM (*C. militaris*) exhibits similar bioactive compounds to its wild counterpart, with some studies even suggesting higher

concentrations of certain bioactive compounds in cultivated CM compared to *C. sinensis*. Consequently, large-scale cultivation of CM has become increasingly prevalent in recent years.[4]

CM contains various bioactive compounds, including polysaccharides, polyphenols, γ-aminobutyric acid (GABA), and ergothioneine, among others.[5] Among these, adenosine (ADE) and cordycepin (COR) are considered the most important bioactive compounds contributing to the medicinal value of CM.[6] COR is a nucleoside analogue of adenosine, consisting of a purine base linked to a ribofuranose sugar via a β-N9-glycosidic bond; it is also known as 3'-deoxyadenosine and is one of the four nucleosides found in RNA.[7] ADE is a purine nucleoside composed of adenine linked to a ribose sugar through a β-N9-glycosidic bond.[8] Modern medicine attributes various significant pharmacological properties to CM, including: antioxidant activity, attributed to its high concentration of antioxidants that protect cells from free radical damage; anticancer properties, with several studies suggesting that CM can inhibit cancer cell growth and enhance the efficacy of cancer therapies;[9] anti-inflammatory effects,[10] and pulmonary fibrosis alleviation, as CM helps dilate the bronchi and increase airflow, improving respiratory function and treating pulmonary diseases;[11] and immunostimulatory effects.[12]

Numerous studies have explored the extraction of bioactive compounds from CM. For example, in 2017, Hsiao, F. S. H et al. used hot water extraction to obtain bioactive compounds from the fruiting bodies of *C. militaris* and investigated their anti-inflammatory activity, suggesting that the extract could serve as a potential novel feed additive for immunomodulation in farm animals.[13] Organic solvents such as ethanol, methanol, acetone, and ethyl acetate have also been widely used, as seen in studies by H.C. Nguyen in 2019,[14] Pintathong et al. in 2021 on by-products from cultivated *Cordyceps*,[20]

and Sornchaithawatwong, Chayanid et al. in 2022, who used both ethanol and water to extract bioactive compounds from *C. militaris.*[15,21] Other methods include supercritical CO_2 extraction,[16] microwave-assisted extraction,[17,23] ultrasound-assisted extraction.[18] Overall, efficient extraction methods for valuable bioactive compounds from CM are continually being developed and investigated.

To date, comprehensive studies on the simultaneous extraction of ADE and COR using ultrasound-assisted extraction and a thorough investigation of the effects of multiple variables are lacking. Therefore, this study conducts a comprehensive analysis of the effects of individual factors and employs response surface methodology to determine the combined effects of these factors on the simultaneous extraction of ADE and COR.

Materials and methods

Chemicals

All chemicals used were of analytical grade. Adenosine and cordycepin reference standards were purchased from TRC (Ontario, Canada). HPLC-grade methanol and acetonitrile, and deionized water were obtained from J.T. Baker (Phillipsburg, USA). Formic acid (FA) was supplied by Sigma-Aldrich (Taufkirchen, Germany). *Cordyceps militaris* was provided by Thien An Cordyceps Co., Ltd. (Go Cong Tay, Tien Giang, Vietnam).

Sample preparation

Sample collection

Cordyceps militaris fruiting bodies were cultivated by Thien An Cordyceps Co., Ltd. After harvesting, samples were freeze-dried, ground into a fine powder, and stored at 4 °C until use.

Extraction procedure

Approximately 1.0 g of homogenized *C. militaris* powder was accurately weighed into a 50 mL centrifuge tube and 20 mL of deionized water was added. The mixture was thoroughly shaken and sonicated using an ultrasonic bath (Elma S300H, 500 W, 37 kHz, Germany) at 300 W and 45 °C for 30 minutes. Subsequently, the extract was centrifuged for 5 min at 4000 rpm, the resulted supernatant was filtered through a 0.45 μm membrane filter, and diluted 20-fold prior to HPLC-DAD analysis to determine the concentrations of ADE and COR.

