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# Comparing Quality and Functional Properties of Protein Isolates from Soybean Cakes: Effect of De-Oiling Technologies

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**Abstract:** Driven by growing concerns about food supply and the environment, research on alternative protein sources has become increasingly important. In this context, de-oiled seed cakes, particularly soybean cakes, have emerged as a promising option. However, the conventional methods, such as organic solvent extraction, from which these cakes are obtained present several limitations. This study aims to evaluate the efficiency of supercritical fluid extraction (SFE) as an alternative method for de-oiling soybean seeds and obtaining related protein isolates. By using SFE for de-oiling, it was possible to achieve 19% more protein isolates from soybean cakes than the conventional de-oiling method using hexane. Moreover, protein isolates from the SFE de-oiled cake reported significantly improved ($p < 0.05$) emulsifying abilities and water absorption capacity. Gel electrophoresis and differential scanning calorimetry indicated the presence of a higher concentration of proteins in their native state in the SFE de-oiled flour. Finally, results from the sulfhydryl group content, surface hydrophobicity, and protein dispersibility index also supported these conclusions. The SFE process produced de-oiled soybean cakes with superior functional characteristics and lower environmental impact. Thus, this study provided important information for the food industry to develop more sustainable and healthier production methods.

**Keywords:** solvent extraction; green extraction; alternative protein sources; oilseed co-products; functional properties



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## 1. Introduction

The growing demand for environmental sustainability has led research and industry to a paradigm shift from animal to plant-based proteins. Given the need to adopt sustainable and environmentally friendly technologies, there is a growing interest in extraction technologies applied to vegetable seeds. Renowned as a rich source of plant-based protein, oil extraction from vegetable seeds leads to interesting co-products, commonly called "cakes." These defatted cakes are characterized by high protein content and significant nutritional value and represent an attractive and low-cost alternative to animal-based proteins [1–4].

In the production of de-oiled cakes, the extraction of oil from vegetable seeds plays a critical role. Conventional methods of oil extraction, such as mechanical pressing and solvent extraction with hexane, are prevalent but fraught with inefficiencies and environmental concerns. For instance, mechanical pressing is notably inefficient for soybean seeds, which are characterized by low oil content. Conversely, solvent extraction, despite its effectiveness in oil removal, often leads to protein denaturation, thereby affecting the functional quality of the resultant cakes [5–9]. This study investigates the potential of supercritical fluid extraction (SFE) as an alternative to conventional methods to de-oil

soybean cake. SFE exploits the unique properties of $CO_2$ in its supercritical state, exhibiting both liquid and gaseous properties. This condition facilitates the penetration of $CO_2$ into the vegetable cake and the efficient extraction of oil. Using $CO_2$ as a solvent, SFE offers several advantages: its non-toxic nature ensures environmental compatibility, and the moderate temperatures involved in the process preserve the integrity and functionality of the obtained extracts [10–12]. Studies demonstrated the feasibility and effectiveness of using SFE for oil extraction from seeds [13–15]. The potential functional properties associated with oils extracted by SFE include high nutritional value, high antioxidant activity, and bioactive compounds. The process allows to preservation of the natural antioxidants present in the oils, such as phenolic compounds and tocopherols, which can help neutralize harmful free radicals in the body. Moreover, oils obtained through SFE contain bioactive compounds, such as sterols, phytosterols, and polyphenols, which may offer various health benefits, such as anti-inflammatory, antimicrobial, and anti-cancer properties [16]. Several studies also showed the potential of SFE for the recovery of oils from soybean seeds [17]. However, just a few investigations were carried out on the defatted residue obtained from SFE. Recently, a research study proved that the defatted soybean cake presented interesting physicochemical and functional properties, suggesting the potential of using the powder as an alternative ingredient to a costly soy protein isolate commonly used in the food industry [18].

To the best of the authors' knowledge, no studies have investigated and compared the physicochemical and functional properties of both the defatted soybean cake and the protein isolate obtained from the cake by de-oiling it using two different technologies, namely SFE and conventional solvent extraction.

The hypothesis of this study is that SFE, given its moderate processing conditions, is more efficient and environmentally sustainable than conventional extraction methods. Thus, this study aims to comprehensively compare the functional properties of proteins derived from de-oiled cakes obtained using conventional solvent extraction and SFE. The proximate composition of the de-oiled cakes obtained via both hexane extraction and SFE was determined, focusing on the extraction yield of protein isolates. Differential scanning calorimetry was applied to assess the thermal stability and denaturation behavior of both protein isolates. Moreover, the study delved into the functional properties of protein flours and isolates, including their foaming and emulsifying abilities and their water and oil-holding capacities. A subsequent correlation analysis explored the interrelationships between these functional properties.

Through this comprehensive approach, the study aimed to enhance the understanding of SFE as an efficient and sustainable method for producing high-quality de-oiled soybean cakes.

## 2. Materials and Methods

### 2.1. Materials

Soybean seeds (not GMO and harvested in Europe) were kindly provided by Cereal Docks S.p.A. (Camisano Vicentino, Italy). All chemicals used were of analytical grade and were purchased from Sigma-Aldrich (Milan, Italy). Distilled water (Milli-Q, Merck Millipore, Darmstadt, Germany) was used to prepare all solutions and dispersions.

### 2.2. Samples Preparation

Soybean seeds were ground with a hammer mill (Laboratory Mill 3100, Perten Instruments, with 800 µm mesh size), resulting in fine flour with the following particle size measured with an orbital shaker composed of four trays with mesh size ranging from 1000 to 100 micron (Retsch GmbH, Verder Scientific, Germany): 14.8 ± 1.5% between 250 and 500 µm, 31.0 ± 2.3% between 100 and 250 µm and 53.5 ± 3.2% lower than 100 µm. The proximate composition of soybean seeds was 9.2% moisture, 37.4% protein, 21.6% crude fiber, 19.7% lipids, and 4.6% ashes.

### 2.3. Soybean De-Oiling Methods

#### 2.3.1. Solvent Extraction

De-oiling of soybean seeds was performed using an industrial plant at Cereal Docks S.p.A. (Camisano Vicentino, Vicenza, Italy). Initially, the seeds underwent a softening step. To this purpose, the seeds were passed through a horizontal softening pot at 70 °C for a period of up to 30 min. In a second step, softened seeds were subjected to flaking using an extruder to destroy the cell walls and increase the surface area, making the seeds more porous. The material was then extracted using hexane in a continuous countercurrent extractor at 68 °C. After extraction, the meal was desolventized using steam at 120 °C. The process was conducted in a toaster, with the steam flow running countercurrent to the meal flow to improve the efficiency of hexane desorption. Finally, the meal was cooled to ambient temperature and stored.

