Effects of natural deep eutectic solvents' hydration level, choice of hydrogen bond donor and application of ultrasound on the extraction, anti-nutritional components, structural properties and functionality of canola protein isolates

Karimi, Abouzar; Choi, Juwuan; Samaranayaka, Anusha; Bhowmik, Pankaj; Chen, Lingyun

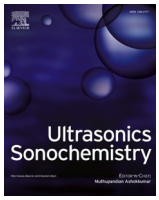
Publisher's version / Version de l'éditeur:
https://doi.org/10.1016/j.ultsonch.2025.107618
Ultrasonics Sonochemistry, 122, C, pp. 1-16, 2025-10-12

Ultrasonics Sonochemistry 122 (2025) 107618

Contents lists available at ScienceDirect

Ultrasonics Sonochemistry

journal homepage: www.elsevier.com/locate/ultson



Effects of natural deep eutectic solvents' hydration level, choice of hydrogen bond donor and application of ultrasound on the extraction, anti-nutritional components, structural properties and functionality of canola protein isolates

Abouzar Karimi [a], Juwuan Choi [a], Anusha Samaranayaka [b], Pankaj Bhowmik [b], Lingyun Chen [a,*]

[a] Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton T6G 2P5 AB, Canada
[b] Aquatic and Crop Resource Development, National Research Council of Canada, 110 Gymnasium Place, Saskatoon S7N0W9 SK, Canada

ARTICLE INFO

Keywords:
Canola/rapeseed
Natural deep eutectic solvents
Protein extraction
Ultrasound
Protein structure and functionality

ABSTRACT

In a systematic attempt to improve the cost-effectiveness and knowledge of the topic of plant protein extraction using natural deep eutectic solvents (NDESs), 18 extraction treatments, half of which paired with sonication, were performed to extract canola protein isolates using two groups of NDESs: choline chloride-glucose-water (CG) and choline chloride-water (C), with water contents ranging from 30 to 90 %. The highest protein extraction efficiencies were observed at water contents of 60 % for CG group (50.28 %) and 90 % for C group (45.48 %), while sonication improved these efficiencies to 60.68 % and 58.11 %, respectively. Increased solvent hydration and sonication also effectively reduced phenolic compounds and phytic acid contents, especially in the CG group. Despite increasing the solvents' viscosity and density, the combination of glucose and water as hydrogen bond donors proved more effective for protein extraction than water alone, especially for larger fractions such as cruciferins and aggregates. Furthermore, while protein secondary structure remained mostly intact, variations in solvents' water content, and sonication, affected the tertiary structure and particle size distribution, with the strength and flexibility of the NDESs' nanostructure possibly affecting the protein conformation and aggregation. Regarding functionality, sonicated isolates showed an average of 8.31 % lower aqueous solubility across the pH range of 3–7, along with more than double the emulsion stability and a lower foam stability at pH 3, compared to non-sonicated isolates. Overall, pushing NDESs to their upper hydration limit, proper selection of hydrogen bond donors, and the application of ultrasound can improve the cost-effectiveness and quality of extracted canola protein isolates.

1. Introduction

Global food production is responsible for ~35 % of all greenhouse gas emissions, with ~60 % of these emissions generated from animal husbandry and animal feed production [1]. Curbing these emissions is essential for preventing catastrophic and irreversible changes in the planet's climate. Therefore, the urgency of partially replacing animal proteins with alternative proteins in the human diet is now felt more than ever. Among these alternative sources, canola/rapeseed (*Brassica napus*) offers a highly nutritious protein with a reported average PDCAAS of 0.95, which is close to soy protein (0.98) and higher than pea (0.84) and lentil (0.73) proteins [2]. This protein can be obtained from the by-product of canola oil extraction, canola meal (containing 30–50 % protein), which makes it even more valuable in terms of sustainability [3]. However, extraction of canola protein from the meal has long been considered challenging due to issues such as the heat-induced denaturation during oil extraction process, and the presence of high contents of anti-nutritional components in the meal, such as glucosinolates, phytic acid and phenolic compounds [4]. Therefore, to be able to use canola meal as a reliable source of protein, a shift toward less invasive oil extraction techniques, as well as the development of green and efficient extraction methods is essential.

* Corresponding author.
E-mail address: lingyun.chen@ualberta.ca (L. Chen).

https://doi.org/10.1016/j.ultsonch.2025.107618
Received 11 March 2025; Received in revised form 26 August 2025; Accepted 10 October 2025
Available online 12 October 2025

One of the least explored and recently trending techniques for protein extraction is the use of deep eutectic solvents (DESs), which are composed of a specific ratio of hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD) molecules. DESs are prepared in a few simple steps, which often include mixing the HBAs and HBDs at high temperatures to obtain a uniform liquid with a melting point below the melting points of the solvents' individual components [5]. Depending on their formulation, DESs may be able to facilitate the extraction of proteins through the formation of various types of non-covalent interactions with the protein structure [6]. Furthermore, these solvents could be tuned to potentially maximize the extraction of proteins from complex food matrices, while minimizing the extraction of other, interfering components [7]. If the components of DESs are selected from naturally occurring molecules, they can be sub-categorized as natural deep eutectic solvents (NDESs). These solvents could offer a more environmentally friendly and safer option for protein extraction compared to most traditional methods, such organic solvents, or acid and alkali treatments [8,9]. All of the mentioned advantages have resulted in a surge in research regarding the use of DESs for protein extraction, with recent examples focusing on protein from microalgae [10], fava bean [11], tiger nut meal [12], sesame meal [13], oat [14] and canola [15]. However, despite their promising potential, the components used for preparation of DESs are still generally more expensive than the conventional solvents. This, along with the insufficient research on the effects of DESs on protein extraction, structure and functionality, are the two key challenges in the way of the large-scale, industrial adaptation of DESs for protein extraction.

There are two strategies for making protein extraction using DESs more cost-effective: reducing the cost of the solvent or improving the protein extraction yield. The first strategy could be achieved by using less-expensive HBAs and HBDs, with two prominent examples being choline chloride and sugars (such as glucose), respectively [16]. While both of these components are inexpensive, widely available and GRAS, the potential of choline chloride-sugar DESs for extraction of proteins has rarely been studied before. Nevertheless, the one molecule that is the most affordable component for development of DESs is water. Water can act as both HBD and HBA, and is often added to DES formulations to fine-tune properties such as viscosity and polarity [11]. However, the majority of studies have been somewhat conservative when it came to incorporating high contents of water in the DES formulation, possibly because it is often stated that increasing the content of water could disrupt the structure of DESs [6,17]. While this notion is true, the effects of these disruptions on the extraction of proteins may not necessarily be negative. Furthermore, recent studies have shown that in combination with certain contents of choline chloride, water can act as the sole HBD to develop NDESs [18,19,20]. However, to the best of the authors' knowledge, the potential of choline chloride-water mediums for protein extraction has never been studied before. Overall, since water has a tremendous potential to make protein extraction using DESs more sustainable and affordable (in terms of both solvent preparation and downstream recovery), the effects of its incorporation into DES structures should be further explored.

The second strategy (improving the protein extraction yield to increase the cost-effectiveness of DESs) can be achieved through the use of novel assisting technologies in combination with DES extraction, among which ultrasound treatment has proven effective in improving the extraction efficiency, functional properties and digestibility of plant proteins [21,22,23]. The combination of DESs and ultrasound treatment for protein extraction has been studied in a few plant sources, including brewing waste [24], lime peels [25], pumpkin [26] and pomegranate [27]; however, no studies on canola protein have been published to this day.

Based on the identified gaps and opportunities, this study was designed to explore three main objectives: to evaluate the effects of NDES water content, the use of glucose and water versus water alone as HBD, and the application of ultrasound treatment on the extraction and properties of canola protein. To evaluate these objectives, two groups of solvents with varying water contents of 30–90 % were developed: one containing choline chloride and glucose at the ratio of 1:1, and the other containing choline chloride only (total of eight solvents). After the evaluation of their physical properties, these eight solvents, along with pure water, were then used to extract 18 different protein isolates, half of which were obtained using ultrasound treatment. The isolates were then subjected to comprehensive analyses of their protein content, extraction efficiency, anti-nutritional components and color, as well as their molecular and structural properties, with a focus on the individual and combined effects of the mentioned objectives on protein characteristics. Finally, the functional properties of selected isolates at three pH levels of 3, 5 and 7 were also evaluated.

2. Materials and methods

2.1. Materials

Canola meal (cold-pressed, with oil extraction temperatures maintained below 40 °C) was purchased from Highwood Crossing Foods Ltd. (AB, Canada). After grinding and defatting, the protein content of the meal was measured at 37.73 ± 0.48 %, while the contents of anti-nutritional components were measured as follows: 10.71 ± 0.18 mg/g of sinapic acid equivalent (SAE) for total phenolic content (TPC), 41.18 ± 0.33 mg/g for phytic acid, and 8.12 ± 0.57 mmol/kg for glucosinolates. A Milli-Q Advantage A10 purification system (EMD Millipore Corporation, MA, USA) was used to produce ultrapure water. Choline chloride, D-glucose and all the other chemicals were purchased from Sigma Aldrich (ON, Canada) and used without further purification.

2.2. Solvent abbreviation system and preparation

A total of nine solvents were employed for the extraction of canola protein, eight containing solid NDES components and water, and one consisting of pure water. The first eight solvents were divided into two groups. The first group, CG, was prepared using choline chloride and glucose at a mass ratio of 1:1 and varying water contents of 30 %, 45 %,60 %, 75 % and 90 %. The second group, C, contained only choline chloride, with water contents of 30 %, 60 % and 90 %. Protein extraction using the nine solvents was performed both without (CG and C) and with (UCG and UC) the application of ultrasound (extraction details explained in section 2.4). Therefore, the following 18 protein extraction treatments were performed: CG30, CG45, CG60, CG75, CG90; UCG30, UCG45, UCG60, UCG75, UCG90; C30, C60, C90; UC30, UC60, UC90; water and water + ultrasound.

For the preparation of solvents, the components of each solvent were weighed on an analytical scale and transferred into Erlenmeyer flasks. The flasks were then placed on a Super-Nuova™ Multi-Position Digital Stirrer equipped with a Type K thermocouple probe (Thermo Fisher Scientific, MA, USA) and mixed at 80 °C and 400 RPM for approximately 10 min, until uniform, clear liquids were obtained. The solvents were then left to cool on the bench to room temperature before being subjected to further analyses.

2.3. Solvent characterization

The dielectric constant of CG and C solvents was measured using a ZVA67 vector network analyzer (10 MHz to 67 GHz, Rohde & Schwarz, MD, USA) equipped with a coaxial probe and calibrated with water and acetone, at the frequency of 800 MHz. pH measurements were carried out using an F20 pH meter (Mettler Toledo, OH, USA). Solvent conductivity was measured using an Oakton PC 700 benchtop meter (Cole-Parmer, IL, USA). Viscosity measurements were performed via a Discovery HR-3 rheometer (TA Instruments, DE, USA) equipped with a Peltier Concentric Cylinder at shear rates of 0.01 to 90 s^{-1} over 3 min. The slopes of the shear stress versus shear rate graphs were then

calculated to acquire the viscosity of the solvents. Density measurements were performed by weighing 1 ml of each solvent on an analytical scale. All analyses were conducted at room temperature.

2.4. Extraction of canola protein isolates

Canola meal was added to each solvent at a meal:solvent mass ratio of 1:10. The mixtures were then placed on a Super-Nuova™ Multi-Position Digital Stirrer equipped with a Type K thermocouple probe (Thermo Fisher Scientific, MA, USA) to maintain the temperature of the mixtures at around 40 °C during extraction. Protein extraction was carried out for 2 h at the stirring rate of 800 rpm. While CG and C treatments were left on the stirrer for the full duration of extraction, UCG and UC treatments were temporarily removed for ultrasound application using an ultrasound homogenizer model JY92-IIN (Ningbo Scientz Biotechnology, ZJ, China) equipped with an 8 mm probe. Sonication parameters were based on preliminary optimizations, as presented in supplementary material 1, and performed for 20 min (pulse mode, 2 s on and 2 s off, actual sonication duration of 10 min) at a frequency of 20 kHz and power density of 3 W/g of solvent. The temperature of the mixtures was allowed to rise during sonication treatment to around 64–68 °C. However, after sonication, the mixtures were placed in an ice bath to reduce the temperature to 40 °C and then returned to the stirrer. After finishing the extraction, the mixtures were centrifuged for 10 min at 25 °C and 3350 g, and the obtained supernatants were diluted with water and poured into dialysis bags with molecular cut-off of 3.5 kDa. Dialysis was carried out at 4 for 5 days with frequent water changes and conductivity measurements to ensure the proper execution of the process. Finally, the contents of the dialysis bags were freeze-dried, and the obtained protein isolates were kept at −18 °C until further analyses.

2.5. Protein content and extraction efficiency

Protein content evaluations were carried out using a Nitrogen Analyzer model FP-428 (Leco Corporation, MI, USA), with a protein conversion factor of 5.7 [28,29].

Protein extraction efficiency was calculated via the following equation:

$$\textit{Protein extraction efficiency}(\%) = \frac{\textit{Protein weight of the isolate}}{\textit{Protein weight of the meal}} \times 100$$

2.6. Evaluation of anti-nutritional components

Total phenolic content was measured according to a method described by Szydłowska-Czerniak et al. [30]. The method was based on the Folin–Ciocalteu assay, involving the reaction of this reagent with phenolic compounds in the presence of sodium carbonate, followed by absorbance measurement at 725 nm. TPC was calculated based on the formula obtained from a sinapic acid calibration curve.

Phytic acid content was measured according to a method described by Akbari & Wu [31]. This method was based on the reaction of phytic acid with ammonium iron (III) sulfate in an acidic environment, followed by the addition of 2.2′-bipyridine and absorbance measurement at 519 nm. Phytic acid content was calculated using the formula obtained from a phytic acid calibration curve.

Glucosinolate content was measured according to a method described by Jezek et al. [32]. Briefly, glucosinolates were subjected to alkaline degradation to produce 1-thioglucose, which was then reacted with ferricyanide. The absorbance of the solution was measured at 420 nm within 15 s. Content of glucosinolates was calculated using the formula obtained from a sinigrin calibration curve.

2.7. Isolate color evaluation

Color measurements were performed based on CIELAB color space using a CR-410 chroma meter (Konica Minolta Inc., Tokyo, Japan). A white plate was used to calibrate the machine first, and the L* (lightness), a* (green [−] to red [+]) and b* (blue [−] to yellow [+]) values of protein isolates were recorded at room temperature.