Quantification of ADE and COR

ADE and COR concentrations were quantified using an HPLC-DAD system (Agilent 1260) equipped with a DAD detector at a wavelength of 260 nm, following a previously published method.[27] Chromatographic conditions included a C18 column (ZORBAX Eclipse XDB-C18, Agilent) (250 mm × 4.6 mm × 5 μm), a mobile phase consisting of water: methanol (85:15), a flow rate of 1.0 mL/min, and an injection volume of 25 μL.

Single-factor experiments to investigate the effects on ADE and COR yields

Preliminary screening of solvent ratio (water: ethanol)

The extraction procedure (as described in section 2.2.1) was performed with varying ethanol: water ratios (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, and 0:100). ADE and COR concentrations were determined using HPLC-DAD to select the optimal solvent ratio.

Investigation of solid-to-solvent ratio

Following the selection of the optimal solvent ratio, the impact of the solid-to-solvent ratio was investigated using ratios of 1:10, 1:20, 1:30, and 1:40. Extracts were filtered through a 0.45 μm membrane filter, diluted, and

analyzed via HPLC-DAD (each experiment was performed in triplicate). The optimal solid-to-solvent ratio was determined based on ADE and COR yields.

Effect of extraction temperature

After determining the optimal solvent and solid-to-solvent ratios, the effect of extraction temperature on ADE and COR yields was examined at temperatures of 15 °C, 30 °C, 45 °C, 60 °C, and 75 °C, using the extraction procedure described in section 2.2.1. Extracts were centrifuged, filtered (0.45 μm), diluted, and analyzed using HPLC-DAD (triplicate measurements).

Effect of ultrasonic power

The impact of ultrasonic power on extraction efficiency was assessed at power levels of 100 W, 200 W, 300 W, and 400 W. Other parameters were maintained at the previously selected levels. Extracts were centrifuged, filtered (0.45 μm), diluted, and analyzed by HPLC-DAD (triplicate measurements).

Effect of number of extractions

Approximately 1.0 g of homogenized *C. militaris* powder was weighed into three 50 mL centrifuge tubes, with 20 mL of water added to each. Each tube underwent the same extraction process: shaking, sonication (at 45 °C and 300 W), and 30-minute sonication for one, two, or three extractions. Extracts were centrifuged, filtered (0.45 μm), diluted, and analyzed using HPLC-DAD with triplicate measurements.

Optimization of the extraction process

Screening of factors using Plackett-Burman design

In experimental processes, numerous factors may influence outcomes, but only some have significant impacts. Key factors, which greatly affect

results, are prioritized to achieve an efficient model. Thus, the first step in optimization is identifying these critical factors. The Plackett-Burman design is a widely used method for screening important factors in multi-variable experiments. It is particularly effective in initial stages, enabling rapid identification of significant factors with minimal experimental runs.[19] The number of experimental runs (N) is a multiple of 4, where N is close to the number of factors (k) being investigated, allowing for a maximum of k = N - 1 factors to be screened.

This design primarily estimates the main effects of each factor. Instead of testing a wide range of values for all factors, the Plackett-Burman design reduces the number of experiments by testing each factor at two levels: high (+) and low (-), arranged in a cyclical pattern. Collected data is statistically analyzed to determine the influence of each variable on the response, experimental error, and the degree of influence of each variable on the response. The magnitude of a variable's influence is reflected in the difference between the average response at the high and low levels. Statistical tests are then used to determine the significance of each variable's effect. The Plackett-Burman design for screening the factors was based on the results of the preliminary single-factor experiments. The ranges for each condition were derived from previous studies, aiming to identify the most influential factors affecting the extraction efficiency of ADE and COR among the five factors investigated.[20]

Following the single-factor experiments, a Plackett-Burman design was employed to screen for the most influential factors affecting ADE and COR extraction yields. Each factor was assessed at two levels: low (-1) and high (+1). The factors included: A (Ethanol: water ratio), B (solid-to-solvent ratio), C (number of extractions), D (temperature), and E (ultrasonic power). Each experimental run was performed in triplicate, and ADE and COR concentrations were used as response variables. Data were analyzed using

Minitab 16.1 (free version) to identify the key factors influencing the target compound yields.

