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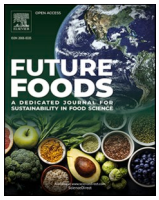

Defatting strategies for chia protein production: effects on physicochemical properties

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ABSTRACT

The growing demand for sustainable plant-based proteins has highlighted chia seeds (Salvia hispanica L.), offering 18–32 % protein content. This study evaluates protein extraction efficiency and functional properties across two types of chia product: organic partially defatted flour (O) and conventional whole full-fat seeds (C). Defatting methods included mechanical cold pressing (O_PD), hexane extraction (O_HEX, C_HEX), supercritical CO_2 with ethanol (C_SCF), and a non-defatted control (C_ND). Hexane extraction achieved the highest protein content (O_HEX: 67.1 %; C_HEX: 63.9 %) and foaming stability (76.85 %, 75.00 %), with minimal residual fat (0.10–0.40 %). Supercritical CO_2 yielded the highest protein recovery (44.6 %) and emulsion capacity (203.17 %), while O_PD and C_ND showed superior solubility at neutral pH. Functional properties varied significantly: O_HEX exhibited the highest water-holding capacity (794.86 %), and C_HEX demonstrated peak oil-binding capacity (473.92 %). These findings shows that both seed types and defatting method critically influence protein yield and functionality. While hexane extraction maximizes efficiency, its environmental drawbacks position supercritical CO_2 as an eco-friendly alternative, despite solubility trade-offs. This study provides actionable insights for optimizing plant-based protein ingredients by balancing extraction efficiency, functional performance, and sustainability goals across chia seed sources and processing technologies.

1. Introduction

The interest in plant-based proteins is growing due to their nutritional benefits, sustainability, and potential applications in the food and pharmaceutical industries. Chia seeds (*Salvia hispanica* L.) have proven to be a promising source of plant-based protein, known for their high protein content and functional properties. Chia seeds contain 18–27 % protein, with some sources even reporting protein levels as high as 32 % (Ferreira et al., 2023), which is significantly higher than other grains such as wheat, corn, or rice. Optimizing protein extraction methods is essential to maximize yield and preserve the functional properties that are critical for industrial applications. The functional properties of chia proteins include solubility, water- and oil-binding capacity (WHC/OBC), foaming ability and stability, emulsifying properties, making them versatile ingredients for various food applications. Traditional defatting techniques often rely on solvent-based methods such as hexane extraction; however, these methods have limitations, including environmental concerns and potential effects on protein quality. Cold pressing is an alternative method that effectively removes most of the oil by mechanically pressing the seeds, resulting in a fat content of approximately 7 % in the chia cake compared to 33 % in the seeds (Ferreira et al., 2023). Other extraction methods include supercritical carbon dioxide (SCF) extraction. SCF is an environmentally friendly method that serves as an alternative to organic solvent extraction, allowing oil separation while preserving valuable components (Ishak et al., 2021; Xu et al., 2011). Studies have shown that oil yield can be increased by raising pressure from 220 to 340 bar, with optimal yields achieved at a pressure of 335 bar (Ishak et al., 2021). Despite these advancements, there are gaps in understanding how different defatting techniques impact protein yield, purity, and functional properties such as solubility and emulsifying capacity (Ixtaina et al., 2011). Additionally, few studies have examined the application of chia seed proteins in food systems(Chen and Luo, 2024) most research focuses on chia oil rather than proteins (Chen and Luo, 2024; Khushairay et al., 2023). There is also a lack of

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comprehensive evaluations comparing traditional and alternative defatting methods in terms of efficiency, environmental impact, and industrial scalability (Fernandes et al., 2021; Ishak et al., 2021). Research should focus on optimizing chia seed protein extraction methods to achieve higher yields without compromising quality (Ishak et al., 2021; Ixtaina et al., 2011). For example, proteins isolated through alkaline extraction at pH 12.0 have been reported to achieve a protein yield of 19.10 % with a protein content of 74.53 %(Khushairay et al., 2023), while also yielding the highest levels of essential amino acids (36.87 %), hydrophobic amino acids (33.81 %), and aromatic amino acids (15.54 %) (Khushairay et al., 2023). The research contributes to closing critical gaps in the literature by providing insights into effective and sustainable defatting techniques while identifying optimal conditions for SCF (Ishak et al., 2021). The findings will also have practical implications for industries developing plant-based products with high protein content and improved functional properties. For instance, by-products such as chia cake rich in minerals (6 %), proteins (27 %), and fibers (48 %) can be utilized effectively (Ferreira et al., 2023). Additionally, extracted proteins can serve as emulsifiers or gelling agents in meat product formulations like sausages or meatballs, demonstrating their versatility in food applications (Chen and Luo, 2024).

This study aims to evaluate and compare various defatting methods such as mechanical defatting via cold pressing, solvent extraction with n-hexane, and Supercritical Fluid Extraction using CO_2 as the main solvent and ethanol as a co-solvent and analyze their effects on protein extraction efficiency from chia seeds. Furthermore, the study hypothesized that defatting methods not only influence protein extraction efficiency but also alter the structural and functional characteristics of chia proteins, with solvent-based methods expected to induce greater conformational changes due to stronger lipid removal capacity.

2. MATERIAL AND methods

2.1. Chemicals and materials

All the chemicals used were of analytical grade unless stated otherwise. Water used in all experiments was distilled water with a conductivity <2 μS. The plant material, consisting of Chia (*Salvia hispanica L.*), was purchased from two suppliers: **o**rganic partially defatted chia flour (Ölmühle Solling, Germany) and **c**onventionally farmed whole chia seeds (Yayla, Turkey)

2.2. Proximate Analysis of chia seeds

As a starting point for fat and protein extraction, this study utilized two types of chia seed raw materials: conventional whole chia seeds from Yayla, Turkey, and organic, partially defatted chia flour from Ölmühle Solling, Germany. The general composition of these raw materials according to the supplier's statements is provided in Table 1, highlighting notable differences in fat and protein content between the two sources. For all extraction procedures, the initial fat and protein concentrations of the starting materials were carefully considered, as these parameters significantly influence both the efficiency of subsequent defatting and the yield and functionality of the extracted protein fractions. This approach enabled a systematic comparison of how different defatting methods (mechanical pressing, hexane extraction, and supercritical CO_2 extraction) affect protein yield and functional properties.

Table 1
Proximate Analysis of Different Raw Chia Seeds.

