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Green Protein Extraction from Hazelnut Press Cake: Yield, Efficiency, and Secondary Structure Analysis

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Abstract: Hazelnut (*Corylus avellana* L.) oil production generates press cakes, a by-product rich in plant-based proteins. As demand for alternative proteins grows, sustainable extraction methods like deep eutectic solvents (DESs) are essential for reducing the environmental impact. Alkali solubilization/isoelectric precipitation (ALKIS) is a widely used method for protein extraction due to its simplicity and cost-effectiveness. Despite that, ALKIS extraction has limitations due to the alteration in protein functionality, a reduction in protein stability, and significant wastewater production. This study compares DESs with ALKIS for extracting proteins from hazelnut press cake. ALKIS resulted in higher protein content (42.53 ± 17.45% vs. 8.55 ± 1.68%), while DES extraction showed higher efficiency (56.41 ± 2.4% vs. 45.16 ± 5.32%). FTIR analysis revealed significant structural differences: DES-extracted proteins exhibited higher β-sheet content (α-helix peak: 31.55 ± 6.97% vs. 18.13 ± 1.15%, $p = 0.0302$), indicating enhanced stability, whereas ALKIS-extracted proteins had more random coil structures (β-sheet peak: 57.92 ± 3.12% vs. 34.07 ± 12.45%, $p = 0.0324$), suggesting partial denaturation. The preservation of native protein structures in DES extraction is likely due to hydrogen bonding networks that stabilize proteins during processing.

Keywords: plant-based protein; green chemistry; protein extraction; hazelnut oil; side streams; by-products; deep eutectic solvents; DES; green extraction; waste

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Academic Editor: Alberto Barbiroli

Received: 18 February 2025
Revised: 6 March 2025
Accepted: 7 March 2025
Published: 9 March 2025

Citation: Anzà, B.; Fraterrigo Garofalo, S.; Lapolla, A.; Fino, D. Green Protein Extraction from Hazelnut Press Cake: Yield, Efficiency, and Secondary Structure Analysis. *Separations* **2025**, *12*, 63. https://doi.org/10.3390/separations12030063

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1. Introduction

Food systems contribute to approximately one-third of global greenhouse gas (GHG) emissions, making them the second largest producer of GHG [1]. In this scenario, shifting from a linear economic model to a circular one would be advantageous for utilizing food by-products and side-streams while also creating nutritional value to sustain the increasing population, which is expected to reach 10 billion by 2050 [2].

Hazelnut (*Corylus avellana* L.) belongs to the birch family, is extensively grown in numerous countries owing to its significant economic and ecological value [3] and is used in confectionery and bakery products. Italy is the second largest producer of hazelnuts, with agricultural activities predominantly concentrated in distinct regions, including Piemonte, Lazio, Campania, and Sicilia [4]. On the one hand, the rising interest in this crop stems from the increasing demand of the processing industry [5], which contributes to its nutritional and economic value. However, while the global area dedicated to hazelnut cultivation expands, the quantity of hazelnut by-products is also significantly increasing [3].

Hazelnut kernels constitute less than half of the total nut mass. In contrast, numerous by-products, including skin, shells, husks (green leaf cover), and leaves, are generated

during harvesting and processing [6]. Hazelnut oil production generates a by-product called hazelnut press cake [7], which is a good source of plant-based proteins [8,9].

Identifying the optimal extraction technique for specific plant matrices is the critical first step for up-cycling plant proteins from agri-food side streams/by-products. Indeed, plant proteins can be extracted from oil production side streams using various methods. Currently, common extraction techniques include solid/liquid extractions, particularly water-based methods (e.g., alkaline or acidic extraction), followed by isoelectric precipitation. Recent advancements have led to the development of more efficient and sustainable extraction methods aimed at minimizing the drawbacks of traditional approaches. Conventional methods, such as aqueous, salt, solvent, detergent, and alkali extractions, often result in protein degradation. Several factors, including extraction duration, solvent choice, pH levels, and temperature, can reduce protein yield and quality [10].

