Ultrasonic Oil Infusion

Ultrasonic energy, broadly defined as sound above the range of human hearing (approximately 20,000 cycles/s or 20 kHz), can be used in several ways during the production of cannabis to ensure that the product is of the highest quality. In addition to providing a tutorial on how ultrasonic equipment works, this article describes how the equipment is applied in laboratory practices and manufacturing. Examples include extraction procedures, cleaning ion targets in mass spectrometers, preparing oil emulsions for edible or drinkable products, degassing oil (removing trapped air) to maintain stable oil volume during selling, degassing high performance liquid chromatography (HPLC) solvents, bubbling off ethanol before oil distillation, and removing gums and waxes from glassware used in production.

Ultrasonic energy is commonly associated with ultrasonic cleaning. As explained by Edward W. Lamm in his article “The Development of Ultrasonic Cleaning” (1), its history dates to the early 1930s and work done at Radio Corporation of America (RCA) laboratories in New Jersey. The first practical applications, according to Lamm’s article, were introduced in the 1950s, and were operated at 18–40 kHz. “Up until the late 1980s most of the commercially available systems operated at 25–40 kHz,” Lamm stated.

Today, ultrasonic cleaners are available in several frequencies, including 25, 45, 80, and 130 kHz. Units are also available offering dual-frequency options.

Ultrasonic energy is used in research, product development, and manufacturing operations. Typically, these involve homogenizing, emulsifying, dispersing, dissolving or mixing difficult samples, and degassing liquids to remove trapped air.

Extraction Procedures

Ultrasonic energy is a proven technique to achieve fast, safe extraction. For example, it is a method often specified in United States Pharmacopeia (USP) monographs to extract active pharmaceutical ingredients from carriers for content uniformity and potency assay tests.

In cannabis production, most regulated markets require all cannabis products to be tested for efficacy (active ingredients, such as cannabinoids and terpenoids), as well as for contaminants (such as pesticides, mycotoxins, heavy metals, microbes, and residual solvents).

Cannabis products include plant material (mostly flowers and trimmed leaves), concentrated extracted essential oils (concentrates, waxes, and oils), and infused products (edibles such as candies, chocolates, baked goods, transdermal patches, suppositories, and beverages).

Clearly, accurate test results depend on efficient, reproducible extraction from these often complex matrices, and sonication is one way many cannabis laboratories seek to achieve those goals. Sonication is valuable because it deposits energy into the solvent–matrix system, effectively speeding the process of extraction and dissolution.

Mass Spectrometry
Mass spectrometers are typically the workhorse instruments in a cannabis laboratory. Many laboratories have inductively coupled plasma–mass spectrometry (ICP-MS), liquid chromatography–tandem mass spectrometry (LC–MS/MS) and gas chromatography (GC)–MS/MS instruments all in the same laboratory.

Since cannabis contains viscous oils and resinous compounds of moderate to high molecular weight, ion sources and associated components can get contaminated with organic residues that are difficult to remove. In particular, contamination of electrodes that steer the ions leads to defocusing and loss of signal.

Sonication is often the most efficient method to clean these parts. Likewise, the chromatography injectors and inlets can also become contaminated and clogged with the resins and residues and sonication in a nonpolar solvent is often the method of choice to clean these components as well.

General Cleaning with Sanitation
For cleaning applications, ultrasonic energy is used to remove contaminants from the surfaces of virtually any product that can be safely immersed in a water-based biodegradable ultrasonic cleaning solution. Cleaning solution formulas, dilution recommendations, and operating procedures are available for specific cleaning tasks.

Components of Ultrasonic Cleaning Equipment
There are multiple manufacturers of ultrasonic cleaning and processing equipment. Regardless of the manufacturer, common components include
A tank, usually stainless steel, to hold the cleaning solution or water that is typically mixed with a surfactant

- A generator to supply power
- Ultrasonic transducers bonded to the bottom of the tank
- A mesh-bottomed basket (standard or optional) to hold products being cleaned or processed without contacting the bottom of the tank
- A lid (standard or optional) to reduce cleaning solution evaporation and noise

Selection criteria then can move to

- Cleaning tank size (length, width, and depth)
- A control panel that can range from a simple on–off switch to highly sophisticated options including timers, thermostats, and features including sweep, normal, pulse, degassing, and adjustable ultrasonic power and frequency.

One might ask about the need for a more sophisticated unit. The answer is simple. It provides cannabis processors with the ability to develop and customize optimum processing steps to achieve consistent, high-quality product from a variety of sources.

How the Process Works

When activated, the equipment's generator powers the transducers to vibrate at their designed ultrasonic frequency. This vibration causes the tank bottom to vibrate as a membrane that produces countless microscopic vacuum bubbles.

In applications such as cleaning glassware, these bubbles implode with tremendous force in a process called *cavitation*. This cavitation quickly and safely blasts loose contaminants and carries away even the most tenacious residue. Products are cleaned with a solution formulation designed for the application.

In a processing application, products are contained in Erlenmeyer flasks, test tubes, or beakers. These containers are lowered, but not fully immersed, into a water or surfactant solution. Ultrasonic energy passes through the glass walls of the containers to act on the contents.

This approach achieves the homogenizing, emulsifying, degassing, and other cannabis processing steps in a fast, efficient, and environmentally friendly way.

Comparing Ultrasonic Cannabis Production and Quality Control to Alternative Methods
In 2015, the National Hemp Association published an article in *Hemp News* titled "Five Major Types of Cannabis Extraction" (2). In the article, Rien Havens, PhD, CTO, Really Helping, PBC, stated that in the winter of 2014 he began research to develop the optimal methods of hemp extraction. "It was quite a ride. I had in mind three main goals," said Havens. "No use of fossil fuels, low energy footprint, and cost effectiveness."

Here, we paraphrase Havens' findings as published in the article (2). Readers may wish to access the full article for additional details.

- Ethanol produced a relatively good quality extract, but the solvent and energy costs were high. It did not produce the desired results of volume and speed. Residual solvent in the final product was also a drawback.
- A closed-loop hydrocarbon extractor is inexpensive to set up, but uses fossil fuels, almost always contains cancer-causing components like benzene, and often there are metal filings and welding debris in the solvent tanks.
- A supercritical carbon dioxide extractor was able to produce a high quality extract with very high terpene retention, a great color, taste, speed, and selectivity. The downside of this approach includes high overhead and unruly energy consumption.
- Critical water extraction is "green" with no added solvents but clean water. There is no solvent loss, or cost, and the volume and cost of the extractor makes it a good candidate for industrial hemp extraction.
- A truly solventless method is sonic and ultrasonic waves in the plant matter that push the product out through vibration. This method can also be scaled up, like water extraction on a budget, and produces a very nice, high-quality extract.

A Closer Look at Ultrasonic Equipment for Cannabis Production
Ultrasonic cleaner tanks are available in multiple sizes in terms of length, width, and depth. When processing in flasks and beakers, a shallow-depth tank is a good choice with a length and width that allows the processing of several containers at once.

The following sections provide a more detailed illustration of how the process works. This example describes the use of a 37-kHz ultrasonic cleaner based on its tank configuration and operating features.

Remember that the transformation or extraction process avoids chemical degradation that can be caused by excessive heat or mechanically induced damage.

Extraction and Processing Steps

Product is placed in flasks along with a recommended solvent. Flasks are partially immersed in a sonicator bath containing a surfactant.

The tank configuration of the ultrasonic unit used in this process is especially designed to quickly and safely accomplish extraction and further processing. The inside dimensions of the shallow basket, 17.9 x 9.8 x 2.2 in. (LxWxH), facilitate positioning of multiple smaller containers or larger beakers. Flask clamps are used to affix flasks to the mesh-bottom basket; test tube holders are also available.

The equipment described was also selected because of its high ultrasonic power per unit volume. This feature permits the preparation process to be completed before heat buildup, a natural result of ultrasonic energy, which can degrade product. If heat is a concern, a useful accessory is a cooling coil to prevent temperature increase. The cooling coil must be attached to a source of recirculating cold liquid such as a laboratory chiller.

Another suggestion for producers is to look for an ultrasonic unit equipped with a microprocessor-controlled ultrasonic generator that adjusts to the load; a degas mode to remove trapped air, and a timer that displays set and remaining time.

Other useful features include the ability to operate in a fixed frequency (also called normal) mode that is ideal for breaking up product and a sweep mode that provides uniform distribution of ultrasonic energy when it is used to clean glassware and other processing equipment (see below). The sweep mode delivers a small positive and negative fluctuation in ultrasonic frequency throughout the bath.

An Extraction Sequence

Water and a surfactant are added to the fill line of the sonicator tank. The unit is turned on and the degas function is activated to both mix the solution and drive off trapped air. This step should take about 10 min.

The product is lowered into the bath and the unit is set to operate in the normal mode. The generator provides ultrasonic energy in the bath that passes through flask walls. This step mixes, disperses, emulsifies, homogenizes, and dissolves the samples. The unit will shut down at the end of the timed cycle.

Operators will soon develop their own “techniques” or “standard operating procedures” for their processing cycles.