Raw Material	Fat [%]	Carbohydrates [%]	Protein [%]	Fiber [%]	Dry Matter [%]
Organic Chia Flour (O)	6.9	4.5	28.3	44.5	91.4
Conventional Chia Seeds (C)	31.4	1.6	23.2	31.9	92.8

The two raw materials resulted into five samples used for protein extraction:

O_PD: Organic partial defatted chia flour, mechanically defatted
O_HEX: Organic partial defatted chia flour further defatted using n-hexane as solvent
C_ND: Conventionally farmed whole chia seeds, ground not defatted
C_HEX: Conventionally farmed whole chia seeds, ground and defatted using n-hexane
C_SCF: Conventionally farmed whole chia seeds, ground and defatted using Super Critical Fluid extraction

2.3. Defatting Methods

2.3.1. Conventional Defatting method (HEX)

For defatting n-hexane was used in a ratio of 1:10 (w/v) at 40 °C and stirred at 500 rpm for 3 h before filtering it through a 16 μm filter. This step was repeated up to a total of three defatting cycles to ensure a high level of defatting and until the n-hexane was clear (Olivos-Lugo et al., 2010).

2.3.2. Partial Defatting method (PD)

Partial defatting was performed by the supplier (Ölmühle Solling, Germany) using a twin screw press. However, the manufacturer, model, and set parameters of the press are not disclosed due to the supplier's internal company policies. This process reduced the initial fat content from 31.4 % in the raw seeds to 6.9 % in the partially defatted flour.

2.3.3. Defatting by supercritical CO_2 (SCF) method

Before extraction, chia seeds (Yayla, Turkey) were ground using a coffee grinder to reduce particle size and increase surface area to improve mass transfer efficiency. Approximately 50 g of seeds were ground for 10 s until the majority of the particles passed through a 0.5 mm sieve. During supercritical CO_2 extraction, 20 g of ground chia seeds were placed in the extraction column of a 500 mL capacity laboratory-scale SFE device. 20 mL of 100 % ethanol was used as a co-solvent to increase extraction efficiency. The extraction was carried out at 300 bar pressure and 50 °C temperature, and the process time was determined as 300 min. However, when the pre-process pressure increases and post-process pressure decrease stages were included, the total process time was approximately 7.5 h (Ishak et al., 2021).

2.4. Protein Extraction

Chia flour obtained from the different defatting methods was mixed with distilled water at a ratio of 3 % (w/w) and homogenized for 90 s at 21,000 rpm using an Ultra-Turrax. The mixture was hydrated for 24 h at 21 °C, followed by heat treatment for 1 hour at 60 °C at its natural pH (around pH 8) on a heatable magnetic stirrer. The solution was stirred for 2 h at 21 °C (pH 10) and centrifuged at 4750 × *g* for 20 min at 4 °C. The supernatant was adjusted to pH 4 for C samples and pH 3.75 for O samples, stirred for 30 min, and centrifuged again under the same conditions. The sediment was neutralized to pH 7 by stirring for 30 min at 21 °C before freeze drying.

2.5. Characterization of chia protein samples

2.5.1. Total Nitrogen content

Total nitrogen content was determined using the Kjeldahl method (Dirr & Özmutlu, 2024). Approximately 0.1 g of the sample was weighed using nitrogen-free weighing boats (Cytiva, Marlborough, MA, USA) and

transferred into a Kjeldahl digestion tube. Then, 20 mL of 98 % sulfuric acid and one Missouri Kjeldahl catalyst tablet (Merck KGaA, Germany) were added. Samples were digested at 400 °C for approximately 3 h using a Kjeldahtherm and Turbosog apparatus (C. Gerhard GmbH & Co. KG, Germany). After digestion, the tubes were cooled to 60 °C, diluted with 100 mL distilled water, and steam-distilled with 80 mL of 33 % NaOH solution for 8 min at 100 °C using a Vapodest system (C. Gerhard GmbH & Co. KG, Germany). The distillate was collected in 4 % boric acid solution and titrated back to the initial pH with 0.1 M HCl using an automatic titrator (Titroline 500, SI Analytics, Germany). Protein content was calculated by using the following Eq. (1) with a standard protein factor of 6.25.

$$P(\%) = \frac{C_{eq} * (V - V_{BL}) * M * F * 100\%}{m_{sample} * DM} \quad (1)$$

where C_{eq} is the normality of the titration solution in mol/L, V is the volume of the titration solution consumed for the sample in liters, V_{BL} is the volume of the titration solution consumed for the blank in liters, M is the molar mass of nitrogen in grams per mole, m_{sample} is the initial weight of the sample in grams, F is the protein factor (6,25) and DM is the dry matter percentage of the sample.

2.5.2. Total Fat content

The Soxhlet extraction was performed using n-hexane as a solvent. 20 g of ground chia seed was introduced into a cellulose tube and 200 mL of solvent was added into the extraction flask. Extraction was carried out in cycles for approximately 3 h at the boiling point of the solvent. Afterwards the solvent was evaporated, and yield was calculated.

2.5.3. Protein Yield

Protein yield was calculated to assess the efficiency of the extraction process (Nynäs et al., 2021). The protein yield was calculated by using Eq. (2).

$$PY(\%) = \frac{m_{sample,out} * P_{out} * DM_{out}}{m_{sample,in} * P_{in} * DM_{in}} \quad (2)$$

$m_{sample,out}$ refers to the mass (g) of the final extracted protein concentrate, while P_{out} and DM_{out} denote its protein content (%) and dry matter content (%), respectively. $m_{sample,in}$ represents the initial mass (g) of the chia-based raw material subjected to extraction, with while P_{in} and DM_{in} corresponding to its initial protein and dry matter content.

2.5.4. Soluble Protein content and isoelectric point of chia protein

Samples (0.4 g each) were dispersed in 40 mL water (1 % w/v) within 50 mL centrifuge tubes, hydrated by shaking at 130 rpm and 25 °C for 24 h. The dispersions were adjusted to target pH values (2–12 initially, then in 0.25 increments to determine the isoelectric point) using NaOH or HCl (5–0.1 M) without exceeding a total volume of 1.25 mL. After adjusting the pH, the samples were stirred for two hours, rechecked for pH, centrifuged at 2862 RCF for 20 min at 25 °C, and supernatants were collected (Bernardino-Nicanor et al., 2014). Soluble protein content was measured by a modified Bradford assay (Bradford, 1976) using bovine serum albumin (BSA) standards (125–1000 μg/mL) for calibration. Briefly, 50 μL of each supernatant was mixed with 1.5 mL Bradford reagent, incubated for 10 min, and absorbance read at 595 nm. Samples exceeding the assay range were diluted appropriately. Measurements were conducted in five replicates, and protein solubility was calculated accordingly using formula (3).

$$PS\ [\%] = \frac{C * DF * V}{m * 10^6 * \frac{DM}{100} * \frac{P}{100}} * 100\% \quad (3)$$

C represents the concentration of the BSA standard at the absorbance of the sample (μg/ml); DF is the dilution factor; V is the volume of water (ml) used to hydrate the sample; m denotes the weight of the initial powder sample (g); DM indicates the dry matter content (%) of the initial powder; and P is the protein content (%) of the sample expressed on a dry matter basis. The factor 10^6 was used to convert grams to micrograms.