#### 2.3.2. Supercritical Fluid Extraction

Ground soybean seeds were de-oiled using a high-pressure pilot plant (Superfluidi s.r.l., Padova, Italy) equipped with a 1 L capacity extractor vessel and two gravimetric separators. The high-pressure vessel included an 800-mL extraction cylinder sealed with porous stainless steel mesh filters on both ends, allowing carbon dioxide to pass through while retaining solids inside the cylinder. Approximately 120 g of soybean flour was loaded into the extraction cylinder for each batch. The solvent, liquid carbon dioxide ($CO_2$), was supplied from a storage vessel pre-cooled to 4.0 °C using a cooling heat exchanger. It was then pumped into the extraction chamber using a high-pressure diaphragm pump (Lewa LDC–M–9XXV1, Milan, Italy) at a flow rate of 2 L/h. The extraction conditions were set to 40 °C and 30 MPa for 4 h, as reported in previous studies [19].

### 2.4. Soybean Protein Isolates

Soybean protein isolates were prepared by dispersing 30 g of defatted soybean flour in 750 mL of distilled water. The mixture was then transferred into a 1 L jacketed glass reactor maintained at a constant temperature of 60 °C. After temperature stabilization, the mixture was stirred for 30 min at 500 rpm using an overhead stirrer (IKA Eurostar 20, IKA, Staufen, Germany).

Subsequently, an aliquot of this mixture was then centrifuged at 12,000× *g* for 15 min at 20 °C (Thermo Scientific SL 16 R Centrifuge, Waltham, MA, USA). The supernatant was then filtered through Whatman filter paper grade 1 and concentrated to obtain the molasse. The pellet collected on the filter paper was resuspended in distilled water at a 1:25 ratio, and the pH was readjusted to 9.0 using 0.1 M NaOH solution. A second extraction cycle was repeated under the same conditions, and the two molasses were combined. The second collected pellet was again resuspended in distilled water to yield a solubilized protein mixture. The pH of this mixture was then adjusted to pH = 4.5 by adding HCl solution (0.5 M). The solution was then cooled to 10 °C by recirculating cold water in a jacked vessel and allowed to stand for 10 min. The precipitated material was collected by centrifugation at 8000× *g* for 15 min, resuspended, and homogenized in distilled water using an UltraTurrax (T25, IKA). Finally, the resulting protein suspension was freeze-dried (Epsilon 2-6D LSCplus, Martin Christ, Osterode am Harz, Germany). The process was performed by freezing the samples at −40 °C for 4 h and subsequently setting a primary drying for 24 h at a temperature of 5 °C and a vacuum of 1.01 mbar, followed by a secondary drying phase for 24 h at a temperature of 15 °C and a vacuum of 0.05 mbar. The dried protein isolates were then pulverized using a pestle and mortar.

### 2.5. Proximate Analysis of De-Oiled Soybean Flours

The proximate composition of the defatted soybean flours, including moisture, ash, fat, crude fiber, and protein content, was determined using standard methods as outlined in [20]. For moisture content, 5 g of the sample was heated at 103 °C for 4 h in a ventilated

oven. The sample was then allowed to cool to ambient temperature, stored for 45 min in a dryer, and subsequently weighed.

Ash content was determined by weighing 5 g of the sample in a crucible and heating it to 550 °C for at least 6 h in a muffle furnace. Then, the crucible was cooled down in a dryer and weighed.

For crude fiber analysis, 1 g of the sample was treated with boiling sulfuric acid solution to remove sugars and starches. The mixture was filtered and treated with a potassium hydroxide solution. The residue from this mixture was obtained by filtration using sintered glass. It was then washed with water and acetone, dried, weighed, and incinerated at 550 °C for 3 h. The weight loss from incineration indicated the crude cellulose content.

Crude protein content was determined using Kjeldahl's method [20]. The method indirectly quantified the amount of protein by the total nitrogen content. To obtain the crude protein content, the nitrogen content was multiplied by the factor 6.25 on a dry basis.

### 2.6. Protein Profiling via Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Protein profiling of the defatted flours and their corresponding protein isolates was accomplished using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To initiate this procedure, defatted flours were dehydrated for 4 h in 0.05 M phosphate buffer at pH 7.0, achieving a protein concentration of 10 mg/mL. Subsequently, the rehydrated flours were homogenized using an Ultraturrax device at 12,000 rpm for 2 min. The resulting slurry was designated as the total protein fraction. The remaining supernatant, after centrifugation at 12,000× *g* for 15 min at 20 °C, was designed as the soluble protein fraction and rehydrated in 0.05 M phosphate buffer at pH 7.0, resulting in an approximate protein concentration of 4 mg/mL. The total protein fraction and soluble protein isolates were combined in a 1:1 volume ratio. These combined fractions were mixed with Laemmli buffer consisting of 65.8 mM Tris-HCl (pH 6.8), 26.3% glycerol, 2.1% sodium dodecyl sulfate, and 0.01% bromophenol blue. In addition, 100 mM dithiothreitol (DTT) was included in the buffer for reducing conditions, whereas DTT was omitted for non-reducing conditions. Subsequently, the mixtures were heated at 95 °C for 5 min with agitation at 300 rpm using an Eppendorf thermomixer. The samples were then allowed to cool and then subjected to centrifugation at 2000× *g* for 5 min. Ten microliters of the resulting supernatants were loaded into pre-cast 4–20% Mini-PROTEAN® TGX gels (Bio-Rad Laboratories, Hercules, CA, USA), which were positioned within a Miniprotean® TetraCell electrophoresis cell. The cell was filled with running buffer (10× Tris/Glycine/SDS, Bio-Rad Laboratories) and connected to a PowerPac™ basic power supply (Bio-Rad Laboratories, Hercules, CA, USA). Molecular weight standards, specifically Precision Plus Protein™ Kaleidoscope™ prestained protein standards (Bio-Rad Laboratories), were employed, covering the range of 10–250 kDa. Electrophoresis was performed at 150 V for a duration of 1 h. Following electrophoresis, the gels were subjected to fixation using a solution consisting of 40% MeOH and 10% acetic acid for 15 min. Subsequently, they were stained with a staining solution comprising 0.1% Coomassie Brilliant Blue G-250 in 20% MeOH and 10% acetic acid for 30 min. After staining, the gels underwent destaining through multiple cycles employing the same fixing solution until a clear background was observed in the gels. Finally, the gels were scanned using a Gel Doc EQ system.