On the other hand, newer, more sustainable approaches, such as ultrasound-assisted solid/liquid extraction [11], microwave-assisted solid/liquid extraction [12], enzymatic extraction [13], and deep eutectic solvent (DES) solid/liquid extraction, are currently under investigation. In response to the limitations described, deep eutectic solvents (DESs) have gained recognition as environmentally friendly solvents [14]. DESs are formed by blending two or more ionic or non-ionic compounds at specific molar ratios (via heating and stirring, mechanical forces, sonication, or microwave radiation) to lower their individual melting points to a common eutectic point. The advantages of DESs are their biodegradability, non-toxicity, non-flammability, and sustainability, as they primarily comprise natural organic substances [15]. Furthermore, their low vapor pressure and minimal volatility make them a suitable replacement for organic solvents [16,17]. Recently, DESs have been utilized as solvents in both conventional and cutting-edge technologies for the extraction of phenolics [18], flavonoids [19], vitamins [20], lignin [21], sugars [22], and other compounds. DESs have been acknowledged as an eco-friendly and sustainable medium for protein extraction.

Specifically, DES mixtures were used for the extraction of protein from rapeseed meal [23]; sesame meal [24]; canola [25,26], sunflower, flax, and camelina meals [27]; seabuckthorn seed meal [28]; and hempseed press cakes [29]. This study aimed to conduct a comparative analysis between traditional protein extraction methodologies and DES-based protein extraction techniques, with a specific focus on evaluating yields, purity, and the secondary structure of the extracted proteins. Indeed, to our knowledge, no study has investigated the possibility of extracting proteins using the described DES from hazelnut press cakes as in this paper. The novelty of this study lies in investigating a DES mixture that has not been previously tested on hazelnut press cakes.

2. Materials and Methods

2.1. Materials

Hazelnut press cake flour (HPCF), a by-product of hazelnut cold press oil extraction, was obtained from Vortex SRL (Torino, Italy). Choline chloride (≥98.0%) and oxalic acid (98.0%) were purchased form Sigma Aldrich (St. software (St. Louis, MO, USA). Petroleum ether was purchased from Thermo Fischer Scientific (Waltham, MA, USA). All other chemical reagents used were of analytical grade and purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Proximate Composition of HPCF

Total lipid content was determined via the Soxhlet method using petroleum ether for 3 h. Moisture content was measured by drying the sample at 105 °C in a drying oven until a constant weight was achieved. Ash content was evaluated via dry ashing in a muffle furnace at 550 °C overnight. Protein content was determined using the Dumas method

using an elemental analyzer (Elementar vario Macro Cube). Total carbohydrate content was calculated by subtracting the sum of lipid, moisture, ash, and protein contents from 100%. All values are expressed as percentages. The analysis was performed in triplicates, and the values are expressed as mean ± standard deviation.

2.3. Deep Eutectic Solvent (DES) Preparation

Choline chloride and oxalic acid were mixed at a 1:1 molar ratio in a 250 mL round-bottom flask sealed with aluminum foil. The mixture was stirred in an oil bath at 80 °C for 1 h until a clear liquid was obtained [15].

2.4. Protein Extraction from HPCF

2.4.1. Acid–Alkali (ALKIS) Extraction

Acid–alkali extraction was performed as described by Tsermoula et al. [30], with some modifications. Three grams (3 g) of HPCF was mixed with distilled water at a solid-to-liquid (S/L) ratio of 1:10 (g/mL). Protein solubilization was achieved by adjusting the pH to 9.0 using 1 N NaOH, differing from what previously done by Tsermoula et al. [30], who used pH 11.0. The mixture was then vacuum filtered, and the liquid fraction containing the solubilized proteins was acidified to pH 4.0 using 1 N HCl (compared to pH 5.5 in [30]) and kept undisturbed for 1 h to allow for protein precipitation at room temperature. The protein precipitate was collected via centrifugation at 4500 rpm for 10 min at 20 °C, whereas Tsermoula et al. employed 4000× *g* for 20 min at 4 °C. The resulting protein pellet was neutralized to pH 7.0 with 1 N NaOH and then dried at 40 °C in a drying oven until a constant weight was achieved, and it was subsequently analyzed.

