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Article

Production of Protein-Rich and Cannabinoid-Free Extracts from Industrial Hemp by Combined Solvent-Free Ultrasound- and Microwave-Assisted Extraction

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ABSTRACT: Protein-rich cannabinoid-free extracts were prepared from hemp (*Cannabis sativa* L.) inflorescences using an ultrasound probe system and an ultrasound-microwave extractor. The cannabinoid content was evaluated by liquid chromatography-diode array detection-mass spectrometry (LC-DAD-MS^n) analysis, assessing tetrahydrocannabinol (THC)'s non-detectability in most of the extracts and hemp residues. Extract characterization through a Leco nitrogen analyzer and by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis highlighted a higher protein content and interesting profile with respect to untreated hemp. Ultrahigh-performance liquid chromatography-fluorescence detection (UHPLC-FLD) analysis on the most promising extracts quantified all the flavor active free amino acids, with "umami" taste-rich glutamic acid as the most abundant one. In this regard, according to the hierarchical cluster and principal component analysis, the ultrasound technique was the most influential factor. The outcomes of this study support the use of cannabinoid-free hemp inflorescence extracts obtained by sustainable processes as novel vegetal protein sources.

KEYWORDS: *Cannabis sativa L., ultrasound probe, ultrasound-microwave extractor, cannabinoid-free extracts, hemp proteins, hemp free amino acids*

1. INTRODUCTION

The versatility and multifunctionality of *Cannabis sativa* L. (Cannabaceae), along with its complex chemical profile, largely contribute to extend studies and research on this crop to better investigate its bioactive constituents, such as cannabinoids, phenolics, and terpenes, which can both interact with cannabinoids and alter their effects. The identification and enhancement of recovery techniques for phenolic and terpenoid compounds are crucial, given their significance in the pharmaceutical and food sectors.[1] Among cannabis metabolites, cannabinoids, which are formed through the fusion of phenolics and terpenes, are the most renowned, including cannabidiol (CBD) and δ-9-tetrahydrocannabinol (THC). The latter is known as a psychotropic substance with numerous adverse effects, including heightened anxiety, cholinergic deficits, and suppression of the immune system. Conversely, there is growing support for the extraction and use of nonpsychotropic cannabinoids, such as CBD and other minor cannabinoids. This approach valorizes the utilization of nonpsychotropic cannabis varieties (hemp), which have traditionally been cultivated primarily for their high-quality natural fiber.[2] Given the current environmental and economic scenario, which has prompted a shift toward using raw plant materials to reduce the reliance on petrochemical-derived products, industrial hemp has gained renewed interest. The industry of hemp yields numerous byproducts from various underutilized plant components, including inflorescences, seeds, and residues from defibration or extraction processes, such as dust, shives, and seed meal. These diverse byproducts contain high-value substances, enabling multiple ways of valorization for this crop. Notably, hemp demonstrates considerable potential as a source of food and feed.[3]

Along with the conventional maceration or percolation, a broad range of novel techniques is being developed to obtain value-added products from hemp.[4] For the purpose, microwave-assisted (MAE), ultrasound-assisted (UAE), pressurized liquid (PLE), supercritical fluid (SFE),[5−10] and subcritical water extraction (SWE),[6] as well as deep eutectic solvents (DES)[11] have been employed. As an example, MAE and pulsed electric fields (PEF) were employed before UAE on defatted hemp seed cake to obtain extracts with antioxidative phenolic compounds.[12] UAE and MAE stand out as highly adaptable and efficient extraction methods, offering benefits in extraction duration, yields, solvent consumption, and consistency compared to conventional extraction techniques. UAE involves two physical processes: the penetration through cell walls and the flushing of cellular contents following wall rupture. Reducing the size of plant material can increase the number of cells exposed to ultrasound-induced cavitation. This process

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can encourage swelling and hydration, leading to an expansion of cell wall pores. Consequently, the diffusion process and mass transfer are enhanced. The cavitation effect ultimately results in the breakdown of plant cells.[13] In relation to MAE, this form of radiation can quickly increase the matrix's temperature if the solvent has a high dielectric constant, which measures how effectively the absorbed microwave energy can be transformed into heat within a material under an applied electric field. Due to these constant values, water emerges as the optimal solvent for MAE. When combined with other solvents typically employed for extracting plant bioactive components, it can enhance their polarity indices and, as a result, the dielectric constant of the mixture.[14]

C. sativa, particularly its seeds, is rich in various components beyond cannabinoids, terpenes, and polyphenols. These include dietary fiber, proteins, and lipids. Hemp represents a viable alternative to conventional oil crops such as rapeseed, sunflower seed, cottonseed, soybean, and peanut, containing a considerable lipid level of approximately 25−35% (w/w). The seeds also comprise all the essential amino acids (EAAs), which constitute 20−25% of their protein content. Furthermore, saccharides make up 20−30% of the dry matter, while insoluble fiber accounts for 10−15%.[15] Hemp seeds are nutritionally valuable and offer potential benefits against neurodegenerative and cardiovascular conditions, as well as cancer. This is largely due to their essential fatty acids, notably α-linolenic acid (omega-6) and linoleic (omega-3), which are often found in an advantageous 3:1 ratio. Furthermore, hemp seeds serve as a valuable source of highly digestible protein, suitable for consumption by both humans and animals.[16] It is worth noting that the Finola hemp variety, a Finnish cultivar, is the first to be categorized as an oilseed crop and is particularly appropriate for seed production. This cultivar is particularly short and characterized by rapid flowering and limited THC content.[17]

The increasing demand for renewable and sustainable protein sources is driven by concerns about the environmental impact of animal protein production and the rising popularity of vegetarian and vegan lifestyles. Consequently, there is a growing focus on developing eco-friendly technologies to extract high-value proteins from plant-based byproducts. These innovative approaches aim to achieve cost-effective and environmentally sustainable production methods while adhering to a zero-waste model.[18] Currently, the use of proteins from hemp in food products is still in its infancy; however, this crop can represent a new and sustainable protein source for animal feeds, as an alternative to the conventional soybean meal, canola meal, or fish meal, whose utilization in livestock nutrition will be further limited by increasing prices.[19]

On this basis, this work was centered on novel UAE and MAE of proteins from Finola hemp inflorescences by using an UAE probe system and an UAE-MAE pilot-scale extractor, without the use of organic solvents. The different extracts obtained by the two extraction techniques performed alone and in combination were evaluated for the protein and free amino acid (FAA) profiles. First, the suitability for their potential use for nutrition and supplementation purposes was assessed, due to the nondetectability of cannabinoids able to give psychotropic effects.