2.5.5. Color Analysis

Digital color measurement was conducted using DigiEye equipment and software (VeriVide Ltd, Leicester, UK) in diffuse mode to eliminate specular reflections(Limbo and Piergiovanni, 2006). Measurements were performed with a standardized D65 light source, simulating daylight, and a 10° observation angle. Color coordinates (L*, a*, b*) were determined according to the CIELAB system, and the total color difference (ΔE) between samples was calculated using Formula (4). The perceptual interpretation of ΔE was based on the boundaries provided in Table 2.

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2} \quad (4)$$

2.5.6. Foaming Properties

Foaming properties of protein concentrates were determined following the method described by Brishti et al. (Brishti et al., 2017). To determine the foaming capacity, 0.5 g of a dry sample was placed in a 100 mL graduated cylinder with 25 mL of distilled water. The initial volume (V_0) was recorded before homogenizing at 10,500 rpm for 90 s. After homogenization, the total volume (V_{TOTAL}) and foam volume (V_{FOAM}) were measured.

Foaming stability was assessed by leaving the graduated cylinder undisturbed at room temperature for 30 min. After this period, the remaining foam volume ($V_{30min\text{-}FOAM}$) was recorded. The foaming capacity (FC) and foaming stability (FS) were calculated using the following Eqs. (5) and (6) (Brishti et al., 2017):

$$FC\ (\%) = \frac{V_{Foam}}{V_0} * 100 \quad (5)$$

$$FS\ (\%) = \frac{V_{30-Foam} - V_{Foam}}{V_{Foam}} * 100 \quad (6)$$

2.5.7. Emulsifying Properties

Emulsifying properties of protein concentrates were determined following the method described by Brishti et al., with slight modifications (Brishti et al., 2017). A sample of 0.5 g was weighed into a plastic graduated cylinder, mixed with 40 mL distilled water, and homogenized at 10,000 rpm for 30 s using a high-shear homogenizer (Ultra-Turrax, Germany) at room temperature. The initial volume (V_0) was recorded. Then, 40 mL of sunflower oil was gradually added, and the mixture was homogenized again at 18,000 rpm for 120 s at room temperature. After standing undisturbed for 30 min, the volume of the emulsified phase (V_{30min}) was measured. Emulsion stability was assessed after leaving the emulsion undisturbed for 24 h at room temperature, after which the remaining volume (V_{24h}) was recorded. Emulsifying capacity (EC) and emulsion stability (ES) were calculated using the following Eqs. (7, 8):

$$EC\ (\%) = \frac{V_{30min}}{V_0} * 100 \quad (7)$$

$$ES\ (\%) = \frac{V_{24h}}{V_{30min}} * 100 \quad (8)$$

Table 2
ΔE values as perception boundaries.

$\Delta E < 0.2$	no perceptible difference
$0.2 < \Delta E < 0.5$	indicates a slight difference.
$0.5 < \Delta E < 2$	small difference
$2 < \Delta E < 3$	fairly perceptible difference
$\Delta E < 6$	perceptible difference
$6 < \Delta E < 12$	strong difference
$\Delta E > 12$	different colors

2.5.8. Water Holding and oil binding capacity (WHC & OBC)

Water Holding and Oil Holding Capacity of protein concentrates were determined following the method described by Garcia-Vaquero et al., with slight modifications (Garcia-Vaquero et al., 2017). Initially, a sample weighing 0.5 g (dry weight-DW) is placed into a centrifuge tube and recorded as W_0. The centrifuge tube containing the sample is then weighed to obtain W_1. To facilitate hydration, 10 mL of distilled water/oil is added to the tube, followed by mixing for one minute to ensure uniform mixing. The mixture is then allowed to rest at 25 °C for two hours, with intermittent mixing every 10 min to optimize water absorption. Subsequently, the sample undergoes centrifugation at 2862 relative centrifugal force (RCF) for 30 min to separate the supernatant from the sediment. After discarding the supernatant, the tube is left to drip on a paper towel for 10 min to remove any excess water or oil. Finally, the centrifuge tube containing the sediment is weighed again (W_2). The WHC / OBC is calculated using formula (9):

$$WHC \Big/ OBC\left(\frac{g\ H_2O/g\ sunflower\ oil}{g\ protein\ concentrate}\right) = \frac{W_2 - W_1}{W_0\ x\ DW} \times 100 \quad (10)$$

where W_0 is the weight of the dry sample, W_1 is the weight of the centrifuge tube and dry sample, and W_2 is the weight of the centrifuge tube and the pellet.

2.5.9. Protein Solubility of chia protein concentrate

The protein solubility of the concentrates is measured and calculated as described in Chapter 2.5.4, with the modification that the pH is fixed at 7.

2.5.10. Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy was also performed for chia protein concentrate with different defatting methods by using IR Affinity-1 Spectrometer with Attenuated Total Reflectance (ATR) attachment (Shimadzu Sci. Ins., Kyoto, Japan). The measurements were collected within a 500–4000 cm^{-1} spectral range, and 16 scans were applied at a resolution of 128 cm^{-1}.

2.5.11. Statistical Analysis

For the statistical analysis, Minitab 21 (Version 10 Minitab Inc., State College, PA, USA) was used with a significance level of $\alpha = 5$ %. The Anderson–Darling test was used to determine the normality of the data. If the *p*-value is <0.05, the null hypothesis, which assumes the data follow a normal distribution, is rejected. Conversely, if the *p*-value is 0.05 or greater, there is no sufficient evidence to reject the hypothesis of normality. To assess variance homogeneity, the Levene test is used. The null hypothesis states that the variances are equal. If the *p*-value is less than the α level, the null hypothesis is rejected, indicating that there is no reliable evidence that the variances are equal. A single-factor ANOVA is used to compare the mean values, where the null hypothesis is that all means are equal. If the *p*-value is smaller than the α level, the null hypothesis is rejected, suggesting that at least one mean value is different. To identify the specific differences, Tukey's Honest Significant Difference (HSD) post-hoc test was conducted. In the resulting groups, those sharing the same smaller letter have no significant difference.