### 2.7. Differential Scanning Calorimetry

For DSC analysis, the defatted flour (150 mg/mL) and soy protein isolates (100 mg/mL) were dispersed in phosphate buffer (50 mM, pH 7.0). The dispersions were allowed to hydrate for 4 h and then homogenized using Ultraturrax operating at 12,000 rpm for 2 min. A differential scanning calorimeter (DSC 250, TA Instruments, New Castle, DE, USA) equipped with a refrigerated cooling system (Refrigerated Cooling System 90, TA Instruments) and operated with TRIOS 5.2 software (TA Instruments) was used. High-purity nitrogen gas served as the purging gas, flowing at a 50 mL/min rate throughout

the analysis. The DSC instrument was calibrated using indium (melting temperature of 156.6 °C). For the analysis, approximately 10 mg of each sample was accurately weighed and placed into aluminum pans (Tzero cells), which were then hermetically sealed. The samples were initially equilibrated at 35 °C for 5 min. Subsequently, a heating ramp was applied, increasing the temperature from 30 to 125 °C at a rate of 10 °C/min. An empty pan was used as a reference. The resulting thermograms were analyzed using the TRIOS software (TA Instruments).

### 2.8. Surface Hydrophobicity

Soy protein isolates and defatted flour samples were prepared at 30 and 70 mg/mL concentrations to assess surface hydrophobicity. These samples were first dispersed in distilled water and then hydrated for 4 h. Following hydration, the flour samples were homogenized for 10 min at 8500 rpm using Ultraturrax. Centrifugation was then performed at 800× *g* for 10 min to separate the protein precipitate from the isolates and flour samples. The protein concentration in the isolates and flour supernatants was adjusted to 0.5–0.005 mg protein/mL. This was achieved through serial dilutions employing a 50 mM phosphate buffer (pH 7.0).

The surface hydrophobicity was determined using a fluorescence assay [21]. Briefly, 2 mL of each diluted sample was mixed with 10 µL of an 8 mM solution of 1-anilino-8-naphthalene-sulfonate (ANS), which was used as a fluorescence probe. This mixture was then incubated at 20 °C in a dark environment. Fluorescence intensity was quantified using an Infinite 200 PRO multimode plate reader (Tecan Trading AG, Männedorf, Switzerland). Excitation and emission wavelengths of 380 and 490 nm, respectively, were employed for the measurements. The H0 index was determined by calculating the slope of the fluorescence intensity versus protein concentration plot. Each sample was subjected to triplicate measurements for robustness and accuracy.

### 2.9. Free Sulfhydryl Groups

Using a spectrophotometric assay, free sulfhydryl groups were quantified using Ellman's reagent, 5,5-dithio-bis-2-nitrobenzoic acid (DTNB). Both the flour supernatant and protein isolates were prepared following the procedure described for the surface hydrophobicity measurement. However, the protein concentration was adjusted to 2 mg/mL using 50 mM Tris-HCl buffer with a pH of 7.0. A volume of 1 mL of the sample was then mixed with 20 µL of a 10 mM DTNB solution prepared in methanol. This mixture was then kept in the dark for 15 min. Subsequently, centrifugation at 10,000× *g* for 3 min was performed, and the absorbance was recorded at 412 nm using a spectrophotometer (Agilent Technologies, Milan, Italy). The concentration of sulfhydryl groups was calculated using a molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$ for DTNB based on the following equation:

$$\mu mol\ SH\ groups/g = \frac{\left(\frac{ln\frac{I_z}{I_0}}{-\varepsilon x\lambda}\right) \times D \times V}{m} \quad (1)$$

where $I_z/I_0$ is the transmittance, ε is the molar extinction coefficient, $\lambda$ is the path length, $D$ is the dilution factor, $V$ is the cell volume, and m is the protein mass in grams. Blank samples were measured and subtracted from the absorbance values to calculate net absorbance.

### 2.10. Pasting Properties of Defatted Flours and Isolates

To evaluate the pasting properties, a Discovery Hybrid Rheometer HR-2, equipped with a starch pasting cell and a Peltier Concentric Cylinder (TA-Instruments, Milan, Italy), featuring an impeller rotor (bob diameter: 32.40 mm; bob length: 12 mm) was employed. Temperature control was achieved using a recirculating chiller (LT ecocooler 150, Grant Instruments, Cambridge, UK). Defatted soy flours were initially dispersed in distilled water (15 wt.%), stirred for 1 h, and then hydrated for 16 h at 4 °C. The pH was then adjusted to 7.0, and the resulting slurries were transferred to a stainless-steel cup (36 mm diameter)

for analysis. The gap between the rotor and cup was 5.5 mm. The experimental procedure consisted of several steps: an initial isothermal holding at 50 °C for 5 min, a subsequent heating ramp to 95 °C at a rate of 2 °C/min, and a final isothermal step for 5 min at 95 °C. A cooling phase was then executed, bringing the temperature down to 50 °C at a rate of 2 °C/min. An isothermal step was then performed for 5 min at 50 °C. The viscosity of the samples was measured throughout both the heating and cooling phases. A fixed shear rate of 15 rad/s was applied.

### 2.11. Protein Dispersibility Index

The protein dispersibility index (PDI) was determined, as reported by other authors [22]. In summary, the samples of soy protein isolates (30 mg/mL) and defatted flour (70 mg/mL) were dispersed in distilled water and allowed to hydrate for 4 h. The samples were homogenized for 10 min at 8500 rpm using Ultraturrax. After homogenization, both flour and isolate samples were centrifuged at 20 °C, 800× $g$ for 10 min, and the resulting supernatants were collected.