2. MATERIALS AND METHODS

2.1. Plant Material

The Finola hemp variety's female inflorescences were sourced from a farm in Newrow, County Kildare, Ireland. The cultivation took place in a 6-acre field that underwent plowing and shallow sowing. The soil in the field was slightly alkaline and arable. Fertilization was carried out using a compound mixture containing potassium, phosphorus, and nitrogen. Hemp was planted in early May and harvested at the end of September. Irrigation was not needed due to sufficient rainfall.

2.2. Moisture Content Determination

The moisture content of air-dried hemp inflorescences was evaluated based on AOAC method 930.15,[20] consisting of utilizing a hot-air oven at 135 °C for 2 h. Additionally, to verify the results, a LECO TGM 800 Thermogravimetric Analyzer was used. As a result, a moisture content of 12% wet basis was determined.

2.3. Ultrasound and Microwave Extraction

Dry inflorescences of Finola hemp were ground, and 40 g of the biomass was immersed in 800 mL of distilled water for 30 min. Then, the mixture was subjected to ultrasound-assisted extraction (UAE), employing an ultrasonic generator (UIP 1000 hdT, Hielscher Ultrasound Technology, Germany) with a 1.8 cm-diameter probe (20 kHz), submerged for 2 cm into the mixture (Figure 1a). The latter was then sonicated for 10, 20, and 30 min, at 40 W (20%) and 200 W (100%) ultrasound amplitude levels at 4 °C, respectively. Subsequently, an additional series of experiments was conducted by UAE, MAE, and simultaneous ultrasound-microwave extraction (UMAE), using a pilot-scale equipment model E200 (Idco SAS, Marseille, France) (Figure 1b). Specifically, 50 g of ground hemp inflorescences in 1000 mL of distilled water was processed for 10 min at 200 W of ultrasound, 1000 W of microwave (equivalent to 100% ultrasound and microwave amplitude deliverable by the instrument, respectively), and both ultrasound and microwave simultaneously. In conventional experiments, hemp inflorescences and water in equal proportions underwent a stirring extraction for 24 h at 22 °C. The list of samples' names and conditions for their extraction is reported in Table 1. Each extraction was conducted in duplicate, and the resulting mixtures were filtered using a muslin cloth before being centrifuged with a Thermo Scientific Sorvall LYNX 6000 superspeed centrifuge, at 8000*g* for 20 min, at 4 °C. Consequently, the aqueous and solid residues were separated, weighed, and stored at −20 °C prior to freeze-drying in an L-300 Continuous Pro Modular, Buchi, Switzerland. A portion of the aqueous residues from ultrasound probe (USP) and conventional extractions (100 g) was not immediately freeze-dried but combined with ethanol (1:3) and left overnight at 4 °C to promote protein precipitation. Following this, the mixtures were subjected to centrifugation at 10,000*g* for 20 min at 4 °C, and the precipitates were isolated from the ethanolic solution and freeze-dried.

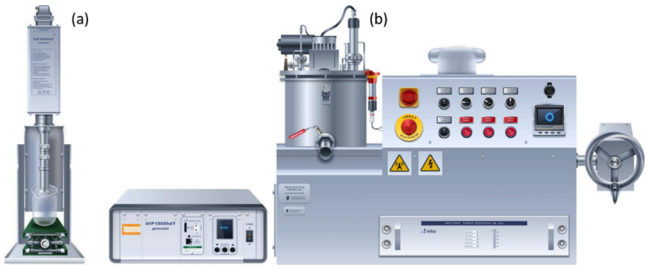

Figure 1. Ultrasound probe (a) and ultrasound-microwave extractor system (b).

2.4. LC-DAD-MS^n Analysis of Cannabinoids

LC-DAD-MS^n analysis for cannabinoid detection and quantification was carried out on freeze-dried aqueous and solid residues. In brief, 500 mg of the samples was combined with 10 mL of a 50:50 methanol−water solution, subjected to sonication for 15 min, and

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Table 1. Samples' Names and Conditions for Their Extraction

treatment	sample	extraction conditions
conventional	Con	stirring, 24 h, 22 °C
ultrasound probe (USP)	100USP10	100% ultrasound amplitude, 10 min, 4 °C
	100USP20	100% ultrasound amplitude, 20 min, 4 °C
	100USP30	100% ultrasound amplitude, 30 min, 4 °C
	20USP10	20% ultrasound amplitude, 10 min, 4 °C
	20USP20	20% ultrasound amplitude, 20 min, 4 °C
	20USP30	20% ultrasound amplitude, 30 min, 4 °C
E200 extractor	100MW10	100% microwave amplitude, 10 min
	100US10	100% ultrasound amplitude, 10 min
	100MW +US10	100% ultrasound + microwave amplitude, 10 min