Optimization of the extraction process using Box-Behnken design

The Box-Behnken design (BBD) is a three-level fractional experimental design developed by Box and Behnken. It combines elements of a two-level full factorial design and an incomplete block design. In each block, some factors are investigated at all combinations of the full factorial design, while others are held at the center level.[20] BBD positions experimental runs at the center points of the edges and the center of the design space, visualized as a polyhedron, and excludes corner points of the experimental region. This exclusion is beneficial when corner points are impractical or costly to implement, saving time and resources while ensuring comprehensive coverage. BBD typically includes several center points, allowing for the evaluation of factor effects at intermediate levels and improving model accuracy.[21] Compared to the Central Composite Design (CCD), BBD is more economical as it involves fewer levels for each factor and avoids extreme maximum or minimum levels.

Based on the results of the Plackett-Burman screening, a BBD was employed to determine the optimal conditions for ADE and COR extraction. This model aimed to optimize extraction efficiency, yield, and product quality. Data analysis was performed using Design Expert software, version 13.0 (Stat-Ease Inc., Minneapolis, Minnesota, USA).

Results and discussion

Method validation for the quantification of ADE and COR

Initially, the method for quantifying ADE and COR in the extracts was validated. Figure 1 shows the retention times of ADE and COR as 6.08 and 7.04 min, respectively. This separation allows for the independent and accurate quantification of each analyte without interference from other

components. The difference in retention times between the two compounds can be attributed to the presence of an additional hydroxyl (-OH) group in the ADE structure compared to COR. This -OH group interacts less strongly with the stationary phase (C18 column), resulting in faster elution of ADE compared to COR, this consistent with previous work.[22]

Calibration curves were constructed for both ADE and COR (Figure 2). Within the concentration range of 0.2 to 20 mg/L, a high degree of linearity was observed between peak area and analyte concentration, with highest R^2 values. Recovery (R %) ranged from 97.9 % to 106.3 % for ADE and from 94.3 % to 108.2 % for COR, demonstrating the high accuracy of the method. Furthermore, the limits of detection (LOD) were determined to be 0.16 mg/L for ADE and 0.2 mg/L for COR, while the limits of quantification (LOQ) were 0.5 mg/L for ADE and 0.70 mg/L for COR, indicating the high sensitivity of the analytical method. These results confirm the suitability of the HPLC-DAD method for the quantitative analysis of ADE and COR in the extracts.

