DOI: 10.17344/acsi.2024.8822 *Acta Chim. Slov.* **2025,** *72,* 34–44

*Scientific paper*

# High-pressure Ultrasonic-Assisted Extraction of Cannabinoids from Industrial Hemp Leaves: Structure Analysis and Antioxidant Activity

**Hang Li[1,2], Tian-Xiao Yang[3], Qing-Sheng Zhao[2,*], Yuan Zhu[2] and Bing Zhao[2,*]**

[1] *School of Biological Science and Technology, University of Jinan, Jinan, 250022, China*

[2] *State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing, 100190, China*

[3] *Department of Biomedicine, Beijing City University, Beijing, 100094, China*

* *Corresponding author: E-mail: bzhao@ipe.ac.cn, qszhao@ipe.ac.cn*
*Tel./Fax: +86-010-62574372*

*Received: 05-24-2024*

## Abstract

Cannabinoids are very valuable natural products of industrial hemp. In this work, the high-pressure ultrasonic-assisted extraction (HUE) technique was employed for the first time to extract cannabinoids from industrial hemp leaves. A maximum extraction yield (60.02 ± 0.09 mg/g) was obtained through the single-factor experiments, which was much higher compared to ultrasonic-assisted extraction (UE, 42.88 ± 0.02 mg/g) and stirring extraction (SE, 35.35 ± 0.07 mg/g). The optimized HUE conditions were: extraction time was 40 min, EtOH concentration was 60%, extraction temperature was 60 °C, ultrasonic power was 400 W, $R_{L/S}$ was 20 mL/g, stirring rate was 400 rpm, and extraction pressure was 0.6 MPa. The total phenolic content (TPC, 244.36 ± 0.84 mg GAE/g extract) and the total flavonoids content (TFC, 0.27 ± 0.02 mg RE/g extract) of the HUE extract were also the highest. The HUE mechanism was revealed to some extent by several characterizations including surface morphology that HUE could deeply destroy the structure of leave particles. Furthermore, the *in vitro* antioxidant activity of HUE extract was the best among the three extracts. The $EC_{50}$ values against DPPH and ABTS were 0.28 mg/mL and 0.25 mg/mL, respectively. In summary, this work confirmed that HUE was a suitable method for extracting cannabinoids and the obtained extract with a high content of cannabinoids had great application values.

**Keywords:** Hemp; Cannabinoids; High-pressure ultrasonic-assisted extraction; Antioxidant activity; Characterization

## 1. Introduction

Hemp (*Cannabis sativa* L.) has been used for thousands of years as an economic crop. There were many records about hemp in Chinese ancient medical works, such as Shengnong's Herbal Classic and The Yellow Emperor's Canon of Internal Medicine. Ancient Egypt, ancient India, and some European countries also had a history of using hemp to treat eye diseases and pain.[1] Because of the addictive property of the tetrahydrocannabinol (THC) component, the application of hemp was once heavily restricted. However, with the development of the research on cannabinoids, it has been found that their biological activities are very good. Meanwhile, the content of THC can be reduced to less than 0.3% through existing techniques, to obtain industrial hemp.[2] Therefore, the industrial hemp industry is growing rapidly.

Cannabinoids are a class of phenolic terpenoid compounds, which are mainly produced in hemp flowers, leaves, and stems.[3] Cannabidiol (CBD) and THC are the two main cannabinoids. Up to now, cannabinoids have been reported to possess many pharmacological activities, such as anticancer, neuroprotective, antioxidative, anti-inflammatory, antiepileptic, antipsychotic, antibacterial, and immune-suppressive activities.[4–8] Therefore, cannabinoids have great application value in the fields of functional food, drug, and daily chemicals.

Some conventional organic solvents including methanol, butane, chloroform, and hexane were used for the extraction of cannabinoids from hemp.[9, 10] But the disadvantages of solvent extraction are the low extraction yield and solvent toxicity, which could not meet the green chemistry requirement. According to the available reports, several new extraction techniques, such as supercritical fluid extraction,[11] microwave-assisted extraction,[12] pressurized liquid extraction,[13] and subcritical extraction[14] have been found to simplify the process, reduce pollution, and improve the extraction yield of cannabinoids. However, these techniques all require high cost and can not be applied in practical production in a short time. Moreover, unusual extraction conditions might can damage complete features of effective constituents.

High-pressure ultrasonic-assisted extraction (HUE) is an emerging extraction technique combining ultrasonic-assisted extraction with high pressure, which has the advantages of high efficiency, relatively low cost, high extraction yield, and good reproducibility.[15] HUE can facilitate extraction in two ways. On the one hand, the use of high pressure can enhance ultrasonic power by creating shear forces, which promote the destruction of the cell wall and membrane through cavitation mechanical effects.[16] And then the exudation and diffusion of intracellular substances from cells into the solvent are accelerated. On the other hand, high pressure can facilitate the dissolution of active components and reduce the viscosity and surface tension of the solvent. These characteristics make HUE particularly suitable for the extraction of bioactive ingredients. It was reported that *Hovenia dulcis* polysaccharides were extracted by HUE and an extraction yield of 11.81% was obtained.[17] In summary, HUE can improve extraction efficiency and reduce production cost, which is of great significance for industrial production that needs to deal with a large number of raw materials. In the future, HUE is expected to be further combined with other emerging technologies to form a more efficient extraction process. And with the continuous maturity of technology, the manufacturing cost of HUE equipment is expected to gradually reduce. Therefore, the HUE technique has good practical industrial applicability and broad future prospects. Besides, the HUE technique has not yet been employed to extract cannabinoids from industrial hemp.

Hence, the purpose of this study was to explore the application effect of HUE for the extraction of cannabinoids from industrial hemp leaves. HUE was used for extracting cannabinoids and the extraction conditions were optimized by single-factor experiments. Ultrasonic-assisted extraction (UE) and stirring extraction (SE) were employed for comparison of extraction effects with HUE. Several physicochemical characterizations were carried out to find evidence related to the extraction mechanism of HUE. Besides, the antioxidant activity of the obtained extracts was investigated. This work expanded the application scope of HUE and the experimental results could