2.8. Protein molecular and structural analyses

2.8.1. Size-exclusion high-performance liquid chromatography (SE-HPLC)

A method described by Defaix et al. [33], with modifications, was used to evaluate the apparent molecular weights (M_w) and percentage of protein fractions in the isolates. Protein solutions were prepared in a mobile phase containing 54.9 % water, 45 % acetonitrile and 0.1 % trifluoroacetic acid (TFA) at a concentration of 1 mg/ml. After 2 h of shaking at room temperature, the solutions were subjected to a mild sonication treatment and then passed through a 0.45 µm syringe filter. SE-HPLC was performed using an Agilent series 1100 system (CA, USA) equipped with a BioSep-SEC-S2000 300 × 7.8 mm, 5 µm column (Phenomenex, CA, USA) over 30 mins at a flow rate of 0.6 ml/min and a detection wavelength of 214 nm. The mobile phase (54.9 % water, 45 % acetonitrile and 0.1 % TFA) was used at a constant composition (isocratic elution) during each run. A protein standard solution, using proteins from a gel filtration markers kit from Sigma-Aldrich (12–200 kDa) [33], was analyzed under identical conditions and a logarithmic calibration curve was plotted using the retention times of each standard component for M_w calculations. Furthermore, the percentages of protein fractions were calculated by measuring the area under the identified protein peaks using OriginLab Origin 2024b software (MA, USA).

2.8.2. Fourier-transform infrared spectrum (FT-IR)

Mixtures of 1 mg of protein and 99 mg of KBr were ground into a fine powder and pressed to form transparent disks. The disks were then placed inside a Nicolet 6700 spectrophotometer (Thermo Fisher Scientific Inc., MA, USA) and the FT-IR spectra were recorded with a number of scans of 128 and a resolution of 4 cm^{-1}. The spectrum of a pure KBr disk was also obtained and subtracted from protein spectra as background. Fourier self-deconvolution on the Amide I region (1700–1600 cm^{-1}) of the spectra was performed via Omnic 8.1 software, at a bandwidth of ~ 25 cm^{-1} and an enhancement factor of 2.6. The percentages of protein secondary structure were calculated via second derivative analysis and peak fitting using PeakFit v4.12 (SeaSolve software Inc., Framingham, USA).

2.8.3. Intrinsic fluorescence

Protein suspensions were prepared in phosphate buffered saline (PBS, pH 7.4) at a concentration of 1 mg/ml and analyzed using a SpectraMax M3 spectrophotometer (Molecular Devices, CA, USA). The excitation wavelength was set to 280 nm, and emission spectra were collected over the range of 290–400 nm with a slit of 2 nm at 25 °C.

2.8.4. Particle size distribution

Protein suspensions at a concentration of 10 mg/ml were shaken at room temperature for 24 h, and their particle size distribution was measured using a Mastersizer 3000 (Malvern Instruments Ltd., Malvern, UK). Measurements were performed at room temperature, with the refractive indices of protein and dispersant (water) set to 1.45 and 1.33, respectively.

2.9. Evaluation of protein functionality at pH levels of 3, 5 and 7

2.9.1. Aqueous solubility

Protein aqueous solubility was evaluated based on a method described by Yang et al. [34], with some modifications. 200 ml of protein suspensions at a concentration of 10 mg/ml were adjusted to pH 3, 5

and 7 using 0.1 M and 1 M NaOH and HCl solutions. The suspensions were then vigorously shaken at room temperature for 2 h and centrifuged at 3250 g for 30 min at 25 °C. The obtained supernatants were then freeze-dried, and the nitrogen content of the dried material was measured using a Nitrogen Analyzer model FP-428 (Leco Corporation, MI, USA). Protein aqueous solubility (%) was measured according to the following equation,

$$Aqueous\ solubility(\%) = \frac{Nitrogen\ content\ of\ the\ dried\ supernatant}{Nitorgen\ content\ of\ the\ protein\ isolate} \times 100$$

2.9.2. Emulsifying properties

The emulsifying activity index (EAI) and emulsion stability index (ESI) of protein isolates were calculated according to [35] and [36] with a few modifications. Mixtures of 6 ml of protein suspension at a concentration of 10 mg/ml (adjusted to pH 3,5 and 7 using 0.1 M and 1 M, NaOH and HCl solutions) and 2 ml of canola oil were homogenized at room temperature using a T18 high-speed homogenizer (IKA, NC, USA) for two minutes at a speed of 25,000 RPM. Immediately after homogenization, 50 μL of the emulsion was removed from the bottom of the container and combined with 5 ml of 0.1 % SDS solution under gentle shaking, and the absorbance of the mixture was measured at 500 nm to obtain A_0. The emulsion was then left at room temperature for 10 min, after which another 50 μL of sample was taken from the bottom of the container, mixed with 5 ml of 0.1 % SDS solution and measured for absorbance at 500 nm to obtain A_1. EAI was measured according to the following equation,

$$EAI\left(\frac{m^2}{g}\right) = \frac{2 \times 2.303 \times A_0 \times DF}{C \times \theta \times 10000}$$

where A_0 was the absorbance immediately after homogenization, C was the protein concentration (g/ml), DF was the dilution factor (100), and θ was the volume of oil fraction (0.25). Furthermore, ESI was calculated according to the following equation,

$$ESI(min) = A_0 \frac{\Delta t}{\Delta A}$$

where A_0 was the absorbance immediately after homogenization, Δt was the time between the first and the second emulsion sampling (10 min) and ΔA was the difference in the absorbance of the two samples.

2.9.3. Foaming properties

The foaming capacity (FC) and foam stability (FS) of protein isolates were measured according to [34] and [37], with some modifications. Protein suspensions at a concentration of 10 mg/ml (adjusted to pH 3,5 and 7 using 0.1 M and 1 M, NaOH and HCl solutions) were vigorously mixed at room temperature for 2 min at a speed of 15,000 RPM using a T18 high-speed homogenizer (IKA, NC, USA). The total volume of each sample was measured immediately after mixing, and again after 30 min. FC and FS were calculated according to the following equations,

$$FC(\%) = \frac{V_1 - V_0}{V_0} \times 100$$

$$FS(\%) = \frac{V_2 - V_0}{V_0} \times 100$$

where V_0 was the volume of the suspension before homogenization, V_1 was the total volume immediately after homogenization and V_2 was the total volume 30 min after homogenization.

2.10. Statistical analyses

The statistical analyses were carried out using OriginLab Origin 2024b (MA, USA). All the experiments were repeated at least three times, and the data were reported as mean ± standard deviation. Mean comparisons were carried out using the one-way analysis of variance (ANOVA) followed by Tukey's test at the significance level of 0.05. The use of the words "significant" and "significantly" throughout the paper is an indication of the statistical significance at the level of 0.05.

3. Results and discussion

3.1. Solvent characterization and categorization

Fig. 1 presents the graphs for viscosity, dielectric constant, conductivity, density and pH of the two groups of employed solvents, choline chloride-glucose-water (CG) and choline chloride-water (C). Increasing the water content from 30 % to 90 % in both solvent groups resulted in a decrease in viscosity and density and an increase in dielectric constant values. Water molecules can disrupt the strong network of hydrogen bonding between the NDES components, which can reduce both viscosity and density of the system. Water is also a more polar molecule compared to both choline chloride and glucose, and its addition can increase the dielectric constant, and subsequently, the polarity of the NDESs system [38]. In terms of conductivity, an increasing trend was observed in both solvent groups up to 60 % of solvent water content. However, further increase in the water content resulted in a reduction of conductivity. These observations are in accordance with previous reports [20,39], and could be related to the solvent composition and the degree of ionic dissociation. Specifically, increasing the water content to 60 % may have increased the ionic dissociation of the NDESs components by reducing their association with the NDESs structure. However, above 60 % of water, a reduction in the concentration of ions in the NDESs system could have caused the observed drop in conductivity, regardless the extent of ionic dissociation [20]. In terms of pH, CG solvents demonstrated an increasing trend with increasing water content, while C solvents showed a decreasing trend.

It was also observed that substitution of glucose with choline chloride (the key difference between the C and the CG formulations) resulted in an increase in the conductivity of C solvents compared to their respective CG solvents. This substitution also decreased the viscosity, density and polarity of the C solvents. The large and compact structure of glucose molecules and their ability to form multiple hydrogen bonds could have contributed to the higher viscosity and density of the CG solvents. When glucose was removed from the system, the smaller water molecules were left to act as the sole HBD and may have created a more flexible network of hydrogen bonding with choline chloride, resulting in the reduction of viscosity and density of the C solvents [6]. Finally, C30 and C60 showed higher pH levels compared to CG30 and CG60, while the pH of C90 was lower than that of CG90.

The nanostructure of NDESs is composed of clusters of solute molecules, formed within a strong, heterogeneous network of NDES-NDES hydrogen bonding [40]. When water molecules are introduced into this system, they gradually occupy the hydration shell of the solutes, break some of the NDES-NDES hydrogen bonds and form their own network of NDES-water and water-water hydrogen bonding. The clustered structure of NDES and its intermolecular bonding still exists within these systems and forms separate regions from water due to solvophobic effects. However, when the water content exceeds a certain threshold, the hydration shell of the solutes becomes fully saturated with water and the system transitions from a hydrated NDES to an aqueous solution of the NDES components [41]. The hydration threshold varies depending on the NDES components and their ratio. In the case of NDESs used in this study, for choline chloride-glucose systems with a ratio of 1:1, Zafarani-Moattar et al. [42] reported the elimination of the NDES-NDES hydrogen bonding at the water content ~ 75 %. For choline chloride-water NDESs, this threshold was reported to be ~ 80 % by Zhang et al. [18]. Therefore, based on water content, the employed solvents in this study were divided into three zones. The first zone, NDESs, included the CG and C solvents with water contents of 30 %, 45 % and 60 %. The

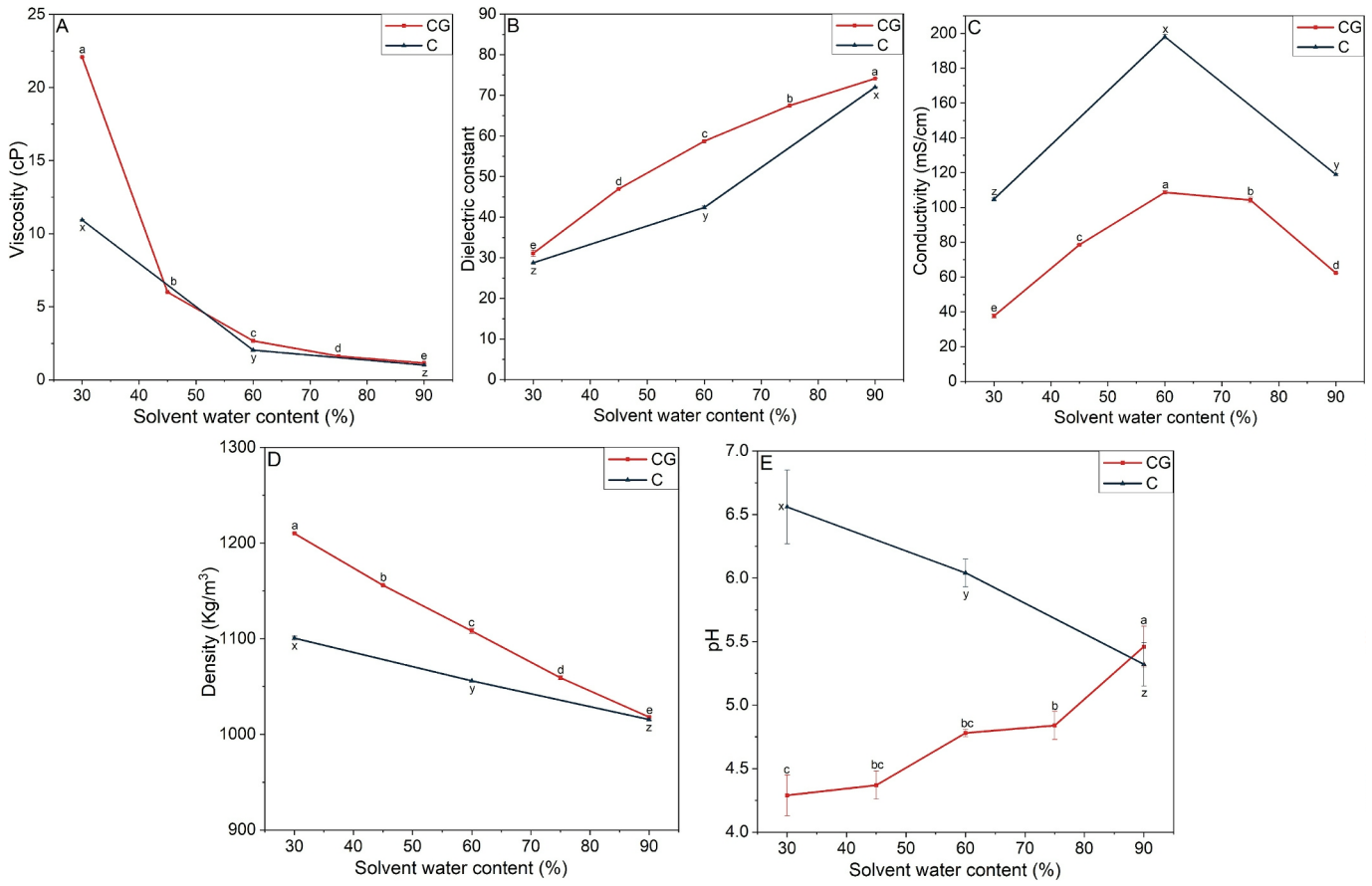


Fig. 1. Plots showing the viscosity (A), dielectric constant (B), conductivity (C), density (D) and pH (E) of CG and C solvents. Different letters (a-e for CG group and x-z for C group) indicate significant differences ($p < 0.05$) between the solvents in each group.

second zone was named aqueous solutions and contained the CG and C solvents with water contents of 75 % and 90 %, while the third zone was the pure water used for protein extraction. While changes in the conductivity values appeared to correspond with the defined solvent zones (conductivity peaked at 60 % of water and then decreased after the transition from NDES to aqueous solution at 75 % of water), the rest of the solvents' physical properties appeared to be independent of these zones.