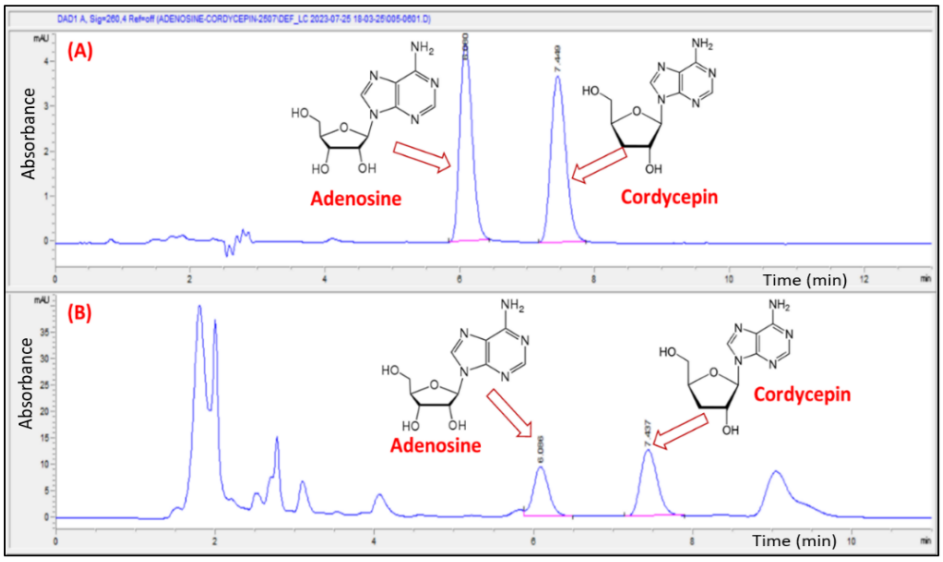

Figure 1. Representative HPLC-DAD chromatograms of (A) the standard solution at a concentration of 20 mg/L and (B) the sample extract.

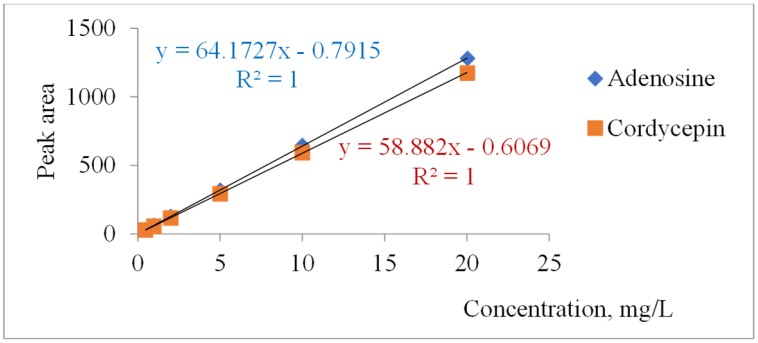

Figure 2. Calibration curve of ADE and COR.

Effects of single-factor variables on ADE and COR yields

Effect of ethanol: water ratio

The ethanol-to-water ratio plays a crucial role in the extraction efficiency of ADE and COR. As illustrated in Figure 3A, water content significantly affects the extraction efficiency of ADE. Notably, ADE yield is highly dependent on the ethanol-to-water ratio, achieving its maximum at 100 % water content, whereas the COR yield remains unchanged regardless of variations in the water-to-ethanol ratio. This discrepancy can be attributed to the distinct solubilities of ADE and COR in water and ethanol. COR demonstrates good solubility in both solvents, rendering its extraction yield independent of the solvent ratio. At room temperature (25 °C), the solubility of COR in deionized water is approximately 4.0 mg/mL, increasing to 6.0 mg/mL at 60 °C. In 95 % (v/v) ethanol, the solubility of COR is 1.8 mg/mL and 2.5 mg/mL at 25 °C and 60 °C, respectively.

Conversely, ADE exhibits poor solubility in ethanol. Notably, ADE is insoluble in anhydrous ethanol. The solubility of ADE in deionized water is 5.1 mg/mL at 25 °C and increase with tempeture.[23] This explains the significant increase in ADE extraction yield with increasing water content. The findings for COR are consistent with those reported by Thanh et al., but

the results for ADE diverge markedly. Specifically, Thanh observed that as the ethanol concentration increased from 40 % to 80 %, the ADE yield rose correspondingly from 343.6 ± 14.4 to 423.9 ± 17.8 μg/g.[24] Based on these results, water was chosen as the solvent for subsequent experiments.

Effect of solid-to-solvent ratio

The influence of the solid-to-solvent ratio on ADE and COR extraction efficiency is illustrated in Figure 3B. Increasing the solvent ratio from 1:10 to 1:20 resulted in a significant increase in ADE and COR yields. This is likely because the increased solvent volume enhances the dissolution of these compounds from the solid material.[25] Further increasing the solvent ratio to 1:40 did not lead to significant changes in ADE and COR yields, suggesting that a saturation point had been reached where the solvent had dissolved the maximum possible amount of ADE and COR. Based on these findings, a solid-to-solvent ratio of 1:20 was selected for further experiments.