then the supernatant was centrifuged and utilized for the analysis. The used stationary phase was an Agilent SB-C18, 1.8 μm, 4.6 × 50 mm column, and the mobile phases consisted of water with 1% v/v formic acid (A), acetonitrile (B), and methanol (C), employed in gradient elution beginning with 85% A and 15% B. In 11 min, the gradient attained 70% B and 30% C, and then at 15 min, it reached 100% C and stayed isocratic up to 25 min. At 26 min, the gradient reached 85% A and 15% B and was kept isocratic for the other 3 min. The flow rate was 0.7 mL/min, and CBD, cannabidiolic acid (CBDA), cannabinol (CBN), and THC were used as reference standards. After the column, the flow was split using a passive "T" connection and eluent was split half to a diode array detector (Agilent 1260 diode array) and half to a mass spectrometer (Agilent/Varian MS 500). The diode array collected spectra in the range 200−600 nm. The mass spectrometer was endowed with an electrospray (ESI) ion source working in positive and negative ion modes for the neutral and acidic cannabinoids, respectively. The applied parameters were as follows: capillary 4500 V, drying gas pressure 25 psi, spray shield 600C, RF loading 80% capillary 80 V, temperature of drying gas 310 °C. Spectral acquisition was in the range of m/z 120−1500. As reference compounds CBDA, CBD, and THC were used. The calibration curves were obtained in positive ion mode for THC and CBD acquiring the m/z value of 315 corresponding to the $[M + H]^+$ ion while the CBDA curve was obtained in negative ion mode acquiring m/z 357 corresponding to the $[M - H]^-$ ion.

To establish the suitability of the method, CBD (1.0 mg/mL), CBN (1.0 mg/mL), and THC (1.0 mg/mL) standard solutions (Sigma-Aldrich Cerilliant) were diluted using methanol to a concentration of 10 μg/mL of each analyte. Once prepared, solutions were kept at −20 °C until use. The solutions were used for the determination of system suitability test. Furthermore, a standard solution of CBDA was prepared at 10 μg/mL and used in the same way. Six injections were performed for the solutions, and reproducibility was evaluated considering the percent RSD of retention time and peak area. For THC, the RSD values for retention time and peak area were 0.25 and 0.76%, respectively. For CBD, the RSD values for retention time and peak area were 0.70 and 0.97%, respectively, while for CBDA, the RSD values for retention time and peak area were 0.45 and 0.90%, respectively.

To establish the linearity, standard solutions using methanol as solvent in the range of 0.5, 5, 10, and 20 μg/mL of CBD, THC, and CBDA were prepared. Solutions were injected in triplicate. Linear regression analysis was performed, and calibration curves were the following: for CBD, $y = 1.95 \times 10^5 x - 8.57 \times 10^3$ ($R^2 = 0.9999$); for THC, $y = 2.253 \times 10^5 x - 8.34 \times 10^3$ ($R^2 = 0.9999$); for CBDA, $y = 7.74 \times 10^4 x - 6.4 \times 10^3$ ($R^2 = 0.9997$). To establish accuracy and recovery of the method, spiked solutions of hemp extracts at a final concentration of 10 μg/mL were injected. The percent recovery of THC, CBD, and CBDA from the test sample was determined. CBD recovery was 99.3%, THC recovery was 98.6%, and CBDA recovery was 99.9%.

The limits of detection (LOD) were 0.05 and 0.06 μg/mL for CBD and THC, respectively, while it was 0.08 μg/mL for CBDA. The limits of quantification (LOQ) were 0.15, 0.18, and 0.24 μg/mL for CBD, THC, and CBDA, respectively.

For the quantification, peak areas in the samples were used to calculate the cannabinoid amounts using the proper calibration curve, and the amount of cannabinoids was calculated as weight percentage on the vegetal material (% w/w).

2.5. Protein Content Determination

Protein content evaluation was carried out by a nitrogen analyzer (FP-628 Leco Instrument, Leco Corporation, USA) employing the Dumas principle (nitrogen content × 6.25). The method and protein recovery rate calculation (eq 1 below) were adopted from Das et al.[21] All the freeze-dried aqueous residues, solid residues, and precipitates were assessed, coming from USP and E200 treatments, as well as the conventionally extracted samples and the untreated hemp inflorescences, with the starting plant material being only subjected to freeze drying. The analyses were performed in duplicate.

$$\text{protein recovery rate} = \frac{\text{protein content in extracts} \times \text{amount of extracts obtained (g)}}{\text{protein content in hemp samples used for extraction (g)}} \times 100\% \quad (1)$$

2.6. LC-MS/MS Analysis of Proteins

Protein digestion was performed by the filter-aided sample preparation method (FASP) with modifications as outlined in Carvalho et al.[22] Briefly, 50 μg of samples was loaded into a Vivaspin 500 centrifugal concentrator with 10,000 Da MWCO, followed by the addition of 200 μL of 2% (w/v) SDS in 25 mM ammonium bicarbonate (AmBic). Centrifugation of the samples was carried out at 14,000*g* for 20 min. The proteins were rinsed with 200 μL of 8 M urea 25 mM AmBic using a centrifuge at 14,000*g* for 20 min.

The samples were reduced by introducing 200 μL of 50 mM dithiothreitol (DTT) in 8 M urea and 25 mM AmBic and using an ultrasonic microplate horn assembly for 5.25 min (seven cycles: 30 s active and 15 s inactive, 25% UA, 20 kHz UF). Subsequently, the FASP devices were centrifuged for 20 min at 14,000*g*. The proteins were alkylated by the addition of 100 μL of 50 mM iodoacetamide (IAA) in 8 M urea and 25 mM AmBic solution. The alkylation process was sped up using the ultrasonic microplate horn assembly for 5.25 min (seven cycles: 30 s active and 15 s inactive, 25% UA, 20 kHz UF). Lastly, 100 μL of 1:30 trypsin in 12.5 mM AmBic solution was introduced, and the protein was digested by the ultrasonic microplate horn assembly for 5.25 min (seven cycles: 30 s active and 15 s inactive, 25% UA, 20 kHz UF). The peptides were dried and preserved at −20 °C until following analysis by Nano-LC-MS/MS.