The total nitrogen content was measured using the Kjeldahl method to assess the crude protein content. The protein dispersibility index (PDI) was calculated as the percentage of soluble protein relative to the initial protein content in the defatted flour or protein isolate according to the following equation:

$$\mathrm{PDI}(\%) = \frac{\textit{Dispersible protein (supernatant)}}{\text{Total protein in the sample(defatted flour or isolate)}} \times 100 \quad (2)$$

### 2.12. Foaming Capacity and Stability

The measurement of foaming capacity and stability was performed using a method adapted from a research study [23]. Approximately 2.5 g of samples were suspended in 50 mL distilled water and homogenized using an Ultraturrax operating at 10,000 rpm for 3 min at room temperature. The samples were then transferred to a graduated cylinder, and the total volume was recorded immediately. Changes in foam volume within the graduated cylinder were recorded at 0, 15, and 60 min after homogenization. Foaming capacity (FC) and stability (FS) were calculated using the following equations:

$$\mathrm{FC}(\%) = \frac{V_2 - V_1}{V_1} \times 100 \quad (3)$$

$$\mathrm{FS}(\%) = \frac{V_3}{V_2} \times 100 \quad (4)$$

where $V_1$ is the volume of the suspension (mL) before stirring, $V_2$ is the volume of the suspension (mL) after stirring, and $V_3$ is the volume of the foam (mL) after it was allowed to stand.

### 2.13. Emulsifying Ability and Stability

The emulsifying ability and stability were determined by adapting a method previously described [23,24]. In brief, 5 g solution containing 0.5% ($w/w$) protein and 5 g of sunflower oil was homogenized at 7000 rpm for 30 s. Subsequently, 50 µL of the solution was extracted from the bottom of the samples at intervals of 0, 10, and 30 min. The extracted samples were then diluted with 7.5 mL of sodium phosphate buffer (10 mM, pH 7.0) containing 0.1% sodium dodecyl sulfonate (SDS) and mixed for 10 s. The absorbance of the mixed samples was measured by spectrophotometry at 500 nm in plastic cuvettes (1 cm path length) using 0.1% SDS solution as a blank reference. The emulsifying ability (EA) and stability (ES) were calculated at time intervals of 0, 10, and 30 min using the equations [25]:

$$\mathrm{EA}(\mathrm{m}^2 \times \mathrm{g}^{-1}) = \frac{2 \times 2.303 \times \mathrm{A}_0 \times D}{\mathrm{C} \times \varphi \times 10{,}000} \quad (5)$$

$$ES(\%) = \frac{A_t}{A_0} \times 100 \quad (6)$$

where C is the protein concentration (g/mL) before emulsification, φ is the oil volume fraction ($v/v$) of the emulsion, $D$ is the dilution factor, $A_0$ is the initial absorbance, and $A_t$ the absorbance at time t.

### 2.14. Water and Oil Holding Capacity

To measure water (WHC) and oil holding capacity (OHC), 100 mg of soybean protein sample and 200 mg of defatted soybean flour were thoroughly mixed in 4 g of water (for water holding capacity) and 4 g of soybean oil (for oil holding capacity). The mixtures were homogenized at 7000 rpm for 30 s to ensure uniform dispersion. For WHC measurements, the homogenized samples were centrifuged at 18,000× $g$ for 15 min at room temperature. The weight of the resulting wet pellet was recorded. The pellets were then dried for 24 h at 40 °C and weighed. *WHC* was calculated using the following equation:

$$WHC(g/g) = \frac{W_{wet\ pellet} - W_{dry\ pellet}}{W_{powder}} \quad (7)$$

where $W_{wet\ pellet}$ and $W_{dry\ pellet}$ are the weights of the wet and dry pellets, respectively, and $W_{powder}$ is the weight of the initial sample.

For the *OHC* measurements, homogenized samples were centrifuged at 4800× $g$ for 15 min at room temperature. The weight of the resulting oily pellet was recorded, and the OHC was calculated as follows:

$$OHC(g/g) = \frac{W_{oily\ pellet} - W_{dry\ pellet}}{W_{powder}} \quad (8)$$

where $W_{oily\ pellet}$ and $W_{dry\ pellet}$ are the weights of the oily pellet and dry pellets, respectively, and $W_{powder}$ is the weight of the initial sample.

### 2.15. Statistical Analysis

The experimental measurements were conducted in a completely randomized design (CRD) with at least three replicates for each test. Data were expressed as mean ± standard deviation, calculated from the replicate measurements. Differences between mean values were assessed using one-way ANOVA followed by Tukey's multiple-range test. The significance of differences was defined at the $p < 0.05$ level.

## 3. Results and Discussion

### 3.1. Proximate Composition of De-Oiled Flours and Protein Isolates

Figure 1 shows the processing steps for producing soybean isolates using hexane and SFE de-oiled methods. Starting from soybean seeds, both methods produced de-oiled flours with comparable protein content on a dry basis ($p > 0.05$). Table 1 presents the proximate composition of soybean flours and isolates obtained by the two defatting methods. Results indicate that solvent extraction did not significantly affect the flour's protein content. These findings are consistent with existing literature, which reported negligible differences in protein content between these two defatting methods, suggesting that both extraction techniques did not compromise the protein content of soybean flour [8,9]. The other components quantified in the de-oiled flours and the protein isolates were also not significantly different.