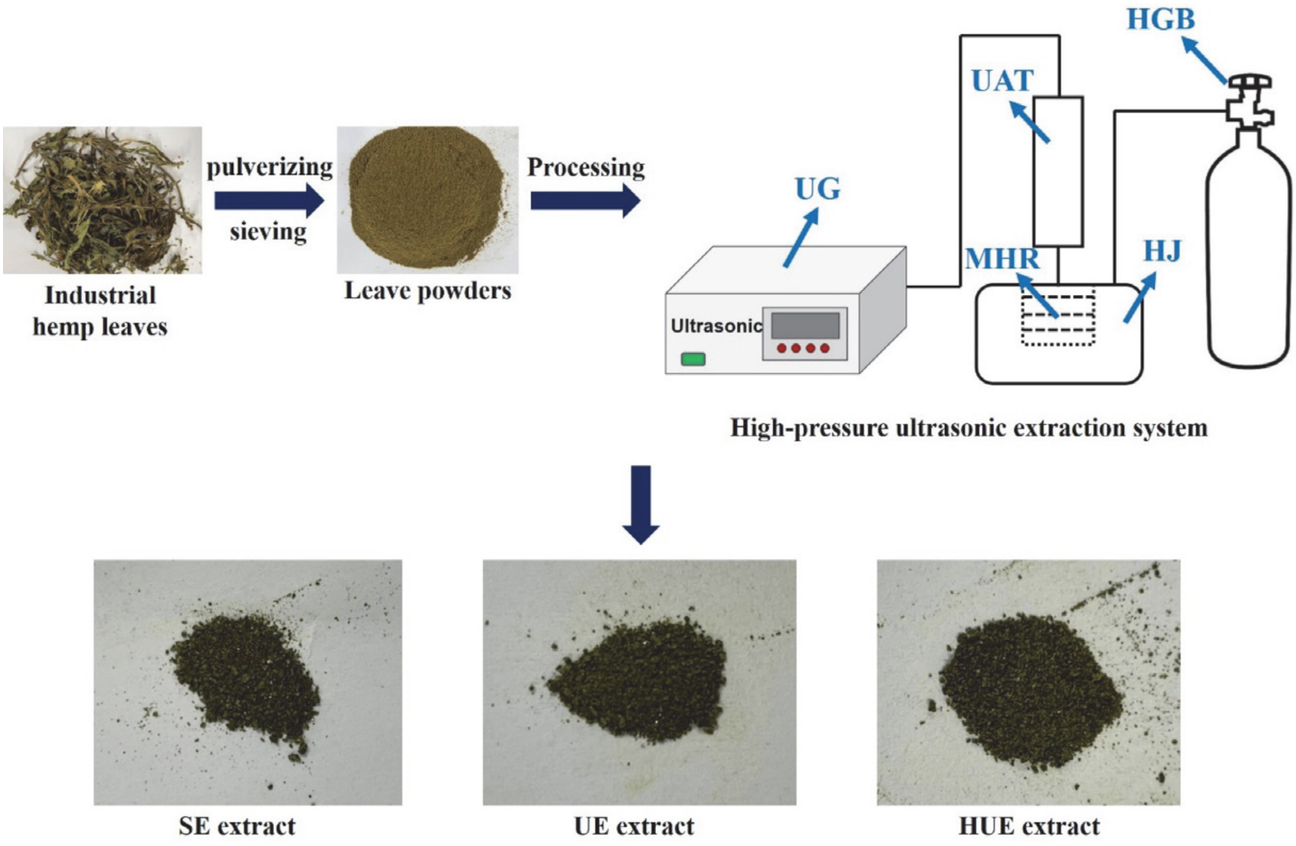

**Fig. 1.** Schematic representation of the preparation of HUE extract.

promote the potential utilization of HUE in the field of industrial hemp.

# 2. Materials and Methods

## 2. 1. Materials and Reagents

Industrial hemp leaves and hemp full-spectrum oil (used as standard) were supplied by Yunnan Hempmon Pharmaceutical Co., Ltd. (Yunnan, China). Because of the instability, cannabidiolic acid (CBDA) is easily transformed into CBD via decarboxylation. Therefore, the leaves were pre-processed for 1 h at 120 °C to achieve the decarboxylation of CBDA. Subsequently, as shown in Fig. 1, the leaves were treated with high-speed crushing and filtered through 60-mesh sieves to obtain leave powders for further extraction.

2,2'-azinabis(3-ethyl-benzothiazoline-6-sulfonic acid)diammonium salt (ABTS, 98%, CAS: 30931-67-0), 1,1-diphenyl-2-picrylhydrazyl (DPPH, 98%, CAS: 1898-66-4), and chromatographically pure reagents were bought from Aladdin Biochemical Technology Co., Ltd (Los Angeles, USA). KBr (CAS: 7758-02-3) was purchased from Macklin Biochemical Technology Co., Ltd (Shanghai, China). Rutin (CAS: 153-18-4), gallic acid (CAS: 149-91-7), and other analytically pure chemicals were supplied by Sinopharm Group Co., Ltd. (Shanghai, China).

## 2.2. Extraction of Cannabinoids by HUE

The high-pressure ultrasonic extraction equipment described in Fig. 1 was used in this work. The ultrasonic frequency is 20 kHz. It consists of an ultrasonic generator (UG), a high-pressure gas bottle (HGB), an ultrasonic amplitude transformer (UAT), a heating jacket (HJ), and a magnetic high-pressure extraction reactor (MHR). Besides, MHR also includes an inlet valve, a vent valve, and a safety valve. The UAT which was immersed in the extraction solution was employed to generate ultrasound with different powers.

Cannabinoids were extracted from industrial hemp leaves using this equipment. Briefly, 2 g leave powders were blended with 40 mL 60% (*v*/*v*) EtOH and the obtained mixture was added to the MHR. Meanwhile, the MHR began to be heated by the heating jacket to 60 °C. Nitrogen was injected into the MHR through the HGB. When a pressure of 0.6 MPa (monitored by the safety valve) was achieved, the ultrasonic generator with an ultrasonic power of 400 W was started and the magnetic stirring rate of solution was set at 500 rpm. The whole extraction time was 40 min. Finally, the solution was taken out and centrifuged for 5 min at 4000 r/min. The obtained supernatant liquid was lyophilized by a freeze dryer (FD-1A-50, BioCool Experimental Instruments, China) to obtain the HUE extract. The extraction residue was also dried for further use. The above parameter is an example of single-factor experiments.

### 2. 2. 1. Single-factor Experiments of HUE

The extraction of cannabinoids from industrial hemp leaves was optimized through single-factor experiments. Extraction pressure (0.1, 0.2, 0.4, 0.6, 0.8 MPa), extraction time (10, 20, 30, 40, 50 min), ultrasonic power (100, 200, 300, 400, 500 W), EtOH concentration (30, 40, 50, 60, and 70%, *v*/*v*), liquid to solid ratio ($R_{L/S}$, 5, 10, 20, 30, 40 mL/g), extraction temperature (30, 40, 50, 60, 70 °C), and stirring rate (100, 200, 300, 400, 500 rpm) were separately investigated to evaluate the effect of a single variable on the extraction yield of cannabinoids ($Y_C$). Then an optimal extraction condition of HUE was obtained for extracting cannabinoids. The formula for calculating the $Y_C$ was presented in Eq. (1).

$$Extraction\ yield\ of\ cannabinoids\ (mg/g) = \\ = \frac{weight\ of\ extracted\ cannabinoids}{weight\ of\ used\ leave\ powders} \tag{1}$$

### 2. 2. 2. Extraction of Cannabinoids by UE and SE

Ultrasonic-assisted extraction (UE) and stirring extraction (SE) were employed in this work to compare the extraction effect with HUE. To ensure the reliability of the experiment results, the extraction process was still carried out through the above mentioned high-pressure ultrasonic equipment. For UE, only high pressure was not involved and ordinary pressure was adopted in the UE process. For SE, high pressure and ultrasonic treatments were not adopted, and cannabinoids were extracted only by magnetic stirring. The extraction conditions of UE and SE were also determined by single-factor experiments. The $Y_C$, structure, and bioactivity of HUE, UE, and SE extracts were compared in subsequent experiments.