Nano-LC-MS/MS analysis was performed at the Laboratory of Biological Mass Spectrometry - Isabel Moura (LBMS-IM), University NOVA of Lisbon, using an EASY-nLC II system coupled to an Impact HD mass spectrometer (Bruker Daltonics), equipped with a CaptiveSpray nanoBooster source with acetonitrile as a dopant, following Carvalho et al.'s description.[22] This platform was used specifically for protein analysis and was different from the LC-DAD-MS^n system employed for cannabinoid profiling. Peptides were resuspended in 250 μL of 3% (v/v) acetonitrile (ACN) with 0.1% (v/v) aqueous formic acid (FA). After a 5 min vortex homogenization, samples were ultrasonically treated for 10 min at 100% amplitude and 35 kHz frequency. 1 μL of the sample containing 200 ng of peptides was loaded onto the EASY-nLC II system with an EASY-Column precolumn, 2 cm, ID100 μm, 5 μm, C18-A1, and an EASY-Column analytical column, 10 cm, ID75 μm, 3 μm, C18-A2. Chromatographic separation employed a linear 0−35% buffer B (90% v/v ACN and 0.1% v/v FA) gradient at 300 nL/min flow rate over 90 min. MS acquisition was performed in data-dependent mode, comprising cycles

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Table 2. Cannabinoid Content Revealed by LC-DAD-MS^n Analysis on USP Extracts, along with Conventional Extracts and Untreated Hemp[a,b]

sample	% CBDA ± SD		% CBD ± SD		% THC ± SD	
	aqueous residue	solid residue	aqueous residue	solid residue	aqueous residue	solid residue
Con	0.185 ± 0.017a	0.158 ± 0.090a	0.060 ± 0.006a	0.051 ± 0.030a	0.003 ± 0.001a	n.d.
100USP10	0.260 ± 0.019b	0.232 ± 0.050b	0.086 ± 0.007b	0.086 ± 0.021b	0.001 ± 0.001a	0.001 ± 0.001a
100USP20	0.279 ± 0.059b	0.208 ± 0.030b	0.092 ± 0.020b	0.068 ± 0.010b	0.001 ± 0.001a	n.d.
100USP30	0.299 ± 0.053b	0.215 ± 0.03b	0.099 ± 0.018b	0.070 ± 0.010b	0.001 ± 0.001a	0.001 ± 0.001a
20USP10	0.317 ± 0.055b	0.213 ± 0.010b	0.106 ± 0.019b	0.070 ± 0.015b	n.d.	n.d.
20USP20	0.294 ± 0.032b	0.209 ± 0.010b	0.097 ± 0.011b	0.068 ± 0.010b	0.001 ± 0.001a	n.d.
20USP30	0.304 ± 0.010b	0.235 ± 0.050b	0.101 ± 0.003b	0.077 ± 0.020b	0.001 ± 0.001a	n.d.
untreated hemp		0.160 ± 0.007a		0.052 ± 0.002a		n.d.

[a]Cannabinoid content is expressed as % on dried weight of plant material (w/w). [b]Note: The data were the mean of three replicates ($n = 3$); data in the same column with the same lowercase letter are not significantly different ($P > 0.05$); SD indicates the standard deviation; n.d. means not detected.

Table 3. Cannabinoid Content Revealed by LC-DAD-MS^n Analysis on E200 Extracts, along with Conventional Extracts and Untreated Hemp[a,b]

sample	% CBDA ± SD		% CBD ± SD		% THC ± SD	
	aqueous residue	solid residue	aqueous residue	solid residue	aqueous residue	solid residue
Con	0.134 ± 0.010a	0.145 ± 0.040a	0.043 ± 0.003a	0.046 ± 0.010a	0.002 ± 0.001a	n.d.
100US10	0.325 ± 0.071b	0.207 ± 0.030b	0.108 ± 0.024b	0.068 ± 0.010b	0.002 ± 0.001a	n.d.
100MW10	0.326 ± 0.036b	0.257 ± 0.010b	0.109 ± 0.012b	0.085 ± 0.020b	0.003 ± 0.002a	n.d.
100MW+US10	0.225 ± 0.094c	0.226 ± 0.050b	0.074 ± 0.032a	0.074 ± 0.020b	0.004 ± 0.003a	n.d.
untreated hemp		0.160 ± 0.007a		0.052 ± 0.002a		n.d.

[a]Cannabinoid content is expressed as % on dried weight of plant material (w/w) [b]Note: The data were the mean of three replicates ($n = 3$); data in the same column with the same lowercase letter are not significantly different ($P > 0.05$); SD indicates the standard deviation; n.d. means not detected.

of MS (2 Hz), followed by MS/MS (8−32 Hz) with a 3.0 s cycle time, and active exclusion. Fragmented precursors were excluded for 0.5 min after a MS/MS spectrum, with an intensity threshold for fragmentation of 2500 counts. Additionally, active exclusion set to 1 allowed reconsideration if a precursor's intensity increased 3-fold. Spectra were collected in the 150−2200 m/z range. Raw files were processed in MaxQuant v2.2.0.0 with default settings and searched using the Andromeda search engine against the NCBI Cannabis database, followed by data processing in Perseus v1.6.15.0.