3. RESULTS AND discussion

3.1. Total Nitrogen content, protein yield and fat content of chia protein concentrate

Chia protein concentrates obtained by various defatting techniques displayed in Table 3 showed significant differences in total protein content. A significant increase in protein content was observed in the samples subjected to defatting process. The highest protein content was found in O_HEX and C_HEX. Solvent-based defatting removes oil effectively and increases protein concentration. Although it is expected that the protein content will increase with the removal of oil, certain fractions of proteins may be lost or undergo structural changes (Capellini et al., 2017; Kim et al., 2021). In C_SCF sample, the protein content remained slightly lower compared to HEX method. This can be explained by the fact that although supercritical CO_2 extraction reduces protein loss by preserving protein stability better, it does not have as high an oil removal capacity as solvent-based methods (Mateo-Roque et al., 2024). Since the mechanism of supercritical CO_2 to remove oils during the process is not as aggressive as solvents, a protein fraction containing more oil may remain (Prasad et al., 2023). In O_PD sample, the protein content remained relatively high compared to solvent-based methods. The absence of chemical solvents during the mechanical defatting process may have minimized protein loss. However, since the mechanical defatting process is not as effective as solvents, a certain amount of oil may still remain in the protein, which kept the protein content lower than solvent-based methods. The non-defatted chia control sample (C_ND) had the lowest protein content. This is due to the high oil content of chia flour in its raw form and the lower percentage of the protein fraction in the total mass (Tavarini et al., 2016). This result shows that defatting has a significant effect in increasing the protein content.

Table 3
Protein Content and Yield of extracted chia protein with different defatting techniques.

Sample ID	Protein Content [%]	Protein Yield [%]
O_PD	62.1 ± 3.7^a	38.2 ± 8.6^a
O_HEX	67.1 ± 2.4^a	27.1 ± 5.3^a
C_ND	30.8 ± 1.6^b	39.5 ± 12.6^a
C_HEX	63.9 ± 0.6^a	37.8 ± 2.2^a
C_SCF	57.8 ± 5.8^a	44.6 ± 2.1^a

Different lowercase letters within the same column indicate statistically significant differences between values ($p < 0.05$).

In terms of yield, the highest one was found in the chia sample defatted with supercritical fluid extraction (C_SCF). The low temperature and solvent-free structure of supercritical CO_2 may have increased recovery by preserving the stability of proteins (Mateo-Roque et al., 2024). Since denaturation or solubility loss of proteins is less during the supercritical CO_2 process, total protein recovery may have increased. In addition, since the fat removal mechanism of supercritical CO_2 may be more selective, protein fractions may have been less damaged compared to solvents (Elst et al., 2003). The mechanical defatting process may have minimized protein loss, but it may not have been as effective as solvent-based methods in increasing protein concentration due to incomplete removal of fat. However, O_HEX showed lower protein recovery, likely due to solvent-induced loss or denaturation. Although solvent-based methods increase the protein content, they may cause loss of some protein fractions (Senarathna and Malalgoda, 2024). This is due to the solvent dissolving certain protein fractions and removing them from the medium or to some proteins being denatured during the process. Organic solvents such as n-hexane may directly interact with the hydrophobic regions of proteins, reducing their solubility and causing some proteins to be lost during extraction (Senarathna and Malalgoda, 2024). As a result, lower protein yields were obtained.

When the effects of defatting processes on the oil content of chia samples were examined and the results shown in Table 4. It was determined that different methods showed significant differences in terms of oil removal efficiency. The lowest final extraction oil content was observed in samples subjected to hexane defatting. O_HEX and C_HEX achieved the highest oil removal rate compared to their initial oil contents. This result shows that solvent-based methods remove oil fractions quite effectively (Capellini et al., 2017). The fact that oil was almost completely removed also explains why protein solubility remained low despite the high protein content. In samples treated with hexane, hydrophobic regions of proteins may be exposed, and aggregation formation may increase, negatively affecting protein solubility (Gravel et al.,

Table 4
Total Fat Content of samples before and after protein extraction.

	Fat Content (%)	
	Pre-Extraction	Post- Extraction
O_PD	5.75±0.14^{c}	7.70±0.24^{c}
O_HEX	1.78±0.04^{d,e}	0.10±0.05^{e}
C_ND	37.95±1.67^{b}	54.40±1.96^{a}
C_HEX	7.70±0.22^{e}	0.40±0.28^{e}
C_SCF	5.90±0.29^{c}	5.10±0.74^{c,d}

Different lowercase letters within the same column indicate statistically significant differences between values ($p < 0.05$).

2021; Qing et al., 2022; Rajan et al., 2021). In C_SCF, the oil content remained higher compared to hexane defatting method. Supercritical CO_2 is a selective method in oil extraction and may not provide complete removal of oils which may be associated with the fact that supercritical CO_2 cannot remove certain fractions of lipids as effectively as solvent-based methods (Elst et al., 2003; Mateo-Roque et al., 2024; Prasad et al., 2023). Despite this, the sample treated with supercritical CO_2 had lower oil content compared to O_PD and provided the highest protein yield. In O_PD, the fat content was found to be higher than solvent-based methods but close to supercritical fluid extraction. Since no solvent or chemical substance was used during the mechanical degreasing process, complete removal of the oil may not have been possible. However, this method minimized protein loss and provided a relatively high protein content. Nevertheless, the inability to completely reduce the oil content may have a protective effect on protein solubility, and therefore the O_PD sample exhibited higher solubility than solvent-based samples. C_ND had the highest oil content which can be directly related to being the sample with the highest protein solubility. The presence of oil may play a role in increasing solubility by limiting protein-protein interactions(Boatright and Hettiarachchy B˙, 1995). However, the high oil content diluted the protein ratio and caused the lowest protein content.

3.2. Soluble Protein content and isoelectric point of chia seed

The solubility of chia protein concentrates was analyzed to evaluate the effect of pH on protein solubility and to determine the isoelectric point (pI), where minimum solubility occurs.