3.2. Protein extraction mechanisms, efficiency and content

3.2.1. CG and UCG treatments

Fig. 2 presents the protein extraction efficiency and protein content of extracted isolates. For choline chloride-glucose-water solvents, CG30 showed an extraction efficiency of 46.40 % (Fig. 2A). The efficiency increased to 48.82 % and 50.28 % for CG45 and CG60, respectively. However, further increases in solvent water content led to a significant drop in extraction efficiency, with values of 47.51 % and 43.05 % for CG75 and CG90, respectively. The lowest efficiency was observed for water extraction at 29.95 %, which is almost identical to the values reported by Fetzer et al. [43]. Compared to the CG treatments, the application of ultrasound consistently improved the extraction efficiency of UCG treatments by 10.40–11.52 % across different water contents. A trend similar to the extraction efficiency of CG solvents was also observed in UCG solvents, with the efficiency of 57.74 % for UCG30, followed by the peak in efficiency at 60.68 % for UCG60 and a reduction to 59.14 % and 54.44 % for UCG75 and UCG90, respectively. Combined ultrasound and water extraction yielded an efficiency of only 31.78 %, a small increase of 1.83 % compared to water extraction without ultrasound.

Previous studies have discussed the effects of viscosity, density and polarity of the DESs in the extraction of proteins. While the reduction in viscosity and density could improve the DES's mobility and diffusion properties, an appropriate polarity value could maximize the solubilization and extraction of target proteins [5,38]. In this study, a positive relationship was observed, in both CG and UCG treatments, between the protein extraction efficiency and the hydration level of NDESs up 60 % of water, which is close to its reported upper hydration limit [42]. It was also shown that this increase in hydration resulted in reduced viscosity and density and increased polarity, which could partly contribute to the improved protein extraction efficiency. However, once the solvent transitioned to an aqueous state (75 % of water), even though the viscosity and density went further down, the extraction efficiency began to decline. While this could be related to the solvent's polarity moving out of the appropriate range to solubilize the maximum amount of canola protein, it could also highlight the importance of preserving the nanostructure of NDESs, and its effect on maximizing protein extraction. These results also indicate that pushing NDESs to their upper hydration limit could not only drastically reduce the solvent's initial cost and later recovery expenses but also may not severely affect protein extraction efficiency, and in the case of CG solvents, could even improve it. Another study by Hewage et al. [11] reported that increasing the content of water in choline chloride-glycerol NDESs from 0-40 % maximized fava bean protein extraction efficiency at 65.42 %; however, water contents above 40 % were not evaluated.

Furthermore, ultrasound treatment demonstrated consistent effectiveness in improving the extraction of canola protein when combined with CG solvents. The localized, tremendous shear forces and high temperatures caused by the ultrasound-induced cavitation can break down plant cell walls and improve the diffusion of NDESs into the cells

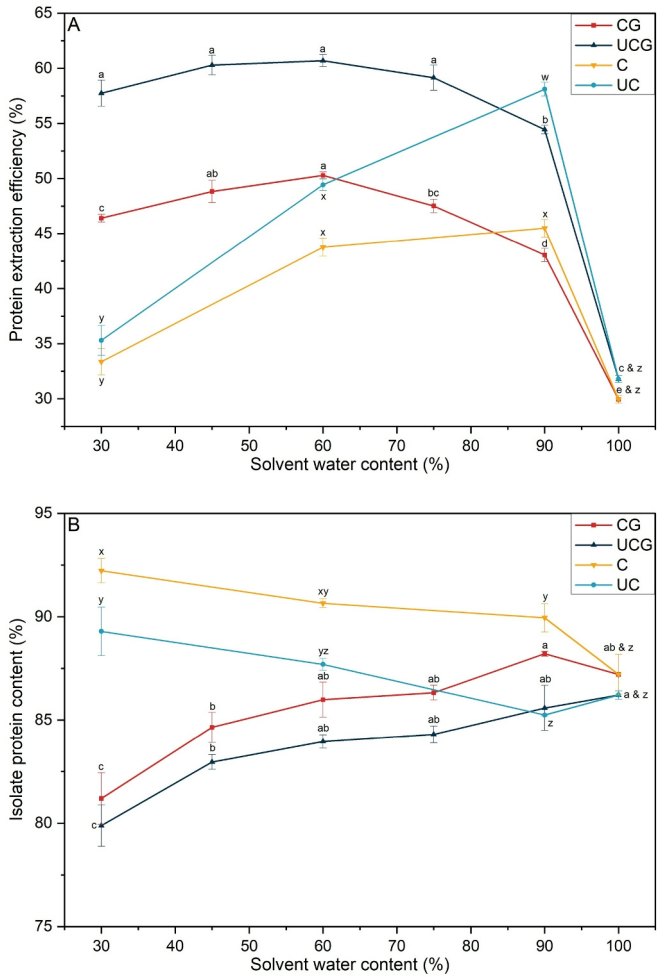


Fig. 2. Protein extraction efficiency (A) and protein content (B) of canola protein isolates. Different letters (a-e for CG and UCG groups and w-z for C and UC groups) indicate significant differences ($p < 0.05$) between the isolates in each group.

where proteins are stored [21]. These effects may also break down protein aggregates, thereby improving their solubility and extraction. Cavitation can also produce reactive hydrogen and hydroxyl radicals from water. These radicals, in combination with the aforementioned physical effects, may impact the proteins (and other components in the medium) in various ways, including alterations to their higher structure, functionality and digestibility [22,44]. The typical range of ultrasound power density for extraction of plant proteins is reported to be 1–10 W/g. Therefore, the applied power density of 3 W/g in this study could be considered a mild treatment [21], and was selected to maximize the preservation of protein's native structure and functionality, while simultaneously improving its extraction efficiency. While research on ultrasound-assisted extraction of canola protein is limited, a study by Dong et al.[45], reported an extraction efficiency of 76.83 % using non-pressed, solvent-defatted meal under optimized conditions of alkaline extraction (pH 11.5), ultrasound power of 450 W (power density not reported), and a meal:solvent ratio of 1:24.

Unlike the extraction efficiency, which peaked at 60 % of solvent water content, the protein content of CG isolates (Fig. 2B) showed a generally increasing trend, from 81.19 % for CG30 to its peak at 88.20 % for CG90 and 87.21 % for water extracts. This increase could be related to the reduction of CG solvents' viscosity and density, which may improve diffusion and solubilization properties and increase the extraction of proteins over non-protein fractions [17]. Furthermore, the protein content of UCG isolates also showed a consistent increase from 79.88 % for UCG30 to 85.58 % for UCG90 and 86.21 % for water + ultrasound isolates. However, sonication was found to reduce the protein content of UCG isolates by an average of 1.93 % compared to CG isolates. This could be related to the breakdown of plant cell wall materials caused by ultrasound-induced cavitation and subsequent release of more non-protein fractions (mostly carbohydrates) into the extraction medium [21]. Overall, the increase in protein content of CG isolates suggested another potential benefit of increasing the hydration level of NDESs for protein extraction.

3.2.2. C and UC treatments, and comparison between the two solvent groups

For choline chloride-water solvents, C30 showed an extraction efficiency of only 33.35 %. The efficiency increased to 43.76 % for C60 and peaked at 45.48 % for C90, with no significant difference between the latter two. While sonication also improved the extraction efficiency of UC treatments, unlike UCG treatments, these improvements were inconsistent across different water contents and measured at 1.94 %, 5.67 % and 12.63 % for UC30, UC60 and UC90, respectively. The lowest extraction efficiency among UC treatments was observed at 35.29 % for UC30, while the highest was recorded at 58.11 % for UC90.

Therefore, the aqueous solution of choline chloride proved to be a more effective medium for extraction of canola protein compared to choline chloride-water NDESs, especially when paired with ultrasound treatment. This could be related to the improvement of protein solubility via the salting-in effect [4]. While this is the first report on the extraction of plant proteins using aqueous solutions of choline chloride, Fetzer et al. [43] reported a canola protein extraction efficiency of ~ 50 % using both 0.5 M and 1 M solutions of NaCl, which was ~ 4.5 % higher than the extraction efficiency of C90 at 0.8 M of choline chloride concentration. However, sonication notably improved the extraction efficiency of UC90 by 12.63 %, highlighting the promising potential of this technology when combined with salt-extraction.

In terms of protein content, a decreasing trend was observed from 92.22 % for C30 to 89.94 % for C90. A similar trend was also observed in UC isolates, with a significant decline from 89.29 % in UC30 to 85.24 % in UC90. Sonication also reduced the protein content of UC isolates by an average of 3.53 % compared to C isolates.

As mentioned earlier, a comparison between the physical properties of NDESs showed that C30 and C60 had lower viscosity and density compared to CG30 and CG60. The difference is especially stark in the case of C30, which exhibited half the viscosity, a much lower density (1100.7 Kg/m^3 versus 1210.15 Kg/m^3), and almost the same polarity compared to CG30. This, however, did not translate into improved protein extraction efficiency, and CG30 still offered 13.05 % higher efficiency compared to C30. These observations highlighted the importance of the choice of HBD in protein extraction, which, under the conditions of this study, proved to be more influential than the mere physical properties of NDESs. Furthermore, even the application of ultrasound was not as effective in improving the extraction efficiency of UC treatments compared to UCG treatments, especially regarding UC30. Judging from the reduction of protein content in UC isolates compared to C isolates, it could be said that sonication was still exerting its cavitation-induced effects on the cells, and the low extraction efficiencies were likely due to the inability of choline chloride-water NDESs to adequately solubilize canola protein. Overall, while CG solvents proved more effective within the NDES zone, C solvents extracted more protein upon transition to the aqueous state.

Compared to a similar NDES from our previous study (choline chloride to glucose ratio of 1:2 + 25 % water), CG30 demonstrated improvements of 2.23 % and 5.76 % in extraction efficiency and protein content of canola protein isolates, respectively [15]. These enhancements could be related to the adjustments made in the HBA:HBD ratio, as well as the content of water. Another observation was that while the NDES developed with glycerol (same formulation as above, except for the HBD) showed significantly lower viscosity and density and similar

polarity compared to NDESs developed with glucose and sorbitol, all three solvents demonstrated similar extraction efficiencies of around 44 %. This finding correlates with the above-mentioned point about the more prominent influence of HBD selection over the physical properties of NDESs. Furthermore, with extraction efficiencies of approximately 60 %, both UCG60 and UC90 treatments came close to the highest extraction efficiency of our previous study (68.34 %), which was achieved using alkaline extraction at pH 12.

3.3. Isolate antinutritional components and color analysis

3.3.1. Content of phytic acid and glucosinolates

In terms of phytic acid content (Fig. 3A), CG30 showed the highest level at 44.98 mg/g, followed by a significant decrease to 29.04 mg/g in CG45 isolates. The lowest level of phytic acid was observed in CG60 at 26.94 mg/g; however, further increase in solvent water content to 75 %, 90 % and 100 % resulted in a slightly increasing trend in the contents of phytic acid. No significant difference was observed between the phytic acid contents of CG isolates extracted with more than 45 % of solvent water content. A similar trend was also observed in UCG isolates, with the highest phytic acid level of 40.15 mg/g in UCG30 and the lowest level of 21.12 mg/g in UCG60. The binding of phytic acid to protein and its subsequent presence in the final isolate is strongly dependent (among other factors) on the ionic strength of the medium [46]. Herein, An inverse correlation was observed between the conductivity of CG solvents and phytic acid contents of CG and UCG isolates, which is in agreement with previous reports [47]. Sonication was also effective in reducing the contents of phytic acid in UCG isolates by an average of ~ 13.5 % compared to CG isolates, with this reduction being relatively consistent across different water contents. This may be related to the effects of cavitation, which can both disintegrate the phytic acid [48], and disrupt its interactions with protein by inducing change in the protein structure [46]. On the other hand, the phytic acid content of C isolates showed a consistent increase from 10.82 mg/g in C30 to 26.03 mg/g in C90 isolates. UC isolates also showed a similar trend; however, sonication did not reduce the phytic acid content of UC isolates to the same extent as in UCG treatments, possibly because lower amounts of phytic acid were extracted in the medium to begin with. Overall, C solvents extracted less phytic acid than CG solvents, which may be related to their higher conductivity [47].

Regarding the content of glucosinolates, while the meal contained 8.12 ± 0.57 mmol/kg, no detectable traces of glucosinolates were found in any of the protein isolates. While Canadian canola typically contains low contents of glucosinolates to begin with, most of the extracted glucosinolates were also possibly removed during the dialysis procedure, resulting in their extremely low levels in the final isolates [49].

3.3.2. Total phenolic content

The TPC of CG isolates (Fig. 3B) demonstrated a decreasing trend, from 3.83 mg/g in CG30 to 2.99 mg/g in CG90. A similar trend, along with very close values, was also observed in the TPC of C isolates. The isolate extracted with water, however, showed the highest TPC at 4.68 mg/g. Previous studies on the extraction of phenolic compounds using choline chloride-based NDESs have reported the negative effects of water addition on the extraction yield [50]. For instance, Liu et al. [51] reported that increasing the water content of NDESs above 20 % resulted in reducing the extraction efficiency of sinapine thiocyanate, possibly due to the weakening of the NDES's hydrogen bonding network. Furthermore, increasing the water content could also prevent the salts from participating in the nanostructure of NDESs, thereby enabling them to occupy the hydration shell of the proteins more effectively and disrupt the protein–phenolic interactions further. However, once the solvent transitioned to pure water, canola meal was subjected to a completely different medium, in which the absence of choline chloride may have contributed to increased interactions between protein and phenolic compounds, resulting in higher phenolic content in the final isolate. Overall, since phenolic compounds of canola are known to cause dark color and undesirable flavor in the final protein product [4], their reduction could be considered as another benefit of increasing the water content of NDESs.