2.7. FAA Profile Determination

Hemp aqueous residues and the most promising ethanol precipitates were assessed for the FAA profile. Before analysis, the extracts (10 mg each) were pretreated with 1.5 mL of 0.1 M HCl, by placing the mixture in a sonication bath for 5 min and then in a centrifuge for 15 min at 10,000g. The supernatant was isolated, and a volume of 200 μL was combined with 200 μL of 12.5% trichloroacetic acid (TCA) in an Eppendorf tube. The mixture was subjected to incubation at 4 °C for 1 h and centrifugation at 10,000g for 20 min. The neutralization of the supernatant was achieved with 1 M NaOH and mixing it with an equal volume of the internal standard aminocaproic acid (50 μM). Before injection, filtration of the sample was carried out through a 0.2 μm nylon membrane filter. The amino acid composition analysis was performed on an UHPLC-FLD apparatus (Thermo Ultimate 3000 RS, Thermo Scientific, Massachusetts, USA) coupled to an Agilent AdvanceBio AAA column (100 mm l. × 3.0 mm i.d. × 2.7 μm particle size; Agilent Technologies, California, USA) fitted to a guard cartridge. Derivatization of amino acids was conducted with an o-phthalaldehyde/3-mercaptopropionic acid mixture in the autosampler. Mobile phase A was represented by 10 mmol/L Na_2HPO_4 in 10 mmol/L $Na_2B_4O_7$ decahydrate (pH 8.2), and mobile phase B consisted of ACN, methanol, and water mixture (45:45:10, v/v/v). A flow rate of 0.620 mL/min was used with a gradient program as follows: 0−0.35 min, 2% B; 0.35−13.4 min, 57% B; 13.4−13.5 min, 100% B; 13.5−15.7 min, 100% B; 15.7−15.8 min, 2% B; 15.8−18.0 min, 2% B. An excitation wavelength of 340 nm and an emission wavelength of 450 nm were set to perform the fluorescence detection. For the quantification of the amino acids in the samples, an internal standard calibration method using authentic amino acid standards from Sigma-Aldrich (Copenhagen, Denmark) was employed (eight calibration points using 2.5−100 μM of every amino acid authentic standard). The results were declared as mg free amino acid per g sample.[23]

2.8. Statistical Analysis

All measurements were conducted in triplicate, unless otherwise indicated. The SPSS 24 (SPSS Inc., Chicago, Illinois, U.S.A.) was used to perform a one-way ANOVA on the levels of protein content and recovery rates, with extraction methods as the main effect. Tukey's test done by the same software was utilized to compare the means of the two levels between treatments, with the statistical significance set at $P < 0.05$. Minitab software was employed to conduct principal component analysis (PCA) on free amino acid levels, while hierarchical clustering analysis (HCA) of free amino acids was executed using ClustVis. Regarding LC-MS, MaxQuant software v2.2.0.0 (https://www.maxquant.org) was used to carry out relative label-free quantification. All raw file processing was made with default parameters in a single run.[24] Database searches were conducted through the Andromeda search engine[25] with the NCBI (National Center for Biotechnology Information) Cannabis. Data were processed using Perseus (version 1.6.15.0, https://www.maxquant.org/perseus) with default settings.[26] Statistical analyses were performed using a multiple-sample Anova test with permutation-based FDR (S0 = 0 and FDR 0.05).

3. RESULTS AND DISCUSSION

3.1. LC-DAD-MS^n Analysis of Cannabinoids in Aqueous and Solid Residues

The amount of CBDA, CBD, and THC was measured in the aqueous and solid residues and compared with the content in

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Table 4. Protein Content and Recovery Rates Related to Aqueous Residues and Ethanol Precipitates (PPT)[a]

samples	protein extracts (g)	protein content in aqueous residues (%, w/w)	recovery rate in aqueous residues (%)	precipitates (g)	precipitated protein (%)	recovery rate in precipitates (%)
Con	8.62 ± 2.78a,b	34.26 ± 0.67a,b	43.12 ± 15.04a	2.88 ± 1.88a	43.25 ± 7.38a	20.76 ± 0.16a
100USP10	5.21 ± 0.72a	33.40 ± 3.97a,b	26.91 ± 9.33a	1.67 ± 1.56a	43.91 ± 3.70a	13.12 ± 0.13a
100USP20	7.69 ± 0.87a	36.88 ± 0.75a	38.97 ± 3.23a	1.85 ± 1.34a	52.81 ± 5.56a	13.74 ± 0.08a
100USP30	7.10 ± 2.01a	34.46 ± 1.87a	36.77 ± 14.74a	1.99 ± 1.85a	46.37 ± 1.38a	15.82 ± 0.15a
20USP10	4.92 ± 0.34a	36.46 ± 2.41a,b	26.24 ± 4.41a	0.97 ± 0.53a	44.77 ± 2.09a	6.84 ± 0.40a
20USP20	5.66 ± 0.34a,b	38.97 ± 1.05a,b	31.09 ± 4.02a	1.13 ± 0.52a	47.96 ± 4.11a	7.96 ± 0.31a
20USP30	5.40 ± 0.91a,b	38.15 ± 1.60a,b	30.43 ± 7.59a	1.02 ± 0.45a	44.46 ± 0.51a	7.10 ± 0.39a
100MW10	7.87 ± 0.26a,b	32.91 ± 1.69a,b	28.70 ± 3.33a			
100US10	11.08 ± 0.66b	35.55 ± 0.52a,b	43.09 ± 1.42a			
100MW +US10	7.45 ± 0.83a	30.58 ± 0.41b	26.26 ± 5.92a			
untreated hemp		21.64				

[a]Note: All the analyses were conducted in three replicates ($n = 3$); data in the same column with the same lowercase letter are not significantly different ($P > 0.05$); protein extracts are freeze-dried aqueous extracts; precipitates are ethanol-precipitated protein from aqueous extracts; precipitated protein is the protein content in precipitates.