Effect of extraction temperature

Increasing the temperature during extraction generally improves extraction yield by enhancing the solubility of extractable compounds in the solvent and increasing the diffusion rate. However, excessively high temperatures can lead to decomposition of the extracted compounds, potentially decreasing the yield.[26] Figure 3C shows that increasing the extraction temperature from 15 °C to 45 °C led to increased ADE and COR yields, reaching a maximum at 45 °C. However, further increasing the temperature to 60 °C resulted in a significant decrease in ADE yield, while the decrease in COR yield was slightly less pronounced at 75 °C. This highlights the crucial role of extraction temperature in ADE and COR extraction efficiency, particularly for ADE. This could be because increased temperature enhances the solubility of various compounds in the solvent,

including ADE and COR. Additionally, higher temperatures increase the diffusion rate of molecules, facilitating faster movement within the extraction solvent, improving contact between the solvent and the material and thus enhancing the extraction of more bioactive compounds in less time consuming.[27] Therefore, within the temperature range of 15 °C to 45 °C, enhanced solubility of these compounds increases the extraction yield. However, at excessively high temperatures (above 60 °C), ADE may undergo partial degradation, or the -OH group may participate in redox reactions, forming by-products and thus reducing the yield. While COR is also affected by high temperatures, the impact is less significant compared to ADE. Previous studies by some authors also showed that temperature also affects the ADE and COR content in the extract.[24,28] Based on these results, 45 °C was selected as the extraction temperature for subsequent experiments.

Effect of ultrasonic power

Ultrasonic energy is vital for extraction and homogenization processes, as it disrupts plant cell membranes and breaks hydrogen bonds and Van der Waals interactions, facilitating the release of bioactive compounds.[29–31] Figure 3D illustrates the influence of ultrasonic power on extraction efficiency, showing that increasing the power from 100 W to 400 W enhanced the yields of ADE and COR, with the maximum yield achieved at 300 W. This improvement is attributed to the increased energy from ultrasound, which enhances cell disruption and expands the solvent-sample contact area, optimizing the extraction of bioactive compounds. Additionally, high-intensity ultrasound promotes the dissolution of ADE, COR, and other bioactive compounds in the solvent. Therefore, a value of 300 W was selected as the optimal ultrasonic power for subsequent experiments.

Furthermore, ADE, COR, and other bioactive compounds are more readily dissolved in the solvent under the action of high-intensity

ultrasound.[32] Therefore, an ultrasonic power of 300 W was selected as the optimal power level for subsequent experiments.

Effect of number of extractions

This study found that the number of extractions did not significantly affect the extraction efficiency of ADE and COR. The maximum yields of ADE and COR were achieved after the first extraction. This result aligns with previous findings reporting that ultrasound-assisted water extraction of ADE and COR from *Cordyceps* achieved maximum extraction efficiency after a single extraction. However, other studies using Soxhlet extraction with 50 % ethanol from insect pupae showed that extraction yields increased with the number of extractions, reaching a maximum after three extractions.[1] Based on our experimental results, a single extraction was chosen to minimize time and cost, and to facilitate scalability for potential large-scale applications.