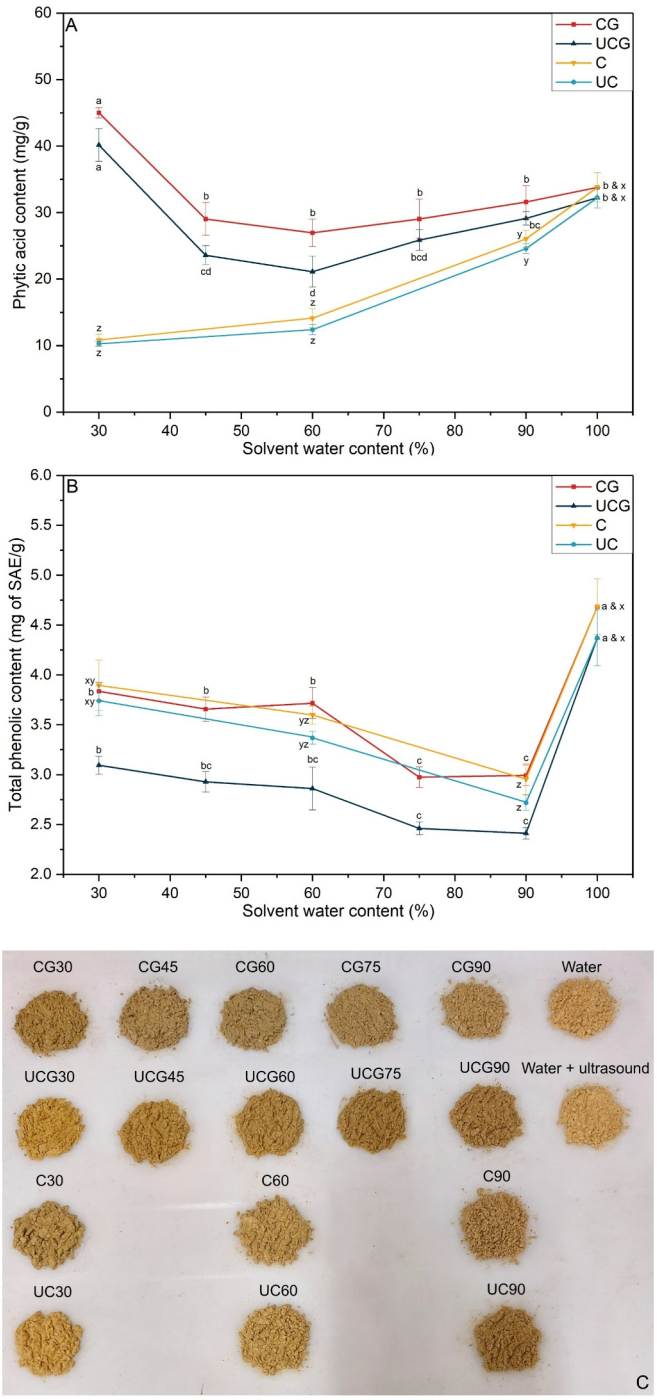


Fig. 3. Phytic acid (A) and total phenolic (B) contents of canola protein isolates, along with their color and visual appearance (C). Different letters (a-d for CG and UCG groups and x-z for C and UC groups) indicate significant differences ($p < 0.05$) between the isolates in each group.

While the application of ultrasound did not change the aforementioned trends, it caused an overall decrease in the TPC of UCG, UC and water + ultrasound isolates compared to their respective non-sonicated counterparts. UC Isolates presented an average reduction of ~ 6.1 %, whereas a more noticeable average reduction of ~ 20 % was observed in UCG isolates. Ultrasound treatment is often employed as an assisting

technology to improve the extraction yield of phenolic compounds [52], and it has possibly increased the extraction of phenolics during our protein extraction procedure as well. However, ultrasound is also known to induce changes in the higher structure of proteins, which could ultimately affect the protein–phenolic interactions. These interactions are responsible for the majority of phenolic retention during the dialysis procedure. Therefore, the modified structure of proteins in the ultrasound-treated samples possibly had less affinity toward phenolic compounds, which facilitated their removal during dialysis. Another possibility is the cavitation-induced oxidation of phenolic compounds into quinones, followed by the formation of covalent bonds with other components in the meal, namely carbohydrates, which may have contributed the observed reductions in TPC [53]. This could also justify the much higher average reduction of TPC in UCG isolates (extracted via glucose-containing NDESs) compared to UC isolates, since the presence of glucose could have promoted the glycosylation of phenolic compounds and their subsequent removal during dialysis [54].

3.3.3. Color analysis

The color analysis of CG isolates (Table 1) revealed an upward trend in both L* and a* values from CG30 to CG90, which, from a visual standpoint, indicated that increasing the content of water resulted in the extraction of lighter, less greenish isolates (Fig. 3C). The highest L*, a* and b* values were observed in water extracts, which produced a notably brighter and yellower isolate compared to CG isolates. A similar trend was also observed in the color parameters of C isolates; however, they demonstrated higher L* values compared to CG isolates. On the other hand, application of ultrasound led to a decreasing trend in L* and b* values, and a fluctuating trend in a* values in both UCG30-90 and UC30-90 treatments. Visually, a progression toward darker, less yellowish colors was observed in both UCG and UC treatments. Sonication also altered the color parameters of water + ultrasound extract, though this isolate remained visually similar to the non-sonicated water extract. It was also observed that ultrasound generally increased both a* and b* values (except for UC90 treatment), resulting in isolates that appeared more yellow and less green. The color of canola protein isolates has been associated with the presence of phenolic compounds, including phenolic acids and condensed tannins, as well as other pigments in the final product [37,55]. A variety of factors, including the type and degree of interactions between these components and proteins during extraction and purification steps, and their content and ratio in the final protein product can determine the isolate's final color. Overall, compared to water extracts, the presence of NDES components at any concentration resulted in the production of isolates with darker color. Most of the NDES extracted canola protein isolates from our previous study also showed closely resembling colors to those reported here, except for protein isolates from choline chloride-urea-water NDES, which was a noticeably brighter and yellower product. However, all of our NDES extracted isolates so far have offered a substantially better color compared to the dark-green color of alkaline extracted isolates at pH 12 [15].

3.4. Protein molecular and structural characterizations

Fig. 4 presents the SE-HPLC chromatograms, as well as FT-IR and intrinsic fluorescence spectra of CG, UCG, C and UC isolates with 30 %, 60 % and 90 % of water, along with water and water + ultrasound isolates. Fig. 5 demonstrates the volume-weighted particle size distribution of the mentioned isolates. Particle size distribution parameters, percentages of extracted protein fractions and contents of the protein secondary structure are also provided in Table 1, Table 2 and Table 3, respectively. It is worth noting that the preliminary data from the isolates extracted using 45 % and 75 % of solvent water content fell predictably between the results of the adjacent water contents and did not alter the overall observed trends. For this reason, these data were excluded from this section.

3.4.1. Molecular weights and the percentage of extracted protein fractions

The majority of protein in canola consists of two main storage fractions, cruciferin (12S globulin) and napin (1.7-2S albumin). Cruciferins possess a hexameric structure with a molecular weight of 300–350 kDa. Each cruciferin hexamer is composed of two trimers and stabilized via non-covalent bonding, while each cruciferin monomer (~50 kDa) is composed of two polypeptides, α (~30 kDa) and β (~20 kDa), connected via disulfide bonds [4]. Unlike cruciferin, napin is a monomeric protein with a molecular weight of 12–16 kDa, composed of two, long (~9 kDa) and short (~4 kDa) polypeptides, which are also connected via disulfide bonds [56,57]. Canola protein also contains minor oil-body proteins, namely steroleosins (~40 kDa), caleosins (~30 kDa) and the predominant fraction, oleosins (~18 kDa) [29].

Fig. 4A-D presents the SE-HPLC chromatograms of canola protein isolates. The highly positive charge of canola protein in the acidic mobile phase (pH ~ 2) disrupted the non-covalent interactions and dissociated the protein subunits and/or aggregates, resulting in the elution of well-separate peaks for all protein fractions [33]. Based on the calculated molecular weights of the observed major peaks, all chromatograms

Table 1
Color and particle size distribution parameters of canola protein isolates. Different letters (a-e for CG and UCG groups and w-z for C and UC groups) indicate significant differences ($p < 0.05$) between the isolates in each group.

Protein isolates	Color parameters			Particle size distribution parameters (μm)			
	L* (lightness)	a* (green → red)	b* (blue → yellow)	D[4,3]	D10	D50	D90
CG30	57.51 ± 0.27^{d}	-0.97 ± 0.01^{f}	21.23 ± 0.29^{c}	63.68 ± 0.52^{c}	4.89 ± 0.06^{d}	46.24 ± 0.19^{a}	143.00 ± 1.41^{c}
CG45	59.31 ± 0.08^{cd}	-0.35 ± 0.01^{e}	19.40 ± 0.005^{e}	–	–	–	–
CG60	58.67 ± 0.50^{d}	0.02 ± 0.02^{d}	20.29 ± 0.20^{d}	75.08 ± 0.68^{b}	14.76 ± 0.05^{b}	56.96 ± 0.15^{b}	153.60 ± 1.51^{c}
CG75	60.37 ± 0.22^{c}	0.77 ± 0.01^{c}	21.94 ± 0.28^{b}	–	–	–	–
CG90	62.24 ± 1.27^{b}	1.33 ± 0.03^{b}	22.22 ± 0.33^{b}	196.40 ± 5.31^{a}	16.02 ± 0.59^{a}	163.80 ± 4.32^{d}	425.00 ± 12.62^{b}
C30	61.14 ± 0.15^{z}	-0.45 ± 0.02^{z}	22.31 ± 0.12^{z}	50.92 ± 0.95^{z}	4.39 ± 0.02^{z}	34.60 ± 0.48^{z}	115.00 ± 1.41^{z}
C60	61.97 ± 0.45^{zy}	0.08 ± 0.02^{y}	21.84 ± 0.31^{z}	110.60 ± 5.31^{y}	25.00 ± 0.50^{x}	74.82 ± 1.28^{y}	221.2 ± 21.41^{y}
C90	62.33 ± 0.22^{y}	1.56 ± 0.03^{x}	21.88 ± 0.20^{z}	213.60 ± 7.56^{x}	34.22 ± 1.21^{w}	160.2 ± 5.54^{w}	478.6 ± 19.98^{x}
W	$68.63 \pm 0.34^{a\ \&\ x}$	$2.87 \pm 0.07^{a\ \&\ w}$	$27.56 \pm 0.06^{a\ \&\ y}$	$202.8 \pm 10.20^{a\ \&\ x}$	$8.62 \pm 0.48^{c\ \&\ y}$	$148.2 \pm 2.58^{c\ \&\ x}$	$479.2 \pm 33.11^{a\ \&\ x}$
UCG30	65.30 ± 0.99^{b}	0.64 ± 0.04^{d}	29.84 ± 0.31^{a}	83.06 ± 0.54^{c}	15.82 ± 0.04^{a}	69.02 ± 0.13^{c}	163.80 ± 0.83^{c}
UCG45	60.19 ± 0.49^{c}	0.81 ± 0.04^{c}	24.97 ± 0.15^{c}	–	–	–	–
UCG60	59.46 ± 0.36^{cd}	0.74 ± 0.02^{cd}	23.38 ± 0.59^{d}	100.28 ± 1.57^{b}	16.22 ± 0.08^{a}	78.50 ± 0.48^{b}	205.80 ± 3.34^{b}
UCG75	58.56 ± 0.32^{de}	0.72 ± 0.01^{cd}	24.14 ± 0.15^{cd}	–	–	–	–
UCG90	57.96 ± 0.39^{e}	1.23 ± 0.01^{b}	22.30 ± 0.20^{e}	89.56 ± 1.71^{c}	15.66 ± 0.39^{a}	67.78 ± 0.30^{c}	176.60 ± 6.73^{c}
UC30	66.75 ± 0.57^{x}	1.99 ± 0.08^{x}	28.33 ± 0.12^{y}	79.40 ± 0.99^{yz}	19.94 ± 0.11^{x}	63.74 ± 0.33^{y}	158.60 ± 0.89^{yz}
UC60	63.12 ± 0.49^{w}	0.84 ± 0.01^{z}	23.40 ± 0.22^{z}	87.56 ± 1.15^{y}	19.42 ± 0.10^{x}	70.80 ± 0.33^{x}	176.80 ± 2.77^{y}
UC90	61.27 ± 0.35^{z}	1.29 ± 0.03^{y}	23.25 ± 0.06^{z}	69.24 ± 2.92^{z}	11.68 ± 0.13^{z}	49.76 ± 0.49^{z}	136.20 ± 4.20^{z}
UW	$70.29 \pm 0.26^{a\ \&\ w}$	$2.55 \pm 0.06^{a\ \&\ w}$	$28.32 \pm 0.11^{b\ \&\ y}$	$241 \pm 11.24^{a\ \&\ x}$	$15.94 \pm 0.87^{a\ \&\ y}$	$176.6 \pm 7.26^{a\ \&\ w}$	$566.8 \pm 26.78^{a\ \&\ x}$

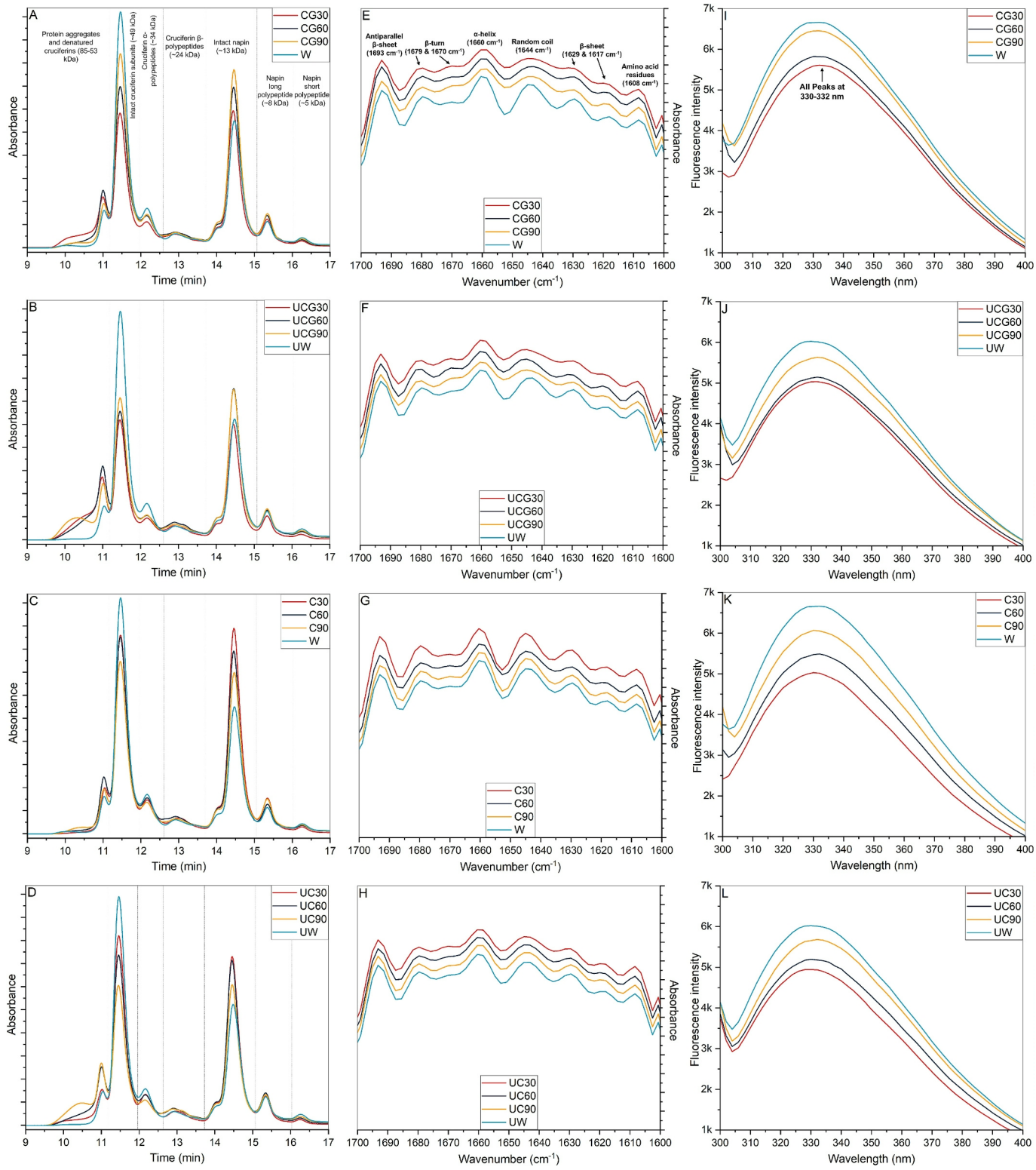


Fig. 4. SE-HPLC chromatograms (A-D), FT-IR spectra (E-H), and intrinsic fluorescence spectra (I-L) of canola protein isolates. Panels A, E and I represent samples from the CG group; panels B, F and J represent samples from the UCG group; panels C, G and K represent samples from the C group; and panels D, H and L represent samples from the UC group.