Fig. 1 shows the overall solubility trends across different pH values, while Fig. 2 highlights the pH range where the low solubility was

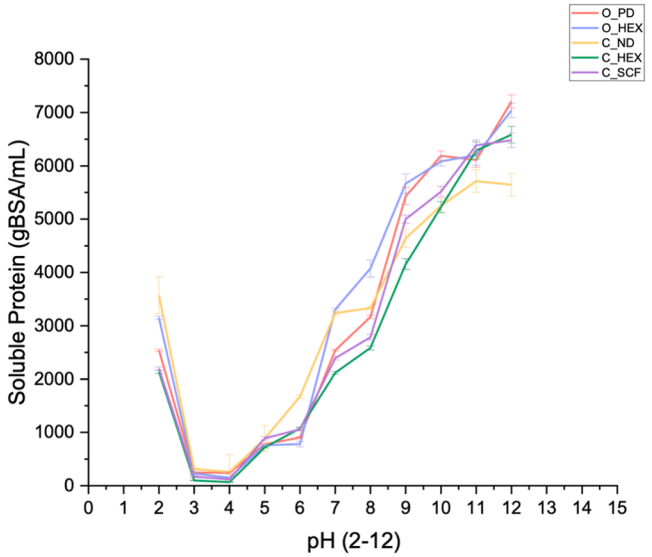

Fig. 1. Solubility curve of chia proteins with different defatting methods.

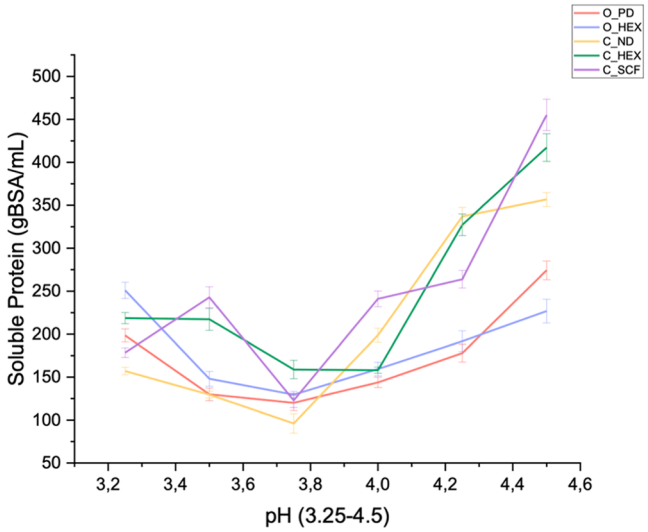

Fig. 2. Isoelectric point determination curve of chia proteins with different defatting methods.

observed and the lowest solubility point is called isoelectric point. The results indicate that protein solubility follows a U-shaped curve, with maximum solubility observed at highly acidic (pH ~2) and highly alkaline (pH ~12) conditions. This pI value is similar to other plant proteins (Gorissen et al., 2018; Hafez et al., 1985; Inyang and Iduh, 1996; Khalid et al., 2003; Mu et al., 2009), showing that chia proteins also dissolve differently at different pH levels because of their charge. As shown in Fig. 2, all defatted chia protein samples exhibited a sharp decrease in solubility at intermediate pH values, reaching their minimum solubility at pH 4 where it is their isoelectric point (pI). At this pH, protein molecules carry no net charge, reducing electrostatic repulsion and promoting aggregation, which results in precipitation and minimal solubility(Guckeisen et al., 2019; Kozlowski, 2016). The solubility patterns differed between O-type and C-type chia protein concentrates, indicating that the intrinsic protein composition and structural properties of each chia type play a role in solubility behavior. In general, C-type proteins showed higher solubility compared to O-type proteins across most pH values which may be attributed not only to differences in protein composition but also to the nature of the raw material (flour vs. whole seed) and its pre-processing history. This suggests that C-type proteins may have a higher proportion of soluble fractions, such as albumins, or a structure that allows better hydration and dispersion in aqueous environments. The defatting method also influenced overall solubility across the pH range. Hexane-defatted (HEX) samples exhibited the lowest solubility values across most pH levels, suggesting that hexane treatment may have altered protein structure, increased hydrophobic interactions and reduced the availability of solubilizing groups (Senarathna and Malalgoda, 2024). In contrast, SCF-treated proteins showed relatively higher solubility, particularly in extreme acidic and alkaline conditions, indicating that supercritical CO_2 extraction may have induced fewer structural modifications, preserving the solubility potential of the proteins. Interestingly, the non-defatted (ND) samples (C_ND) exhibited the highest solubility at nearly all pH levels, suggesting that the presence of native lipids might help maintain a more hydrated and dispersed protein structure. This observation aligns with findings in other plant proteins, where lipid-protein interactions influence solubility properties(Guckeisen et al., 2019). (Table 5)

3.3. Color Analysis of chia protein concentrate

The color characteristics of chia protein concentrates were significantly affected by the extraction and defatting methods applied. Contrary to the expected increase in lightness (L*) after oil removal (Coelho

Table 5
Colour Analysis of Chia Protein Concentrate.

	L*	a*	b*	ΔE
O_PD_Raw	50.7 ± 0.1^{e}	5.0 ± 0.0^{b}	17.5 ± 0.0^{e}	2.61 ± 0.02^{e}
O_PD_Protein	51.3 ± 0.0^{d}	4.3 ± 0.0^{e,f}	19.9 ± 0.0^{a}	
O_PD_HEX_ Raw	63.2 ± 0.0^{c}	3.2 ± 0.0 ^{g}	14.7 ± 0.0 g	5.74 ± 0.05^{c}
O_PD_HEX_Protein	45.0 ± 0.0 g	4.9 ± 0.0^{c}	18.2 ± 0.1^{c}	
C_ND_ Raw	27.1 ± 0.1^{j}	6.1 ± 0.0^{a}	10.7 ± 0.0^{h}	3.36 ± 0.05^{d}
C_ND_Protein	47.8 ± 0.0^{f}	4.8 ± 0.0^{d}	19.3 ± 0.0^{b}	
C_HEX_ Raw	74.2 ± 0.0^{a}	1.8 ± 0.0^{h}	8.6 ± 0.0 ^{j}	7.48 ± 0.28^{b}
C_HEX_Protein	43.3 ± 0.3^{h}	4.2 ± 0.1^{f}	18.0 ± 0.1^{d}	
C_SCF_ Raw	68.4 ± 0.3^{b}	1.8 ± 0.0^{h}	9.9 ± 0.0^{i}	11.46 ± 0.04^{a}
C_SCF_Protein	39.5 ± 0.0^{i}	4.3 ± 0.0^{e}	15.2 ± 0.0^{f}	

Different lowercase letters within the same column indicate statistically significant differences between values ($p < 0.05$).

and Salas-Mellado, 2018; Ndiritu et al., 2017), some protein samples, particularly those treated with hexane and supercritical fluids, exhibited lower L* values compared to their raw chia seeds. This darkening effect suggests that pigment removal was not fully achieved, or alternatively, that protein denaturation during extraction may have led to structural changes facilitating pigment entrapment. Similar observations were reported by López et al. (2018), who noted that pigments such as polyphenols may not be removed but instead bound within the protein matrix, reducing their optical contribution despite being retained in the system (López et al., 2018; Seczyk et al., 2019).