Cleaning Processing Glassware and Other Equipment
Substantial investments may be made in cannabis processing glassware and other tools. Because of the nature of the process, difficult-to-remove deposits adhere to the inside of flasks, test tubes, and beakers. Overall, cleaning is also recommended to ensure a quality product.

Cleaning internal surfaces can be accomplished by filling the container with a suitable biodegradable cleaning solution and, as with the extraction and processing steps, placing the container in the water–surfactant solution and activating the degas mode and ultrasound. Cavitation passes through the glass walls to loosen and remove the strongly adhering residues. These residues are then discarded and the containers can be rinsed for further use.

Small instruments can be placed in the mesh tray. In this case, the water–surfactant solution is removed and replaced with a degassed biodegradable formulation designed for glassware. In this instance, cleaning should be accomplished using the sweep mode to provide more-uniform cleaning.

To thoroughly clean internal and external surfaces of processing equipment, a larger ultrasonic cleaner is required with a suitable depth to enable full immersion of the equipment.

Biodegradable concentrates for labware are available in acidic, basic, and neutral formulations depending on the nature of the contaminants to be removed. All of the formulations come with material safety data sheets and use instructions including dilution recommendations and cleaning temperatures.

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Ultrasonic-ethanol pretreatment assisted aqueous enzymatic extraction of hemp seed oil with low Δ9-THC

Abstract

In this study, ultrasonic-ethanol pretreatment combined with AEE was developed for oil extraction from hemp seeds. The oil yield reached a maximum of 23.32 % at 200 W ultrasonic power and 30 min ultrasonic time, at this point, the degradation rate of Δ9-THC was 83.11 %. By determining the composition of hemp seed before and after pretreatment, it was shown that ultrasonic-ethanol pretreatment reduced the protein content of the raw material. An enzyme mixture consisting of pectinase and hemicellulase (1/1/1, w/w/w) was experimentally determined to be used, and the AEE extraction conditions were optimized using the Plackett-Burman design and the Box-Behnken. The optimal conditions were determined to be pH 5,

total enzyme activity of 37,800 U/g, liquid–solid ratio of 10.4 mL/g, enzyme digestion temperature of 32 °C, enzymatic time of 189 min, and oil recovery of 88.38 %. The results of confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) showed that the emulsion formed during ultrasonic ethanol pretreatment was not uniformly distributed, and the droplets appeared to be aggregated; and the irregular pores of hemp seed increased after pretreatment. The contents of Δ9-THC and CBN in the extracted oil samples were 9.58 mg/kg and 52.45 mg/kg, respectively. Compared with the oil extracted by Soxhlet extraction (SE), the oil extracted by this experimental method was of better quality and similar in fatty acid composition.

1. Introduction

Hemp (Cannabis sativa L.) is a monoecious herbaceous plant from the Cannabaceae family. It is one of the oldest cultivated crops in human history [1]. Hemp is gaining global attention due to the diverse applications of its roots, stems, leaves, flowers, seeds, and fibers. It is rich in nutritional components, including approximately 20–25 % protein, 20–30 % carbohydrates, and 28–35 % oil [2]. Hemp seed oil is a valuable product derived from hemp due to its high content of polyunsaturated fatty acids (PUFAs), including linoleic acid, α-linolenic acid, and oleic acid. Its PUFAs content is over 80 %, which is significantly higher than other vegetable oils [3], [4]. At the same time, it also has a good balance of linoleic acid (C18:2; n-6) and α-linolenic acid (C18:3; n-3) ratio (between 2:1 and 3:1). Hemp seed oil has the potential to reduce cardiovascular diseases, cancer, and autoimmune diseases due to its high levels of polyunsaturated fatty acids [5]. Additionally, it contains various bioactive compounds such as polyphenols, phytosterols, vitamins, and minerals [6].

The main active components in hemp are cannabinoids, including Δ9-tetrahydrocannabinol (Δ9-THC), cannabidiol (CBD), cannabinol (CBN), etc [7]. Modern pharmacological research suggests that Δ9-THC is a psychoactive substance with hallucinogenic and addictive properties [8]. CBD is non-addictive and has significant therapeutic potential, particularly in the treatment of mental disorders. CBN has been associated with several physiological functions, including anti-inflammatory and analgesic properties [9]. While most studies have confirmed that hemp seed oil is a high quality edible oil comparable to fish oil, concerns about its safety for consumption have received considerable attention both nationally and internationally in recent years due to its origin from traditional hemp or industrial hemp plants [10]. In various countries and regions worldwide, a series of regulations have been gradually introduced, mandating that the THC content in hemp seed oil and its products must not exceed specified limits. In the United States and Canada, the cultivation of hemp varieties with THC content exceeding 0.3 % is not permitted. Additionally, both the European Food Safety Authority and the European Industrial Hemp Association require that the THC content in hemp seeds and related oil products should be below 10 mg/kg [11]. Similarly, Australia, Belgium, and South Korea have established regulations stipulating that the maximum THC content in hemp seed oil should not exceed 10 mg/kg [12]. Therefore, it has become imperative to investigate efficient and environmentally friendly methods for the extraction of low Δ9-THC hemp seed oil. Current methods of extracting vegetable oils include solvent extraction, pressing and supercritical fluid extraction. Solvent extraction is highly efficient, but the significant residual

organic solvents pose significant risks to both human health and the environment [13]. Pressing technology, while mature and cost effective, results in lower oil yields, and high residual oil content in seed cakes, and can cause denaturation of hemp seed proteins during prolonged pressing, leading to waste of hemp resources [14]. Supercritical Fluid Extraction is an emerging method of oil extraction, known for its environmental friendliness and for producing high quality, non-toxic oil with no harmful residues. However, it is often considered costly and less suitable for widespread commercial production [15]. As a result, the food industry urgently needs to find an economically efficient hemp seed oil extraction technology to maximise the utilisation of hemp seed resources. Aqueous Enzymatic Extraction (AEE) of vegetable oils offers numerous advantages, including mild reaction conditions, superior oil quality, high by-product utilisation and environmental friendliness [16]. It utilizes water as a medium and incorporates enzymes to hydrolyse oil seed cell walls to extract oil, making it a viable alternative for oil extraction [17]. AEE has previously been used to extract for oil from various sources, including peanuts [18], soybeans [19], and walnuts in previous studies [20]. Despite its many advantages, AEE is still limited by low yield, long extraction times, and the occurrence of severe emulsification during the extraction process. In order to improve the efficiency of oil extraction, various methods have been developed to accelerate enzymatic hydrolysis, including pretreatment of raw materials before extraction and post-processing after extraction. However, these post-processing strategies are time-consuming, involve complex procedures, and have low yields, which limit the large-scale industrial application of AEE [21].

Ultrasonic technology, as an emerging method of oil extraction, has characteristics such as short processing time, environmental friendliness, and mild conditions. It has already found extensive applications in various industries [22]. Studies have shown that ultrasonic pretreatment of plant tissues, causes rapid extrusion, and collision between cells, resulting in the cavitation effect [23], which in turn destroys plant cell walls and changes the state of intracellular substances, making substrates more susceptible to enzyme attack, facilitating the release of oil within plant cells [24]. Bruno found that ultrasonic pretreatment unfolded fish protein and exposed enzyme binding sites, increasing the rate of enzyme hydrolysis and, consequently, the yield of fish oil extraction [25]. Furthermore, Chakma et al. investigated the effect of ultrasonic on the enzymatic degradation mechanism and found that ultrasonic combined with enzymatic energy effectively degraded bisphenol A and ciprofloxacin [26]. In summary, ultrasonic pretreatment is a simple process with mild conditions, that reduces the time required for aqueous enzymatic extraction and increases the extraction yield.

To the best of our knowledge, current research into hemp seed oil extraction techniques is extensive, focusing primarily on methods such as pressing and individual aqueous enzymatic extraction. However, these approaches often fail to address the issue of achieving a high oil yield. In addition, limited attention has been paid to the assessment of Δ9-THC content in hemp seed oil before and after extraction. In this work, hemp seed oil was extracted by pretreatment and aqueous enzymatic method. The Δ9-THC content in the oil samples subjected to different pretreatments was evaluated and the effect of pretreatment on Δ9-THC levels in the oil was investigated. Additionally, changes in sample composition and microstructure before and after pretreatment were investigated to gain further insight into the effects of different pretreatment methods on extraction characteristics. Preliminary studies

were conducted on the effect of enzyme types (enzyme mixtures and single enzymes) on oil recovery rates. Potential factors influencing the AEE process were screened using the Plackett-Burman design (PBD) and optimised using the Box-Behnken design (BBD). A comparison of the physicochemical properties and fatty acid composition of the extracted oils was also carried out.

The aim of this experiment was to study an environmentally friendly and efficient method of extracting hemp seed oil, to extract the maximum amount of hemp seed oil, and to control the content of Δ9-THC below various regulations in order to meet people's demand for hemp seed oil.

2. Materials and methods

2.1. Materials and chemicals

The shelled hemp seeds used in this experiment were purchased from Bama, Guangxi, China. They were thoroughly hulled, cleaned, and dried at room temperature until a uniform weight was reached. Prior to extraction, they were ground in a mill, sieved through a 60-mesh sieve, and stored at 4 °C until further use.