were divided into seven zones, corresponding to different protein fractions in the isolates. The first zone consisted of either one fronting peak, or two overlapping peaks with the molecular weights in the range of 85–53 kDa. The peaks in this zone were previously observed when canola protein was subjected to extreme denaturing and oxidizing conditions, and possibly represent protein aggregates covalently bonded to other protein or non-protein components, as well as unfolded cruciferin subunits that were eluted earlier than intact subunits from the column [15,58]. The next three zones, with peaks eluting at approximately 11.20, 11.50 and 12.25 min, corresponded to the molecular

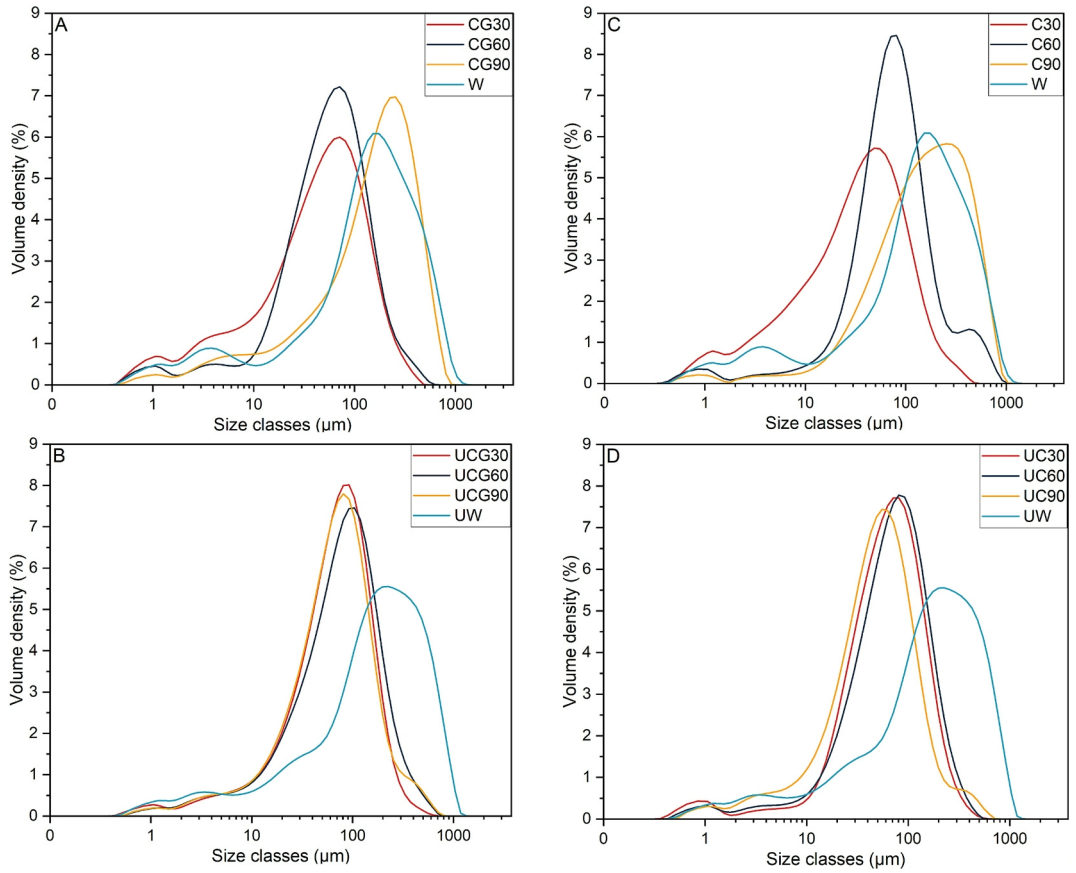


Fig. 5. Volume-weighted particle size distribution of canola protein isolates. Panels A and B represent samples from the CG and UCG groups, respectively; and panels C and D represent samples from the C and UC groups, respectively.

Table 2
Percentage of extracted protein fractions in each canola protein isolate. Different letters (a-d for CG and UCG groups and x-z for C and UC groups) indicate significant differences ($p < 0.05$) between the isolates in each group.

Protein isolates	Protein aggregates and denatured cruciferins (%)	Intact cruciferin subunits (%)	Cruciferin α-polypeptides (%)	Cruciferin β-polypeptides (%)	Intact napin (%)	Napin long polypeptides (%)	Napin short polypeptides (%)
CG30	14.35 ± 0.40^{a}	31.61 ± 2.20^{b}	5.98 ± 0.18^{b}	6.53 ± 0.006^{a}	33.10 ± 1.36^{b}	6.67 ± 0.25^{a}	1.71 ± 0.008^{a}
CG60	10.35 ± 0.11^{b}	32.08 ± 0.31^{b}	6.22 ± 0.39^{b}	6.58 ± 0.26^{a}	35.80 ± 0.72^{ab}	6.96 ± 0.02^{a}	1.95 ± 0.14^{a}
CG90	7.48 ± 0.19^{c}	34.32 ± 1.11^{b}	6.34 ± 0.12^{b}	5.89 ± 0.16^{ab}	36.80 ± 0.74^{a}	6.95 ± 0.20^{a}	2.18 ± 0.25^{a}
C30	4.99 ± 1.14^{y}	35.21 ± 0.92^{z}	6.79 ± 0.29^{z}	4.98 ± 0.07^{z}	40.00 ± 0.26^{x}	6.73 ± 0.39^{z}	1.27 ± 0.06^{z}
C60	7.74 ± 0.70^{yz}	35.30 ± 0.63^{z}	6.71 ± 0.17^{z}	6.29 ± 0.18^{y}	35.73 ± 0.07^{y}	6.55 ± 0.75^{z}	1.65 ± 0.24^{yz}
C90	8.24 ± 0.24^{z}	32.80 ± 0.89^{z}	6.49 ± 0.21^{z}	6.13 ± 0.01^{y}	36.54 ± 0.80^{y}	7.69 ± 0.16^{z}	2.08 ± 0.12^{xy}
W	$5.36 \pm 0.19^{d\ \&\ z}$	$43.44 \pm 0.46^{a\ \&\ y}$	$8.11 \pm 0.22^{a\ \&\ y}$	$5.44 \pm 0.13^{b\ \&\ z}$	$29.20 \pm 0.15^{c\ \&\ z}$	$5.89 \pm 0.19^{b\ \&\ z}$	$2.52 \pm 0.26^{a\ \&\ x}$
UCG30	21.81 ± 0.40^{a}	28.29 ± 0.17^{b}	5.72 ± 0.02^{b}	6.77 ± 0.25^{ab}	29.81 ± 0.72^{a}	6.09 ± 0.01^{a}	1.44 ± 0.11^{b}
UCG60	18.86 ± 1.05^{b}	27.81 ± 1.90^{b}	5.47 ± 0.34^{b}	7.41 ± 0.20^{a}	32.01 ± 2.91^{a}	6.61 ± 0.22^{a}	1.78 ± 0.37^{b}
UCG90	16.74 ± 0.19^{b}	28.05 ± 0.40^{b}	5.06 ± 0.02^{b}	6.60 ± 0.01^{b}	33.97 ± 0.37^{a}	7.35 ± 0.44^{a}	2.20 ± 0.16^{ab}
UC30	7.56 ± 0.50^{y}	36.51 ± 0.95^{xy}	6.75 ± 0.21^{y}	5.04 ± 0.02^{z}	36.85 ± 0.23^{z}	6.07 ± 0.07^{z}	1.19 ± 0.08^{z}
UC60	12.38 ± 0.13^{x}	31.76 ± 0.49^{yz}	6.13 ± 0.004^{y}	6.37 ± 0.19^{y}	35.03 ± 0.58^{z}	6.73 ± 0.09^{z}	1.56 ± 0.12^{yz}
UC90	17.22 ± 0.03^{w}	28.83 ± 0.30^{z}	5.34 ± 0.10^{z}	7.12 ± 0.04^{x}	32.21 ± 0.20^{z}	7.17 ± 0.03^{z}	2.08 ± 0.24^{xy}
UW	$4.36 \pm 0.69^{c\ \&\ z}$	$41.38 \pm 3.15^{a\ \&\ x}$	$7.40 \pm 0.20^{a\ \&\ x}$	$5.34 \pm 0.09^{c\ \&\ z}$	$31.65 \pm 2.95^{a\ \&\ z}$	$7.04 \pm 0.87^{a\ \&\ z}$	$2.77 \pm 0.11^{a\ \&\ x}$

Table 3
Percentages of protein secondary structure components in each canola protein isolate. Different letters (a and b for CG and UCG groups and x-z for C and UC groups) indicate significant differences ($p < 0.05$) between the isolates in each group.

Protein isolates	Protein secondary structure (%)				
	β-sheet	Random coil	α-helix	β-turn	Anti-parallel β-sheet
CG30	30.25 ± 1.09^{a}	16.71 ± 0.40^{a}	17.38 ± 0.43^{a}	28.07 ± 0.22^{a}	7.57 ± 0.02^{a}
CG60	29.29 ± 0.23^{a}	16.73 ± 0.08^{a}	17.54 ± 0.20^{a}	28.56 ± 0.31^{a}	7.85 ± 0.36^{a}
CG90	29.77 ± 0.66^{a}	16.95 ± 0.19^{a}	17.69 ± 0.15^{a}	28.23 ± 0.20^{a}	7.33 ± 0.10^{a}
C30	30.85 ± 0.58^{z}	15.86 ± 0.56^{z}	16.47 ± 0.94^{z}	28.51 ± 0.02^{z}	8.29 ± 0.93^{z}
C60	29.25 ± 0.18^{z}	16.81 ± 0.02^{z}	17.64 ± 0.05^{z}	28.83 ± 0.05^{z}	7.45 ± 0.20^{z}
C90	29.66 ± 0.01^{z}	16.66 ± 0.01^{z}	17.57 ± 0.06^{z}	28.70 ± 0.08^{z}	7.37 ± 0.01^{z}
W	30.51 ± 0.83$^{a\ \&\ z}$	16.73 ± 0.08$^{a\ \&\ z}$	17.63 ± 0.11$^{a\ \&\ z}$	28.09 ± 0.36$^{a\ \&\ z}$	7.02 ± 0.27$^{a\ \&\ z}$
UCG30	30.23 ± 1.11^{a}	16.62 ± 0.27^{a}	17.36 ± 0.41^{a}	28.15 ± 0.35^{ab}	7.61 ± 0.07^{a}
UCG60	28.52 ± 0.03^{a}	16.78 ± 0.11^{a}	17.63 ± 0.10^{a}	29.04 ± 0.24^{a}	7.99 ± 0.06^{a}
UCG90	29.87 ± 0.07^{a}	17.09 ± 0.10^{a}	17.78 ± 0.02^{a}	28.10 ± 0.04^{b}	7.14 ± 0.10^{b}
UC30	29.34 ± 0.02^{z}	16.79 ± 0.01^{z}	17.56 ± 0.02^{yz}	28.66 ± 0.01^{y}	7.62 ± 0.01^{y}
UC60	29.30 ± 0.08^{z}	16.99 ± 0.05^{z}	17.60 ± 0.02^{y}	28.44 ± 0.01^{yz}	7.49 ± 0.01^{y}
UC90	29.58 ± 0.18^{z}	16.40 ± 0.01^{z}	17.38 ± 0.02^{z}	28.56 ± 0.10^{y}	8.05 ± 0.07^{x}
UW	30.44 ± 0.23$^{a\ \&\ z}$	16.65 ± 0.12$^{a\ \&\ z}$	17.66 ± 0.09$^{a\ \&\ y}$	28.26 ± 0.04$^{ab\ \&\ z}$	6.96 ± 0.15$^{b\ \&\ z}$

weights of around 49, 34.5 and 23.5 kDa and were assigned to intact cruciferin subunits, cruciferin α-polypeptides and cruciferin β-polypeptides, respectively. The final three zones, with peaks eluting at approximately 14.62, 15.5 and 16.45 min, corresponded to the molecular weights of around 13, 8 and 5 kDa, and were assigned to intact napin, napin long polypeptides and napin short polypeptides, respectively. Regarding the oil-body proteins, the tiny shoulder peak eluted at around 14 min, corresponding to the molecular weight of ~ 16.5 kDa, could be attributed to oleosins, while the concentration of the rest of the oil-body proteins were possibly too low to produce visible peaks [29]. Since these proteins constitute only a minor fraction of canola protein isolates, they were not accounted for in the calculations of the percentage of protein fractions [43].