The reduction in a* values (red-green axis) following protein extraction also supports this result. Rather than indicating a loss of pigments, the decrease in redness may result from conformational rearrangements or aggregation of proteins, which mask or restrict pigment visibility (Feng et al., 2023). This is consistent with findings in other oilseeds where protein–polyphenol interactions or protein unfolding reduce pigment exposure without removing the pigment itself (Feng et al., 2023; Seczyk et al., 2019).

In contrast, an increase in b* values (yellow-blue axis) was observed in most protein concentrates. This trend is unlikely to stem from the release of pre-existing pigments but is probably explained by the formation of new chromophore compounds during processing. As Teh et al. (2014) observed in defatted oilseed meals, heat and solvents can drive Maillard-type reaction products or oxidized pigment–protein complexes or polymerization reactions that enhance yellow-brown coloration (Teh et al., 2014).

When ΔE, which expresses the magnitude of color changes of the samples before and after extraction, is evaluated, it is evident that especially supercritical fluid extraction and solvent-based methods cause significant color changes. The highest ΔE values were observed in the samples subjected to supercritical fluid extraction. This may be due to the fact that the supercritical CO_2 extraction process removes the pigments of chia seeds more with its high pressure and dissolving power (Jarén-Galán et al., 1999). On the other hand, milder methods such as mechanical extraction have less effect on color and exhibit lower ΔE values.

3.4. Protein Solubility of the chia protein concentrate at neutral pH

The effects of chia protein concentrates subjected to various defatting methods on protein solubility were investigated. The results shown in Table 6 indicate that the defatting process significantly alters the solubility of proteins. The highest protein solubility was observed in the non-defatted chia sample (C_ND). The presence of oil may play a role in increasing the solubility by limiting protein-protein interactions (Alzagtat and Alli, 2002). Solvent-based defatting processes significantly reduced protein solubility, particularly in the hexane-defatted sample (O_HEX), which exhibited the lowest solubility. Here, solvent defatting unexpectedly reduced solubility, likely due to structural disruption and aggregation (Kim et al., 2021). Solvents can increase the formation of

Table 6
Protein Solubility of the extracted chia protein at pH 7.

	Protein Solubility @ pH 7 (%)
O_PD	46.42±0.65^{b}
O_HEX	39.86±0.60^{d}
C_ND	56.26±1.35^{a}
C_HEX	44.57±0.83^{c}
C_SCF	43.40±1.07^{c}

Different lowercase letters within the same column indicate statistically significant differences between values ($p < 0.05$).

aggregation by exposing the hydrophobic regions of proteins. Hydrophobic interactions and disulfide bonds found in the natural structure of proteins may change during contact with the solvent, which may disrupt the folding of proteins and reduce the solubility (Qing et al., 2022; Rajan et al., 2021). The presence of oil may play a role in increasing the solubility by limiting protein–protein interactions or by helping preserve the native protein structure, which might be altered during defatting treatments (Alzagtat and Alli, 2002). Lipids may provide a solubility-enhancing protection on the surface of proteins; however, removal of lipids by defatting may reduce the solubility by making it difficult for proteins to interact with water. In particular, proteins treated with solvents may lose their hydrophilic surface groups and become trapped in their interior, which limits their interaction with water.

The defatted chia sample with supercritical fluid extraction (C_SCF) showed higher solubility compared to solvent-based methods. However, the high pressure used during supercritical CO_2 extraction may affect the conformational structure of proteins. The disruption of protein-lipid interactions due to pressure and the refolding of proteins may lead to the exposure of hydrophobic regions (Geng et al., 2024). As a result, the interaction with water decreases and the solubility may decrease. In addition, supercritical CO_2 may cause proteins to approach their isoelectric point by decreasing the pH of the medium; this may promote precipitation and reduce the solubility (Hofland et al., 2000). The mechanical defatting method (O_PD) caused less structural change compared to solvent-based processes and provided relatively high solubility. Since no high temperature or solvent was used during mechanical treatments, the structure of the proteins may have been less affected. However, since protein-lipid interactions were not completely disrupted during mechanical defatting, the stability of the proteins is likely to be partially preserved.

3.5. Foaming Properties of chia protein concentrate

The effects of different chia sources (O and C) and defatting methods on foaming capacity (FC) and stability (FS) of chia proteins were evaluated and the results were given in Table 7.

Organic chia flour (O) generally exhibited higher foaming capacity and stability than those from conventional chia flour (C); however, this difference may also be influenced by the physical form and prior processing of the raw materials, rather than solely by source. In particular, O_HEX showed the highest foam capacity and stability among all groups. In contrast, C_ND showed no foaming capacity and thus no foaming

Table 7
Foaming Properties of extracted chia protein with different defatting techniques.

Sample ID	Foaming Capacity (%)	Foaming Stability (%)
O_PD	17.95±2.22^{b}	35.00±8.66^{b}
O_HEX	32.10±2.14^{a}	76.85±1.60^{a}
C_ND	0.00±0.00^{c}	0.00±00^{d}
C_HEX	14.81±0.00^{b}	75.00±0.00^{a}
C_SCF	32.05±2.22^{a}	12.04±0.80^{c}

Different lowercase letters within the same column indicate statistically significant differences between values ($p < 0.05$).

stability. The differences between chia types are probably due to variations in protein chemical and physical properties and functional properties. O type sample may have improved foam properties by reducing surface tension more effectively.

In terms of defatting methods, HEX method improved the foaming properties in both chia types, while SCF method provided high results only in foaming capacity, but stability values were found to be low. In addition, no foaming was observed in the ND group where oil removal was incomplete, suggesting that fat may have suppressed the surface-active properties of proteins and completely prevented foam formation (Shen et al., 2020).

HEX may enhance foaming by removing phospholipids and increasing protein content. As the non-polar nature of hexane exposes the hydrophobic regions of proteins by removing phospholipids and other soluble components (Feyzi et al., 2017; L'Hocine et al., 2006). This may have led to higher foam capacity and stability.