Cellulase (EC 3.2.1.1, activity 400,000 U/g, optimum pH range 3.5–5.5, temperature range 30–55 °C), hemicellulase (EC 3.1.1.73, activity 20,000 U/g, optimum pH range 4.0–8.0, temperature range 30–50 °C, from Bacillus subtilis), pectinase (EC 3.2.1.15, activity 500,000 U/g, optimal pH range 3.5–5.5, temperature range 30–55 °C) were purchased from Shanghai Yuan Ye Biotechnology Co, Ltd, Shanghai, China. Neutral protease (EC 3.4.24.28, activity 50,000 U/g, optimal pH range 6.0–8.0, temperature range 30–50 °C, from Bacillus subtilis), acid protease (EC 3.4.23.18, activity 50,000 U/g, optimal pH range 2.0–4.0, temperature range 35–55 °C, from Aspergillus niger), and alkaline protease (EC 3.4.21.14, activity 200,000 U/g, optimal pH range 9.0–11.0, temperature range 40–60 °C, from Bacillus licheniformis). A mixture of Δ9-THC, CBD and CBN, and a mixture of fatty acid methyl esters (FAME), were purchased from Sigma Aldrich Co., Ltd Saint Louis, MO, USA. The other chemicals were used as analytical reagents and were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China).

2.2. Preparation of hemp seed

A 60 % (v/v) ethanol solution (30 mL) was prepared and 10 g of shelled, dried and crushed hemp seed powder was added. The resulting suspension was then subjected to ultrasonic pretreatment at different power levels (0, 100 W, 200 W, 300 W, 400 W, 500 W) and for different durations (10 min, 20 min, 30 min, 40 min, 50 min) using an ultrasonic apparatus (SCIENTZ-IID, China) with a frequency of 25 kHz and power ranging from 20 to 1000 W. The resulting suspension was then centrifuged, the precipitate retained and dried.
The oils obtained from the different treatments were designated as follows: the oil extracted without addition of ethanol and without ultrasonic pretreatment was designated as Control; the oil extracted with addition of ethanol but without ultrasonic pretreatment was designated as Et; the oil extracted with ultrasonic pretreatment but without ethanol was designated as UL; and

the oil extracted with ultrasonic pretreatment in the presence of ethanol was designated as UL-Et.

2.3. Determination of basic nutritional components

The hemp seeds were analysed for their composition according to the international standards of the International Organization for Standardization (ISO), including the determination of moisture (ISO 771:2021) [27], protein (ISO 5983–1:2005) [28], ash (ISO 749:1977) [29], and lipids (ISO 11085:2015) [30]. In addition, the reducing sugars content of hemp seeds was determined according to the standards of the People's Republic of China (GB 5009.7–2016) [31].

2.4. Confocal laser scanning microscope (CLSM) analysis of hemp seed emulsion

The microstructure of the hemp seed emulsions produced during the AEE process was visually observed using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss Jena, Germany). First, 10 mg of Nile Blue and 1 mg of Nile Red were dissolved in 1 mL of isopropanol, thoroughly mixed, and then filtered through an organic membrane. Next, 20 μL of the staining solution was added to 1 mL of the emulsion in complete darkness, and after 30 min, 10 μL of the sample was applied to a glass slide. The emulsion was observed and images were taken using both 10 × and 20 × objectives,the scale was 40 μm.

2.5. Extraction of hemp seed oil

2.5.1. Ultrasonic-pretreatment combined with AEE of hemp seed oil (UCAEE)

The UCAEE process consists of pretreatment and aqueous enzymatic extraction. After various pretreatments as described above, an enzyme of specific activity was added to both the pretreated and untreated mixtures. The pH of the mixture was adjusted to the optimum range for enzyme activity using 0.1 mol/L HCl and 0.1 mol/L NaOH. The mixture was then incubated at the required temperature for a specified time. The mixture was then spun at 6000 × g for 20 min to isolate the free oil from the oil–water emulsion layer. The emulsion layer was subjected to freeze–thaw cycles to isolate the free oil, which was then collected [32]. The free oil was collected with anhydrous sodium sulphate and weighed. The oil yield was calculated according to the following formula:

2.5.2. Solvent extraction (SE)

The oil yield was determined using the standard Soxhlet extraction method. Twenty grams of hemp seed powder was extracted in 200 mL of n-hexane at 90 °C for 6 h using a Soxhlet extractor. The resulting mixture was then filtered, and the n-hexane removed using a rotary evaporator at 45 °C under vacuum. The extracted oil was further dried to constant weight under vacuum. The oil yield was calculated as 100 % per 100 g of seeds. The oil obtained by this process was stored at 4 °C for subsequent experiments.

2.6. Optimization of the AEE process

In this paper, the influence of single and mixed enzymes on the recovery of hemp seed oil was first investigated. Subsequently, the PBD method was used to determine the significance of

each variable on oil recovery, while the significant variables were further optimised using the BBD method.

2.6.1. PBD screening of significant variables

PBD is an efficient two-level experimental design method that allows for rapid screening of the most significant factors from a large number of factors with a relatively small number of experiments [33]. In this experiment, PBD was used to identify significant variables among the following and to investigate the effect of these independent variables on hemp seed oil production: pH (X1: 3–5), total enzyme activity (X2: 20,000 U/g-60,000 U/g), liquid-to-solid ratio (X3: 6–14 mL/g), enzyme hydrolysis temperature (X4: 30–70 °C), and enzyme hydrolysis time (X5: 120–240 min). These independent variables were investigated for their effects on the yield of hemp seed oil. The experimental parameters and their actual values are shown in Table S1. Each variable was run12 times at different combinations of low and high levels. The variables selected by the PBD design can be described by the following model:

In the equation, was the dependent response (oil recovery), was the constant coefficient of the model, was the linear regression coefficient for factor , was an independent variable, and was the number of variables. Factors with a confidence level greater than 95 % ($P < 0.05$) were considered to have a significant effect on oil recovery and were used for further optimisation using BBD.

2.6.2. Optimization of AEE by BBD

Based on the PBD design, it was found that enzyme activity (X2), liquid-to-solid ratio (X3), enzymatic hydrolysis temperature (X4), and enzymatic hydrolysis time (X5) significantly influenced the oil extraction rate. In this work, the BBD was used to further analyse the interaction of these variables and to determine the optimum conditions for the extraction of hemp seed oil. The experimental design is shown in Table S2, and a full quadratic equation model was fitted using Design Expert 9.0.4.5 software. The four variables were evaluated at three levels of coding, resulting in a total of 29 experiments (including three replications of the central point). The predicted response function was described by a second order polynomial equation:

In the equation, represented the response value (oil recovery). 、 、 , and were the model's constant coefficient, linear coefficients, interaction coefficients, and quadratic coefficients, respectively. and were different coded variables, and represented the number of variables.

2.7. Determination of Δ9-THC and CBN content in hemp seed oil

The determination of Δ9-THC and CBN in hemp seed oil samples obtained from different pretreatment methods was performed using high performance liquid chromatography (HPLC, 1260II, Agilent Technologies, USA). First, 1 mL of the mixed standard solution was transferred to a 20 mL amber volumetric flask and diluted to the mark with chromatographic-grade methanol, resulting in a standard solution of 50 mg/L, which was stored under light protected

conditions at temperatures below −20 degrees Celsius. The samples were then mixed with a methanol-hexane mixture (90:10, v/v) and extracted with stirring for 60 min. After extraction, the mixed solution was centrifuged and a second organic phase extraction was performed. Finally, the two extraction solutions were combined, and after vortex filtration, the Δ9-THC and CBN content was determined. The liquid chromatography analysis conditions included the use of an Eclipse Plus C18 column (5 μm, 100 mm × 4.6 mm), a column temperature of 35 °C, a flow rate of 1.0 mL/min, and an injection volume of 10 μL. The mobile phase consisted of an aqueous solution of 0.1 % formic acid and acetonitrile (1:3), held for 10 min. The measurement was performed at a wavelength of 220 nm.

2.8. Determination of the physicochemical properties and color of the oil samples

The physicochemical properties of hemp seed oil extracted by UCAEE and SE were determined using the standard methods of the American Oil Chemists' Society (AOCS) [34], including measurement of saponification value, iodine value acid value, and peroxide value. The color of the oil samples was measured using a Konica Minolta CM-5 spectrophotometer. Calibration was performed using a CM-A213 zero calibration board for the black standard, and distilled water for the white standard, in a 10 mm CM-A98 glass colorimetric dish. In this research, the Hunter Lab scale was used for color evaluation, measuring L*, a*, and b*. In the Hunter Lab scale, L* represents the brightness on a scale from black (0) to white (1 0 0), a* represents the hue from green (values below 0) to red (values above 0), and b* represents the hue from blue (values below 0) to yellow (values above 0).