## 2. 3. Quantitative Analysis of Cannabinoids

Ultraviolet-visible (UV-vis) analysis was used to complete the quantification of cannabinoids by a UV-2802

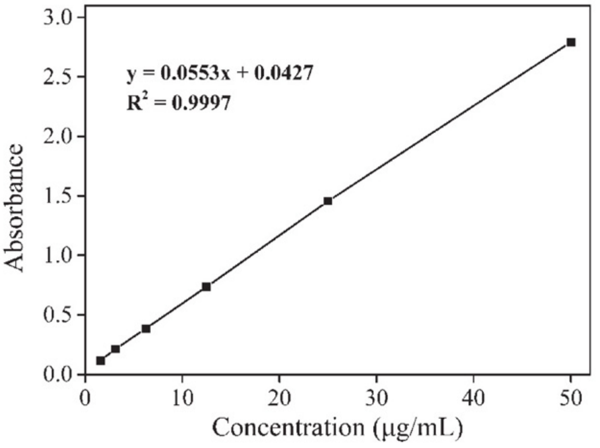

**Fig. 2.** The calibration curve of hemp full-spectrum oil.

UV-vis spectrophotometer (Unico Instrument, China). 2 mL extracting solution with an appropriate dilution was added to a quartz colorimetric dish and then the dish was detected at 220 nm to obtain the absorbance (Abs) of the samples. As displayed in Fig. 2, hemp full-spectrum oil was used as the standard and the calibration curve was y = 0.0553x + 0.0427 ($R^2 > 0.999$, 0–50 µg/mL), where *y* and *x* were Abs and concentration.

## 2. 4. Determination of Total Phenolic Content (TPC) and Total Flavonoids Content (TFC)

The TPC of the extracts was measured through the Folin-Ciocalteu method.[18] Briefly, 1 mL Folin-Ciocalteu reagent was mixed with 1 mL sample solution (500 µg/mL). 3 mL $Na_2CO_3$ solution (20%, *w*/*w*) was then blended with the obtained solution. After mixing well, the solution was treated with a 50 °C water bath for 30 min. The Abs of 765 nm was determined by UV-vis spectroscopy. Gallic acid was the reference and the calibration curve was y = 0.0349 + 0.0137x ($R^2 > 0.99$, 0–125 µg/mL), where *y* and *x* were Abs and concentration. The data were displayed as mg of gallic acid equivalents (GAE) per g of extracts.

The TFC of the extracts was measured according to a commonly used method with minor changes.[19] Briefly, 0.3 mL $NaNO_2$ solution (5%, *w*/*w*) was blended with 4 mL sample solution. 0.3 mL $Al(NO_3)_3$ solution (10%, *w*/*w*) was added to the obtained solution after 6 min. Next, 3 mL NaOH solution (4%, *w*/*w*) and 15 mL deionized water were also added after 6 min. Finally, the Abs of 510 nm was obtained after 10 min. Rutin was the reference and the calibration curve was y = 0.0363 + 1.8049x ($R^2 > 0.99$, 0–750 µg/mL). TFC was expressed as mg of rutin equivalents (RE) per g of samples.

## 2. 5. Fourier Transform Infrared Spectroscopy (FT-IR) Analysis

FT-IR spectra of the extracts were scanned by a NICOLET iS50 FT-IR spectrometer (Thermo Scientific, USA) in the range of 4000 $cm^{-1}$ and 400 $cm^{-1}$ with an optical resolution of 4 $cm^{-1}$. The number of scans was 64. Each extract was blended uniformly with KBr with a quality ratio of 1:100 and then pressed into a thin sheet for detection.

## 2. 6. Powder X-ray Diffraction (PXRD) Analysis

PXRD curves of the extracts were collected to compare their crystalline or amorphous structures. The detection was carried out on an Empyrean powder X-ray diffractometer (PANalytical B.V., The Netherlands) using Cu Kα (wavelength = 1.5406 Å) at 40 mA/40 kV. The diffraction data were recorded between 5° and 80° with a step size of 0.01°.

## 2. 7. Surface Morphology Analysis

The surface morphology of the extracts and extraction residues was acquired via a Gemini 300 field emission scanning electron microscope (FE-SEM, ZEISS, Germany). Before the observation, the sample powders were uniformly adhered to a brass sample stage using a double-sided carbon tape and then the sample stage was metallized with gold sputter under vacuum condition to remove the charging effect.

## 2. 8. *In vitro* Antioxidant Activity

The antioxidant activity of different extracts was investigated by free radical scavenging assays. DPPH and ABTS free radicals were frequently used in scientific research and both were employed for this work. For quick detection, 96-well plates were used. In the DPPH scavenging assay, 100 µL prepared DPPH solution (0.2 mmol/L) was blended with 100 µL sample solution of different concentrations. The plate was incubated in the dark for 30 min and the Abs of 520 nm was measured by a Multiskan FC microplate reader (Thermo Scientific, USA). The scavenging ability of the extracts was calculated as the percentage of clearance by Eq. **(2)**. The $EC_{50}$ value (Concentration for 50% of maximal effect) was also obtained by the scavenging curve.

$$Scavenging\ rate\ (\%) = \left(1 - \frac{A_S - A_C}{A_B}\right) \times 100 \qquad (2)$$

Where $A_C$ is the Abs of control group, $A_S$ refers to the Abs of sample group, and $A_B$ represents the Abs of the blank group.

For ABTS, the test was conducted based on a reported method with some changes.[20] The ABTS stocking solution was prepared in advance. After 12 h, it was diluted until the Abs of 0.7 was obtained. Subsequently, 200 µL ABTS solution was mixed with 50 µL sample solution of different concentrations. After being incubated in the dark for 6 min, the Abs of 700 nm was recorded. The ABTS scavenging capacity was evaluated by Eq. (2).

## 2. 9. Statistical Analysis

The results were presented as mean ± SD (n = 3). Statistical significance was performed by ANOVA. Values of $p < 0.05$ were considered statistically significant.

# 3. Results and Discussions

## 3.1. Single-factor Experiments of HUE for Cannabinoids

### 3. 1. 1. Effects of Extraction Time on $Y_C$

When the extraction conditions were as follows: Ultrasonic power = 300 W, extraction pressure = 0.6 MPa, EtOH concentration = 60%, extraction temperature = 30