2.4.2. Deep Eutectic Solvent (DES) Extraction

DES extraction was performed as described by Cao et al. [24]. HPCF was mixed with the prepared DES with a solid/liquid (S/L) ratio of 1:20. The mixture was transferred into a 250 mL round-bottom flask, sealed with aluminum foil, and stirred in an oil bath at 60 °C for 60 min. After cooling, the mixture was centrifuged at 4500 rpm for 10 min to separate the solid and liquid fractions. The supernatant, containing solubilized proteins, was collected, while the solid residue was discarded. The supernatant was transferred into a beaker, and absolute ethanol was added at a ratio of 1:50 (g/mL). The mixture was maintained at 4 °C for 18 h to allow for protein precipitation. The precipitated proteins, which settled at the bottom of the beaker, were collected via centrifugation at 4000 rpm for 10 min. The protein precipitate was rinsed with deionized water and washed three times to remove residual salts. Each washing step was followed by centrifugation at 4000 rpm. Finally, the purified protein was dried at 40 °C until it was constantly weighed and analyzed.

2.4.3. Determination of Protein Yield and Extraction Rate

Dumas' method was used to determine the protein content in the extracted sample. The yield and the extraction rate were determined using the following equations [24]:

$$Yield\ (\%) = \frac{W_2}{W_1} \times 100 \quad (1)$$

$$Extraction\ rate\ (\%) = \frac{W_2 \times P_2}{W_1 \times P_1} \times 100 \quad (2)$$

W_1 is the weight in g of the HPCF; W_2 is the weight in g of the hazelnut extract; P_1 is the purity of HPCF, and P_2 is the purity of the hazelnut protein. All the values are expressed as percentages. The analysis was performed in triplicates, and the values are expressed as mean ± standard deviation.

2.4.4. Attenuated Total Reflectance–Fourier Transform Infrared (ATR-FTIR)

ATR-FTIR spectra of the samples were acquired in the range of 400–4000 cm^{-1} and at a resolution of 4 cm^{-1} (with 32 total scans) using the Alpha II ATR-IR spectrometer (Bruker Corporation, Billerica, MA, USA). The spectra were visualized, and the baseline was removed and smoothed using "Spectragryph—optical spectroscopy software", Version 1.2.16.1, 2022.

2.4.5. Protein Secondary Structure Quantification

The Amide I region (1600–1700 cm^{-1}) of the spectra was deconvoluted and fitted onto a Gaussian model, and the area was calculated via peak integration using Matlab R2024b. The relative contents of α-helix, β-sheet, random coils, and β-turns were determined according to the peak area at 1650–1660, 1600–1640, 1640–1650, and 1660–1700 cm^{-1} [31].

2.4.6. Statistical Analysis

The results of this study were expressed as mean ± standard deviation (n = 3) and were analyzed using Matlab R2024b. $p < 0.05$ was considered significant after one-way ANOVA and a post hoc Tukey's test was applied to identify specific differences.

3. Results

3.1. Proximate Composition HPCF

The proximate composition of HPCF was determined as described in Section 2.2. Table 1 presents the results of the proximate composition analysis.

Table 1. Proximate composition of hazelnut press cake flour (HPCF).

Proximate Composition (%)	Mean Value (%)	±
Moisture (% *w/w*, wet HPCF basis)	11.82	0.01
Proteins (% *w/w*, dry HPCF basis)	42.44	2.85
Carbohydrates (% *w/w*, dry HPCF basis)	24.69	2.96
Lipids (% *w/w*, dry HPCF basis)	18.87	0.73
Ashes (% *w/w*, dry HPCF basis)	2.18	0.32

The proximate composition of HPCF shows a relatively high protein content (42.44% ± 2.85%), highlighting the potential of HPCF as a valuable protein source. Carbohydrates, also measured on a dry weight basis, account for 24.69% ± 2.96% and are the second most concentrated macromolecule in HPCF.

3.2. Protein Yield and Extraction Rate

The protein yield and extraction rate were calculated as described in Section 2.4, and the results are reported in Figure 1.

Both sets of extractions (ALKIS and DES) showed high variability in the data, more likely due to the heterogeneity of HPCF. The protein yield from ALKIS extraction was significantly higher than that of DES extraction. This is probably due to the difference in the extraction protocols. The differences in protein extraction efficiency among various methods can be attributed to the distinct physicochemical characteristics of the extraction systems. Water, in contrast to DES systems, possesses a lower viscosity. This property enhances molecular movement, enabling particles to diffuse more effectively and interact more readily. As a result, water may facilitate the extraction of proteins more efficiently than DES systems, as shown in previous studies [28,32]. Alkali-soluble acid precipitation relies on electrostatic repulsion at high pH levels, which facilitates the exposure of hydrophobic groups and promotes protein aggregation through hydrophobic interactions. When the

isoelectric point is reached, the electrostatic forces are minimized, with the results of protein precipitation attributed to the minimal charge repulsion and creation of a more compact structure. On the other hand, DES protein extraction relies on hydrogen bond formation with proteins and its ability to solubilize the polysaccharide fraction, the main component of plant biomass, facilitating protein diffusion into the extracellular environment. Despite the differences in yield, the protein extraction rate was significantly higher in the DES protocols, showing greater selectivity of the DES mixture for hazelnut proteins [33].