2.9. Analysis of fatty acid compositions

The fatty acid composition of hemp seed oil extracted by both UCAEE and SE methods was analyzed by gas chromatography-mass spectrometry (GC–MS). Prior to analysis, the extracted oil was converted into fatty acid methyl esters (FAMEs) according to the method reported by [35]. An Agilent 7890 gas chromatograph (GC) equipped with an HP-88 column (30 m × 0.25 mm, 0.20 μm, Agilent, USA) was used for the analysis. Nitrogen was used as the carrier gas at a flow rate of 1 mL/min. The injector and detector (FID) were maintained at 220 °C. The initial temperature was set at 70 °C, with a ramp rate of 15 °C/min to 120 °C, held for 1 min, followed by another ramp rate of 5 °C/min to 175 °C, held for 10 min, and a final ramp rate of 5 °C/min to 220 °C, held for 5 min. The detected chemical components were matched with the mass spectral information and retention indices of various peaks in the NIST02 standard mass spectral library. The relative content of fatty acids in hemp seed oil was determined using the peak area normalization method.

2.10. Scanning electron microscope (SEM)

The microstructure of hemp seeds before and after extraction was observed using a scanning electron microscope (JEOL JSM-7500F, Tokyo, Japan). The samples were coated with a thin layer of gold palladium after vacuum drying. The observations were made under the following conditions: an operating voltage of 15.0 kV and a magnification of 2000×.

2.11. Statistical analysis

The PBD and BBD experiments were performed using Design Expert 9.0.4.5 software from Stat Ease Inc. (Minneapolis, MN, USA), for statistical analysis. All experiments were performed with three replicates, and the actual values of each PBD and BBD run were presented as averages. Other results are presented as mean ± standard deviation. Statistical analysis of mean differences was performed using t-tests in SPSS 16.0 (Statistical Package for Social Science) software (SPSS Inc., Chicago, IL, USA) at a significance level of $p < 0.05$.

3. Results and discussion

3.1. The impact of ultrasonic pretreatment on hemp seed oil

Based on the preliminary experimental results, the AEE conditions after ultrasonic-ethanol pretreatment; were set to which included a pH 5, total enzyme activity of 60,000 U/g, liquid–solid ratio of 6 mL/g, enzymatic hydrolysis temperature of 50 °C, and enzymatic hydrolysis time of 180 min. In this work, ultrasonic was used only for pretreatment and was not used in the actual extraction process in combination with enzymatic hydrolysis. This was because ultrasonic could cause emulsification in systems containing water and oil, and the tendency for emulsification and de-emulsification was greatly influenced by the time and intensity of ultrasonic. Wang et al. conducted research on ultrasonic-assisted aqueous enzymatic extraction of gardenia oil, and they found that prolonged exposure to ultrasonic led to a more significant de-emulsification phenomenon compared to emulsification [36]. This phenomenon was closely related to the intensity of the ultrasonic. In addition, enzyme activity was highly sensitive to both the intensity and time of ultrasonic, and excessive ultrasonic power and time may reduce enzyme activity, resulting in decreased extraction efficiency. At the same time, the continuous generation of heat during the ultrasonic treatment process may affect the quality of the extracted oil [37]. On the other hand, studies using density functional theory demonstrated that ultrasonic could lead to the degradation of Δ9-THC and verified its degradation pathway [38].

Fig. 1a shows the oil yield after different pretreatments. The yield without any treatment was the lowest, and the effect of ultrasonic was more pronounced than that of ethanol. The oil yield increased significantly when ultrasonic was combined with ethanol pretreatment. Therefore, in the present work, ultrasonic-ethanol pretreatment was used to modify the substrate to improve the efficiency of subsequent AEE and reduce the Δ9-THC content in oil. Fig. 1(b,c) illustrates the effect of ultrasonic power and time on the oil extraction rate and Δ9-THC degradation rate in hemp seeds. The oil yield was the percentage of extracted oil quality in hemp seed powder quality. Ultrasonic pretreatment effectively increased the oil yield from hemp seeds. When the samples were treated with ethanol only, without ultrasonic (the point at 0 power in Fig. 1b), the oil yield was 8.19 %. However, with ultrasonic power of 200 W, the maximum oil yield reached 23.32 %. The degradation rate of Δ9-THC degradation rate initially increased with ultrasonic time and then leveled off. At 200 W, it reached 92.14 %. At this point, the Δ9-THC content was much lower than the limits set by most countries and regions for food products (10 mg/kg). Ultrasonic cavitation induces strong mechanical forces that can disrupt the cell wall surface, cause sonoporation, and even damage cells [39]. This process increased cell permeability,

which promoted enzyme entry and lipid release. The results indicated that both too low and too higher power levels had a limited effect on increasing oil recovery rates. Higher power levels generally lead to more intense cavitation effects, thereby improving extraction efficiency. However, excessive ultrasonic power could disrupt bubble dynamics, resulting in diminished cavitation effects [40]. Heidari and Dinani reported similar findings when they applied ultrasonic pretreatment to peanut seeds in n-hexane solvent, achieving a higher extraction rate compared to traditional methods [41]. These findings indicate that while increasing ultrasonic power can enhance oil extraction, it also has certain limitations.

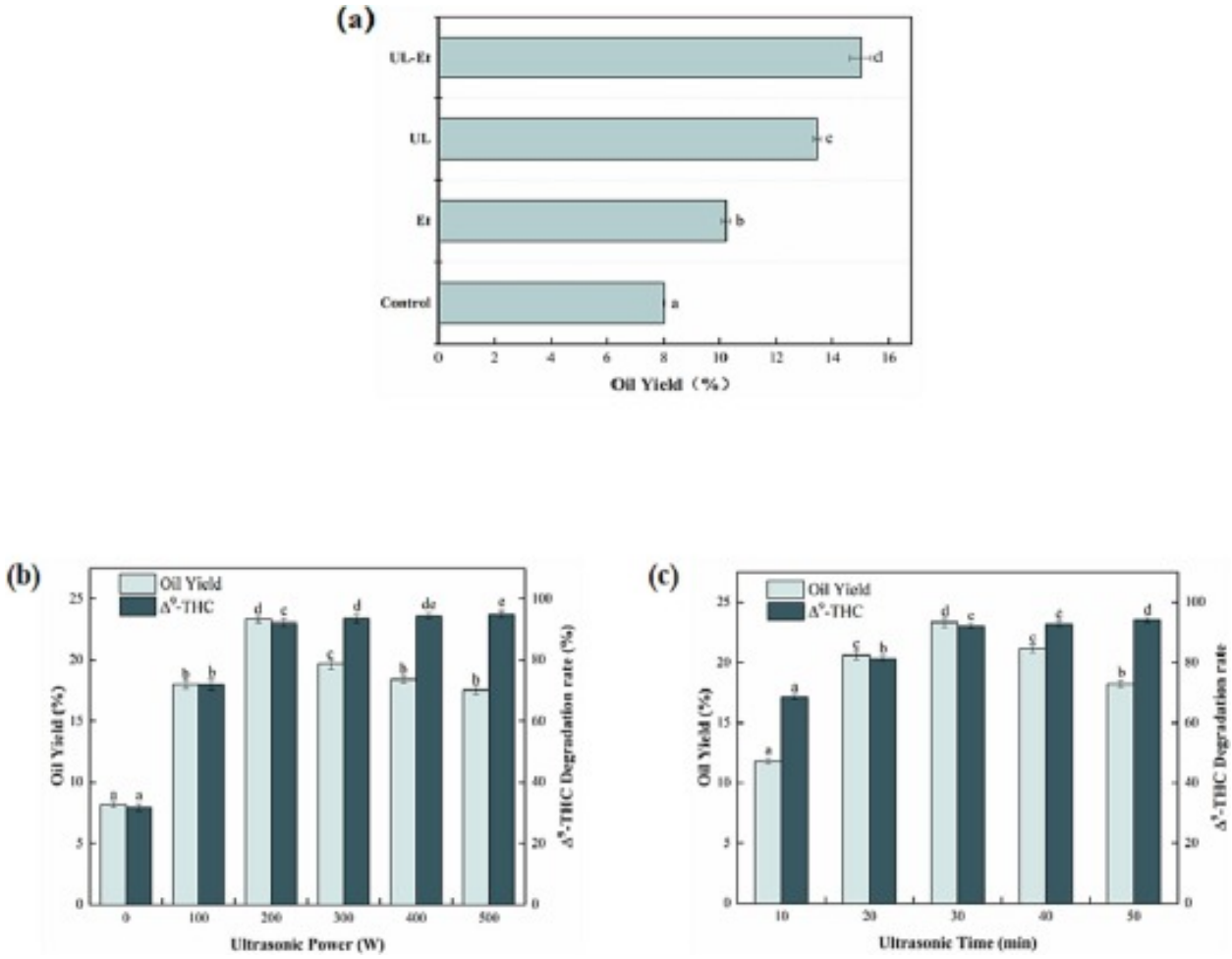

Fig. 1. Yield of hemp seed oil after different pretreatment (a). Effects of power and time of ultrasonic pretreatment on hemp seed oil (b) and (c).