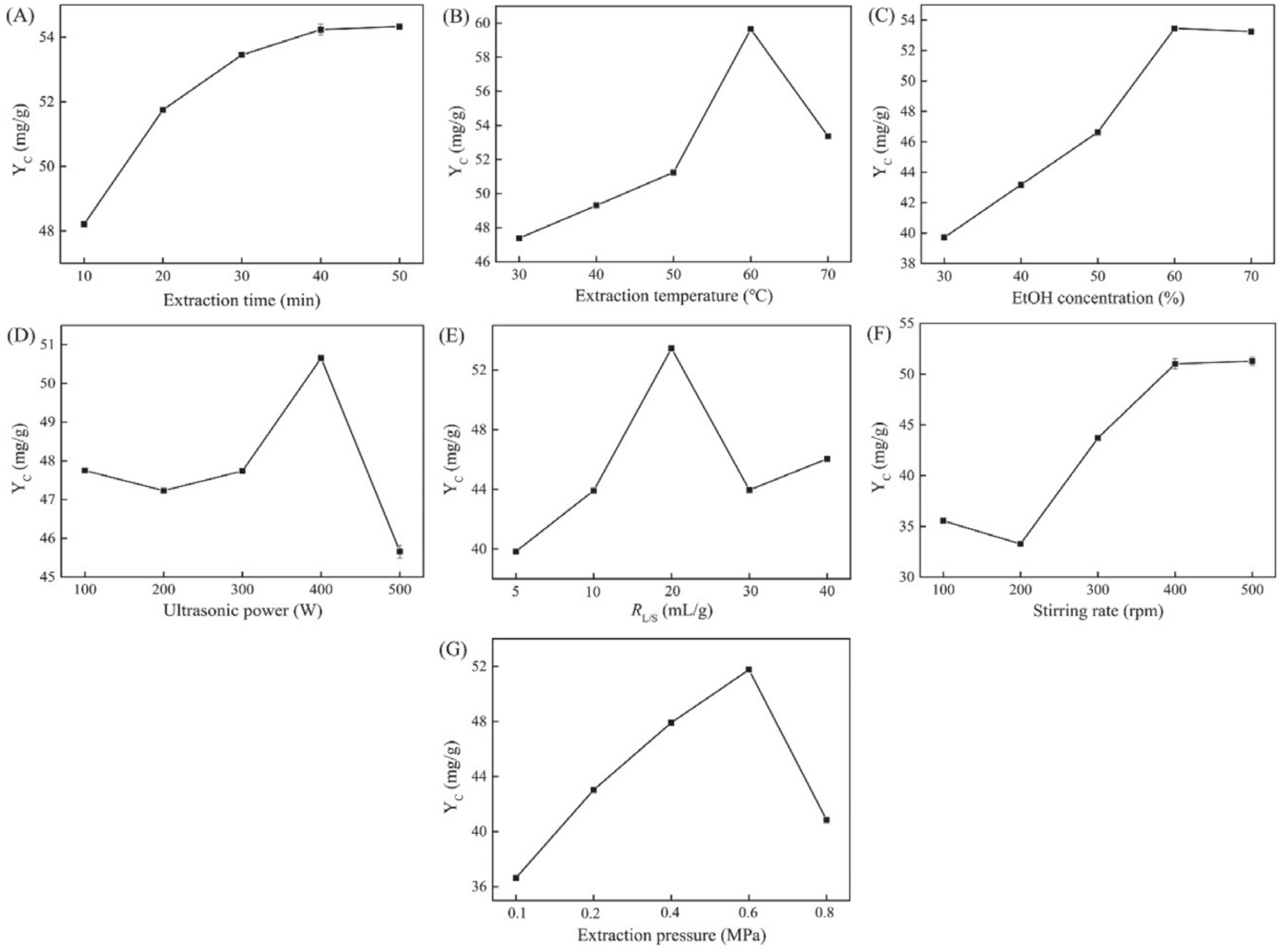

**Fig. 3.** Effects of (A) extraction time, (B) extraction temperature, (C) EtOH concentration, (D) ultrasonic power, (E) $R_{L/S}$, (F) stirring rate, and (G) extraction pressure on $Y_C$.

°C, $R_{L/S}$ = 20 mL/g, and stirring rate = 500 rpm, the effect of extraction time on $Y_C$ was displayed in Fig. 3(A). $Y_C$ gradually increased with the increase of extraction time from 10 min to 40 min and then became stable at 50 min. The maximum $Y_C$ was 54.33 mg/g. with extraction going on, the concentration difference between sample cells and solution was decreasing, then the dissolution of cannabinoids reached the saturation point in the solution. Therefore, nearly no significant difference of $Y_C$ could be found from 40 to 50 min. According to the results, 40 min was determined to be the optimal extraction time for further experiments.

### 3. 1 .2. Effects of Extraction Temperature on $Y_C$

When the extraction conditions were as follows: Ultrasonic power = 300 W, extraction pressure = 0.6 MPa, EtOH concentration = 60%, $R_{L/S}$ = 20 mL/g, extraction time = 20 min, and stirring rate = 500 rpm, the effect of extraction temperature on $Y_C$ was presented in Fig. 3(B). It could be found that $Y_C$ increased to a maximum (59.65 mg/g) at 60 °C and then decreased with the rise of extraction temperature. High temperature could result in the softening of fiber tissues of leave powders and promote the diffusion and dissolution of cannabinoids from cells. Therefore, $Y_C$ increased in the initial stage. However, the thermal stability of cannabinoids is relatively poor and they could easily degrade at high temperature.[21] So, there was a sharp decrease of $Y_C$ from 60 to 70 °C. To avoid the degradation of cannabinoids and obtain the highest $Y_C$, 60 °C was determined to be the optimal extraction temperature for further experiments.

### 3. 1. 3. Effects of EtOH Concentration on $Y_C$

When the extraction conditions were as follows: Extraction pressure = 0.6 MPa, ultrasonic power = 300 W, extraction temperature = 30 °C, $R_{L/S}$ = 20 mL/g, extraction time = 30 min, and stirring rate = 500 rpm, the effect of EtOH concentration on $Y_C$ was exhibited in Fig. 3(C). From the graph, $Y_C$ increased continuously with the rise of EtOH concentration from 30% to 60% and then tended to be constant. The highest $Y_C$ was 53.45 mg/g at a 60% EtOH concentration. Due to the extremely poor water solubility of cannabinoids, it was necessary to employ organic solvents as the cosolvent with water. EtOH was used in this work because of its availability in food.[22] Based on the results, 60% could be a minimum concentration of EtOH, which was suitable to dissolve and extract most cannabinoids. Therefore, 60% EtOH was chosen as the optimal EtOH concentration for further experiments.

### 3. 1. 4. Effects of Ultrasonic Power on $Y_C$

When the extraction conditions were as follows: Extraction pressure = 0.6 MPa, EtOH concentration = 60%, extraction temperature = 30 °C, extraction time = 30 min, $R_{L/S}$ = 20 mL/g, and stirring rate = 500 rpm, the effect of ultrasonic power on $Y_C$ was displayed in Fig. 3(D). The variation trend of $Y_C$ was that it firstly increased to a maximum (50.66 mg/g) at 400 W and then declined from 400 to 500 W. With the ultrasonic power increasing, the sound intensity also increased, which improved the amplitude of sound pressure and liquid negative pressure. The pressure change enhanced the cavitation effect, which was beneficial to the extraction of cannabinoids.[23] Therefore, $Y_C$ increased with the rise of ultrasonic power from 100 to 400 W. Nevertheless, the excessive ultrasonic power could produce lots of vacuoles in solution and they would reduce energy transfer by reflecting the sound wave.[24] Meanwhile, the too high energy of ultrasound could cause the degradation or isomerization of these cannabinoids. So, $Y_C$ had a significant decline from 400 to 500 W. Based on the above analysis, 400 W was determined to be the appropriate ultrasonic power for further experiments.

### 3. 1. 5. Effects of $R_{L/S}$ on $Y_C$

When the extraction conditions were as follows: Extraction pressure = 0.6 MPa, EtOH concentration = 60%, extraction temperature = 30 °C, extraction time = 30 min, ultrasonic power = 300 W, and stirring rate = 500 rpm, the effect of $R_{L/S}$ on $Y_C$ was displayed in Fig. 3(E). The curve indicated that $Y_C$ increased to a maximum (53.45 mg/g) at 20 mL/g and then declined as the $R_{L/S}$ increased. $R_{L/S}$ can determine the concentration gradient between the liquid phase (60% EtOH) and the solid phase (leave powders). The concentration gradient, as the main driving force of material diffusion, is strongly associated with mass transfer.[25] Thus, the suitable $R_{L/S}$ can greatly reduce the mass transfer resistance and increase the extraction of cannabinoids. With the decrease of $R_{L/S}$ from 40 to 20 mL/g, the concentration gradient was getting bigger and the dissolution of cannabinoids was promoted. Therefore, $Y_C$ increased. However, when $R_{L/S}$ was further decreased, the influence of high pressure on the mass transfer in solution was weakened to some extent. And the changes of solution property could also affect the extraction of cannabinoids. So, $Y_C$ had a decreasing trend when $R_{L/S}$ was below 20 mL/g. Based on the data, 20 mL/g was determined to be the optimal $R_{L/S}$.