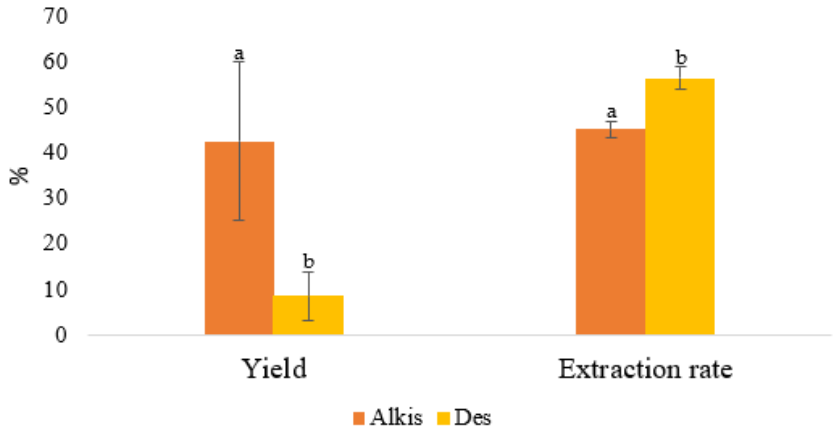


Figure 1. Protein yield and extraction rate obtained via the traditional method (Alkis) and deep eutectic solvent (Des). Data are presented as mean ± standard deviation (n = 3). Different letters above bars indicate statistically significant differences between extraction methods within each parameter (e.g., yield and extraction rate), as determined via one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$).

3.3. Protein Secondary Structure Analysis Via ATR-FTIR

FTIR is a widely used technique for monitoring changes in the functional groups and conformational features of proteins. The distinctive ratios of structural elements (such as α-helix and β-sheet) in proteins are characteristic of their composition. Understanding these secondary structural components is crucial for comprehending protein behavior in various technological applications. Consequently, the FTIR technique is widely employed to assess protein secondary structures [34]. In proteins, the peptide bond serves as the primary IR-active group, exhibiting a dipole moment due to resonance stabilization and a negative inductive effect. The inductive effect's orientation toward the carbonyl oxygen creates a partial negative charge on the oxygen atom and a partial positive charge on the nitrogen atom. Both theoretical predictions and experimental findings identify up to nine amide bands in a protein IR spectrum, labeled A, B, and I-VII. Among these, the Amide I, II, and III regions are considered the most significant [35].

Figure 2 illustrates the FTIR spectra of the HPCF, DES-extracted protein, and ALKIS-extracted protein. The DES extracted protein exhibited amide bands at 3278 cm^{-1}, 2919 cm^{-1}, 2850 cm^{-1}, 1635, 1522, and 1020 cm^{-1}, which correspond to the bending vibrations of free and bound OH and NH groups (amide A); asymmetric –CH2, symmetric –CH3, and –CH2 stretching (amide B); C=O stretching (amide I); N–H bending and C–N stretching (amide II); and C–N stretching and N–H vibration (amide III), in accordance with the literature [36]. As shown in the spectra, an increase in the intensity of the Amide B in the ALKIS-extracted protein may suggest conformational changes in the secondary structure of the protein or the rupture of the proteins into smaller pieces, while an increase in the intensity of the Amide A in the DES-extracted protein indicates the formation of hydrogen bonds [37].