Table 5. Proteins Detected in USP Precipitates (PPT) Using LC-MS/MS[a]

protein type	protein name	molecular weight (kDa)	amino acids	post hoc analysis of the protein content among Con PPT, 20USP20PPT and 100USP20PPT ($P < 0.05$)
albumin	2S albumin-like	13.86	126	Con PPT > 20USP20PPT and 100USP20PPT
	edestin 2	54.01	491	
	edestin 3	56.08	493	Con PPT > 20USP20PPT
globulin	vicilin-like seed storage protein At2g28490	55.89	493	20USP20PPT > Con PPT
	vicilin C72	98.38	840	20USP20PPT > Con PPT and 100USP20PPT
	11-S seed storage protein	56.49	494	Con PPT > 20USP20PPT
enzyme	chlorophyll synthase	42.02	382	
	triosephosphate isomerase, cytosolic	27.94	254	20USP20PPT > 100USP20PPT > Con PPT
	alpha carbonic anhydrase 4	15.07	137	
	glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a	79.86	726	20USP20PPT > 100USP20PPT
defensive protein	NB-ARC domain, LRR domain containing protein	93.06	846	
chlorophyll−protein complex	chlorophyll A−B binding protein	23.41	222	20USP20PPT > Con PPT > 100USP20PPT

[a]Note: All of the analyses were conducted in three replicates ($n = 3$). The blanks in the last column mean that there is no significant difference.

conventional extracts and untreated hemp. The results are presented as the mean of the two replicates of each extract and are displayed in Tables 2 (for USP treatment) and 3 (for E200 treatment). For untreated hemp, as well as all the samples from USP and E200 treatments, THC content was significantly lower than the mandated maximum limit for industrial hemp cultivation, that is, 0.2% on vegetal dried material. The extraction technologies allowed us to obtain extracts almost completely devoid of cannabinoids, especially CBD and THC, which were in very low concentrations and in some samples under detection limits, and a consistent situation was observed for untreated hemp inflorescences. In general, CBDA was slightly more present than CBD in all of the samples, especially in aqueous residues, for both USP and E200 treatments. In these instances, the decarboxylation induced by microwave and ultrasound does not take place in a significant way, contrary to the findings reported in our earlier studies.[27,28] Some research has previously indicated the presence of various cannabinoids in Finola hemp.[29,30] Despite this, the fact that the biomass here employed was found to be very poor in cannabinoids (0.052% of CBD and 0.16% of CBDA) may be due to its intrinsic features and growing conditions, since Finola is selected and employed as an oilseed variety with a very low THC level.[17] Otherwise, the scarce content of these constituents can also be related to the extraction processes and conditions especially due to the use of water as extraction solvent and its poor ability to solubilize cannabinoids. Furthermore, Finola is generally a variety used for fiber production, and the particular material used in this study is grown and collected specifically for seed recovery. The poor amount or the nondetectability of cannabinoids in some of the obtained extracts surely represents a significant advantage if considering the possible application of these products for supplementation and feeding, suggesting their safety for both animal and human consumption. The detection by LC-DAD-MS^n analysis of minimal amounts of cannabinoids in the untreated hemp inflorescences and derived extracts represented the first crucial outcome of this study, with the THC level far below the maximum 0.2% stated for hemp inflorescences by the European law. This is a key aspect and prerequisite for the possible exploitation of these products from a nutritional perspective, highlighting at the same time the potential of UAE and MAE of Finola hemp inflorescences conducted by the advanced UAE probe system and UAE-MAE pilot-scale extractor with only water as the extraction solvent.

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Table 6. Quantitative Results for Amino Acid Composition of Hemp Aqueous Residues and Ethanol Precipitates (PPT)[a]

FAA	samples														
mg/g	100US10	100MW10	100MW+US10	Con	100USP20PPT	20USP20PPT	Con PPT	100USP10	100USP20	100USP30	20USP10	20USP20	20USP30	Untreated Hemp
aspartic acid	2.408	1.402	1.478	0.356	2.042	1.193	2.584	4.256	4.749	5.123	4.635	4.026	3.983	0.408
glutamic acid	2.689	2.052	2.300	7.136	1.436	0.892	3.444	5.024	5.407	5.916	5.631	4.912	4.969	0.576
serine	0.827	0.406	0.444	n.d.	0.223	0.164	0.453	1.616	1.863	1.999	1.638	1.412	1.422	0.047
histidine	0.507	0.436	0.433	1.225	0.314	0.272	0.381	0.782	0.844	0.888	0.793	0.640	0.722	0.235
glycine	0.434	0.221	0.248	2.650	0.209	0.133	0.442	1.127	1.298	1.313	1.080	0.904	0.889	0.065
threonine	0.498	0.166	0.177	n.d.	0.048	0.087	0.284	1.177	1.401	1.352	1.060	0.911	0.877	n.d.
arginine	2.668	1.829	2.099	0.146	1.365	1.253	0.812	3.072	3.712	3.697	3.480	3.255	3.135	0.601
alanine	1.969	1.292	1.437	4.237	0.259	0.186	0.318	2.895	3.314	3.548	3.399	3.072	2.942	0.421
tyrosine	n.d.	0.242	0.255	0.148	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
valine	0.890	0.396	0.386	3.407	0.094	0.057	0.175	1.702	2.020	2.103	1.698	1.453	1.514	0.112
methionine	0.213	n.d.	n.d.	0.331	n.d.	n.d.	n.d.	0.278	0.373	0.414	0.392	0.362	0.325	n.d.
tryptophan	0.125	0.085	0.117	0.031	0.022	n.d.	0.028	0.247	0.353	0.295	0.254	0.212	0.232	n.d.
phenylalanine	1.022	0.416	0.442	2.653	0.108	0.092	0.126	1.438	1.576	1.660	1.568	1.358	1.408	0.086
isoleucine	0.584	0.153	0.156	2.378	0.054	0.018	0.055	1.290	1.448	1.583	1.219	1.021	1.060	0.042
leucine	1.450	0.489	0.448	4.496	0.130	0.059	0.088	2.311	2.507	2.709	2.293	1.957	2.063	0.142
lysine	0.868	0.418	0.444	1.416	0.858	0.859	0.718	1.515	1.837	1.839	1.639	1.439	1.426	0.181
total FAA	17.047	10.004	10.864	30.611	7.045	5.137	9.498	28.730	32.700	34.440	30.779	26.935	26.967	2.760

[a]Note: all of the analyses were carried out with four replicates (n = 4); n.d. means not detected.