### 3. 1. 6. Effects of Stirring Rate on $Y_C$

When the extraction conditions were as follows: Extraction pressure = 0.6 MPa, EtOH concentration = 60%, extraction temperature = 30 °C, extraction time = 20 min, ultrasonic power = 300 W, and $R_{L/S}$ = 20 mL/g, the effect of stirring rate on $Y_C$ was shown in Fig. 3(F). It could be found that $Y_C$ had a continuous increase with the rise of stirring rate from 100 to 400 rpm and then became smooth. The maximum $Y_C$ was 51.27 mg/g at 500 rpm. The increase of stirring rate could accelerate the mass transfer between the leave powders and the solution, promote the formation of a homogeneous solution, and improve the extraction of cannabinoids. The $Y_C$ at 500 rpm was very close to that at 400 rpm (51.02 mg/g). Considering the cost of practical production, 400 rpm was determined to be the appropriate stirring rate for further experiments.

### 3. 1. 7. Effects of Extraction Pressure on $Y_C$

When the extraction conditions were as follows: Stirring rate = 500 rpm, EtOH concentration = 60%, ultrasonic power = 300 W, extraction time = 20 min, extraction temperature = 30 °C, and $R_{L/S}$ = 20 mL/g, the effect of extraction pressure on $Y_C$ was presented in Fig. 3(G). $Y_C$ increased gradually to a maximum (51.75 mg/g) at 0.6 MPa and then decreased with the increase of extraction pressure. As mentioned above, the use of high pressure could enhance the effect of ultrasonic cavitation, which promoted the destruction of cell wall and membrane. Subsequently, the diffusion of cannabinoids from cells into the solvent was accelerated. Besides, high pressure could reduce the viscosity and surface tension of the solvent and facilitate the dissolution of cannabinoids. Based on these reasons, $Y_C$ increased from 0.1 to 0.6 MPa. However, a higher extraction pressure could cause the decomposition of some cannabinoids due to the solution overheating and variation of solution property.[26] So $Y_C$ displayed an obvious decrease from 0.6 to 0.8 MPa. According to the results, 0.6 MPa was determined to be the optimal extraction pressure.

In summary, the optimal extraction conditions of HUE were obtained by the single-factor experiments above. They were as follows: Extraction temperature = 60 °C, extraction time = 40 min, EtOH concentration = 60%, ultrasonic power = 400 W, $R_{L/S}$ = 20 mL/g, stirring rate = 400 rpm, and extraction pressure = 0.6 MPa.

## 3. 2. Comparison of Extraction Effect by Different Extraction Techniques

The optimized extraction conditions in part 3.1 were also applied to the UE and SE. The $Y_C$ under optimal

**Table 1:** Comparison of different extraction techniques HUE, UE, and SE.

| Extraction techniques | SE | UE | HUE |
|---|---|---|---|
| $Y_C$ (mg/g) | 35.35 ± 0.07$^c$ | 42.88 ± 0.02$^b$ | 60.02 ± 0.09$^a$ |
| TPC (mg GAE/ g extract) | 231.70 ± 0.42$^b$ | 231.46 ± 0.00$^b$ | 244.36 ± 0.84$^a$ |
| TFC (mg RE/ g extract) | 0.25 ± 0.01$^{ab}$ | 0.24 ± 0.01$^b$ | 0.27 ± 0.02 |

$^a$ Different letters indicate significant difference when compared between groups.

conditions of the three extraction techniques (HUE, UE, and SE) were displayed in Table 1. As expected, HUE had a much higher $Y_C$ (60.02 mg/g) in comparison with UE (42.88 mg/g) and SE (35.35 mg/g). The results demonstrated the great advantages of HUE. At the target exudation stage, HUE could enhance the ultrasonic effect and produce a synergistic effect between high pressure and ultrasound, further to accelerate the destruction of cell wall and membrane. Besides, at the dissolution stage, the diffusion of target compounds from cells into the solvent is accelerated. The high pressure also could facilitate the dissolution by reducing the viscosity and surface tension of the solvent. Based on the above advantages, HUE had the best extraction effect for cannabinoids. It was also studied that the HUE extraction yield of polysaccharides from ginger leaves was 9.82% while the UE extraction yield was only 8.22%.[15] The referenced data were consistent with our conclusion. Cold ethanol extraction was reported to be used for the extraction of cannabinoids and terpenes.[27] Under the optimized conditions, the extraction yield (g/100 g dry matter) was 19.7% for −40 °C. Supercritical $CO_2$ extraction was employed for bioactive extraction from industrial hemp inflorescences and the highest CBDA content (305.8 µg/mg) was obtained at 320 bar and 40 °C.[28] The reference value of these data was relatively small due to the difference of raw materials and detection.

## 3. 3. Determination of TPC and TFC

To continue the comparison of different extraction techniques, the TPC and TFC of these extracts were determined. Flavonoids and polyphenols are generally regarded to possess strong biological activities. TFC and TPC can usually describe the general chemical composition.[29] As displayed in Table 1, the TPC of SE, UE, and HUE extracts were 231.70, 231.46, and 244.36 mg GAE/g extract, respectively. The TPC of UE and SE extracts were very close. The higher TPC of HUE extract was attributed to the better extraction effect. Meanwhile, according to the higher $Y_C$ of HUE extract, the proportion of cannabinoids in the total phenols could be much higher than UE extract and SE extract. It was reported that 50% acetone extraction could achieve the maximum TPC (53.65 mg GAE per g extract) from defatted hempseeds after optimization.[30] The optimal predicted content of TPC (3.85 mg GA/g oil) could be obtained by microwave-assisted extraction after response surface optimization.[31] Besides, the TFC of SE, UE, HUE extracts were 0.25, 0.24, and 0.27 mg RE/g extract, respectively. The data showed that the flavonoid content of these extracts was very low and there was nearly no difference in TFC between the three extracts. It was reported that the highest TFC (9.28 mg QE $g^{-1}$) was obtained from the leaves and inflorescences of hemp by pulsed ultrasound-assisted extraction.[32] The analysis of TPC and TFC indicated that HUE could improve the active components content of the extracts and had great advantages for cannabinoids extraction.

## 3. 4. FT-IR Analysis

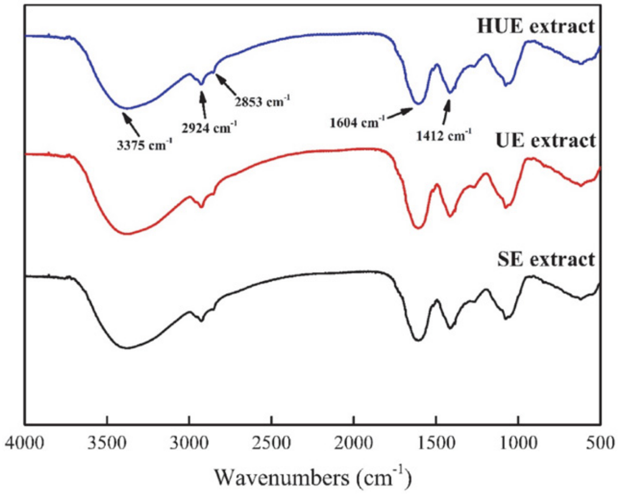

**Fig. 4.** FT-IR spectra of different extracts.

The advantages of HUE for cannabinoids extraction were demonstrated by comparing the different extraction techniques described above. Therefore, several characterizations were then carried out to investigate the extraction mechanism of HUE. FT-IR was used in this work to analyze the functional groups of SE, UE, and HUE extracts. As presented in Fig. 4, the FT-IR spectra of these extracts were very similar. The spectrum of HUE extract was described as an example. The FT-IR spectrum of HUE extract displayed characteristic O-H stretching vibration at 3375 $cm^{-1}$, C-H stretching vibration at 2959, 2924, and 2853 $cm^{-1}$, benzene skeleton vibration at 1604 and 1412 $cm^{-1}$, C-O stretching vibration at 1268 $cm^{-1}$. Most of the absorption peaks were the characteristic absorption of cannabinoids, which demonstrated the existence of cannabinoids in the extracts to some extent. Meanwhile, the presence of hydroxyl peaks was also related to the analysis of total phenols. However, the FT–IR spectra of these extracts were very similar, which hindered the further analysis. To explore the extraction mechanism of HUE, other characterization techniques were adopted in this study.