Table 2 presents the percentage of different protein fractions in the isolates, calculated based on the area under the peaks in each zone. The most prominent changes were observed in the zones corresponding to covalently bonded aggregates and denatured cruciferins, as well as intact cruciferin and napin. Increasing the water content in CG isolates resulted in a significant reduction in the percentage of extracted protein aggregates and denatured cruciferins, from 14.35 % in CG30 to 7.48 % in CG90. Simultaneously, the percentages of intact cruciferin and napin increased from 31.61 % to 34.32 % and 33.10 % to 36.80 %, respectively. This could be related to the increase in solvent polarity, which may reduce its tendency to solubilize larger, possibly more hydrophobic aggregates [59]. However, in water extracts, while the content of aggregates and denatured cruciferins further decreased to 5.36 %, the percentage of intact cruciferin increased to 43.44 % and the percentage of intact napin decreased to 29.20 %. This is an interesting observation, since napins are small, highly hydrophilic molecules and were expected to be present in water extracts in greater percentages compared to salt soluble cruciferins. Furthermore, application of ultrasound resulted in a noticeable increase in the percentage of aggregates and denatured cruciferins, and a reduction in the percentage of intact cruciferin and napin in UCG isolates compared to CG isolates. This could be linked to cavitation-induced effects, such as denaturation of the higher structure of protein due to localized high shear forces and temperatures, as well as promotion of covalent bonding between proteins or between proteins and non-protein meal components, caused by the production of free radicals [21]. Finally, the increase in the percentage of aggregates due to sonication was not observed in water + ultrasound extracts, which showed slightly lower percentages of aggregates and intact cruciferin, and a slightly higher content of intact napin compared to water extracts. This could be related to the inability of water to keep the unfolded and aggregated protein in a solubilized state, as opposed to NDESs or aqueous solutions.

On the other hand, C isolates exhibited an almost opposite trend compared to CG isolates, as increasing the water content led to a significant increase in the percentage of protein aggregates and denatured cruciferins, from 4.99 % in C30 to 8.24 % in C90. At the same time, while the percentage of intact cruciferins remained unchanged at around 28 %, the percentage of intact napin decreased from 40.00 % to 36.54 %. Furthermore, similar to CG vs UCG observations, sonication increased the content of aggregates and decreased the average content of intact cruciferin and napin in UC isolates compared to C isolates. Overall, it appeared that regardless of ultrasound application, C30 was unable to efficiently solubilize larger protein molecules, including aggregates and cruciferins, which possibly led to its notably lower extraction efficiency compared to CG30. This was improved to some extent by increasing the content of water to 60 %; however, C60 still offered a lower percentage of aggregates and denatured cruciferins compared to CG60. These findings highlighted the importance of the choice of HBD for protein extraction using NDESs once again and showed that glucose was considerably more effective in solubilizing the larger protein fractions compared to water. However, once the solvent transitioned into the aqueous solution zone at 90 % of water, the differences between the percentages of protein fractions in CG90 vs C90 and UCG90 vs UC90 became less pronounced, possibly due to the diminished impact of HBD on protein extraction.

3.4.2. *Protein secondary structure*

Fig. 4E-H shows the deconvoluted FT-IR spectra of the amide I region (1700–1600 cm^{-1}) of extracted protein isolates. Peaks in this region originate from the C=O stretching vibrations of the peptide backbone and are very sensitive to changes in the hydrogen bonding. Therefore, they can be used to evaluate alterations in the protein's secondary structure [60]. Herein, all spectra exhibited eight peaks corresponding to anti-parallel β-sheets at 1693 cm^{-1}, β-turns at 1679 and 1670 cm^{-1}, α-helix 1660 cm^{-1}, random coil at 1644 cm^{-1}, β-sheets at 1629 and 1617 cm^{-1} and amino acid residues at 1608 cm^{-1} [61,62]. Visually, apart from some minor differences in peak intensities, all spectra appeared roughly similar, with no noticeable shift in peak positions. This could indicate that the secondary structure of canola protein was not affected to a noticeable extent, neither by the difference in the employed solvents, nor by the application of ultrasound.

Further assessment via the analysis of second derivative and peak fitting revealed the composition of the protein secondary structure (Table 3), which was very similar across all protein isolates and measured at roughly 30 % β-sheet, 16.5 % random coil, 17.5 % α-helix, 28.5 % β-turns and 7.5 % anti-parallel β-sheets. The dominance of β structures in protein isolates could be related to their higher percentages of cruciferins, which have been shown to possess higher contents of β-sheets and β-turns, compared to napins and their predominantly α-helical structure [62]. Furthermore, since the overall contents of cruciferins (60–70 %) and napins (30–40 %) were also found to be close among the protein isolates, similarities between the percentages of protein secondary structure could be another indication of the limited effects of NDESs' water content, choice of HBD or application of

ultrasound, on the secondary structure of extracted canola protein. Lack of significant change in the secondary structure due to sonication was also observed in previous studies on soybean [63] and evening primrose cake [64] proteins. However, some other studies reported changes in the protein secondary structure caused by sonication, which was often characterized by reductions in α-helix and random coil contents and increases in β-sheet contents [44]. Overall, the effects of sonication on protein secondary structure is heavily influenced by the type of protein, sonication parameters and the environment in which the protein is exposed to sonication [65].

3.4.3. Protein tertiary structure

Fig. 4I-L presents the intrinsic fluorescence spectra of the extracted protein isolates. Information obtained from these spectra, such as emission peak and intensity, is indicative of the degree of exposure of aromatic amino acids (mainly tryptophan) to the hydrophilic environment and could be used to track changes in the tertiary structure of canola protein [66]. While the emission peaks of all spectra were close to each other and recorded at 330–332 nm, the main difference between the protein isolates was seen in their emission intensities. It was found that increasing the water content in CG and C solvents from 30 % to 90 %, and eventually to 100 %, resulted in an increase in the fluorescence intensity of canola protein, meaning that more tryptophan residues were exposed to the hydrophilic environment [62]. Previous studies by Li et al. [67] and Zeng et al. [68] showed that the DES clusters tend to surround the protein aggregates and solubilize them through the formation of various non-covalent interactions. The strength and flexibility of this cage-like structure are determined by the strength of the DES-DES network, which could be affected by water. Therefore, in the case of CG30 and C30, the rigid and less flexible structure of these solvents might have forced the extracted proteins to adopt a more compact state, resulting in the entrapment of tryptophan residues within the compressed structure. However, increasing the content of water to 60 %, 90 % and 100 %, and the corresponding reduction in solvent compactness (reflected by the reduction of viscosity and density) may have allowed the higher structure of protein to adopt a more relaxed state, leading to the exposure of more tryptophan residues.

A similar pattern was observed in the emission intensities of UCG, UC and water + ultrasound isolates, indicating that the aforementioned effects of solvent properties on protein tertiary structures occurred regardless of the application of ultrasound. However, the spectra of all sonicated isolates showed lower emission intensities compared to their non-sonicated counterparts, indicating a reduction of tryptophan exposure due to sonication. This could be related to either the degradation of tryptophan, or changes in protein aggregation and subsequent burial of tryptophan residues within the aggregates, both of which could have happened due to cavitation-induced effects [44].

3.4.4. Protein particle size distribution

The volume weighted particle size distribution graphs of all protein isolates (Fig. 5) demonstrated multimodal distributions, with one major peak in the range of 50–300 μm and several smaller peaks in the 0–10 μm range, and in some cases, between 300–500 μm. Analysis of the distribution parameters (Table 1) showed that increasing the water content in both CG and C solvents from 30 to 90 % led to an increase in the D[3,4] (volume-weighted particle mean diameter) of extracted protein isolates. This trend was also apparent in the 10th, 50th and 90th percentile values. Furthermore, the D[3,4] of water-extracted isolates was close to the values reported for CG90 and C90, with no significant difference between them. Protein particle size distribution is affected by various factors, including the protein conformation, the medium in which the protein molecules interact with each other, and the presence of other components in the medium [46,69]. Herein, a pattern similar to that observed in the intrinsic fluorescence spectra, was seen between the D[3,4] of CG and C isolates and the NDESs' water content. The stronger and more compact structure of DES at lower water contents during protein extraction may have affected the particle size distribution by either breaking down existing large aggregates or preventing further aggregation, likely through the formation of the cage-like structure around the proteins. This could explain the lower D[3,4] values observed at lower NDES water contents.

Furthermore, ultrasound treatment was found to substantially affect the distribution characteristics of the protein particles, with D[3,4] values narrowed down to a range of 83.06–89.46 μm for UCG and 69.24–87.56 μm for UC, compared to the much broader range of 63.68–196.40 μm for CG and 50.92–213.60 μm for C isolates. This was also apparent from the more uniform shape of UCG and UC distribution graphs, compared to the broader distributions observed in CG and C isolates. These observations are in accordance with previous reports [70]; however, regarding the effects of sonication on particle mean diameter, it was found that sonication increased the D[3,4] of UCG30, UCG60 and UC30, while reduced the D[3,4] of UC60 and, to a greater extent, UCG90 and UC90, compared to their non-sonicated counterparts (Table 1). These results could indicate that the impact of sonication on particle size may vary depending on the solvent's water content and the type of HBD. They also indicate that the effects of sonication on the alteration of particle distribution characteristics were much more pronounced when the solvent was in aqueous solution zone compared to NDES zone, suggesting that the presence of the nanostructure of NDES may dampened the effects of sonication. Finally, compared to non-sonicated water extracts, sonicated water extracts showed a higher D[3,4], as well as higher D10, D50 and D90 values. Overall, the interpretations provided in the last two sections lay the groundwork for future investigations, and further in-depth research is required to elucidate the effects of ultrasound, as well as the combined effects of NDESs and ultrasound, on protein structure and particle size distribution.

3.5. Protein functionality

Fig. 6 presents the aqueous solubility, as well as the emulsifying and foaming properties of selected isolates with the highest protein extraction efficiencies from each solvent group (CG60, UCG60, C90 and UC90). These functionalities were measured at three pH levels of 3, 5 and 7, since this range is applicable to the majority of food products.

3.5.1. Aqueous solubility

The highest percentages of protein aqueous solubility (Fig. 6A) for CG60 and C90 were observed at pH 3, with values of 80.89 % and 76.77 %, respectively. Increasing the pH to 5 resulted in a reduction of protein solubility to 50.83 % for CG60 and 39.85 % for C90, while further increasing the pH to 7 resulted in an almost similar solubility for CG60 and an increased solubility to 47.37 % for C90. Increased solubility of canola protein at acidic pH has also been observed in previous studies and has been linked to the positive charge of protein at this pH, as well as to pH-induced structural dissociation, especially in cruciferins [15,36]. Overall, while CG60 demonstrated a significantly higher solubility compared to C90 at pH 5, the differences between the two isolates were not significant at pH 3 and 7, and the average solubility of both isolates across the measured pH range was close (60.45 % for CG60 vs 54.66 % for C90). The aqueous solubility of canola protein isolates is mostly determined by the isolate's percentage of protein fractions, degree of protein denaturation and the content of phytic acid [36,46]. The analyses provided in the previous sections suggest a close resemblance between CG60 and C90 in all the mentioned factors, which could justify their close average aqueous solubility. It is worth mentioning that the beneficial effects of the reduced phytic acid content on protein aqueous solubility is further revealed when these results are compared to the results of our previous study, in which an isolate extracted via a similar NDES (choline chloride to glucose ratio of 1:2 + 25 % water) with phytic acid content of 58.36 mg/g showed an average solubility of 33.29 % across the pH range of 3–7, compared to the average solubility of CG60

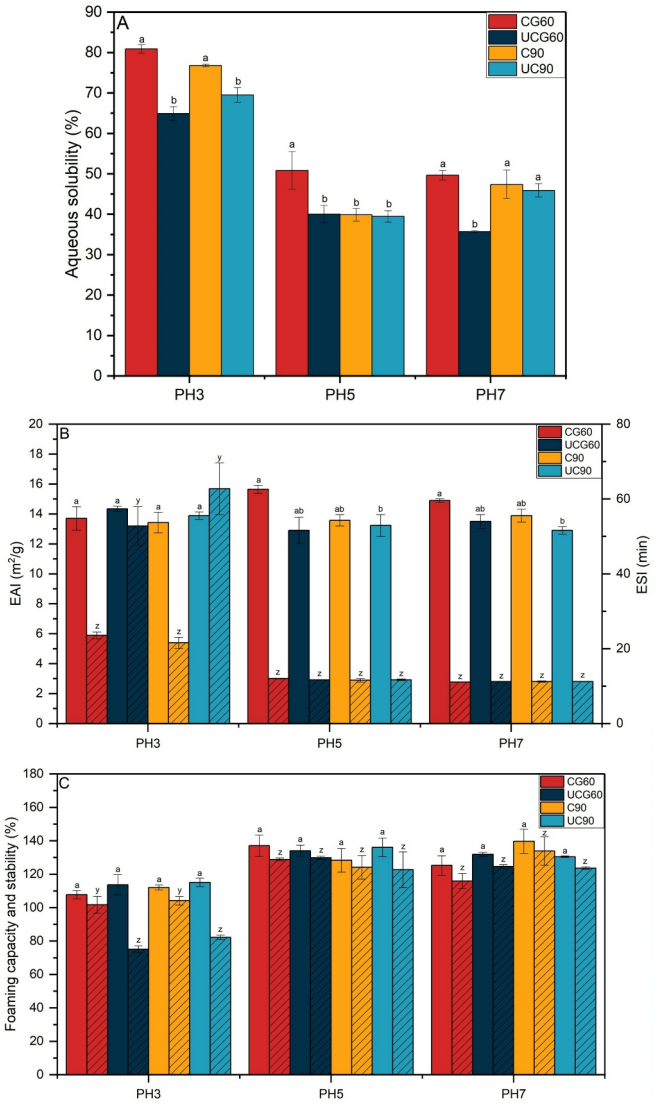


Fig. 6. Aqueous solubility (A), emulsifying properties (B), and foaming properties (C) of CG60, UCG60, C90 and UC90 isolates. In panel A, different letters on each bar indicate significant differences ($p < 0.05$) the isolates at each pH level. In panels B and C, plain bars represent EAI and foaming capacity, and striped bars represent ESI and foam stability. Different letters (a-b for EAI and foaming capacity, and y-z for ESI and foam stability) on each bar indicate significant differences ($p < 0.05$) between the isolates at each pH level.

at 60.45 % with phytic acid content of 26.94 mg/g [15].

On the other hand, ultrasound-treated isolates demonstrated lower percentages of aqueous solubility compared to non-sonicated isolates at all pH levels. For UCG60, a significant 10–15 % reduction was observed across all pH levels. For UC90, a significant reduction of 7.29 % was observed only at pH 3, while at pH 5 and 7, the solubility reduction was not significant, and the values were very close. Therefore, it appeared that the effects of ultrasound on the reduction of protein solubility were less pronounced in C isolates compared to CG isolates. Evaluation of protein structure in the previous section showed that sonication altered the protein tertiary structure and increased the content of covalently bonded aggregates and denatured cruciferins, which could explain the reduced aqueous solubility of ultrasound-treated isolates [21,23].