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3.2. Protein Content Determination

Table 4 describes the protein contents and recovery rates of aqueous residues and ethanol precipitates (PPT). These samples exhibited the most noteworthy values in comparison with the solid residues, whose data are not provided. It is worth highlighting that the employed techniques enabled the production of hemp extracts with superior protein content to that of untreated hemp and, in certain cases, conventional extraction. There is no significant difference ($P > 0.05$) in protein contents and recovery rate of the USP extracts obtained by the application of 20 and 100% ultrasounds amplitude (with a maximum level of 38.97 and 36.88%, respectively). Among E200 samples, extracts obtained by applying ultrasound at 100% amplitude for 10 min had a higher protein content (35.55%), and they exhibited the best recovery rate among all the extracts derived from USP and E200 (43.09%). Precipitation with ethanol of USP aqueous extracts was proven to give an improved protein content in the PPT. In detail, the PPT of the extracts obtained by 20% ultrasound amplitude for 20 min (47.96%) and those of the extracts produced by 100% ultrasound amplitude for 20 and 30 min (52.81 and 46.37%, respectively) showed the highest protein levels. The latter two PPT also demonstrated the greatest recovery rates among all the PPT from the samples treated with US. In summary, the employed methods enabled the recovery of hemp extracts with higher protein concentrations compared to untreated hemp and, in certain cases, to conventional extracts. The protein contents were notably increased by introducing ethanol into the aqueous extracts obtained by probe UAE, which led to facilitated precipitation.

3.3. LC-MS/MS Analysis of Proteins

Since USP extraction provided the highest protein content in PPT, the ones obtained by 100 and 20% ultrasound amplitudes for 20 min underwent LC-MS/MS analysis to identify the protein profile (100USP20PPT and 20USP20PPT). The results (Table 5) revealed that the extracts contained diverse proteins (12), mainly storage proteins, followed by enzymes and others. The number of amino acids for each protein was retrieved from the NCBI database, and subsequently, the molecular weight of each specific protein was determined based on the respective amino acid content. Previous studies have also reported that the total hemp protein predominantly comprises a single globular storage protein known as edestin, which makes up approximately 65% of the protein content. Additionally, the low-molecular-weight albumin fraction constitutes around 25% of the storage proteins. Edestin is characterized by its hexameric structure consisting of three acidic and three basic subunits.[31,32] However, proteins including alpha carbonic anhydrase 4, glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a, and chlorophyll A−B binding protein have been rarely reported earlier. The variation in the content and concentration of proteins in the extracts greatly depends on the hemp variety, cultivation, and processing conditions, and so on.[33] ANOVA revealed that apart from two albumins (2S albumin-like and edestin 3) and one globulin (11-S seed storage protein), 20USP20PPT had significantly ($P < 0.05$) higher protein content than Con PPT and 100USP20PPT. Interestingly, 20USP20PPT presented more proteins than 100USP20PPT. This might be because of the selection of the extraction conditions. Larger amplitudes or longer processing times may be needed in these cases to increase the extraction efficiency and enhance the yield by promoting the transfer of inner components into the solvent. Once this stage is reached and completed, prolonging the US treatment may not be beneficial. The diffusion front moves toward the inner part of the raw material, resulting in a reduced diffusion area and rate. Additionally, the equilibrium in osmotic pressure between the inside and outside of the raw material may be reached as the diffusion rate decreases.[34] Therefore, the extraction yield does not improve further with higher US amplitude, and longer processing times can also potentially lead to the degradation of the extracted compounds. The conditions of ultrasound-assisted hemp protein extraction need to be further optimized. Notably, besides the more common edestin and albumin fractions, other less-known compounds, scarcely detected in hemp so far, were identified in the PPT evaluated through LC-MS/MS protein analysis.

3.4. FAA Profile Determination

Free amino acids are significant flavor precursors in foods, especially in meat products. FAA contribute to developing desirable tastes and sensory properties, which can improve the marketability and consumers' acceptability of high-protein plant-based foods.[35] The outcomes for FAA composition of hemp extracts are represented in Table 6. The 16 amino acids aspartic acid, glutamic acid, serine, histidine, glycine, threonine, arginine, alanine, tyrosine, valine, methionine, tryptophan, phenylalanine, isoleucine, leucine, and lysine were measured in aqueous residues from E200 and USP treatments, and in the best PPT, which were compared with conventional extracts and untreated hemp. It is clear that the extraction processes improve the amino acid profile of extracts, with respect to untreated hemp samples. Indeed, for untreated hemp, an average value of 2.8 mg/g of total FAA was quantified, while much higher contents were detected in E200 and USP-treated samples, and conventional extracts. As depicted in Figure S1 in the Supporting Information, a heatmap was employed for visualizing the free amino acids' distribution in samples following the diverse extraction methods. To examine how the extraction methods influence all the quantified free amino acids from a multivariate perspective, first, the data were processed by HCA (Figure S1). Two main groups can be noticed: one comprises the samples exclusively obtained by ultrasound, while the other cluster encompasses the conventional, untreated, microwave-extracted, and precipitated samples. Considering the impact of extraction methods on the profiles of free amino acids, 100MW10, 100MW+US10, and untreated hemp exhibit positive correlations and are clustered together. In addition, all the data were submitted to PCA. The PCA findings are reported in Figure S2 in the Supporting Information. The X- and Y-axes displayed PC1 and PC2, which accounted for 92.6% of the overall variance, confirming the PCA's effectiveness in explaining the impact of extraction methods on the free amino acid profile presented in this work. The samples were differentiated based on treatments, with ultrasound-extracted samples positioned in the positive region of PC1. Microwave-extracted and precipitated samples displayed a considerable dispersion and were located at the PC1 negative area (Figure S2). These findings aligned with those from the HCA. Consequently, it is evident that the ultrasound extraction exerts a more significant influence than microwave or ethanol precipitation when distinguishing the samples according to their free amino acid composition. Particularly, the total FAA content exceeded 30 mg/g for USP