Fig. 1c shows the influence of ultrasonic treatment duration on oil yield and the degradation rate of Δ9-THC. The oil yield exhibited rapid growth with prolonged ultrasonic treatment, peaking at 30 min and subsequently declining. The degradation rate of Δ9-THC increased over time, with a noticeable effect within the first 30 min. Beyond this duration, the degradation rate stabilized, indicating that the majority of Δ9-THC in the oil had either been absorbed or degraded. This phenomenon can be attributed to the fact that, after 30 min of ultrasonic treatment, most of the Δ9-THC in the oil had been absorbed or degraded. When combined with the findings presented in Fig. 1b, it becomes evident that ethanol pretreatment has the potential to increase oil yield, and ultrasonic pretreatment further enhances this effect. This is

attributed to the ethanol solution's ability to permeate the plant cell walls, dissolving and extracting hydrophobic substances from hemp seeds. During pretreatment, ethanol reabsorbs the impurities suspended in the system into the ruptured cells, thereby reducing solvent permeability. Additionally, as ethanol penetrates further into the cells, its effective area decreases while the distance it covers increases, leading to limited dissolution of amphiphilic substances. Ultrasonic treatment disrupts plant cell walls, enhances mass transfer, and increases solvent penetration, thus facilitating the extraction of hydrophobic compounds by ethanol [42].

3.2. Nutrient composition analysis

Table 1 presents the nutritional composition of hemp seeds with and without ultrasonic-ethanol pretreatment. Following ultrasonic-ethanol pretreatment, there was a decrease in oil content, reducing sugars, and protein content, while moisture and ash content slightly increased. This phenomenon can be attributed to the disruptive effect of ultrasonic waves on cell walls, and the cell permeability enhancement caused by ethanol, resulting in the release of a small amount of oil and protein. Considering that proteins play a crucial role in emulsion formation during the AEE process [43], the decrease in protein content may lead to a reduced formation of emulsions in this process. As a result, the oil yield from hemp seeds after ultrasonic pretreatment was higher compared to untreated hemp seeds [44].

Table 1. Analysis of Basic Components of Hemp Seed.

Content (%)	Untreated	Ultrasonic-ethanol pretreated
oil	42.34 ± 0.64a	38.47 ± 0.10b
protein	26.03 ± 0.61a	22.53 ± 0.11b
moisture	6.48 ± 0.04a	7.51 ± 0.23b
ash	4.19 ± 0.23b	5.04 ± 0.15a
reducing	4.46 ± 0.06a	4.07 ± 0.19b

Different letters in the same row mean a significant difference ($p < 0.05$).

3.3. CLSM analysis of hemp seed emulsion

To explore the effect of pretreatment on hemp seed tissue cells, the microstructure of the pretreatment hemp seed emulsion of the AEE was observed by CLSM. Fig. 2 shows laser confocal microscopy images of hemp seed emulsions formed after different pretreatments. The yellow portions represent protein-oil complexes formed by Nile red-stained hemp seed oil and Nile blue-stained hemp seed protein. From Fig. 2a, it can be seen that the distribution of lipids in untreated hemp seed oil is comparatively uniform, with fewer aggregates of oil droplets and proteins, indicating a relatively stable emulsion. Yang et al. (2021) conducted research on camellia and found that untreated camellia proteins, polysaccharides, and saponins exhibit

abundant electrostatic interactions, resulting in their most uniform distribution [45]. This phenomenon was similarly observed in hemp seed. Fig. 2b shows the distribution state of the emulsion after sonication alone, which has a more homogeneous and smaller particle size distribution compared to the untreated emulsion. In general, the oil droplets in the emulsion are subjected to the cavitation effect and fragmentation produced by ultrasound, dispersing into emulsions with smaller particle sizes and better emulsification performance [46]. However, the addition of ethanol may inhibit this phenomenon, as shown in Fig. 2 c and d, the emulsion formed larger aggregates, which may be due to the solubilization of amphiphiles by ethanol as well as penetration into the cells, resulting in the formation of larger aggregates, leading to a decrease in emulsification performance. When ethanol and ultrasonic pretreatment acted together, the oil release was more complete, the emulsion particle size distribution became non-uniform, and the droplets appeared aggregated, which may be due to the reduction of spatial and electrostatic repulsion between the droplets. Therefore, ultrasonic-ethanol pretreatment can reduce the stability of the emulsion, which can improve the extraction efficiency of the subsequent AEE to some extent.

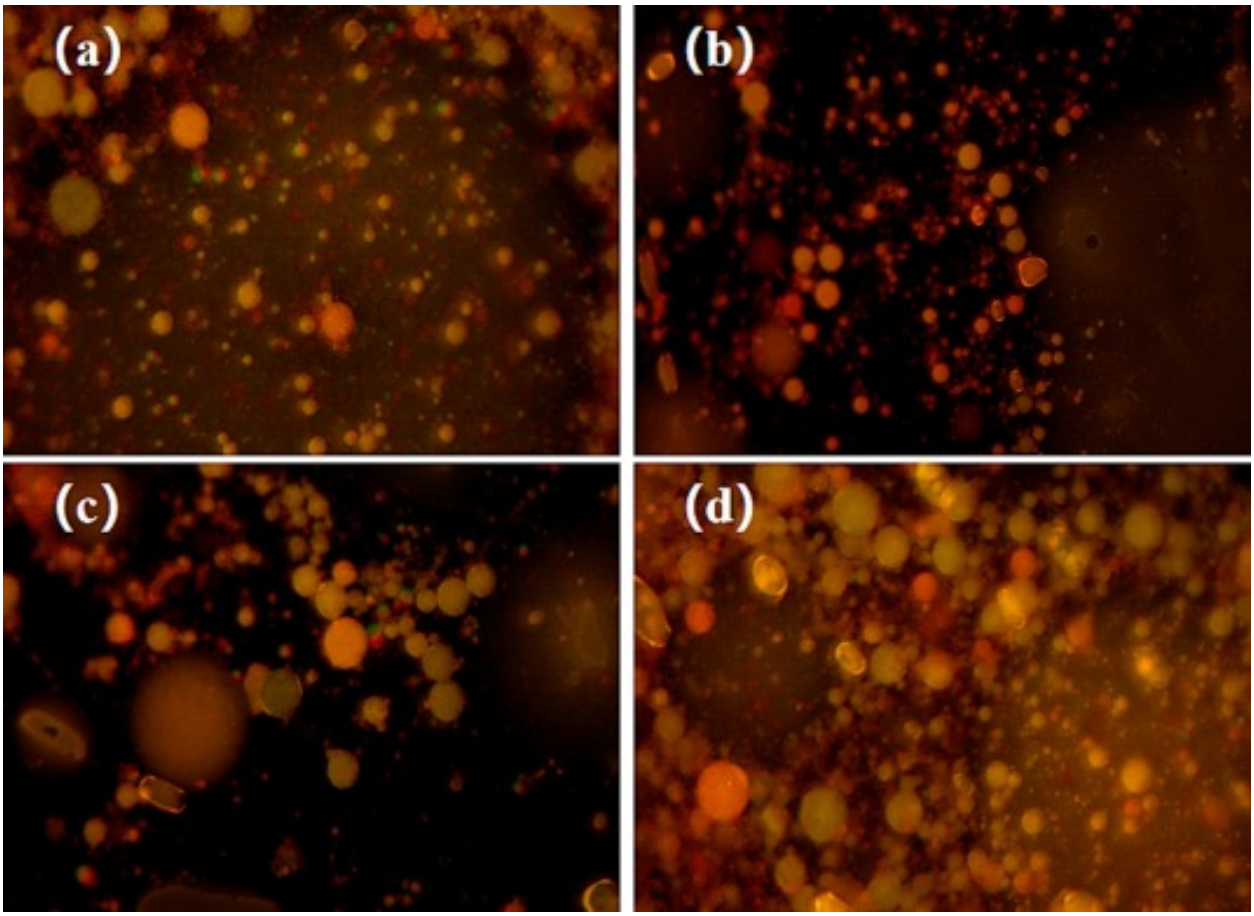

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Fig. 2. The CLSM results of hemp seed emulsion. Note: The four different pretreated emulsions are as follows: Untreated hemp seed emulsion (a); Hemp emulsion without ethanol (60 %) ultrasonic pretreatment (b); Ultrasonic treatment (200 W, 20 min) of hemp emulsion without

ethanol pretreatment (c); Ultrasonic ethanol pretreatment (200 W, 20 min, 60 %) of hemp seed emulsion (d).