3.5.2. Emulsifying properties

The differences between the emulsifying properties of canola protein isolates (Fig. 6B) were especially stark at pH 3 compared to other pH levels. Specifically, while the EAIs of all protein isolates at pH 3 were in the range of 13.42–14.33 m^2/g with no significant differences between them, a substantial improvement was observed in the ESI of sonicated isolates (52.79 and 62.71 min for UCG60 and UC90, respectively) compared to non-sonicated isolates (23.55 and 21.54 min for CG60 and C90, respectively). These improvements, however, were not maintained when the pH of the medium was increased to 5 or 7. While the EAI of all isolates at these pH levels were recorded at 12.89–15.65 m^2/g, with CG60 and C90 showing slightly better EAIs compared to their sonicated counterparts, the ESI of all protein isolates fell within a narrow range of 11.06–12.02 min. Visually, apart from UCG60 and UC90 at pH 3, all the other emulsions developed a noticeable and well-separated creaming layer within a few minutes of preparation.

The emulsification mechanisms of canola protein isolates containing a mixture of cruciferins and napins have been explained in detail in our previous study [15]. In short, while napins quickly dominate the O/W interface, cruciferins tend to remain in the continuous phase, form non-covalent interactions with the napin layer and delay the creaming phenomenon by increasing the viscoelasticity of the continuous phase [71,72]. However, the stabilizing role of cruciferins is highly dependent on the environmental pH, as well as the extent of unfolding of their higher structure. Therefore, due to the improvement in solubility (also supported by aqueous solubility data) and dissociation of the hexameric structure of cruciferins at pH 3, these proteins were possibly more present and interactive in the continuous phase, which could explain the improved ESI of CG60 and C90 at pH 3, compared to other pH levels. Regarding UCG60 and UC90 isolates, the substantial structural changes due to cavitation-induced effects could have further improved the protein's molecular flexibility and amphiphilicity at pH 3, which might be the reason for their significantly higher ESI compared to their non-sonicated counterparts. Similar results were also observed when alkaline extracted canola protein isolate showed a much higher ESI compared to NDES extracted isolates at pH 3, but not pH 5 and 7, possibly due to higher degree of change in the cruciferin's secondary and tertiary structure due to alkaline denaturation [15]. Overall, while the EAI and ESI of CG60 vs C90 showed similar values and trends across different pH levels, the differences were much more pronounced when sonicated isolates were compared to non-sonicated ones.

3.5.3. Foaming properties

Similar to the emulsifying properties, the FC and FS of protein isolates (Fig. 6C) also showed the most noticeable differences at pH 3, compared to other pH levels. Specifically, while the FC of all protein isolates at this pH ranged from 107 to 115 % with no significant differences between them, a significant reduction in FS was observed in sonicated isolates (75.06 % and 82.22 % for UCG60 and UC90, respectively) compared to non-sonicated isolates (101.74 % and 104.13 % for CG60 and C90, respectively). At pH 5 and 7, however, the FC of all isolates was measured in the range of 125–139 % with no significant differences observed. These foams also showed high levels of stability, with all reductions in foam volume remaining below 14 % after 30 min.

Shen et al. [73] studied the air/water interfacial behavior and foaming properties of mixtures of cruciferin and napin and found that while both proteins co-adsorb and interact at the air/water interface, the FS and mechanical properties of the interface are mostly determined by cruciferins, since these proteins were able to create viscoelastic and stiff interfaces, as opposed to the weaker interface produced by napins. Furthermore, sonication was shown to alter the higher structure of cruciferins, which in turn could affect their interfacial adsorption behavior and their interactions with napin at the A/W interface. At pH 7 and 5, these changes alone were possibly not enough to produce significant differences between the FS of sonicated vs non-sonicated isolates. At pH 3, however, the combined effects of the pH-induced and sonication-induced unfolding of the cruciferins may be the reason for the reduction in FS observed in UCG60 and UC90 compared to CG60 and C90, respectively. Overall, the effects of sonication, as well as the combination of sonication and pH on the functional properties of canola

protein isolates need to be explored further through detailed studies, similar to those conducted by Ntone et al. [71,72] and Shen et al. [73].

4. Conclusions

This study provided a comprehensive evaluation of the effects of NDESs' water content, choice of HBD and application of ultrasound, on various features of extracted canola protein isolates, including extraction efficiency, protein and antinutritional content, color, and protein molecular, structural and functional properties. In the case of choline chloride-glucose-water solvents, it was observed that increasing the content of water to the NDES's upper hydration limit (60 %) not only maximized protein extraction efficiency but also improved protein content and reduced the level of extracted phenolic compounds and phytic acid. These findings could encourage researchers to employ DESs with high contents of water for protein extraction, which could not only improve the quality of extracted isolates but also substantially reduce the cost of DES production and potentially facilitate the recovery and reuse of their solid components. Furthermore, a combination of mild ultrasound treatment with choline chloride-glucose-water solvents was shown to significantly improve the extraction efficiency of canola protein and reduce the content of phenolic compounds and phytic acid, highlighting the benefits of this technology for extraction of high-quality canola protein.

Despite having lower viscosities and densities and comparable polarities, choline chloride-water NDESs (30 % and 60 % of water) exhibited considerably lower protein extraction efficiencies than choline chloride-glucose-water NDESs, even when ultrasound was applied. These observations underscored the importance of the choice of HBD and demonstrated that the presence of glucose as HBD, despite its perceived negative effects on the physical properties of NDESs, was crucial for maximizing the extraction efficiency of canola protein. However, an aqueous solution of choline chloride (90 % of water) also proved effective for extraction of canola protein isolates with high protein content and low levels of anti-nutritional components, especially when paired with ultrasound treatments.

A deeper look into the percentage of extracted protein fractions showed that while choline chloride-glucose-water solvents extracted lower contents of covalently bonded aggregates and denatured cruciferins as their water content increased, choline chloride-water solvents exhibited an opposite trend. This led to the consideration that the low extraction efficiency of choline chloride-water NDESs was possibly related to their inability to solubilize large, less soluble protein fractions. Furthermore, while no noticeable differences were observed in the secondary structure of protein, both the solvent water content and the application of ultrasound affected the protein's tertiary structure and particle size distribution. These observations suggested that the strength and flexibility of the network of NDES clusters surrounding the protein, which could be weakened by increasing the water content, might have influenced the protein's conformation and average particle size. In terms of functionality, while sonication reduced the aqueous solubility of proteins at all pH levels, it notably increased their emulsion stability and decreased their foam stability at pH 3, suggesting that the specific application of protein isolates should be considered before employing ultrasound treatment.

Overall, incorporation of high contents of water in the formulation of NDESs, proper selection of HBDs, and the use of ultrasound to assist protein extraction offered clear benefits for improving the extraction efficiency and quality of canola protein isolates. Future studies should focus on statistical optimizations aimed at identifying the best NDES and ultrasound parameters to maximize the protein extraction efficiency and functionality, and to minimize the content of anti-nutritional components. In addition, further elucidation of the effects of the nanostructure of NDESs and sonication on protein structure and functionality could be of great interest. Moreover, while incorporating higher contents of water in NDESs may substantially reduce solvent production costs and improve environmental sustainability, the recovery and reuse of the solid components of NDESs using novel technologies, such as bipolar membrane electrodialysis, should also be further explored.

CRediT authorship contribution statement

Abouzar Karimi: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Juwuan Choi:** Investigation, Formal analysis. **Anusha Samaranayaka:** Supervision, Funding acquisition. **Pankaj Bhowmik:** Supervision, Funding acquisition. **Lingyun Chen:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors are grateful to the Natural Sciences and Engineering Research Council of Canada (NSERC-Discovery) and National Research Council Canada (Collaborative R&D Initiative- HQP Grant). Professor Lingyun Chen would like to thank the Natural Sciences and Engineering Research Council of Canada (NSERC) -Canada Research Chairs Program for its financial support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2025.107618.

References

[1] X. Xu, et al., Global greenhouse gas emissions from animal-based foods are twice those of plant-based foods, Nat. Food 2 (9) (2021) 724–732, https://doi.org/10.1038/s43016-021-00358-x.
[2] S.R. Hertzler, J.C. Lieblein-Boff, M. Weiler, C. Allgeier, Plant proteins: Assessing their nutritional quality and effects on health and physical function, Nutrients 12 (12) (2020) 1–27, https://doi.org/10.3390/nu12123704.
[3] A. Gerzhova, M. Mondor, M. Benali, M. Aider, Study of total dry matter and protein extraction from canola meal as affected by the pH, salt addition and use of zeta-potential/turbidimetry analysis to optimize the extraction conditions, Food Chem. 201 (2016) 243–252, https://doi.org/10.1016/j.foodchem.2016.01.074.
[4] A. Chmielewska, et al., Canola/rapeseed protein–nutritional value, functionality and food application: a review, Crit. Rev. Food Sci. Nutr. 61 (22) (2021) 3836–3856, https://doi.org/10.1080/10408398.2020.1809342.
[5] D.S. Freitas, A. Cavaco-Paulo, C. Silva, Enhancing insights into the phenomena of deep eutectic solvents, Sustain. Mater. Technol. 41 (June) (2024) e01039, https://doi.org/10.1016/j.susmat.2024.e01039.
[6] Y. Zhou, W. Wu, N. Zhang, O. P. Soladoye, Y. Zhang, and Y. Fu, "Deep eutectic solvents as new media for green extraction of food proteins: Opportunity and challenges," Food Res. Int., 161(August) (2022), doi: 10.1016/j.foodres.2022.111842.
[7] S. He, et al., Deep eutectic solvents as extraction media for food-derived biomacromolecules, TrAC - Trends Anal. Chem. 171 (January) (2024) 117521, https://doi.org/10.1016/j.trac.2024.117521.
[8] Y. Dai, J. van Spronsen, G.J. Witkamp, R. Verpoorte, Y.H. Choi, Natural deep eutectic solvents as new potential media for green technology, Anal. Chim. Acta 766 (2013) 61–68, https://doi.org/10.1016/j.aca.2012.12.019.
[9] F.S. Bragagnolo, M.M. Strieder, R.S. Pizani, L.M. de Souza Mesquita, M. González-Miquel, M.A. Rostagno, "Revisiting natural deep eutectic solvents (NADES) as extraction media and ready-to-use purposes, TrAC - Trends Anal. Chem. 175 (December) (2024) 2023, https://doi.org/10.1016/j.trac.2024.117726.
[10] B.A. Pereira, C.T. Matos, L. Costa, L.M. Ferreira, C. Brazinha, Sustainable processing of microalgae protein : design of biphasic partitioning systems based on natural deep eutectic solvents for C-phycocyanin recovery from model aqueous solutions, Sep. Purif. Technol. 353 (June) (2024) 2025, https://doi.org/10.1016/j.seppur.2024.128510.
[11] A. Hewage, O.O. Olatunde, C. Nimalaratne, J.D. House, R.E. Aluko, N. Bandara, Improved protein extraction technology using deep eutectic solvent system for producing high purity fava bean protein isolates at mild conditions, Food