## 3. 5. PXRD analysis

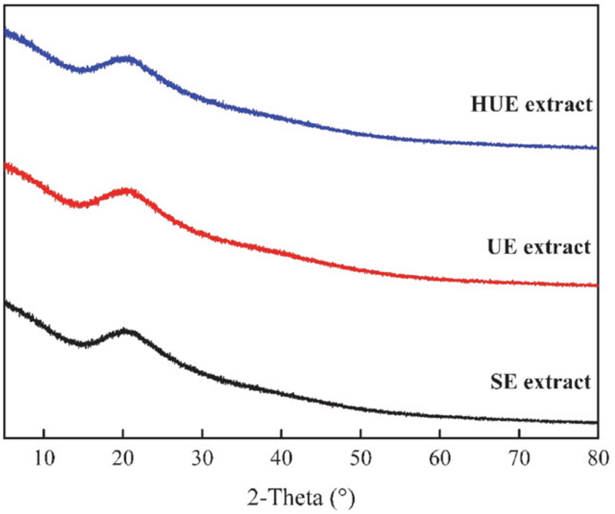

**Fig. 5.** PXRD patterns of different extracts.

PXRD was used in this work to compare the crystalline or amorphous structure of SE, UE, and HUE extracts. The PXRD patterns of them were displayed in Fig. 5. It was found that the PXRD curves of different extracts were very close. They all showed a single wide diffraction peak at about 20°, which indicated that the three extracts all had an amorphous structure. Besides, the similar structures of different extracts demonstrated that different extraction techniques could not influence the amorphous structure of the extracts. To explore the extraction mechanism of HUE, the microscopic analysis was necessary.

## 3. 6. Surface Morphology Analysis

The surface morphology of SER, UER, and HUER was obtained by FE-SEM to explore the extraction mechanism of HUE in more depth. As shown in Fig. 6, the SER displayed a relatively thick sheet-like morphology. The surface of SER powder was smooth and compact, which indicated that the damage to hemp leave powders caused by SE was small. However, the UER showed an irregular plate-like structure with many cracks. Moreover, a large number of small holes could be found on the surface of UER powders at 2000 ×. The production of these cracks and holes was due to that the cavitation effect of ultrasound produced an impact force on the surface, and further the original morphology of the surface was destroyed.[33] Therefore, the extraction effect of UE was better than that of SE. Besides, the surface morphology of HUER changed significantly. The HUER presented a blocky morphology with many folds and cracks on the surface. The formation of the structure was attributed to two aspects. On the one hand, high pressure promoted the solution

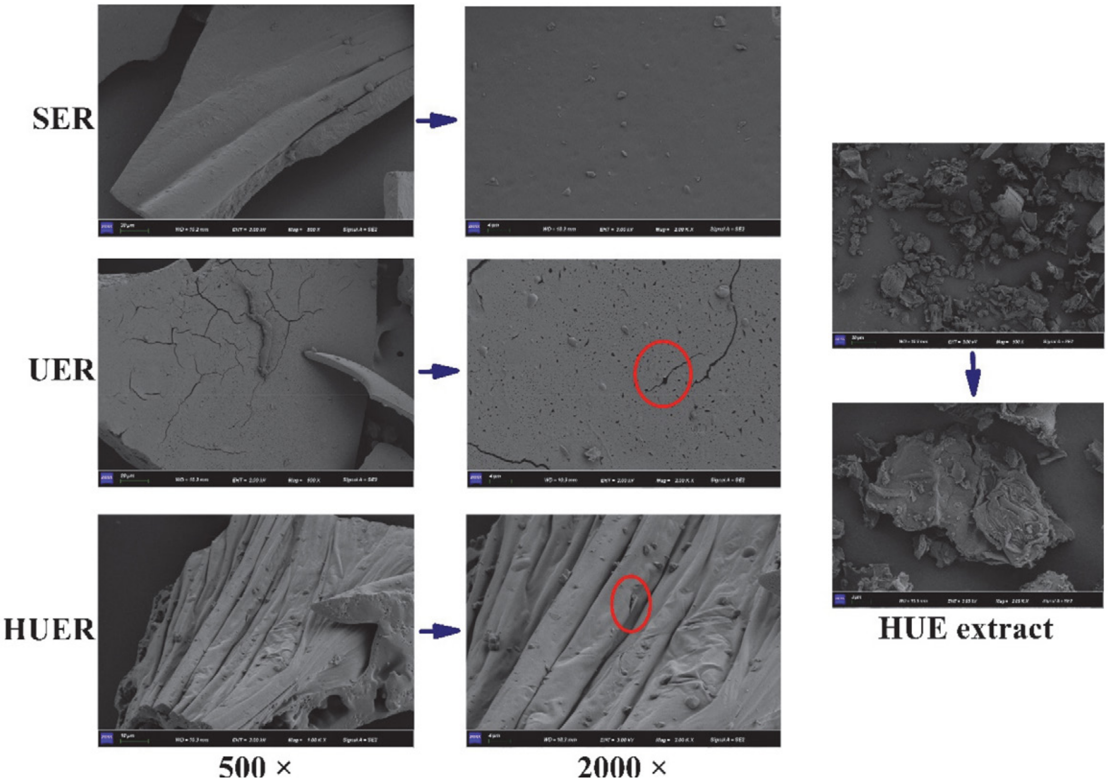

**Fig. 6.** SEM images of SE residue (SER), UE residue (UER), HUE residue (HUER), and HUE extract.

system to squeeze the HUER powders, resulting in a great change of the powder shape and the formation of folds. On the other hand, high pressure could enhance the cavitation effect of ultrasound and further promoted the destruction of powders.[34] Therefore, the extraction effect of HUE was the best among these different extraction techniques. The surface appearance of HUE extract was also displayed. The particle size of HUE extract was about 20~30 μm. It also presented a lumpy structure and many small fragments adhered to a large particle. The extraction mechanism of HUE could be well explained by surface morphology analysis.