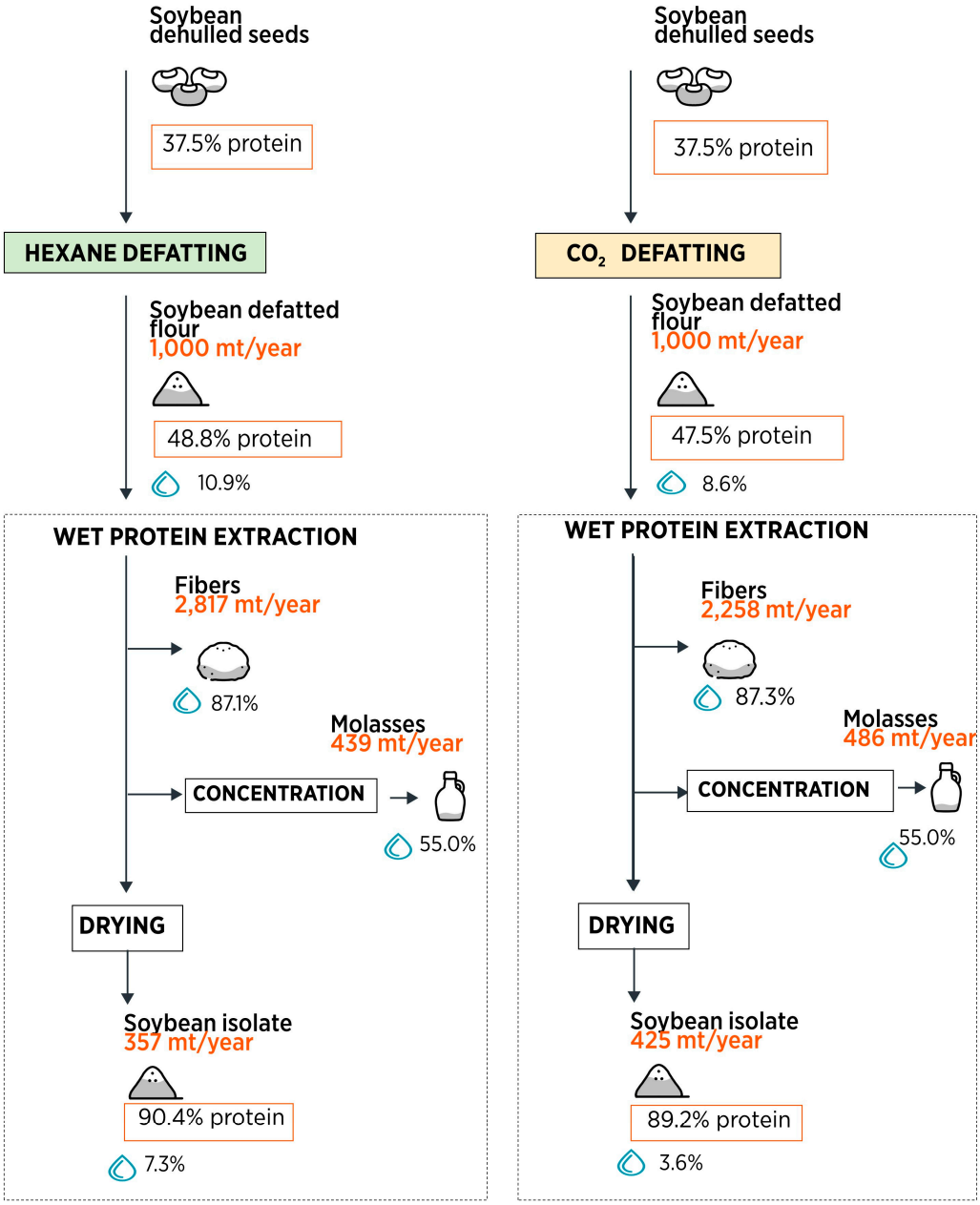


**Figure 1.** Mass balance of soybean protein isolates obtained using hexane and SFE de-oiled methods.

**Table 1.** Proximate composition of defatted soybean flours.

| Composition (%) | Hexane De-Oiled | SFE De-Oiled | Hexane Isolate | Sfe Isolate |
|---|---|---|---|---|
| Moisture | 10.8 ± 1.4 [a] | 8.6 ± 1.2 [a] | 7.3 ± 0.6 [a] | 3.5 ± 1.2 [a] |
| Protein | 48.8 ± 2.1 [a] | 47.5 ± 1.2 [a] | 83.7 ± 0.7 [a] | 86.0 ± 0.4 [b] |
| Lipids | 1.0 ± 0.2 [a] | 0.5 ± 0.4 [a] | 0.3 ±0.1 [a] | 0.2 ± 0.01 [a] |
| Carbohydrate | 29.1 ± 2.2 [a] | 31.7 ± 1.2 [a] | 3.8 ± 0.6 [a] | 5.3 ± 0.1 [b] |
| Crude fiber | 4.2 ± 1.0 [a] | 5.8 ± 0.8 [a] | 0.6 ± 0.01 [a] | 0.6 ± 0.1 [a] |
| Ash | 6.1 ± 0.2 [a] | 5.9 ± 0.2 [a] | 4.3 ± 0.6 [a] | 4.4 ± 0.5 [a] |

Results are expressed as mean values ± standard deviations. Different letters within columns indicate significant differences ($p < 0.05$).

Regardless of the de-oiled method, the mass balance analysis revealed three economically viable streams after protein extraction: fibers, molasses (a liquid stream obtained after

the protein extraction), and protein isolates. In particular, when using the flour de-oiled by SFE, the yield of protein isolates was 19% higher than that obtained from the hexane de-oiled flour. The results were linked to the higher percentage (86.0 ± 0.4%) of proteins solubilized using the SFE de-oiled flour. The values were significantly higher ($p < 0.05$) compared to that of the hexane de-oiled flour (83.7 ± 0.7%). The higher extraction efficiency of the SFE method could compensate for and bypass the drawbacks related to its higher capital expenditures (CapEx) and operating expenses (OpEx) compared with traditional solvent extraction. This suggested a promising potential for high-quality protein production within the food industry while balancing economic and environmental considerations.

### *3.2. Protein Profile*

Electrophoretic analysis, as shown in Figure 2a,b, compares the protein isolates and flours obtained using both hexane and SFE de-oiling methods. The protein isolates from both methods mainly consisted of glycinin and β-conglycinin bands. Further analysis of the defatted flours revealed protein bands with higher intensity in the SFE de-oiled flour, consistent with the increased percentage of solubilized proteins. Notably, during the wet protein extraction phase, samples from both de-oiled flours showed differences in the intensities of some bands, as shown in lines 4 and 5 of Figure 2a. The soluble fraction obtained from the SFE-defatted flour showed more intense bands, particularly at 35 kDa and 20 kDa, which were indicative of the presence of glycinin. In addition, more intense signals were observed at 67, 71, and 50 kDa, corresponding to β-conglycinin. These distinctions in band intensity and protein composition underscore the effectiveness of SFE in protein isolation. The enhanced solubility and extractability of proteins from SFE de-oiled flour likely contributed to these observed differences, reinforcing the potential advantages of SFE in protein extraction and isolation processes.