3.4. Effects of different enzymes on the oil recovery of hemp seed

Owing to the varied compositions of different raw materials, the role of specific enzymes is crucial in enhancing the efficiency of oil recovery [47]. Domínguez et al. have observed that oil present in plant cells is closely associated with proteins and a range of carbohydrates [48]. Consequently, the direct action of enzymes or mechanical disruption of cell structures promotes the release of oil from plant materials into the solution. Fig. 3 illustrates the impact of different enzyme types on oil recovery rates. The oil recovery rates using different types of individual enzymes exceeded the control condition (no enzyme). Under the condition of constant total enzyme activity (40,000 U/g), the addition of cellulase, hemicellulase, and pectinase in a ratio of 1:1:1 (w/w/w) significantly increased the oil recovery compared to the use of the three enzymes separately. This indicated that the mixed enzymes were more favorable for the release of oil from hemp seeds. In plant cells, cellulose and hemicellulose are the primary components of the internal cell wall structure. Hydrolysis of cellulose and hemicellulose by enzymes facilitates the release of oil from the cells [49]. Conversely, pectinase is a component of the middle layer of the cell wall, and intercellular adhesion hinders oil release [50]. Therefore, the addition of mixed enzymes promoted the degradation of cellulose, hemicellulose, and pectin within the cell wall, thereby compromising the integrity of the hemp seed cell structure, and making oil release more efficient. The pH and temperature ranges of cellulase, pectinase, and hemicellulase are similar, facilitating their mutual combination to enhance hydrolysis efficiency. In oil extraction from yellow horn seeds, Li et al. achieved similar results using microwave-assisted enzymatic extraction. The use of mixed enzymes, including cellulase, pectinase, and hemicellulase in a 1:1:1 (w/w/w) ratio, resulted in higher oil recovery rates compared to the use of single enzymes [51]. Therefore, a mixed enzyme combination of cellulase, pectinase, and hemicellulase in a 1:1:1 (w/w/w) ratio was selected for further oil extraction.

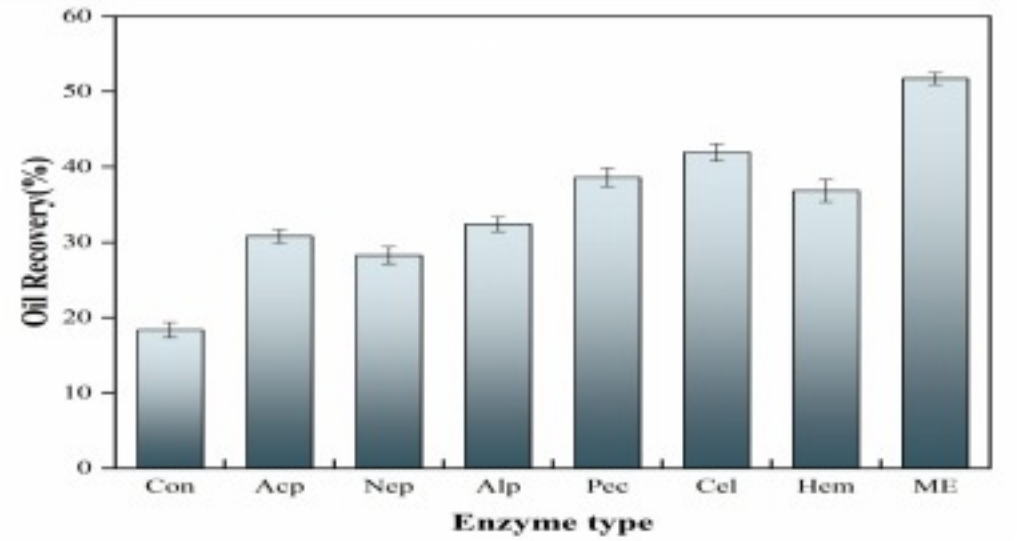

Fig. 3. Effects of different enzymes on oil recovery. Note: Con: control; Acp: acid protease; Nep: neutral protease; Alp: alkaline protease; Pec: pectinase; Cel: cellulase; Hem: hemicellulose; And ME: mixed enzyme (cellulase/hemicellulase/pectinase = 1/1/1, w/w/w). Set different enzymes to the optimal extraction range given by the manufacturer. The enzymolysis temperature and pH of the mixed enzyme were set at 40 °C and 3.5. Other parameters are set as follows: ultrasonic pretreatment power is 200 w, ultrasonic time is 40 min, enzyme concentration is 1.5 %, liquid–solid ratio is 10 mL/g, and enzymolysis time is 60 min.

3.5. Optimization of the AEE process

3.5.1. The main factors were screened by PBD

The design and results of the PBD were presented in Table S1, and the first-order linear equation explaining the impact of various variables on the oil recovery rate of hemp seed (Y) was as follows:

The results suggested that in the 12 sets of experiments, the oil recovery rate ranged from 39.25 % to 64.75 %, highlighting the significant influence of the experimental process on oil recovery. The model's F-value was 73.75, indicating the significance of the model. The coefficient of determination (R2) value of 0.9840 implied that 98.40 % of the variability could be explained by this model. The coefficient of variation (CV) of less than 5 % suggested good model precision, with a CV of 3 % indicating reliability. Variables with a confidence level exceeding 95 % were considered to have a significant impact on the independent variables. From Table S1, it was evident that X2 (enzyme activity), X3 (liquid-to-solid ratio), X4 (enzymatic temperature), and X5 (enzymatic time) had a significant impact on oil recovery ($p < 0.05$), while X1 (pH) did not significantly affect oil recovery ($p > 0.05$).
Typically, pH could affect the extraction rate of oil, with each enzyme having an optimal pH range for activity. However, the optimal pH ranges for the three enzymes used in this study were close. When the pH was within the optimal range, the mixed enzymes exhibited high activity in oil extraction, resulting in a non-significant effect on oil recovery, and it is not included in the subsequent optimization phase.
Enzyme activity is the basis for oil release, and insufficient enzyme activity may not be sufficient to degrade cell wall components. Excessive enzyme activity can lead to enzyme aggregation due to experimental conditions, reducing enzyme activity relative to the solid substrate. During the course of this research, the enzyme dosage varied from 1 % to 3 %, and its effect on oil recovery was considered significant and included in the next phase of optimization.

Regarding the impact of the liquid-to-solid ratio, a low ratio results in high viscosity, making it difficult to homogenize the mixture. Conversely, excess water reduces the affinity of enzyme for the substrate, reducing enzymatic effectiveness and causing difficulties in subsequent processing, negatively affecting oil extraction [52].
Regarding enzymatic temperature, a significant variation in oil recovery rates was observed between 30 °C and 70 °C ($p < 0.05$). Generally, enzymes exhibit peak activity within a specific

optimal temperature range. The behavior of different enzymes can vary notably when they function collectively under identical conditions. Given the distinct temperature ranges of cellulase, pectinase, and hemicellulase used in this study, the optimal temperature range for the enzyme mixture is a cumulative effect of these three enzymes. This underscores the necessity of identifying the most suitable temperature range for the combined enzymes. Concerning enzymatic duration, achieving complete oil release from cell walls necessitates a specific period. An inadequately short extraction time leads to insufficient interaction between enzymes and cells, resulting in lower oil recovery. Prolonging the extraction time improves cell wall disruption and enzymatic hydrolysis, thus enhancing oil recovery. However, overly extended extraction times are impractical and may result in compromised product quality and reduced energy efficiency [15]. Consequently, identifying the optimal enzymatic duration is essential. In subsequent experiments, parameters with minimal impact, like pH, were maintained at 5, while the optimal levels of critical variables (total enzyme activity, liquid-to-solid ratio, enzymatic temperature, and enzymatic time) will be extensively explored using the BBD.

3.5.2. Fitting the model

After PBD screening for main factors, following the principles of the BBD, the four independent variables were designed as four factors with three levels each, resulting in 29 experimental runs (with five repetitions at the center point), and the response surface experimental results were shown in Table S2. The Design Expert 9.0.4.5 software was used to process the experimental results in Table S2, with extraction rate as the response variable. Regression fitting was performed concerning each factor, resulting in the following second-degree polynomial regression equation:

$$Y = 88.22 + 1.48X2 + 1.43X3 - 2.78X4 + 2.88X5 - 4.23X2X3 + 1.00X2X4 - 2.02X2X5 - 0.88X3X4 + 0.050X3X5 - 0.025X4X5 - 8.69X22 - 3.24X32 - 1.84X42 - 6.64X52.$$

The results of the analysis of variance (ANOVA) showed that the model had high credibility with a very low p-value ($P < 0.0001$). At the same time, the lack of fit was not significant ($P = 0.0562 > 0.05$), indicating that the equation fit the data reasonably well and could be used to predict the actual values. Factors X2、X4、X5、X2X3、X22、X32, and X52 had a highly significant impact on the response variable ($P < 0.01$), while factors X3、X2X5, and X42 had a significant influence ($P < 0.05$).

The results clearly showed that enzymatic duration and temperature were the two principal factors significantly influencing the extraction rate, with enzyme activity and the liquid-to-solid ratio also playing notable roles. The interaction between the liquid-to-solid ratio and enzyme activity, as well as between enzymatic duration and enzyme activity, significantly impacted hemp seed oil yield ($P < 0.05$). In contrast, the interaction effects of other factors were not statistically significant in influencing the extraction rate. The regression model's R2 value of 0.9622 and adjusted R2 of 0.9243 indicate that it could account for 96.22 % of the variability in the response variable. In essence, the model demonstrates a strong fit with the experimental data, offering a solid foundation for optimizing experimental conditions and forecasting the optimal process for extraction rate.