In contrast, supercritical defatting (SCF) method may cause denaturation or modifications in protein structure due to the high pressure and temperature applied during the process(Senarathna and Malalgoda, 2024; Sheikh et al., 2023). This may explain the high foam capacity but low stability in samples processed with SCF. This situation can be explained by the weakening of protein-protein interactions due to the use of organic solvents, which prevents the formation of a hard interfacial film. In addition, structural changes in the SCF process, especially the increase in β-sheet and compositional changes in protein fractions, may have contributed to the decrease in FS(Yue et al., 2021).

The low foaming capacity of chia proteins may be due to the inadequate balance between hydrophobic and hydrophilic regions and the negative effect of high net charge (~50 %) on foaming(Vázquez-Ovando et al., 2013). Hydrophilic and/or hydrophobic amino acids could significantly affect the protein foaming and emulsifying properties (Kim et al., 2022). In addition, the low albumin content (10 %) increases this limitation. The denaturation-resistant structures of the dominant protein fractions such as globulin (65 %) and glutelin (20 %) may cause their foaming properties to be limited(Vázquez-Ovando et al., 2013).

3.6. Emulsifying Properties of chia protein concentrate

The effects of different chia types and defatting methods on the emulsion capacity (EC) and stability (ES) of chia proteins were investigated and presented in Table 8. The results showed that both factors had significant effects on the emulsion properties.

Different chia types (O and C) showed differences in terms of emulsion properties. While C showed higher emulsion capacity in general, O showed greater performance especially in terms of emulsion stability which may be due to differences in protein composition between chia types. For example, the higher protein solubility of C may be effective in increasing emulsion capacity since it also means better dispersion of protein molecules in the continuous phase, leading to improved interfacial adsorption and emulsification efficiency. However, this was not reflected in emulsion stability, as O formed more stable emulsions when it was defatted, showing that stability is related not only to solubility but also to other factors such as the hydrophobic-hydrophilic group balance.

The defatting methods affected the emulsion capacity and stability of chia proteins in different ways. SCF provided the highest emulsion capacity which was attributed to the increase of the surface-active properties of the proteins by the removal of phospholipids. However, the stability of the proteins obtained by the SCF method was found to be lower which can be explained by the partial denaturation of the proteins during the process and the weakening of the interfacial film-forming capacity. HEX improved emulsion stability, likely by enhancing protein-protein interactions and film strength. The samples without defatting (ND) showed high emulsion capacity but were poor in terms of stability which may be partially influenced by the presence of lipids, although their composition was not characterized in this study as well as the limited interaction capacity of the untreated proteins.

3.7. OBC and WHC of chia protein concentrate

The effects of different chia types and defatting methods on water holding capacity (WHC) and oil binding capacity (OBC) were investigated and the results presented in Table 9. The two different chia types (O and C) showed significant differences in WHC and OBC.

O had higher WHC and OBC values overall compared to C, indicating that O proteins can interact more strongly with water and fat molecules. In particular, the lower WHC and OBC of the C_ND sample suggested that untreated C proteins might have a more compact or less porous structure. This might be due to the retention of lipids, which limit protein unfolding and reduce the exposure of functional groups involved in water and oil binding. Additionally, stronger hydrophobic interactions and lower solubility contribute to a denser protein network, restricting its ability to interact with surrounding molecules.

Defatting methods significantly affected the WHC and OBC of chia proteins. Defatted samples had higher WHC (Zielińska, 2022) especially hexane treated samples showed the highest WHC values in both chia types, indicating that protein structures changed after hexane treatment to better retain water. Because the removal of lipids disrupted hydrophobic interactions, allowing the protein structure to unfold and expose more hydrophilic groups that can bind water(Kinsella, 1976; Teh et al., 2014). In addition, SCF gave intermediate results in terms of WHC. SCF-treated samples showed lower WHC compared to hexane-treated ones, likely due to the high-pressure and temperature conditions of the supercritical CO_2 extraction process, which can partially denature proteins and alter their structural integrity (Geng et al., 2024; Sheikh et al., 2023). This may lead to protein aggregation or reduced exposure of hydrophilic groups, limiting their ability to retain water. Additionally, SCF extraction might not remove lipids as effectively as hexane, leaving behind some hydrophobic regions that interfere with water absorption.

Generally, samples with high protein solubility can be expected to have high WHC and OBC, because soluble proteins can interact better with water and oil(Nahimana et al., 2024). However, despite the highest solubility of C_ND in this study, its low WHC and OBC indicate that solubility alone does not determine water and oil binding properties. Soluble proteins may bind water but lack sufficient unfolding to trap it effectively (Qing et al., 2022). Samples treated with HEX showed high WHC and OBC values despite decreased solubility. Hexane treatment

Table 8
Emulsifying Properties of extracted chia protein with different defatting techniques.

Sample ID	Emulsion Capacity (%)	Emulsion Stability (%)
O_PD	173.00±1.73^{c}	94.16±1.97^{a}
O_HEX	161.21±3.04^{d}	91.96±5.10^{a}
C_ND	193.94±2.62^{b}	74.23±2.05^{c}
C_HEX	175.39±3.60^{c}	97.66±2.33^{a}
C_SCF	203.17±4.96^{a}	84.01±1.41^{b}

Different lowercase letters within the same column indicate statistically significant differences between values ($p < 0.05$).

Table 9
Water and oil binding capacity of extracted chia protein with different defatting techniques.

Sample ID	WBC (ml/gram)	OBC (ml/gram)
O_PD	639.62±32.79a	366.20±7.27b
O_HEX	794.86±20.81a	346.71±6.18b
C_ND	234.56±4.98c	185.82±1.72d
C_HEX	686.72±4.88a	473.92±6.20a
C_SCF	495.23±16.70b	302.44±3.90c

Different lowercase letters within the same column indicate statistically significant differences between values ($p < 0.05$).

may have increased hydrophobic interactions by denaturing some of the proteins, which may have decreased solubility but increased WHC and OBC. In the SCF treatment, the solubility remained at a moderate level, but WHC and OBC were relatively low. Supercritical CO_2 treatment may have partially changed the structure of the proteins but did not completely denature them (Liu et al., 2021). As a result, although the solubility was prevented from decreasing completely, the water and oil binding capacities of the proteins decreased. The SCF method may not have been as effective as HEX in removing hydrophobic interactions from the proteins, which may have caused WHC and OBC to remain lower compared to the HEX samples.