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extracts recovered using 100% ultrasound amplitude for 30 and 20 min, and 20% ultrasound amplitude for 10 min, surpassing the amounts achieved for conventional samples. Among FAA, glutamic acid was found to be the most prevalent one, mainly in conventional extracts, followed by USP samples, and last E200 ones. The "umami" flavor has been attributed to this particular amino acid in its free form, so this aspect supports the contribution of industrial hemp to this taste in foods.[36] Aspartic acid was another amino acid particularly abundant in USP samples, followed by arginine, alanine, and leucine. Aspartic acid also possesses umami taste, and a high amount of free arginine and other amino acids in the diet was reported to be important in fish nutrition. Moreover, alanine is endowed with a sweet taste.[37] Valine, lysine, serine, phenylalanine, isoleucine, glycine, and histidine were detected in lower quantities, being anyway found in all the extracts. Conversely, the less abundant amino acids, which were absent in certain samples, included threonine, tryptophan, methionine, and tyrosine. Notably, threonine was identified in the extracts by ultrasound and microwave treatments, but it was missing in conventional samples and untreated hemp. Likewise, tryptophan was not found in untreated hemp, but it was quantified in the other samples, while methionine was only detected in certain extracts, especially in USP ones. Free methionine is responsible for the aroma of meat products, and it has been reported that its supplementation in legumes can increase the utilization efficiency of dietary protein for supporting animal growth.[36] Lastly, identification of tyrosine exclusively occurred in conventional and E200 extracts obtained by the application of only microwaves and microwaves with ultrasounds. Similarly, previous research has reported glutamic acid, aspartic acid, arginine, and leucine as some of the mostly prevalent amino acids in hemp seeds.[23,15] The results of the actual study, in compliance with that by Cabral et al.,[23] highlighted that water could be an ideal solvent to obtain proteins from a nutritional point of view, with the advantage of employing innovative extraction techniques on hemp inflorescences as an emerging protein source.[19] The amino acid profile of any food product serves as an indicator of the protein content quality, and in particular free amino acids lead to an increase in the food's nutritional value. Given that, the obtained hemp extracts from inflorescences could be considered as sources of high-value bioactive peptides, due to the detection of interesting amounts of essential amino acids in their free form. In conclusion, the supplementation with industrial hemp or hemp byproducts could represent a good feeding strategy, addressing the raising exploitation of plant protein, fundamental to support the production of protein-rich foods for animal nutrition, replace animal proteins in the human diet, and reduce the impact of intensive animal husbandry on the environment. In the aqueous and precipitated extracts, the presence of all the essential FAA was uncovered, showing a noticeable enhancement in their content compared to untreated hemp and, to some extent, to conventional extracts. The statistical analyses through HCA and PCA, conducted to shed light on differences among extractions and variables contributing to variance, showed that ultrasound extraction was a more influential factor than microwave extraction or ethanol precipitation for the differentiation of the samples based on their FAA composition.

Overall, the simultaneous ultrasound-microwave treatment could not be considered to be globally superior under the conditions tested in this study. In terms of protein extraction, 100MW+US10 produced a lower protein content in the aqueous residue (30.58%, w/w) and a lower recovery rate (26.26%) than 100US10 (35.55% and 43.09%, respectively), and it also underperformed relative to the probe ultrasound treatments. A similar trend was observed for free amino acids, as the total FAA content of 100MW+US10 (10.86 mg/g) was only marginally higher than that of 100MW10 (10.00 mg/g) but markedly lower than 100US10 (17.05 mg/g), conventional extraction (30.61 mg/g), and the USP extracts (26.94−34.44 mg/g). Regarding cannabinoids, all treatments yielded extracts with negligible THC contents, while the simultaneous ultrasound-microwave-treated aqueous residues showed lower CBDA and CBD contents than those of 100US10 and 100MW10 within the E200 system. Therefore, the main advantage of the combined treatment was a modest limitation of cannabinoid aqueous coextraction, whereas ultrasound alone, particularly probe ultrasound and 100US10, was more effective for maximizing protein enrichment and FAA recovery.

In conclusion, the outcomes of the present study can support the employment of cannabinoid-free hemp-derived products, obtained by sustainable extraction processes, as novel sources of proteins and amino acids in alternative to seeds, with the advantage of valorizing the inflorescences which usually represent a waste from industrial processing.

■ ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the manuscript and its Supporting Information, or upon reasonable request.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsfoodscitech.6c00123.

Hierarchical clustering analysis (HCA) and heat map of 16 free amino acids detected in extracted samples with four replicates ($n = 4$) (Figure S1); Principal component analysis (PCA) score plot on the levels of nine detected amino acids in extracted samples with three replicates ($n = 3$) (Figure S2) (PDF)

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Notes

During the revision of this work, the corresponding author used PAPERPAL exclusively in order to HELP REPHRASING THE TEXT FOR MORE CLARITY AND REDUCING THE SIMILARITY INDEX. When using this tool/service, the author always reviewed and edited the content as needed and takes full responsibility for the content of the publication.
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