3.5.3. Response surface analysis

Based on the fitted equations, interaction response surface analysis diagrams were generated for each variable. The results were shown in Fig. 4. The slope of the response surface plots reflects the influence of factors on the response variable. A steeper slope, indicating a more pronounced incline, signifies a greater impact of the interaction between two factors on the response variable. Upon comparing the trends in slope steepness in the response surface plots (Fig. 4), it becomes evident that enzymatic duration (X5) exerts a significant impact on the extraction rate, followed by enzymatic hydrolysis temperature (X4) and enzyme activity (X2). In contrast, the response surface associated with the liquid–solid ratio (X3) appears relatively flat, indicating a comparatively minor influence on the extraction rate. A notably significant interaction effect between the liquid–solid ratio (X3) and enzyme activity (X2) on the recovery rate was observed ($P < 0.01$) (Fig. 4a). When the liquid–solid ratio was low, the extraction rate initially increased and then decreased with varying enzyme activity. A significant interaction effect between enzyme activity (X2) and enzymatic duration (X5) on the recovery rate was observed ($P < 0.05$) (Fig. 4c), with the recovery rate initially increasing and then decreasing with their increments. The interaction among the liquid–solid ratio (X3), enzymatic temperature (X4), and enzymatic duration (X5) did not have a significant impact on the recovery rate.

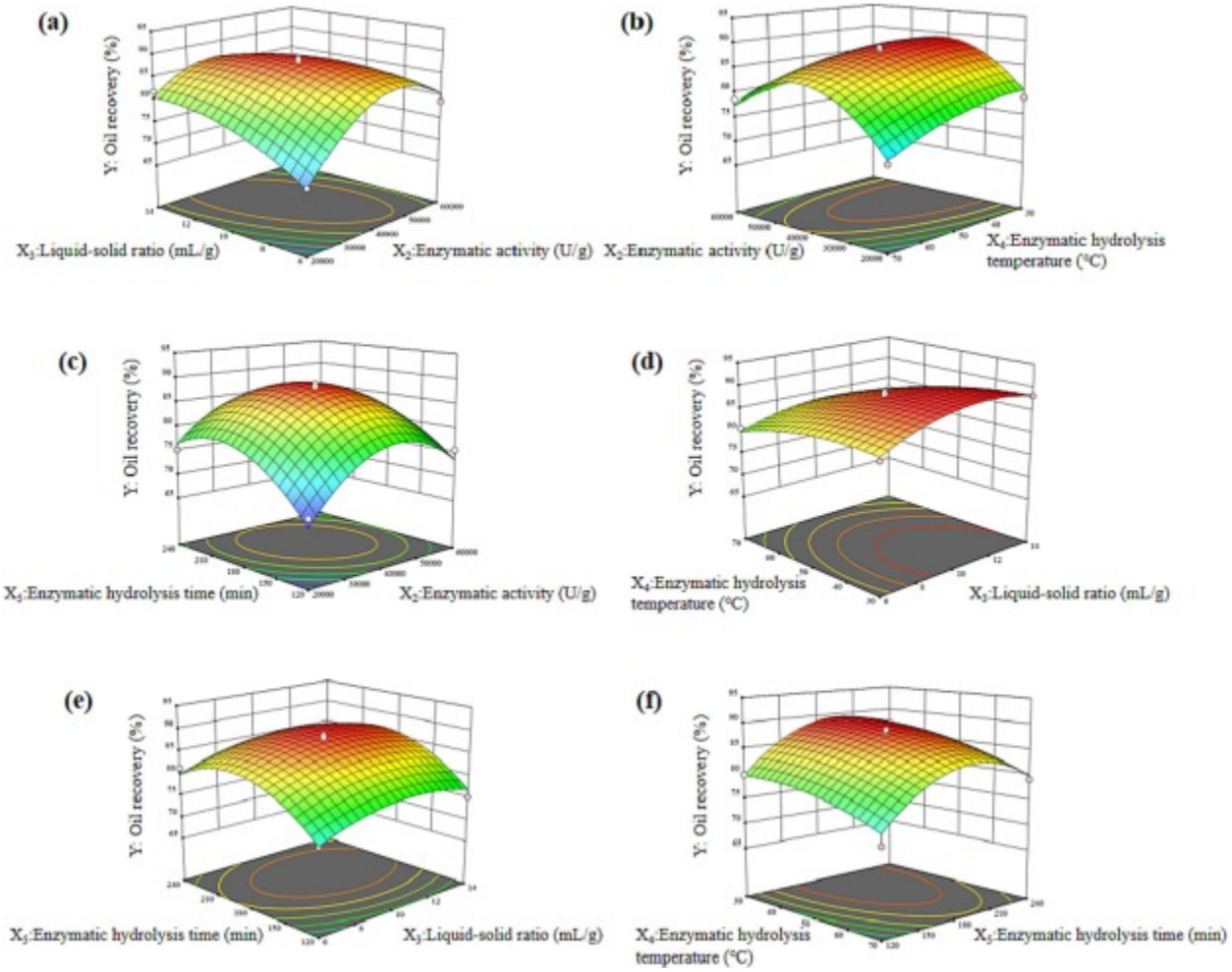

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Fig. 4. Interactive response surface images among various factors. Note: Interaction response surfaces of enzyme activity and liquid–solid ratio (a), enzyme activity and enzymolysis temperature on oil yield (b), enzyme activity and enzymolysis time on oil yield (c), liquid–solid ratio and enzymolysis temperature on oil yield (d), liquid–solid ratio and enzymolysis time on oil yield (e), enzymolysis temperature and enzymolysis time on oil yield (f).

3.5.4. Verification of optimal conditions

In order to assess the model's reliability, experiments were conducted using the established regression model. Under pH 5 conditions, the model provided the following optimal process parameters: total enzyme activity of 37,833 U/g, liquid-to-solid ratio of 10.4 mg/L, enzymatic temperature of 32.2 °C, and enzymatic duration of 188.5 min. These parameters were expected to yield an oil recovery rate of 89.61 %. For practical operational purposes, adjustments were made to the enzyme activity, enzymatic temperature, and enzymatic duration, resulting in values of 37,800 U/g, 32 °C, and 189 min, respectively. Under these modified conditions, the actual oil recovery rate was (88.38 ± 0.59) %, closely matching the predicted recovery rate. Compared with previous studies, the present experiment can ensure a higher oil recovery rate in an environmentally friendly way. For example, LIN et al. obtained an oil recovery of 82.6 % by ultrasound-assisted extraction of cannabis seed oil, which was lower than that of 88.38 % [53]. González et al. used a hydro-enzymatic method to extract oil from walnuts and obtained only 75.4 % oil recovery after response surface optimization [54]. Therefore, the extraction conditions for hemp seed oil, as determined by the regression model, were proven to be reliable and applicable.

3.6. Analysis of Δ9-THC and CBN contents in hemp seed oil

The content of Δ9-THC and CBN in hemp seed oil extracted by AEE after different pretreatment was determined by liquid chromatography, and the content of Δ9-THC and CBN in the oil sample was shown in Table 2. Based on the standard sample results, liquid chromatography analysis was conducted on the extracted oil to determine the respective peak areas corresponding to retention times. The analysis indicates that the oil extracted without any preprocessing contains a Δ9-THC content of 121.9 mg/kg and a CBN content of 3.48 mg/kg. When ultrasonic and ethanol were applied individually to the oil samples, the Δ9-THC content experienced a significant reduction, and ultrasonic was better than ethanol. In the case of combined treatment, the Δ9-THC content in the oil sample was measured at 9.58 mg/kg, which was significantly lower than the established standards for food products in most countries and regions, typically set at 10 mg/kg. This demonstrated that the combination of ultrasonic-ethanol pretreatment could significantly reduce the Δ9-THC content in hemp seed oil.

Table 2. Analysis of Δ9-THC and CBN contents in hemp seed oil.

Different pretreatment	Δ_9-THC (mg/kg)	CBN (mg/kg)

Control	121.90 ± 1.18a	3.48 ± 0.57a
Et	83.21 ± 1.24b	18.74 ± 0.43b
UL	19.96 ± 0.94c	46.31 ± 1.02c
UL-Et	9.58 ± 0.85d	52.45 ± 0.87d

Different letters in the same row mean a significant difference ($p < 0.05$).
Note: Control: without ethanol and ultrasonic pretreatment; Et: with ethanol but without ultrasonic Pretreatment;
UL: with ultrasonic pretreatment but without ethanol; UL-Et: with ultrasonic pretreatment of ethanol system.

Recent studies have shown that Δ9-THC in hemp can be degraded under conditions such as acidity, high temperature, and exposure to light, yielding primarily the non-psychoactive component CBN. Wang et al. used DFT to investigate the degradation pathways and mechanisms of Δ9-THC and discovered that Δ9-THC conversion to CBN is a spontaneous reaction, but a certain amount of energy is required to transform the ground state molecule into an excited state to start the reaction process [38]. Ultrasonic treatment produces cavitation effects, which generate high temperatures and pressures locally. The energy released during ultrasonication serves as the excitation energy to convert the ground state Δ9-THC molecules into their excited state, facilitating molecular transitions, chemical bond transformations, and hydrogen atom release, thereby achieving the degradation of Δ9-THC. The results in Table 2 show that the CBN content in the untreated oil was only 3.48 mg/kg. However, after various pretreatments, the CBN content increased significantly, reaching a peak of 46.31 mg/kg with ultrasonic pretreatment alone. When combined with ethanol treatment, there was a slight decrease to 52.45 mg/kg. This phenomenon is attributed to ultrasound-induced degradation of Δ9-THC into CBN, with ethanol interacting with the seeds and dissolving some cannabinoids. This is also in agreement with Yangsud et al. who found that Δ9-THC in hemp seed oil was degraded to a greater extent under light at 22 °C than under dark conditions at 4 °C, along with an increase in the CBN content of the oil [55]. The above results indicate that ultrasonic ethanol pretreatment is an effective means to significantly reduce the Δ9-THC content of the oil, which has a negative impact on the human body.