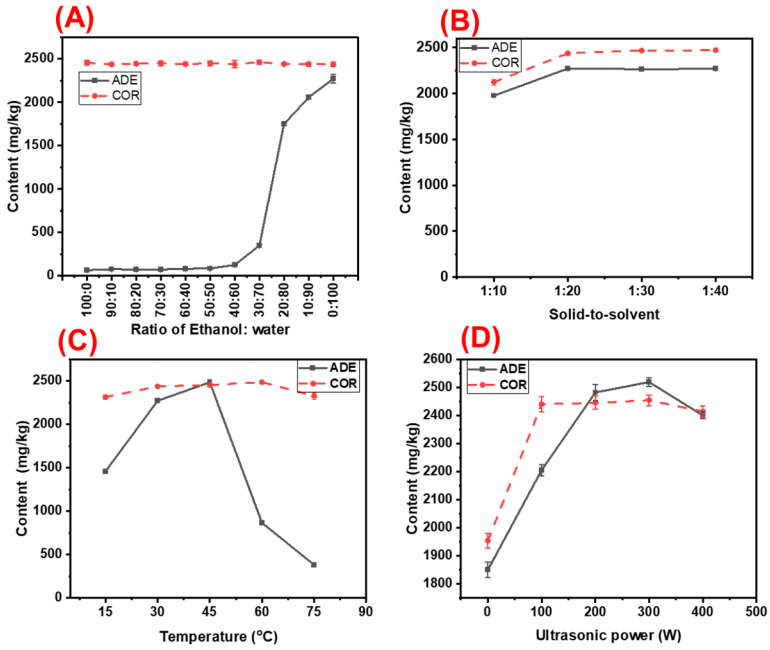

Figure 3. Effects of single-factor variables on ADE and COR yields in various extraction conditions **(A)** Effect of ethanol: water ratio, **(B)** Effect of solid-to-solvent ratio, **(C)** Effect of extraction temperature, **(D)** Effect of ultrasonic power.

Optimization of the extraction process

Results of Plackett-Burman design

The single-factor experiments indicated that COR yield remained relatively constant. Therefore, the focus shifted to optimizing ADE yield. Table 1 presents the results obtained from 12 experiments using the Plackett-Burman design. The data reveal that temperature (D) and alcohol: water ratio (A) significantly affected ($P < 0.01$) ADE extraction efficiency. Ultrasonic power (E) also had a significant effect ($P < 0.05$). In contrast, the solid-to-solvent ratio (B) and the number of extractions (C) showed no significant effect ($P > 0.05$) on ADE extraction efficiency.

Optimization using Box-Behnken design

Based on the Plackett-Burman screening, the three most influential factors affecting ADE extraction efficiency were selected for optimization using a Box-Behnken design (BBD). In this BBD, three levels (-1, 0, +1) were assigned to each factor: X_1 (ethanol: water ratio), X_2 (temperature), and X_3 (ultrasonic power) (Table 2). The remaining factors, solid-to-solvent ratio and number of extractions, were fixed at 1:20 (g/mL) and one extraction, respectively.

The model's suitability was assessed based on the following criteria: (1) p-value < 0.0001, indicating a good fit between the experimental data and the model; (2) adequate precision (AP) > 4.0, indicating an acceptable model prediction capability; (3) lack-of-fit (LOF) > 0.05, indicating that the lack of fit is not statistically significant; and (4) $R^2 > 0.81$, indicating a strong correlation between the model and the experimental data. The optimized model for ADE extraction met all these criteria (p-value < 0.001, AP = 48.71, LOF = 0.2009, and $R^2 = 0.9965$). The adjusted R^2 (0.9920) is close to 1, indicating a strong correlation between the model and experimental data.

Table 1. Experimental design matrix and results according to the Plackett-Burman design.

Run number	Independent variables					ADE	COR
	A	B	C	D	E	Yield (mg/kg) ± SD	Yield (mg/kg) ± SD
1	1	1	1	-1	1	2121 ± 46	2461 ± 20
2	1	1	-1	1	-1	1061 ± 44	2469 ± 27
3	1	-1	1	1	-1	1193 ± 17	2467 ± 34
4	-1	1	1	-1	1	1740 ± 16	2452 ± 55
5	-1	-1	-1	-1	-1	2254 ± 46	2467 ± 70
6	-1	-1	-1	1	1	545 ± 29	2441 ± 17
7	1	-1	-1	-1	1	2093 ± 54	2444 ± 24
8	-1	1	-1	-1	-1	2170 ± 36	2446 ± 45
9	-1	-1	1	1	1	452 ± 9.0	2442 ± 23
10	1	1	-1	1	1	874 ± 17	2443 ± 47
11	-1	1	1	1	-1	418 ± 16	2458 ± 29
12	1	-1	1	-1	-1	2250 ± 20	2473 ± 45