Hydrocoll. 147 (PA) (2024) 109283, https://doi.org/10.1016/j.foodhyd.2023.109283.
[12] X. Jiang, F. Gao, Y. Ma, N. Huo, Y. Guo, Y. Yu, Protein from tiger nut meal extracted by deep eutectic solvent and alkali-soluble acid precipitation: a comparative study on structure, function, and nutrition, Food Chem. 452 (5333) (2024) 139608, https://doi.org/10.1016/j.foodchem.2024.139608.
[13] P. Cao, et al., Extraction of protein from sesame meal : Impact of deep eutectic solvents on protein structure and functionality, LWT 187 (100) (2023) 115366, https://doi.org/10.1016/j.lwt.2023.115366.
[14] J. Yue, Z. Zhu, J. Yi, Y. Lan, B. Chen, J. Rao, Structure and functionality of oat protein extracted by choline chloride–dihydric alcohol deep eutectic solvent and its water binary mixtures, Food Hydrocoll. 112 (September) (2020) 2021, https://doi.org/10.1016/j.foodhyd.2020.106330.
[15] A. Karimi, P. Bhowmik, T.C. Yang, A. Samaranayaka, L. Chen, Extraction of canola protein via natural deep eutectic solvents compared to alkaline treatments: Isolate characteristics and protein structural and functional properties, Food Hydrocoll. 152 (2024) 109922, https://doi.org/10.1016/j.foodhyd.2024.109922.
[16] Q.Q. Koh et al., Corrigendum to 'Sugar-based natural deep eutectic solvent (NADES): Physicochemical properties, antimicrobial activity, toxicity, biodegradability and potential use as green extraction media for phytonutrients' [Sustain. Chem. Pharm. 35 (2023) 101218](S235," Sustain. Chem. Pharm., vol. 37, no. July, 2024, doi: 10.1016/j.scp.2023.101342.
[17] I.D. Boateng, Evaluating the status quo of deep eutectic solvent in food chemistry. Potentials and limitations, Food Chem. 406 (June 2022) (2023) 135079, https://doi.org/10.1016/j.foodchem.2022.135079.
[18] H. Zhang, M.L. Ferrer, M.J. Roldán-Ruiz, R.J. Jiménez-Riobóo, M.C. Gutiérrez, F. Del Monte, Brillouin spectroscopy as a suitable technique for the determination of the eutectic composition in mixtures of choline chloride and water, J. Phys. Chem. B 124 (19) (2020) 4002–4009, https://doi.org/10.1021/acs.jpcb.0c01919.
[19] A.I.M.C. Lobo Ferreira, et al., Extensive characterization of choline chloride and its solid-liquid equilibrium with water, Phys. Chem. Chem. Phys. 24 (24) (2022) 14886–14897, https://doi.org/10.1039/d2cp00377e.
[20] E. Mangiacapre, F. Castiglione, M. D'Aristotile, V. Di Lisio, A. Triolo, and O. Russina, "Choline chloride-water mixtures as new generation of green solvents: A comprehensive physico-chemical study," J. Mol. Liq., vol. 383, no. March, p. 122120, 2023, doi: 10.1016/j.molliq.2023.122120.
[21] M.M. Rahman, B.P. Lamsal, Ultrasound-assisted extraction and modification of plant-based proteins: Impact on physicochemical, functional, and nutritional properties, Compr. Rev. Food Sci. Food Saf. 20 (2) (2021) 1457–1480, https://doi.org/10.1111/1541-4337.12709.
[22] F. Aghababaei, D.J. McClements, M. Hadidi, Ultrasound processing for enhanced digestibility of plant proteins, Food Hydrocoll. 155 (February) (2024) 110188, https://doi.org/10.1016/j.foodhyd.2024.110188.
[23] K. Jahan, A. Ashfaq, K. Younis, O. Yousuf, R.U. Islam, A review of the effects of ultrasound-assisted extraction factors on plant protein yield and functional properties, J. Food Meas. Charact. 16 (4) (2022) 2875–2883, https://doi.org/10.1007/s11694-022-01390-6.
[24] S. Olivares-galv, M. Luisa, and E. Hern, "Approaching the extraction of proteins from brewing wastes using deep eutectic solvents," vol. 189, no. October, 2023, doi: 10.1016/j.lwt.2023.115470.
[25] A. Sánchez-Elvira, E. Hernández-Corroto, M. C. García, M. Castro-Puyana, and M. L. Marina, "Sustainable extraction of proteins from lime peels using ultrasound, deep eutectic solvents, and pressurized liquids, as a source of bioactive peptides," *Food Chem.*, vol. 458, no. February, 2024, doi: 10.1016/j.foodchem.2024.140139.
[26] R.L. Liu, P. Yu, X.L. Ge, X.F. Bai, X.Q. Li, Q. Fu, Establishment of an aqueous PEG 200-based deep eutectic solvent extraction and enrichment method for pumpkin (Cucurbita moschata) seed protein, Food Anal. Methods 10 (6) (2017) 1669–1680, https://doi.org/10.1007/s12161-016-0732-y.
[27] M. Guzmán-Lorite, M.L. Marina, M.C. García, Successive extraction using natural deep eutectic solvents and pressurized liquids for a greener and holistic recovery of proteins from pomegranate seeds, Food Res. Int., vol. 161, no. July, 2022, doi: 10.1016/j.foodres.2022.111862.
[28] S. Bleibach Alpiger, M. Corredig, Pectin polysaccharide contribution to oleosome extraction after wet milling of rapeseed, Food Res. Int. 175 (September 2023) (2024), https://doi.org/10.1016/j.foodres.2023.113736.
[29] M.J. Romero-Guzmán, L. Jung, K. Kyriakopoulou, R.M. Boom, C.V. Nikiforidis, Efficient single-step rapeseed oleosome extraction using twin-screw press, J. Food Eng. 276 (2020), https://doi.org/10.1016/j.jfoodeng.2019.109890.
[30] A. Szydłowska-Czerniak, I. Bartkowiak-Broda, I. Karlović, G. Karlovits, E. Szłyk, Antioxidant capacity, total phenolics, glucosinolates and colour parameters of rapeseed cultivars, Food Chem. 127 (2) (2011) 556–563, https://doi.org/10.1016/j.foodchem.2011.01.040.
[31] A. Akbari, J. Wu, An integrated method of isolating napin and cruciferin from defatted canola meal, Lwt 64 (1) (2015) 308–315, https://doi.org/10.1016/j.lwt.2015.05.046.
[32] J. Jezek, B.G.D. Haggett, A. Atkinson, D.M. Rawson, Determination of glucosinolates using their alkaline degradation and reaction with ferricyanide, J. Agric. Food Chem. 47 (11) (1999) 4669–4674, https://doi.org/10.1021/jf9906026.
[33] C. Defaix, et al., A new size-exclusion chromatography method for fast rapeseed albumin and globulin quantification, Food Chem. 287 (January) (2019) 151–159, https://doi.org/10.1016/j.foodchem.2019.01.209.
[34] J. Yang, G. Liu, H. Zeng, L. Chen, Effects of high pressure homogenization on faba bean protein aggregation in relation to solubility and interfacial properties, Food Hydrocoll. 83 (February) (2018) 275–286, https://doi.org/10.1016/j.foodhyd.2018.05.020.
[35] K.N. Pearce, J.E. Kinsella, Emulsifying properties of proteins: evaluation of a turbidimetric technique, J. Agric. Food Chem. 26 (3) (May 1978) 716–723, https://doi.org/10.1021/jf60217a041.
[36] L. Cheung, J. Wanasundara, M.T. Nickerson, The effect of pH and NaCl levels on the physicochemical and emulsifying properties of a Cruciferin protein isolate, Food Biophys. 9 (2) (2014) 105–113, https://doi.org/10.1007/s11483-013-9323-2.
[37] Z. Zhang, et al., Effect of pH regulation on the components and functional properties of proteins isolated from cold-pressed rapeseed meal through alkaline extraction and acid precipitation, Food Chem. 327 (May) (2020) 126998, https://doi.org/10.1016/j.foodchem.2020.126998.
[38] J. K. U. Ling and K. Hadinoto, "Deep Eutectic Solvent as Green Solvent in Extraction of Biological Macromolecules: A Review," *Int. J. Mol. Sci.*, vol. 23, no. 6, 2022, doi: 10.3390/ijms23063381.
[39] Y. Dai, G.J. Witkamp, R. Verpoorte, Y.H. Choi, Tailoring properties of natural deep eutectic solvents with water to facilitate their applications, Food Chem. 187 (2015) 14–19, https://doi.org/10.1016/j.foodchem.2015.03.123.
[40] S.S. Sakpal, S.H. Deshmukh, S. Chatterjee, D. Ghosh, S. Bagchi, Transition of a deep eutectic solution to aqueous solution: a dynamical perspective of the dissolved solute, J. Phys. Chem. Lett. 12 (36) (2021) 8784–8789, https://doi.org/10.1021/acs.jpclett.1c02118.
[41] O.S. Hammond, D.T. Bowron, K.J. Edler, The effect of water upon deep eutectic solvent nanostructure: an unusual transition from ionic mixture to aqueous solution, Angew. Chemie - Int. Ed. 56 (33) (2017) 9782–9785, https://doi.org/10.1002/anie.201702486.
[42] M.T. Zafarani-Moattar, H. Shekaari, F. Ghaffari, Vapor-liquid equilibria study of the aqueous systems containing choline chloride + glucose or urea and their deep eutectic solvents at 298.15 K and 85 kPa, J. Chem. Eng. Data 64 (11) (2019) 4754–4762, https://doi.org/10.1021/acs.jced.9b00076.
[43] A. Fetzer, T. Herfellner, A. Stäbler, M. Menner, P. Eisner, Influence of process conditions during aqueous protein extraction upon yield from pre-pressed and cold-pressed rapeseed press cake, Ind. Crops Prod. 112 (August 2017) (2018) 236–246, https://doi.org/10.1016/j.indcrop.2017.12.011.
[44] A.S. Sengar, et al., Application of power ultrasound for plant protein extraction, modification and allergen reduction – a review, Appl. Food Res. 2 (2) (2022) 100219, https://doi.org/10.1016/j.afres.2022.100219.
[45] X.Y. Dong, et al., Some characteristics and functional properties of rapeseed protein prepared by ultrasonication, ultrafiltration and isoelectric precipitation, J. Sci. Food Agric. 91 (8) (2011) 1488–1498, https://doi.org/10.1002/jsfa.4339.
[46] R. Wang, S. Guo, Phytic acid and its interactions: contributions to protein functionality, food processing, and safety, Compr. Rev. Food Sci. Food Saf. 20 (2) (2021) 2081–2105, https://doi.org/10.1111/1541-4337.12714.
[47] E. Kaspchak, L.I. Mafra, M.R. Mafra, Effect of heating and ionic strength on the interaction of bovine serum albumin and the antinutrients tannic and phytic acids, and its influence on in vitro protein digestibility, Food Chem. 252 (January) (2018) 1–8, https://doi.org/10.1016/j.foodchem.2018.01.089.
[48] S. Sarkhel, A. Roy, Phytic acid and its reduction in pulse matrix: Structure–function relationship owing to bioavailability enhancement of micronutrients, J. Food Process Eng. 45 (5) (2022) 1–21, https://doi.org/10.1111/jfpe.14030.
[49] J. S. V. P. Thinh Nguyen, "Glucosinolates: Natural Occurrence, Biosynthesis, Accessibility, Isolation, Structures, and Biological Activities," 2020.
[50] W. Lu, S. Liu, Choline chloride–based deep eutectic solvents (Ch-DESs) as promising green solvents for phenolic compounds extraction from bioresources: state-of-the-art, prospects, and challenges, Biomass Convers. Biorefinery 12 (7) (2022) 2949–2962, https://doi.org/10.1007/s13399-020-00753-7.
[51] Z. Liu, T. Sun, J. Liu, N. Liang, L. Zhao, Natural deep eutectic solvent as a green extraction medium for the ultrasonic-assisted extraction of sinapine thiocyanate from black mustard seeds, Microchem. J. 201 (April) (2024) 110549, https://doi.org/10.1016/j.microc.2024.110549.
[52] I.M. Yusoff, Z. Mat Taher, Z. Rahmat, L.S. Chua, A review of ultrasound-assisted extraction for plant bioactive compounds: Phenolics, flavonoids, thymols, saponins and proteins, Food Res. Int. 157 (February) (2022) 111268, https://doi.org/10.1016/j.foodres.2022.111268.
[53] J. Pan *et al.*, "Ultrasound treatment modified the functional mode of gallic acid on properties of fish myofibrillar protein," Food Chem., vol. 320, no. March, 2020, doi: 10.1016/j.foodchem.2020.126637.
[54] L. Jakobek, Interactions of polyphenols with carbohydrates, lipids and proteins, Food Chem. 175 (2015) 556–567, https://doi.org/10.1016/j.foodchem.2014.12.013.
[55] S. Ebert, M. Gibis, N. Terjung, J. Weiss, Survey of aqueous solubility, appearance, and pH of plant protein powders from carbohydrate and vegetable oil production, Lwt 133 (August) (2020) 110078, https://doi.org/10.1016/j.lwt.2020.110078.
[56] J. P. D. Wanasundara, S. Tan, A. M. Alashi, F. Pudel, and C. Blanchard, *Proteins From Canola/Rapeseed: Current Status*. Elsevier Inc., 2017. doi: 10.1016/B978-0-12-802778-3.00018-4.
[57] J.P.D. Wanasundara, T.C. McIntosh, S.P. Perera, T.S. Withana-Gamage, P. Mitra, Canola/rapeseed protein-functionality and nutrition, OCL 23 (4) (2016) D407, https://doi.org/10.1051/ocl/2016028.
[58] S. Tayyab, S. Qamar, M. Islam, Size exclusion chromatography and size exclusion HPLC of proteins, Biochem. Educ. 19 (3) (1991) 149–152, https://doi.org/10.1016/0307-4412(91)90060-L.
[59] M. Vilková, J. Płotka-Wasylka, V. Andruch, The role of water in deep eutectic solvent-base extraction, J. Mol. Liq. 304 (2020), https://doi.org/10.1016/j.molliq.2020.112747.
[60] A. Gerzhova, M. Mondor, M. Benali, M. Aider, A comparative study between the electro-activation technique and conventional extraction method on the

extractability, composition and physicochemical properties of canola protein concentrates and isolates, Food Biosci. 11 (2015) 56–71, https://doi.org/10.1016/j.fbio.2015.04.005.
[61] Q. Li, et al., Physical stability and microstructure of rapeseed protein isolate/gum Arabic stabilized emulsions at alkaline pH, Food Hydrocoll. 88 (July 2018) (2019) 50–57, https://doi.org/10.1016/j.foodhyd.2018.09.020.
[62] S.P. Perera, T.C. McIntosh, J.P.D. Wanasundara, Structural properties of cruciferin and napin of Brassica napus (canola) show distinct responses to changes in pH and temperature, Plants 5 (3) (2016) 64–74, https://doi.org/10.3390/plants5030036.
[63] H. Hu, I.W.Y. Cheung, S. Pan, E.C.Y. Li-Chan, Effect of high intensity ultrasound on physicochemical and functional properties of aggregated soybean β-conglycinin and glycinin, Food Hydrocoll. 45 (2015) 102–110, https://doi.org/10.1016/j.foodhyd.2014.11.004.
[64] M. Hadidi, A. Ibarz, S. Pouramin, Optimization of extraction and deamidation of edible protein from evening primrose (Oenothera biennis L.) oil processing by-products and its effect on structural and techno-functional properties, Food Chem. 334 (July 2020) (2021), https://doi.org/10.1016/j.foodchem.2020.127613.
[65] B. P. Lamsal, "Ultrasound-assisted extraction and modification of plant-based proteins : Impact on physicochemical , functional , and nutritional properties," no. July 2020, pp. 1457–1480, 2021, doi: 10.1111/1541-4337.12709.
[66] N. Zhang, Z. Xiong, W. Xue, R. He, X. Ju, Z. Wang, Insights into the effects of dynamic high-pressure microfluidization on the structural and rheological properties of rapeseed protein isolate, Innov. Food Sci. Emerg. Technol. 80 (July) (2022) 103091, https://doi.org/10.1016/j.ifset.2022.103091.
[67] N. Li, Y. Wang, K. Xu, Y. Huang, Q. Wen, X. Ding, Development of green betaine-based deep eutectic solvent aqueous two-phase system for the extraction of protein, Talanta 152 (2016) 23–32, https://doi.org/10.1016/j.talanta.2016.01.042.
[68] Q. Zeng, Y. Wang, Y. Huang, X. Ding, J. Chen, K. Xu, Deep eutectic solvents as novel extraction media for protein partitioning, Analyst 139 (10) (2014) 2565–2573, https://doi.org/10.1039/c3an02235h.
[69] S. Amin, G.V. Barnett, J.A. Pathak, C.J. Roberts, P.S. Sarangapani, Protein aggregation, particle formation, characterization & rheology, Curr. Opin. Colloid Interface Sci. 19 (5) (2014) 438–449, https://doi.org/10.1016/j.cocis.2014.10.002.
[70] A.R. Jambrak, T.J. Mason, V. Lelas, L. Paniwnyk, Z. Herceg, Effect of ultrasound treatment on particle size and molecular weight of whey proteins, J. Food Eng. 121 (1) (2014) 15–23, https://doi.org/10.1016/j.jfoodeng.2013.08.012.
[71] E. Ntone, et al., Sinapic acid impacts the emulsifying properties of rapeseed proteins at acidic pH, Food Hydrocoll. 125 (November 2021) (2022), https://doi.org/10.1016/j.foodhyd.2021.107423.
[72] E. Ntone, T. van Wesel, L.M.C. Sagis, M. Meinders, J.H. Bitter, C.V. Nikiforidis, Adsorption of rapeseed proteins at oil/water interfaces. Janus-like napins dominate the interface, J. Colloid Interface Sci. 583 (2021) 459–469, https://doi.org/10.1016/j.jcis.2020.09.039.
[73] P. Shen, J. Yang, C.V. Nikiforidis, H.C.M. Mocking-Bode, L.M.C. Sagis, Cruciferin versus napin – Air-water interface and foam stabilizing properties of rapeseed storage proteins, Food Hydrocoll. 136 (PA) (2023) 108300, https://doi.org/10.1016/j.foodhyd.2022.108300.