3.7. Physical and chemical properties and color analysis of oil

Table 3 presents a comparison of the physicochemical properties of oils extracted using the UCAEE and SE methods. The acid value and peroxide value of the oil obtained through the UCAEE method were notably lower than those obtained through the SE method, measuring 1.76 mg KOH/g and 0.93 meq O2/kg, respectively. These values are in compliance with the Codex standard for vegetable oils [56]. The acid value reflects the concentration of free fatty acids in the oil; an excessively high value indicates significant oxidation of the oil. Similarly, the peroxide value is indicative of the oil's oxidation degree, with higher values indicating more pronounced oxidation. This may be attributed to the elevated temperature and extended

extraction time in the SE process, which exacerbated the oil's oxidation. Previous studies have indicated that hemp seeds typically have a higher iodine value compared to most vegetable oils, reflecting their higher degree of unsaturation. Table 3 reveals that the iodine value of the UCAEE-extracted oil exceeded that of the SE extracted oil, signifying a higher proportion of unsaturated fatty acids in hemp seed oil and a more effective extraction. Regarding color, the oil obtained through the UCAEE method appeared as a clear yellow, whereas the oil obtained via the SE method exhibited a darker yellow hue. This difference may be attributed to the incomplete removal of hemp seed shells during the pressing process and the retention of more chlorophyll. However, the UCAEE extraction involved a washing step, allowing some of the oil-absorbed pigments to settle due to the presence of phospholipids, ultimately resulting in a lighter color. It is evident that the UCAEE method produced high quality oil, establishing it as an excellent oil extraction technique.

Table 3. Comparison of Fatty Acid Composition (%) and Physical and Chemical Properties of Hemp Seed Oil Extracted by UAAEE and SE.

Fatty acid composition				Physicochemical properties		
No	Fatty acid	UAAEE	SE	Index	UAAEE	SE
1	Saturated fatty acids C16:0	5.94 ± 0.10a	6.25 ± 0.07a	L* unit	36.32 ± 1.93	36.46 ± 2.17
2	C18:0	5.32 ± 0.	5.53 ± 0.	a* unit	14.75 ±	13.78 ± 0
3	Monounsaturated fatty acids	11.56 ± 0.15a	11.78 ± 0.16a	b* unit	31.89 ± 1.27	32.24 ± 1.39
4	Polyunsaturated fatty acids	54.82 ± 0.31a	54.59 ± 0.12a	Acid value (mg KOH/g)	1.76 ± 0.09 a	2.01 ± 0.10a
5	C18:3	19.25 ± 0.19a	17.35 ± 0.23a	Peroxide value	0.93 ± 0.05b	1.10 ± 0.12a
6	ω-6/ω-3	3.59 ± 0.06a	3.35 ± 0.07b	Iodine value (g	167.80 ± 0.31 a	164.33 ± 0.10b
Total	Saturated fatty acids	11.26	11.78	Saponification value (mg	123.41 ± 0.12 a	123.76 ± 0.21b

Total	Monounsaturated fatty acids	11.56	11.78			
Total	Polyunsaturated fatty acids	74.07	71.94			

Different letters in the same row mean a significant difference ($p < 0.05$).

3.8. Fatty acids composition analysis

Table 3 shows the fatty acid composition of hemp seed oil obtained using the optimal conditions of UCAEE and the SE method. The table reveals that there were no significant differences in the fatty acid composition of hemp seed oil obtained through the two methods, suggesting that the UCAEE method had no adverse impact on the fatty acid composition of hemp seed oil. The analysis indicated that the proportion of unsaturated fatty acids was relatively high, with C18:2 and C18:3 content at 55.92 % and 20.25 %, respectively. These results align with the findings of Juhaimi et al [57]. Furthermore, the ratio of ω-3 to ω-6 fatty acids was closer to the WHO recommended ratio compared to the SE method, aligning better with the principles of human health. Regarding the total fatty acid content, UCAEE exhibited higher levels than SE, consistent with the previously discussed physicochemical results. Considering the analysis of both physical and chemical properties, the UCAEE extracted oil demonstrated a superior ability to preserve its polyunsaturated fatty acids, maintain a more balanced proportion, and ensure the overall quality of the extracted oil.

3.9. SEM analysis

In order to investigate the impact of each pretreatment on hemp seed tissue cells, the microstructure of the pretreated hemp seeds was examined using a scanning electron microscope. Fig. 5 presents SEM images of hemp seeds following different pretreatment methods. From the above four figures (a, b, c, d), it can be seen that the irregular pores between the cell tissues gradually become larger. The surface morphology of untreated hemp seeds (a) appeared relatively regular, with fewer oil droplets, making it less favorable for subsequent oil extraction. Following individual ethanol pretreatment (b) and ultrasonic pretreatment (c), it was observed that the cell surface pores enlarged, leading to an increased presence of oil droplets on the surface. This was because ethanol could remove hemicellulose components from the plant cell walls, reducing cellulose crystallinity and increasing the porosity of the raw material [58]. Fig. 5 (d) shows that the irregular pores on the surface of hemp seeds pretreated with ultrasonic ethanol significantly enlarged, and numerous intact oil droplets adhered to the surface. This was attributed to the disruptive effect of ultrasound on the hemp seed cell walls, facilitating the release of oil and protein from the cells. Ethanol further expedited this process, enhancing the efficiency of the subsequent AEE process.

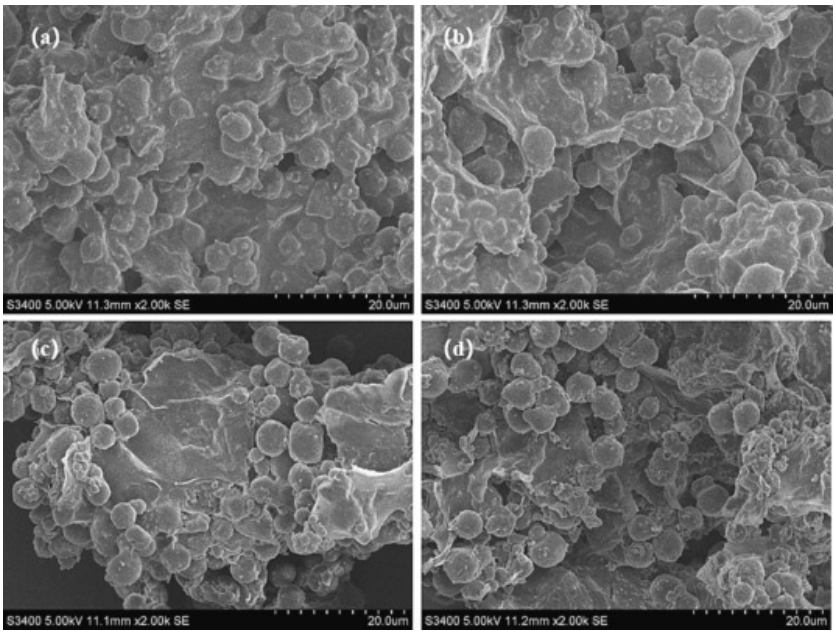

Fig. 5. Microstructure of Hemp Seeds after different pretreatment. Note: Untreated hemp seed powder (a); Hemp seed powder without ultrasonic pretreatment with ethanol (60 %) (b); Ultrasonic treatment (200 W, 20 min) of hemp seed powder without ethanol pretreatment (c); Ultrasonic ethanol pretreatment (200 W, 20 min, 60 %) of hemp seed powder (d).

4. Conclusions

In this study, UCAEE was used to extract oil from hemp seed. Ultrasonic ethanol pretreatment significantly increased the oil yield and the degradation of Δ9-THC from hemp seed. Compared with untreated seeds, ultrasonic ethanol pretreatment affected the basic nutrient composition, surface morphology, and emulsion state of hemp seed, which made the samples more susceptible to enzyme attack, formed more unstable emulsions, and ultimately increased the oil extraction rate. The energy and pressure released by ultrasound degraded Δ9-THC to CBN. In addition, the AEE extraction conditions were optimized and 88.38 % oil recovery was obtained. The oil samples extracted by UCAEE had better quality and higher percentage of polyunsaturated fatty acids compared with the SE method, and the contents of Δ9-THC and CBN in the oil samples were 9.58 mg/kg and 52.45 mg/kg, respectively, which were in accordance with the limit requirements of most countries in the world. In conclusion, ultrasonic ethanol pretreatment can be used as a green technology to improve the hydro-enzymatic extraction process of hemp seed, and at the same time, effectively reduce the content of Δ9-THC in the oil, so that it meets the nutritional needs of people for hemp seed oil.