In terms of OBC, C_HEX sample showed the highest value. This indicated that hexane process increased the oil binding capacity of proteins whose effect may be related to the increased exposure of hydrophobic groups (Teh et al., 2014). Both WHC and OBC can increase when protein unfolding exposes hydrophilic groups for water retention and hydrophobic regions for oil binding, without excessive denaturation. This occurs when defatting or structural modifications enhance porosity and surface area, allowing proteins to interact effectively with both water and oil molecules (Qing et al., 2022). SCF treated and untreated (ND) samples had lower OBC values, which could be explained by the more limited exposure of hydrophobic groups in the protein structure. In addition, OBC value of O_HEX was found to be lower compared to C_HEX, indicating that the effect of hexane treatment on protein structure may vary depending on chia types. (Fig. 3)

3.8. Fourier Transform infrared spectroscopy of chia protein concentrate

FTIR spectroscopy analysis effectively characterized the protein structures of five chia seed samples subjected to different defatting methods. Amide I (1600–1700 cm^{-1}) and Amide II (1517–1550 cm^{-1}) regions were particularly taken into consideration in the analyses which provide important information about the secondary structures of the proteins.

The amide I region (1600–1700 cm^{-1}), primarily associated with the $C = O$ stretching vibrations, serves as a sensitive indicator of changes in protein secondary structures, particularly alpha-helices and beta-sheets (Feyzi et al., 2017; Yue et al., 2021). O_PD was defatted by mechanical treatment and showed broad peaks in the spectrum, indicating that the protein structures remained relatively native. Peaks in the Amide I region at 1650–1653 cm^{-1}, reflecting α-helical structures, are not evident, indicating that mechanical treatment caused minimal changes in the protein structures (Nahimana et al., 2024). Contrastingly, the peaks in Amide I and II regions of O_HEX are much sharper and have higher intensity, especially in the range of 1612–1640 cm^{-1}, indicating β-sheet structures and greater unfolding of protein structures, likely due to the disruption of hydrophobic interactions and exposure of backbone peptide bonds(Nahimana et al., 2024). C_ND showed broader and have lower intensity, indicating that the α-helix and β-sheet structures in Amide I region are more mixed. However, due to the high oil content, broader peaks were formed in the spectrum, which leads to the interpretation that oils mask protein peaks.

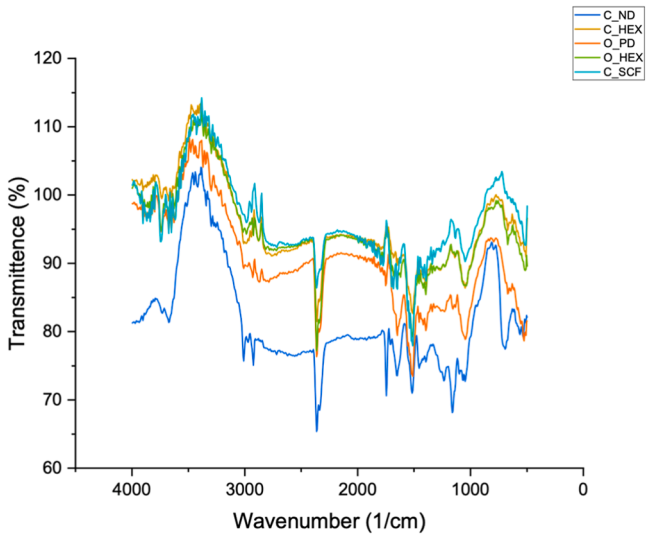

Fig. 3. FTIR Spectra of Chia Protein Concentrate.

The amide II region (1400–1600 cm^{-1}), associated with N—H bending and C—N stretching, also showed differences among samples (Malik and Riar, 2022). The intensity and sharpness of peaks in this region for the solvent-treated samples suggest alterations in the protein backbone, providing evidence of structural denaturation that could affect the functional properties of the proteins. In C_HEX, compared to C_ND, more clear and distinct peaks are observed in Amide I and II regions in this sample. It is understood that n-Hexane effectively removes fats and reveals protein structures more clearly(Pellerin and Doyen, 2024). Moreover, the supercritical fluid-extracted sample (C_SCF) demonstrated the most distinct spectral features in the amide III region (1241–1472 cm^{-1}), which is related to N—H bending and C—N stretching of the amide linkages(Feyzi et al., 2017). SCF appeared to preserve protein structure well, with minimal aggregation or denaturation. Moreover, 2900–3000 cm^{-1} region, which reflects C—H stretching vibrations, showed additional variances among the samples which is crucial for understanding the lipid content and the status of hydrophobic amino acid residues within the protein structure (Shapaval et al., 2019). For instance, peaks in this range were more obvious in the samples treated with n-hexane, suggesting a greater exposure or rearrangement of hydrophobic residues following lipid removal. FTIR results supports the hypothesis that solvent-based defatting methods not only remove lipids more effectively but also potentially disrupt the native packing of proteins, exposing hydrophobic residues to the solvent interface.

4. Conclusion

This study demonstrates that the method of defatting chia seeds significantly influences both protein yield and the functional properties of the resulting protein concentrates. Hexane extraction was the most effective for oil removal, yielding the highest protein content (up to 67.1 %) and superior foaming stability, water-holding, and oil-binding capacities, but it also led to lower protein solubility at neutral pH and presents environmental and food safety concerns due to solvent use. Supercritical CO_2 extraction, while not as efficient in fat removal as hexane, achieved the highest protein yield (44.6 %) and emulsion capacity, and preserved protein solubility better, making it a promising and more sustainable alternative for producing functional plant-based protein ingredients. Mechanical cold pressing, though less efficient at fat removal, resulted in protein concentrates with high protein content (62.1 %), good solubility, and excellent emulsion stability, with functional properties not far behind those of solvent-based methods. The results highlight that mechanical defatting is a viable, practical solution, especially where natural processing and protein solubility are priorities. Ultimately, the optimal defatting method should be selected based on the intended application, balancing protein purity, functional performance, and sustainability considerations. Further research could aim for higher protein concentrations while maintaining or improving functional properties and sustainability.

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Author contribution

Simon Dirr conceptualized and designed the research, conducted the experiments, and supported the manuscript writing process. Elif Gökçen Ateş assisted with the experimental work, wrote the initial draft of the manuscript, and was responsible for the final review and editing. Özlem Özmutlu Karslıoğlu and Mecit H. Oztop supervised the study, managed the overall project administration and funding, and reviewed and edited the final version of the manuscript.

Conflict of interest

The authors declare no competing interest.

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The author(s) utilized ChatGPT to enhance the clarity and language of this work. All content was subsequently reviewed and revised by the author(s), who take full responsibility for the final version of the publication.

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Ethical statement - studies in humans and animals

This research did not involve any studies with human participants or animals performed by any of the authors.

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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.

Supplementary materials

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Data availability

Reposetory is shared

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