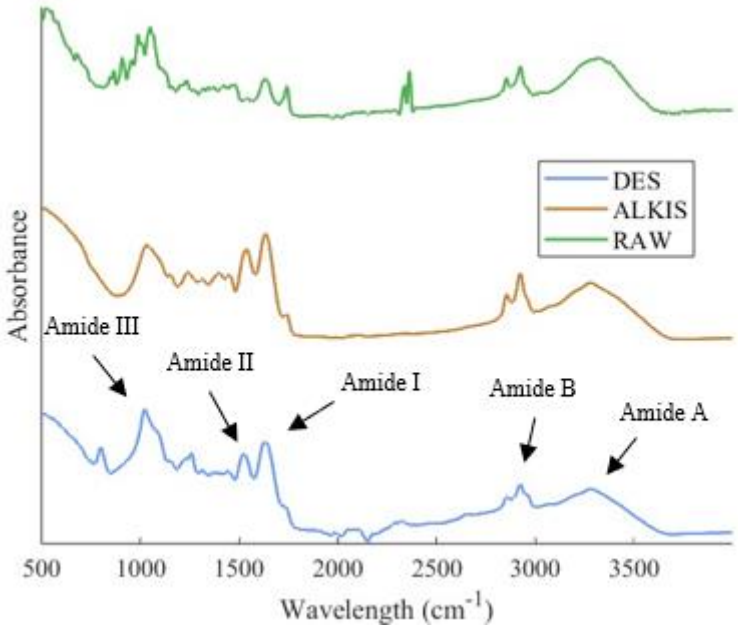


Figure 2. ATR-FTIR spectra of Hazelnut HPCF (green), ALKIS-extracted protein (brown) and DES-extracted protein (blue).

3.4. Secondary Structure of Protein: Amide I Deconvolution

The most frequently utilized band for structural analysis in protein spectra is the Amide I region, spanning 1700–1600 cm^{-1}. This region results from the interplay between C=O stretching and N-H in-plane bending vibrations [38]. Covering the range of 100 cm^{-1}, the Amide I region can be broken down into separate bands that correspond to various hydrogen-bonding patterns, influencing the vibrational energy of amide groups. Since the hydrogen-bonding arrangement of peptide bonds determines the conformation of the polypeptide backbone or secondary structure type, the Amide I region serves as a distinctive marker for a protein's structural composition. The diverse shapes of the Amide I band observed in different proteins indicate their complex structure, with multiple local peaks and troughs representing specific secondary structure elements [33].

Table 2 shows the wavelength range, secondary structure, and peak number identified in the deconvoluted spectra. Figure 3 shows the deconvoluted spectra of the DES/ALKIS-extracted protein samples.

Table 2. Wavelength range, secondary structure, and peak number.

Wavelength Range (cm^{-1})	Secondary Structure	Peak Number
1650–1660	α-helix	Peak 1
1600–1640	β-sheet	Peak 2
1640–1650	random coils	Peak 3
1660–1700	β-turns	Peak 4

The spectral deconvolution of DES- and ALKIS-extracted proteins shows differences in secondary structure composition. Indeed, the DES-extracted protein (Figure 3a–c) exhibits a well-defined deconvolution, with a higher proportion of peaks corresponding to β-sheet structures, which preserve hydrophobic amino acids located in the interior regions of the folded proteins, while α-helix structures typically reside on the exterior of protein molecules [36]. This result may suggest that the use of strong acid/alkaline solutions could cause the unfolding of the protein and consequently a reduction in β-sheet folding. This result is supported by the fact that protein extraction via DES is associated with the presence

of hydrogen bonds that may stabilize the secondary structure of the protein, protecting it from structural changes.