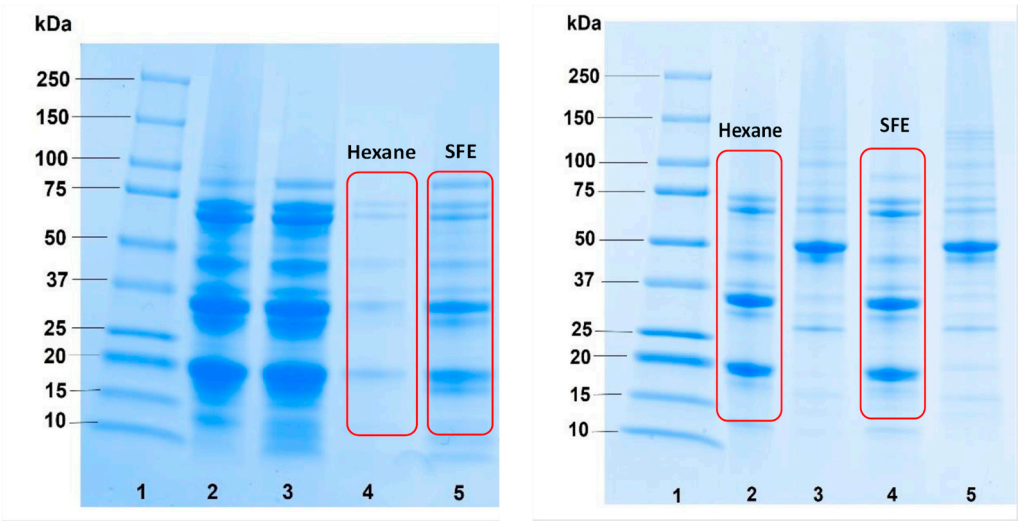


**Figure 2.** (**a**) SDS-PAGE of soy flours under reducing conditions. (1) Mw marker, (2) hexane de-oiled flour, (3) SFE de-oiled flour, (4) soluble fraction of hexane de-oiled flour, (5) soluble fraction of SFE de-oiled flour; (**b**) SDS-PAGE of soy protein isolates from hexane (2, 3) and SFE de-oiled flour (4, 5) under reducing (2, 3) and non-reducing conditions (3, 5).

### *3.3. Differential Scanning Calorimetry Analysis*

Differential scanning calorimetry analysis was performed to assess the thermal stability and denaturation behavior of proteins in both de-oiled soybean flours and isolates. Figure 3 shows the thermogram obtained for the four samples, while Table 2 summarizes the key thermal parameters, including the onset and peak temperatures and the enthalpy change that quantifies protein denaturation. The results revealed significant differences for

β-conglycinin enthalpy and the peak temperature measured for the flours de-oiled using SFE and hexane ($p < 0.05$).

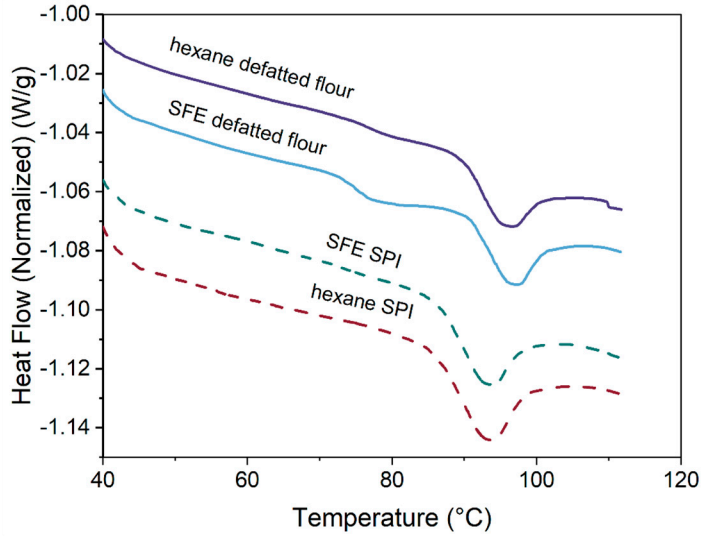


**Figure 3.** DSC thermograms of de-oiled flours and soybean protein isolates obtained from the same flours.

**Table 2.** Thermal properties of de-oiled soybean flours and protein isolates.

| Sample | $T_d$ Onset (°C) | | $T_d$ Peak (°C) | | ΔH (J/g Protein) | |
|---|---|---|---|---|---|---|
| | β-Conglycinin (7S) | Glycinin (11S) | β-Conglycinin (7S) | Glycinin (11S) | β-Conglycinin (7S) | Glycinin (11S) |
| Hexane de-oiled cake | 75.1 ±0.5 [a] | 90.7 ±0.5 [a] | 79.0 ±0.1 [b] | 95.8 ±0.3 [b] | 0.5 ±0.1 [a] | 7.4± 1.3 [a] |
| SFE de-oiled cake | 73.2 ±0.8 [a] | 91.1 ±0.3 [a] | 77.6 ±0.1 [a] | 96.7 ±0.2 [b] | 2.4 ±0.7 [b] | 7.3 ±0.2 [a] |
| Hexane protein isolate | nd | 86.7 ±0.3 [b] | nd | 93.2 ±0.2 [a] | nd | 9.9 ±2.0 [b] |
| SFE protein isolate | nd | 89.1 ±2.4 [b] | nd | 93.2 ±0.1 [a] | nd | 8.8 ±2.0 [b] |

nd = not detected. Results are expressed as mean values ± standard deviations. Different letters within rows indicate significant differences ($p < 0.05$).

Scanning the temperature from 40 to 110 °C, both SFE and hexane de-oiled flours exhibited two distinctive endothermic peaks at temperatures corresponding to the denaturation of β-conglycinin (78 °C) and glycinin (90–95 °C). However, a higher level of denaturation, as evidenced by the lower enthalpy values, was observed in the hexane de-oiled flour. In detail, the enthalpy of β-conglycinin denaturation of SFE de-oiled flour (2.4 ± 0.7 J/g protein) was significantly higher than that in hexane de-oiled flour (0.5 ±0.1 J/g protein). Similar significant differences were observed in glycinin denaturation, supporting the hypothesis that proteins in SFE de-oiled flours experienced less denaturation, thus preserving properties closer to their native states. This finding highlighted the SFE's ability to better retain the native state of proteins during the de-oiling.

Figure 3 also shows the thermograms of the protein isolates obtained from the two types of defatted flours. Only one thermal event was observed in these protein isolates, corresponding to glycinin denaturation. The absence of a second distinguishable thermal transition peak suggested the denaturation of a protein fraction during the wet protein extraction process or post-extraction treatment, where pH, ionic strength conditions, and temperature played major roles [26,27]. This observation also suggested that the drying conditions and temperature or pH during the wet extraction were important factors to consider for the production of protein isolates retaining their maximum native structure and functionality.

### *3.4. Sulfhydryl Group Content, Surface Hydrophobicity, and Protein Dispersibility Index*

The protein dispersibility index (PDI) is a crucial indicator of the functional properties of food proteins, particularly reflecting their solubilization efficacy in aqueous solutions. Table 3 presents data indicating that SFE de-oiled soybean flour exhibited a PDI that was 3.5 times higher than that of hexane de-oiled flour. This significant difference suggested a higher concentration of native protein structures in SFE de-oiled flour, which had significant implications for protein functionality. As outlined in the existing literature, factors influencing PDI include flour composition, lipid removal method, thermal exposure, pH levels, ionic strength, and mixing speed [8,28]. Protein denaturation decreases PDI values. The high PDI values of SFE de-oiled soybean flour could enhance the water-holding capacity and broaden the applications of this defatted flour in diverse food formulations.