The similarity between R^2 and adjusted R^2 demonstrates the model's predictive ability. The predicted R^2 (0.9615) is within 0.2 of the other R^2 values, confirming that the experimentally obtained ADE yields closely match the model's predictions. Furthermore, the F-value (220.44) and the p-value (<0.0001) along with the lack-of-fit (0.2009) demonstrate the excellent fit of the model and its high reliability. All factors had significant impacts on ADE extraction efficiency: water:alcohol ratio ($p < 0.0001$), temperature ($p < 0.0001$), and ultrasonic power ($p = 0.0231$). The equation describes for optimization as follows:

$$ADE\ content\ (mg/kg) = 2{,}413.6 + 419.75 \times X_1 - 636.125 \times X_2 + -67.125 \times X_3 + 350.75 \times X_1X_2 + 238.75 \times X_1X_3 - 182.5 \times X_2X_3 + -228.3 \times X_1^2 - 727.05 \times X_2^2 - 276.55 \times X_3^2$$

Table 2. Box-Behnken design matrix and ADE extraction results.

	Independent Variables			Response Variable (ADE content)	
Run Number	**X_1 Ratio**	**X_2 ^oC**	**X_3 W**	**Observed Mean ± SD, mg/kg**	**Predicted values Mean, mg/kg**
1	100	30	200	2106 ±54	2163
2	90	45	200	2328 ±33	2414
3	100	45	300	2499 ±66	2500
4	90	45	200	2407 ±41	2414
5	90	45	200	2437 ±29	2414
6	80	45	100	1796 ±24	1795
7	80	30	200	2022 ±51	2025
8	80	60	200	109 ±4.0	51.6
9	100	45	100	2212 ±31	2157
10	90	60	100	965 ±27	1024
11	90	45	200	2459 ±48	2414
12	80	45	300	1128 ±20	1183
13	100	60	200	1596 ±34	1593
14	90	60	300	522 ±8.0	524
15	90	45	200	2437 ±33	2414
16	90	30	300	2220 ±50	2162
17	90	30	100	1933 ±47	1931

Table 3 presents the ANOVA results, evaluating the statistical significance of the model.

Table 3. ANOVA analysis result.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	8.522E+06	9	9.469E+05	220.44	< 0.00011	significant
(X_1) Water in Ethanol (%)	1.410E+06	1	1.410E+06	328.14	< 0.0001	
(X_2) Temperature	3.237E+06	1	3.237E+06	753.64	< 0.0001	
(X_3) Ultrasonic power	36046.13	1	36046.13	8.39	0.0231	
X_1X_2	4.921E+05	1	4.921E+05	114.56	< 0.0001	
X_1X_3	2.280E+05	1	2.280E+05	53.08	0.0002	
X_2X_3	1.332E+05	1	1.332E+05	31.02	0.0008	
X_1^2	2.195E+05	1	2.195E+05	51.09	0.0002	
X_2^2	2.226E+06	1	2.226E+06	518.15	< 0.0001	
X_3^2	3.220E+05	1	3.220E+05	74.97	< 0.0001	
Residual	30068.45	7	4295.49			
Lack of Fit	19541.25	3	6513.75	2.48	0.2009	not significant
Pure Error	10527.20	4	2631.80			
Cor Total	8.552E+06	16				

Figure 4 illustrates the combined effects of these three factors on ADE yield. After ANOVA analysis and consideration of the interactions between these factors, optimal conditions were determined at $\alpha = 0.05$ with a confidence interval of 0.01. The model predicted optimal conditions as follows: only water ratio 100:0, temperature 45 °C, and ultrasonic power

300 W, resulting in a predicted ADE yield of 2500 mg/kg. Validation experiments under these optimized conditions resulted in an average ADE yield of 2508 ± 65 mg/kg, and a COR yield of 2454 ± 37 mg/kg, confirming the model's predictive capability.