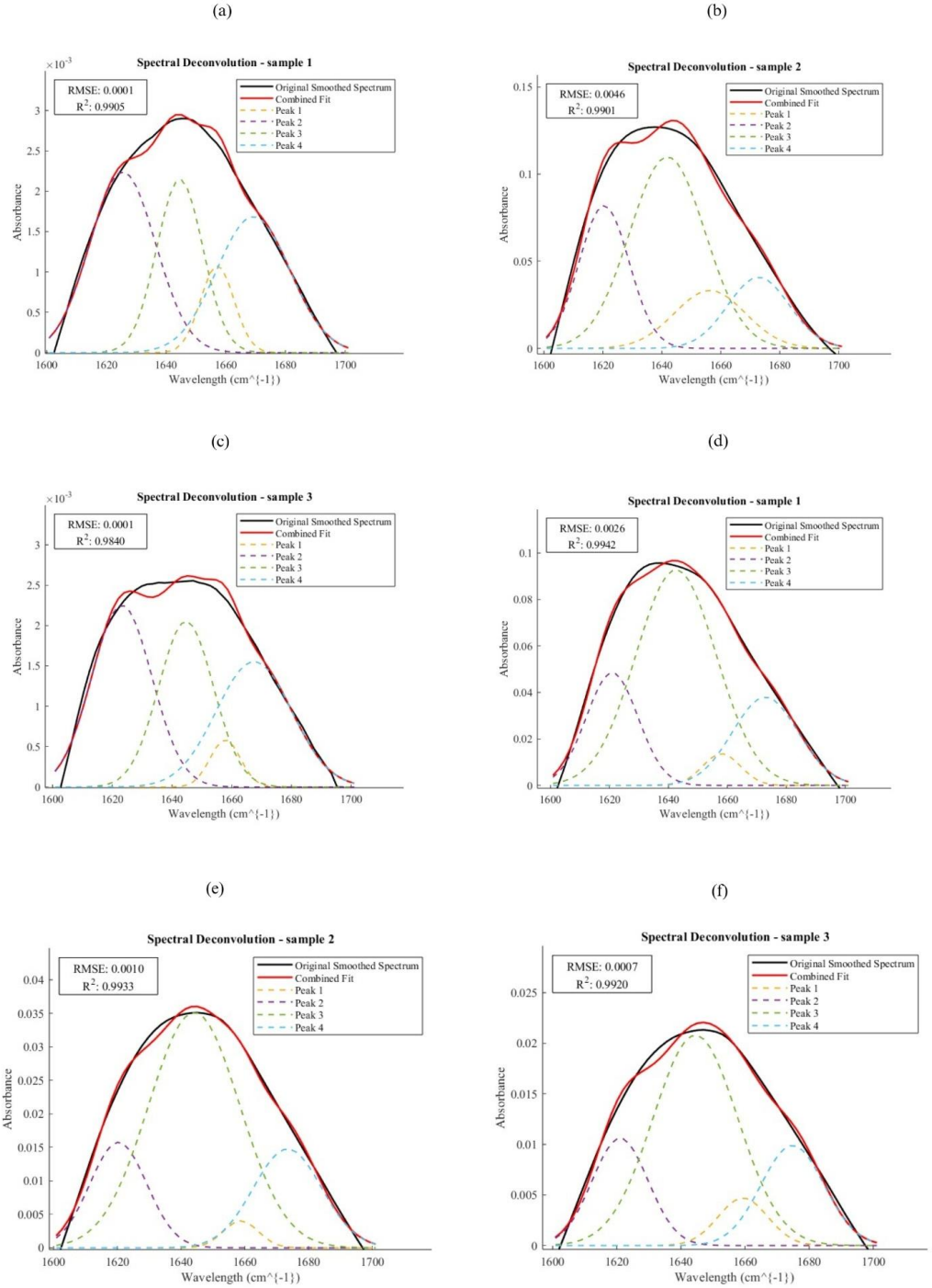


Figure 3. Spectra deconvolution results for each sample. (**a**–**c**) show the DES-extracted samples. (**d**–**f**) show the ALKIS-extracted samples.

In contrast, the ALKIS-extracted protein (Figure 3d–f) presents a different distribution of peaks. Indeed, β-sheet contribution appears lower, while an increased presence of random coil structures may indicate partial protein denaturation. To further confirm this thesis, the percentage of the specific secondary structure was calculated, as reported in Figure 4.