**Table 3.** Functional properties of de-oiled soy flours and protein isolates obtained from them.

| | Units | Hexane De-Oiled Cake | SFE De-Oiled Cake | Hexane Protein Isolate | SFE Protein Isolate |
|---|---|---|---|---|---|
| Surface hydrophobicity | $\times 10^3$ | $134.6 \pm 0.6^{c}$ | $102.4 \pm 0.7^{b}$ | $84.7 \pm 7.4^{b}$ | $62.5 \pm 5.7^{a}$ |
| Free sulfhydryl groups | μmol/g protein | $9.3 \pm 0.2^{d}$ | $6.8 \pm 0.2^{c}$ | $5.8 \pm 0.2^{b}$ | $3.9 \pm 0.1^{a}$ |
| Protein dispersibility index | % | $19.1 \pm 0.7^{a}$ | $68.2 \pm 0.6^{b}$ | $91.5 \pm 3.5^{c}$ | $93.9 \pm 0.9^{c}$ |
| Emulsifying ability | $m^2$/g protein | $52.5 \pm 3.5^{a}$ | $107.5 \pm 2.12^{d}$ | $74.5 \pm 0.7^{b}$ | $90.1 \pm 2.8^{c}$ |
| Emulsifying stability (at 15 min) | % | $48.3 \pm 2.8^{a}$ | $87.4 \pm 2.8^{c}$ | $59.1 \pm 1.4^{b}$ | $64.2 \pm 2.5^{b}$ |
| Emulsifying stability (at 60 min) | % | $9.1 \pm 1.5^{a}$ | $71.2 \pm 1.3^{c}$ | $15.5 \pm 2.1^{a}$ | $51.1 \pm 1.4^{b}$ |
| Foaming ability | % | $110.0 \pm 2.1^{a}$ | $148.0 \pm 2.8^{c}$ | $136.0 \pm 2.12^{b}$ | $134.0 \pm 0.7^{b}$ |
| Foaming stability (at 15 min) | % | $92.9 \pm 3.5^{b}$ | $74.2 \pm 2.8^{c}$ | $68.6 \pm 2.8^{a,b}$ | $60.7 \pm 0.7^{a}$ |
| Foaming stability (at 60 min) | % | $62.6 \pm 3.5^{a}$ | $58.7 \pm 2.8^{a}$ | $52.5 \pm 3.5^{a}$ | $55.5 \pm 0.7^{a}$ |
| Water Holding Capacity | g $H_2O$/g powder | $1.75 \pm 0.5^{b,c}$ | $2.45 \pm 0.2^{c}$ | $0.55 \pm 0.1^{a}$ | $0.87 \pm 0.1^{a,b}$ |
| Oil Holding Capacity | g oil/g powder | $1.05 \pm 0.2^{a}$ | $1.55 \pm 0.2^{a}$ | $1.25 \pm 0.1^{a}$ | $1.38 \pm 0.1^{a}$ |

Results are expressed as mean values ± standard deviations. Different letters within columns indicate significant differences ($p < 0.05$).

Protein isolates derived from both de-oiled methods showed high PDI values, consistent with SDS-PAGE and DSC results. Similar correlations between PDI values, DSC, and SDS-PAGE results were obtained on peanut protein isolates [29,30]. This consistency underscored the minimal presence of denatured protein fractions, indicating the potential of these isolates to improve water holding capacity, extending their applications in various food products.

Table 3 also compares the surface hydrophobicity and concentration of free sulfhydryl groups in de-oiled flours and their corresponding protein isolates. Notably, the hexane de-oiled flour exhibited high values of surface hydrophobicity and sulfhydryl groups, indicating protein denaturation and molecular aggregation. An increase in surface hydrophobicity is typically correlated with a rise in free sulfhydryl group content, reflecting protein unfolding and the exposure of hydrophobic amino acid residues.

### *3.5. Functional Properties*

#### 3.5.1. Foaming and Emulsifying Capacity

Foaming capacity and stability, which are important factors for the suitability of de-oiled flours and protein isolates in food formulations, were significantly higher in SFE de-oiled flour (148 ± 2.8%, $p < 0.05$) than in the hexane one (Table 3). This superior foaming capacity was attributed to the higher concentration of native proteins in SFE de-oiled flour, which acted as effective surface-active agents enhancing foam stability through intermolecular forces [22]. Studies reported that defatted soybean cake possessed the highest foaming stability than other leguminous flours [31,32]. Another study compared different oilseed meals of almond, chestnut, hazelnut, pine nut, pistachio nut, and soybean. The authors found that the foaming capacity and stability of the defatted soybean cake was

40% higher compared to the other oilseed cakes [33]. This aspect is of great relevance to the food industry [34].

Importantly, protein isolates from both de-oiling methods exhibited similar foaming properties, likely because their comparable protein content levels aligned with previous studies [8,28,35].

The emulsifying capacity of the SFE de-oiled flour (107.5 ± 2.1 $m^2/g$) was significantly higher than that of the hexane de-oiled flour (52.5 ± 3.5 $m^2/g$ of protein), as shown in Table 3. This suggested that SFE enhanced emulsifying abilities because of the preservation of protein structure and amino acid composition. These properties are crucial for the development of stable and high-quality emulsified food products [36]. In a previous study, it was reported that SFE-defatted wheat germ protein reported higher emulsifying properties, suggesting it is a good protein additive for use in emulsified foods [37]. Other authors also reported similar results, which showed that the higher protein content of the extracts prepared from SFE oats presented superior emulsifying properties [28].

### 3.5.2. Water and Oil Holding Capacity

SFE de-oiled flour demonstrated superior water-holding capacity compared with hexane de-oiled flour, as detailed in Table 3. This was attributed to the presence of a higher amount of native proteins in the SFE flour, which enhanced the water absorption capacity [8]. On the other side, the oil holding capacities did not show significant differences between the samples ($p > 0.05$). The improved water-holding capacity of SFE-defatted flour could enhance the texture and shelf life of various food products [38]. The higher water holding capacity was in line with previously published studies where a defatted soybean cake was obtained after oil extraction by SFE. The values were linked to the higher surface hydrophobicity of the protein content of the de-oiled cake [18,39].