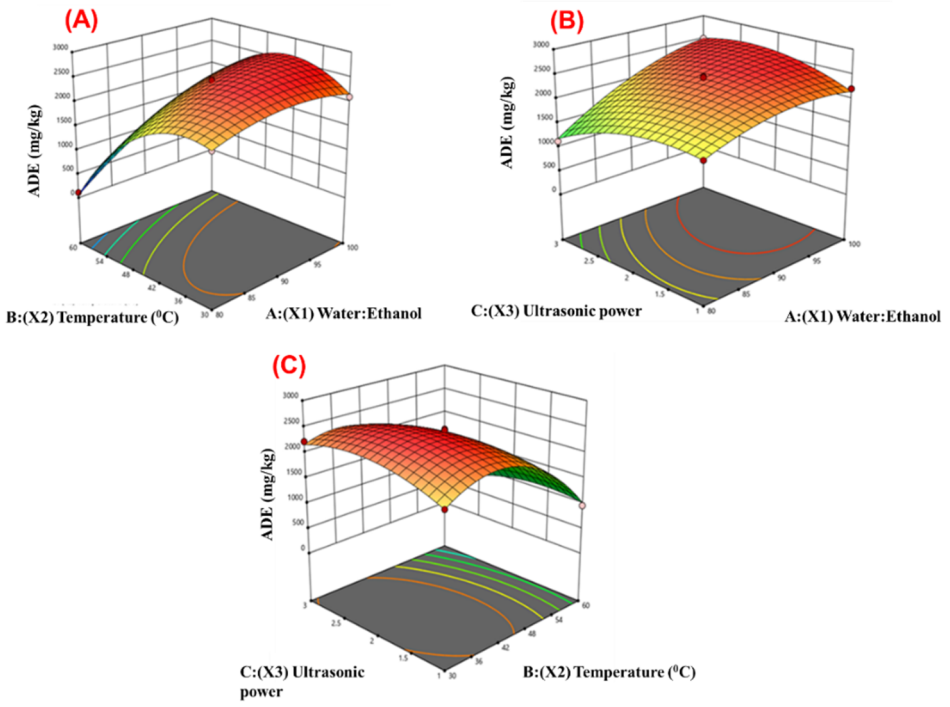

Figure 4. 3D response surface plots showing the combined effects of factors on ADE yield in the extract: (A) effects of temperature and water:ethanol ratio; (B) effects of ultrasonic power and ethanol: water ratio; (C) effects of ultrasonic power and temperature.

Conclusion

This study successfully optimized the extraction of ADE and COR from *Cordyceps militaris* powder using a two-stage approach. A Plackett-Burman design identified significant factors: temperature ($P < 0.0001$), water:alcohol ratio ($P < 0.0001$), and ultrasonic power ($P < 0.0231$). Subsequent optimization using a Box-Behnken design yielded a significant model ($P < 0.001$), with adequate precision (48.71) and non-significant lack-

of-fit (LOF = 0.2009). The optimized model exhibited high fit (R^2 = 0.9965, adjusted R^2 = 0.9920) and predicted optimal conditions: water as a solvent for extraction, a temperature of 45 °C, and 300 W ultrasonic power. Validation experiments under these conditions achieved average yields of 2508 ± 65 mg/kg for ADE and 2454 ± 37 mg/kg for COR, confirming the model's accuracy. This protocol provides a robust and efficient method for extracting ADE and COR from *Cordyceps militaris*, benefiting both industrial applications and further research.

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