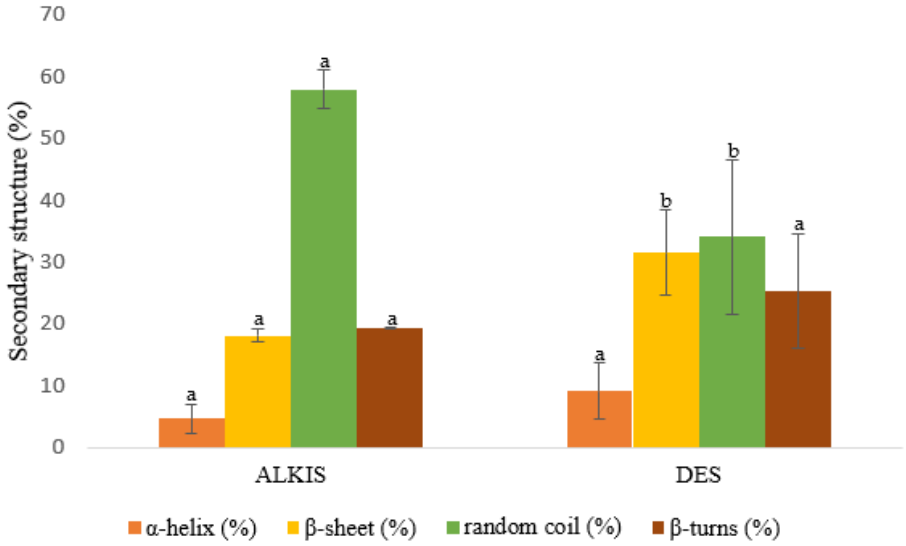


Figure 4. Secondary structure percentage distribution of ALKIS- and DES-extracted proteins. Data are presented as mean ± standard deviation (n = 3). Different letters above bars indicate statistically significant differences among secondary structure types between extraction methods, as determined via one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$).

Quantitative analysis of the Amide I band in infrared spectra can be achieved through curve fitting using deconvoluted and second-derivative spectra, which is one of the methods of protein structure analysis [39].

The deconvolution of the Amide I band and the quantification of the secondary structure of proteins showed significant differences in the secondary structure composition of the ALKIS- and DES-extracted proteins. Indeed, proteins obtained through DES extraction demonstrated a higher proportion of α-helices (9.13%) compared to those extracted via ALKIS (4.67%), indicating better preservation of native structural elements. Furthermore, DES-extracted proteins exhibited an increased β-sheet content (31.55%) in contrast to ALKIS (18.13%), suggesting enhanced structural stability. In contrast, ALKIS-extracted proteins displayed a predominance of random coil structures (57.92%), significantly higher than DES (34.07%), confirming increased structural disorder and potential protein denaturation. Moreover, the β-turn content was more pronounced in DES-extracted proteins (25.26%) compared to ALKIS (19.28%), which may contribute to improved protein folding and functionality. These observations indicate that DES is more effective at maintaining protein structural integrity, possibly due to less harsh extraction conditions that minimize protein denaturation.

4. Conclusions

This research underlines the differences between ALKIS and DES extraction techniques regarding protein yield and structural preservation. ALKIS extraction's superior protein yield can be explained by the smaller viscosity of water compared to DES that makes the extraction more efficient. Nevertheless, despite lower yields, DES extraction exhibited a more specific approach with enhanced protein extraction efficiency, indicating its potential for more effective protein recovery from hazelnut biomass. Consequently, this DES mixture offers the potential for selectively extracting proteins from inexpensive source materials while minimizing the environmental impact in comparison to alternative extraction techniques. Despite that, the extraction yield and the process conditions need to be optimized to maximize protein extraction and minimize the cost of the process. Moreover, FTIR analysis supported the thesis that DES extraction better maintained the proteins' secondary structure compared to ALKIS, showing higher α-helix and β-sheet content,

suggesting improved structural stability and functional integrity. These results imply that DES extraction is more effective in maintaining the functional and structural properties of hazelnut proteins, making it not only the more sustainable solution but also the preferable method in terms of protein structures. Assessing protein secondary structures through various methods is crucial due to its connection with functional characteristics. Changes in protein secondary structures can affect properties like hydrophilicity, hydrophobicity, and structural stability, which are linked to functional attributes such as water retention capacity, surface hydrophobicity, and emulsifying capability [40,41]. Consequently, identifying the appropriate extraction process is essential when repurposing proteins from by-products for diverse applications. This research demonstrates that DESs can preserve protein secondary structures, thus maintaining their functional properties.

Author Contributions: Conceptualization, B.A.; methodology, B.A.; software, B.A.; formal analysis, A.L. and B.A.; investigation, B.A.; resources, B.A.; data curation, B.A.; writing—original draft preparation, B.A.; writing—review and editing, B.A. and S.F.G.; visualization, B.A. and S.F.G.; supervision, S.F.G. and D.F.; project administration, S.F.G. and D.F.; funding acquisition, D.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the NextGenerationEU project NODES (MUR-M4C2 1.5, PNRR), grant number ECS00000036.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments: We thank Vortex for the supply of hazelnut press cake flour.

Conflicts of Interest: The authors declare no conflict of interest.

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