### 3.5.3. Viscoamylographic Profiles

Viscoamylographic analysis revealed that the SFE de-oiled flour displayed a three-fold increase in peak viscosity at 95 °C and a two-fold increase in final viscosity at 55 °C compared to the hexane de-oiled flour, as shown in Figure 4. These findings correlated with the enhanced water-holding capacity of SFE de-oiled flour, which has significant implications for food products such as bakery, pasta, and sauces. The increased viscosity could improve product texture and consistency, potentially reducing the need for additional thickeners and leading to cost savings in food production [38].

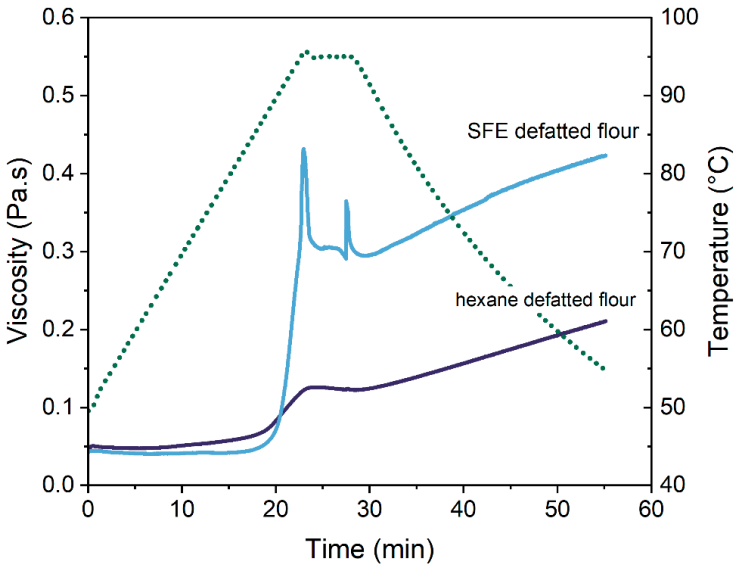


**Figure 4.** Pasting profile of defatted soybean flours.

### 3.6. Correlation Analysis

A correlation analysis was conducted to further understand the interrelationships among the measured functional properties. Figure 5 shows the correlation plot highlighting these relationships. SFE-defatted flour exhibited significantly higher emulsifying ability and stability ($p < 0.05$) with a positive correlation with the oil holding capacity ($R^2 = 0.98$). This study suggested that factors enhancing emulsification could also improve oil retention in these samples.

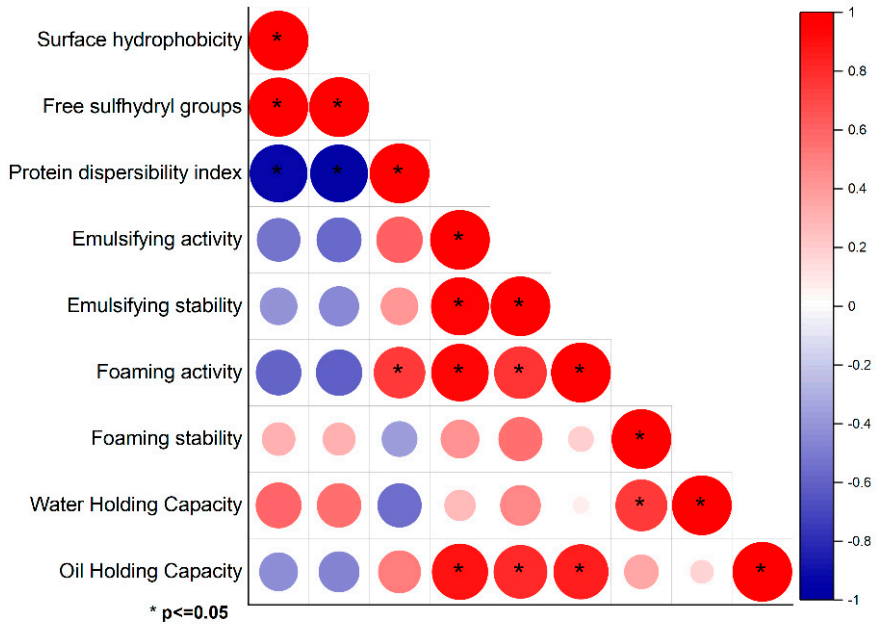


**Figure 5.** Correlation plot between the attributes measured for the samples. On the diagonal, the attributes of the *y*-axis are also reported. Significant or insignificant correlations could be assessed by matching the attributes vertically.

Interestingly, although it was not statistically significant, a correlation was also observed between the water holding capacity and protein dispersibility index. Conversely, an inverse significant correlation was observed between the protein dispersibility index, the surface hydrophobicity, and the free sulfhydryl group content. Samples with higher ease of dissolution in water exhibited lower surface hydrophobicity and reduced free sulfhydryl groups. These findings implied that samples with lower concentrations of native proteins reported increased surface hydrophobicity and free sulfhydryl groups, indicative of protein unfolding and exposure of hydrophobic residues.

## 4. Conclusions

This study demonstrated that SFE is an effective method for obtaining fully de-oiled soybean cakes, yielding a product with a proximate composition comparable to that of industrially extracted flour with hexane. In addition, a significant improvement in protein solubility and extraction yield was observed with soybean cake de-oiled using the SFE method. This improvement was mainly attributed to the reduced protein denaturation obtained at the end of the extraction process, which was supported by our findings.

Furthermore, while protein isolates from both SFE and hexane de-oiled methods shared similar protein profiles, several differences were evident in their functional properties. In particular, the flour de-oiled by SFE exhibited superior characteristics, including higher foaming and emulsifying capacity and stability and better water holding capacity.

Overall, the results of this study provided valuable insights into the preservation of protein quality during soybean cake processing, highlighting the advantages of SFE in obtaining de-oiled flours with unique functional attributes. Furthermore, the potential of

SFE-derived proteins in innovative food formulations is an exciting area for future studies. The findings supported the use of SFE as a green technology for obtaining protein-rich de-oiled plant matrices and encouraged further research to explore the potential of SFE in the field of alternative food proteins. This research aligned with the ongoing goal of applying technologies to sustainably exploit novel protein sources, achieving targeted functional properties. The study contributed to the broader analysis of sustainable practices in food production, answering the needs of the modern food industry to design environmentally safe processes.

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