## 3. 7. *In vitro* antioxidant activity

After analyzing the extraction mechanism by several characterizations, the bioactivity of extracts obtained by different techniques was also compared. The study on biological activity is very important for the application of plant-derived extracts. ABST and DPPH free radicals are frequently used to assess the *in vitro* antioxidant activity of samples. As displayed in Fig. 7(A), the DPPH scavenging activity of SE, UE, and HUE extracts was compared. The three different extracts all had a significant scavenging effect for DPPH radical and the scavenging rate increased with the rise of sample concentration. The scavenging rate of positive control Vc was more than 80% within the tested concentration range (data not shown). At 0.1 mg/mL, the scavenging rate of SE, UE, and HUE extracts was 26.57 ± 0.04%, 25.72 ± 0.05%, 37.87 ± 0.05%, respectively, while at 1.0 mg/mL, the scavenging rate of them was 67.77 ± 0.01%, 72.39 ± 0.01%, and 76.00 ± 0.01%. The data indicated that HUE extract had the strongest DPPH scavenging capacity among the three extracts. Besides, the $EC_{50}$ values of different extracts were 0.35, 0.42, and 0.28 mg/mL, which also demonstrated that the DPPH scavenging capacity of HUE extract was much better than that of other extracts. The results were attributed to the higher TPC content and higher cannabinoids proportion of HUE extract. For ABTS, as shown in Fig. 7(B), the three different extracts all had a significant scavenging effect for ABTS radical and the scavenging rate also increased with the rise of sample concentration. The scavenging rate of positive control Vc was nearly 100% within the tested concentration range (data not shown). At 0.1 mg/mL, the scavenging rate of SE, UE, and HUE extracts was 20.53 ± 2.56%, 23.86 ± 3.39%, and 27.80 ± 8.39%, respectively. At 0.6 mg/mL, the scavenging rate of them was 48.57 ± 0.56%, 73.77 ± 10.66%, 77.37 ± 14.19%, respectively, while at 0.8 mg/mL, the scavenging rate of them was 62.29 ± 4.98%, 74.35 ± 14.71%, and 76.62 ± 4.81%. The data indicated that HUE extract had the best ABTS scavenging capacity among the three extracts. Furthermore, the $EC_{50}$ values of different extracts were 0.62, 0.33, and 0.25 mg/mL, which also confirmed that the ABTS scavenging activity of HUE extract was much better than that of other extracts. The above results confirmed that the *in vitro* antioxidant activity of HUE extract was the best among different extracts. It was mainly due to the higher active components content, especially the higher proportion of cannabinoids of HUE extract.

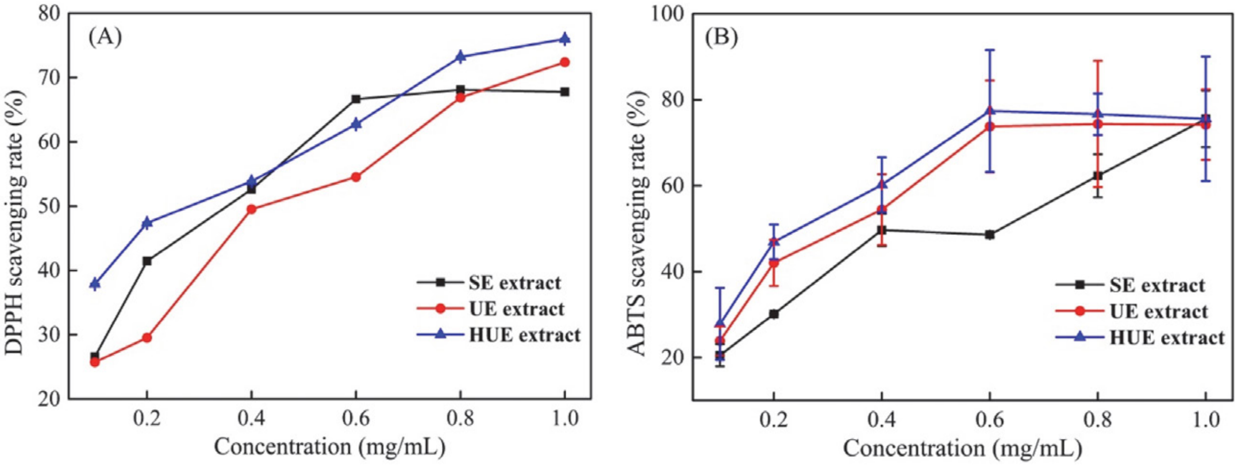

**Fig. 7.** DPPH scavenging activity (A), and ABTS scavenging activity (B) of different extracts ($p < 0.05$, n = 3).

## 4. Conclusions

In this work, the high-pressure ultrasonic-assisted extraction process was developed, and the extraction mechanism and antioxidant activity of the extracts were studied. UE and SE were employed for comparison of extraction effects with HUE. Firstly, the optimization of HUE for cannabinoids from industrial hemp leaves was carried out by single-factor experiments. The optimal conditions were shown below: extraction temperature was 60 °C, extraction time was 40 min, EtOH concentration was 60%, ultrasonic power was 400 W, $R_{L/S}$ was 20 mL/g, stirring rate was 400 rpm, and extraction pressure was 0.6 MPa. $Y_C$ under the conditions was 60.02 ± 0.09 mg/g, which was higher than that of SE (35.35 ± 0.07 mg/g) and UE (42.88 ± 0.02 mg/g). The TPC (244.36 ± 0.84 mg GAE/g extract) and TFC (0.27 ± 0.02 mg RE/g extract) of HUE extract were also the highest. Then the characterizations, mainly SEM, indicated that the excellent extraction effect of HUE was due to that HUE could greatly destroy the structure of leave powders. Besides, the antioxidant capacity of HUE extract was the strongest among different extracts because of the higher proportion of cannabinoids of HUE extract. In short, this research explored the mechanism and application of HUE for cannabinoids extraction. The obtained extract with a high cannabinoids content could be developed as high added-value products in functional foods and other fields.

### Statements and Declarations

The authors declare that they have no potential conflicts of interest.

### Acknowledgments

The authors gratefully appreciate the supports from Yunnan Hempmon Pharmaceutical Co., Ltd.

## 5. References

1. E. B. Russo, *Epilepsy Behav.*, **2017**, *70*, 292–297. **DOI:**10.1016/j.yebeh.2016.09.040
2. X. L. Zhao, X. Y. Wei, Y. Guo, C. S. Qiu, S. H. Long, Y. F. Wang and H. J. Qiu, *Journal of Natural Fibers*, **2021.** **DOI:**10.1080/15440478.2021.1907834
3. S. A. Bonini, M. Premoli, S. Tambaro, A. Kumar, G. Maccarinelli, M. Memo and A. Mastinu, *Journal of Ethnopharmacology*, **2018**, *227*, 300–315. **DOI:**10.1016/j.jep.2018.09.004
4. H. Koltai and N. Shalev, *Cancers*, **2022**, *14*. **DOI:**10.3390/cancers14174299
5. R. Licitra, M. Marchese, V. Naef, A. Ogi, M. Martinelli, C. Kiferle, B. Fronte and F. M. Santorelli, *Biomedicines*, **2022**, *10*. **DOI:**10.3390/biomedicines10081820
6. D. M. Kopustinskiene, R. Masteikova, R. Lazauskas and J. Bernatoniene, *Antioxidants*, **2022**, *11*. **DOI:**10.3390/antiox11040660
7. D. Samanta, *Pediatr. Neurol.*, **2019**, *96*, 24–29. **DOI:**10.1016/j.pediatrneurol.2019.03.014
8. G. H. Briggs, *Faseb J.*, **2018**, *32*, 1. **DOI:**10.1096/fasebj.2018.32.1_supplement.552.6
9. C. Agarwal, K. Mathe, T. Hofmann and L. Csoka, *J. Food Sci.*, **2018**, *83*, 700–710.
10. T. Fei and T. Wang, *J. Am. Oil Chem. Soc.*, **2021**, *98*, 150–150.
11. S. Jokic, I. Jerkovic, V. Pavic, K. Aladic, M. Molnar, M. J. Kovac and S. Vladimir-Knezevi, *Pharmaceuticals*, **2022**, *15*.
12. Z. Drinić, J. Vladić, A. Koren, T. Zeremski, N. Stojanov, B. Kiprovski and S. Vidović, *Journal of Chemical Technology & Biotechnology*, **2020**, *95*, 831–839. **DOI:**10.1002/jctb.6273
13. Y. Nuapia, K. Maraba, H. Tutu, L. Chimuka and E. Cukrowska, *Molecules*, **2021**, *26*. **DOI:**10.3390/molecules26113343
14. S. Fiorito, F. Epifano, L. Palumbo, C. Collevecchio and S. Genovese, *Ind. Crop. Prod.*, **2022**, *183*. **DOI:**10.1016/j.indcrop.2022.114955
15. X. H. Chen, Z. R. Wang, Q. Q. Yang and J. Q. Kan, *J. Food Meas. Charact.*, **2022**, *16*, 85–101. **DOI:**10.1007/s11694-021-01159-3
16. G. A. Pereira, E. K. Silva, N. M. Peixoto Araujo, H. S. Arruda, M. A. A. Meireles and G. M. Pastore, *Ultrason. Sonochem.*, **2019**, *55*, 332–340. **DOI:**10.1016/j.ultsonch.2019.01.010
17. B. Yang, Q. J. Wu, Y. X. Luo, Q. Yang, X. Y. Wei and J. Q. Kan, *Int. J. Biol. Macromol.*, **2019**, *137*, 676–687. **DOI:**10.1016/j.ijbiomac.2019.07.034
18. H. Wang, Q. S. Zhao and B. Zhao, *J. Food Process Eng.*, **2019**, *42*. **DOI:**10.1111/jfpe.13163
19. A. Vega-Galvez, J. Poblete, R. Rojas-Carmona, E. Uribe, A. Pasten and M. G. Goni, *J. Food Sci. Technol.-Mysore*, **2021.** **DOI:**10.1007/s13197-021-05005-8
20. H. Y. Pu, Q. M. Sun, P. X. Tang, L. D. Zhao, Q. Li, Y. Y. Liu and H. Li, *Food Chem.*, **2018**, *260*, 183–192. **DOI:**10.1016/j.foodchem.2018.04.008
21. P. S. Tzimas, E. A. Petrakis, M. Halabalaki, L. A. Skaltsounis and C. Sativa, *Analytica Chimica Acta*, **2021**, *1150*. **DOI:**10.1016/j.aca.2021.338200
22. C. M. Galanakis, V. Goulas, S. Tsakona, G. A. Manganaris and V. Gekas, *Int. J. Food Prop.*, **2013**, *16*, 382–396. **DOI:**10.1080/10942912.2010.522750
23. L. Santos-Zea, J. A. Gutierrez-Uribe and J. Benedito, *Food Engineering Reviews*, **2021**, *13*, 713–725. **DOI:**10.1007/s12393-020-09260-x
24. M. Vinatoru, T. J. Mason and I. Calinescu, *Trac-Trends in Analytical Chemistry*, **2017**, *97*, 159–178. **DOI:**10.1016/j.trac.2017.09.002
25. L. Rakotondramasy-Rabesiaka, J.-L. Havet, C. Porte and H. Fauduet, *Sep. Purif. Technol.*, **2010**, *76*, 126–131. **DOI:**10.1016/j.seppur.2010.09.030
26. L. B. D. Sabino, E. G. Alves, F. A. N. Fernandes, E. S. de Brito and I. J. da Silva, *Food and Bioproducts Processing*, **2021**, *127*, 77–89. **DOI:**10.1016/j.fbp.2021.02.012
27. P. W. Addo, S. Sagili, S. E. Bilodeau, F. A. Gladu-Gallant, D. A. MacKenzie, J. Bates, G. McRae, S. MacPherson, M. Paris, V. Raghavan, V. Orsat and M. Lefsrud, *Molecules*, **2022**, *27*.
28. S. Jokic, I. Jerkovic, V. Pavic, K. Aladic, M. Molnar, M. J. Kovac and S. Vladimir-Knezevi, *Pharmaceuticals*, **2022**, *15*.
29. C. Muscara, A. Smeriglio, D. Trombetta, G. Mandalari, E. La Camera, C. Occhiuto, G. Grassi and C. Circosta, *Phytother. Res.*, **2021**, *35*, 1099–1112. **DOI:**10.1002/ptr.6891
30. C. Benkirane, A. Ben Moumen, M. L. Fauconnier, K. Belhaj, M. Abid, H. S. Caid, A. Elamrani and F. Mansouri, *RSC Adv.*, **2022**, *12*, 25764–25777. **DOI:**10.1039/D2RA04081F

31. D. R. Soroush, S. Solaimanimehr, M. Azizkhani, R. E. Kenari, B. Dehghan, G. Mohammadi and E. Sadeghi, *J. Food Meas. Charact.*, **2021**, *15*, 5191–5202. **DOI:**10.1007/s11694-021-01087-2
32. Z. Kobus, A. Pecyna, A. Buczaj, M. Krzywicka, A. Przywara and R. Nadulski, *Applied Sciences-Basel*, **2022**, *12*. **DOI:**10.3390/app12136747
33. D. Hoo, Z. Li Low, D. Y. S. Low, S. Y. Tang, S. Manickam, K. W. Tan and Z. H. Ban, *Ultrason. Sonochem.*, **2022**, *90*.
34. S. A. Khan, R. Aslam and H. A. Makroo, *J. Food Process Eng.*, **2019**, *42*.

## Povzetek

Kanabinoidi so zelo dragoceni naravni produkti industrijske konoplje. V tej študiji je bila za ekstrakcijo kanabinoidov iz listov industrijske konoplje prvič uporabljena tehnika visokotlačne ultrazvočne ekstrakcije (HUE). Največji izkoristek ekstrakcije (60,02 ± 0,09 mg/g) je bil dosežen z enofaktorskim eksperimentom, kar je bilo veliko višje v primerjavi z ultrazvočno ekstrakcijo (UE, 42,88 ± 0,02 mg/g) in ekstrakcijo z mešanjem (SE, 35,35 ± 0,07). mg/g). Optimizirani pogoji HUE so bili: čas ekstrakcije 40 minut, koncentracija EtOH 60 %, temperatura ekstrakcije 60 °C, moč ultrazvoka 400 W, $R_{L/S}$ 20 ml/g, hitrost mešanja 400 vrt/min in tlak ekstrakcije 0,6 MPa. Tudi vsebnost skupnih fenolov (TPC, 244,36 ± 0,84 mg GAE/g ekstrakta) in skupna vsebnost flavonoidov (TFC, 0,27 ± 0,02 mg RE/g ekstrakta) ekstrakta HUE je bila najvišja. Mehanizem HUE je bil do neke mere razkrit z več karakterizacijami, vključno s površinsko morfologijo, pri čemer bi HUE lahko globoko uničil strukturo listnih delcev. Poleg tega je bila *in vitro* antioksidativna aktivnost ekstrakta HUE najboljša med tremi ekstrakti. Vrednosti $EC_{50}$ proti DPPH in ABTS so bile 0,28 mg/ml oziroma 0,25 mg/ml. Če povzamemo, ta študija potrjuje, da je HUE primerna metoda za ekstrakcijo kanabinoidov in da je imel dobljeni ekstrakt z visoko vsebnostjo kanabinoidov veliko uporabno vrednost.