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Article

Revisiting Conventional Extraction of Protein Isolates from Faba Beans: Recovering Lost Protein from Sustainable Side-Streams

Abraham Badjona [1], Robert Bradshaw [2], Caroline Millman [1], Martin Howarth [1] and Bipro Dubey [1,*]

[1] Advanced Food Innovation Centre, Sheffield Hallam University, Sheffield, S1 1WB, UK
[2] Bimolecular Research Centre, Sheffield Hallam University, Sheffield, S1 1WB, UK
* Correspondence: b.dubey@shu.ac.uk (B.D)

Abstract: Demand for alternative sources of protein ingredients due to increasing population growth, environmental sustainability, and consumer demand is on the rise. However, processes used to generate these new food ingredients must be maximized to ensure a circular economy and a sustainable production system. In this study, the structural and functional properties of faba bean protein isolate and recovered fractions were investigated. Three different protein fractions were recovered with varying protein purities (35–89 %) using different pH conditions of protein fractions. Electrophoresis revealed major differences in protein distribution for protein isolate and recovered protein fractions. Additional FTIR analysis confirmed qualitative screening and differentiation of the different protein fractions based on protein amide regions and fingerprint regions. Faba bean protein isolates and the recovered fraction showed different functional properties with poor water-holding capacity for the albumin fraction. The application of discarded albumin fractions in food applications could improve resource efficiency.

Keywords: Faba beans; protein recovery; FTIR; functional properties; extraction

Introduction

The global population is projected to grow by 40% by 2050, reaching around 10 billion people. However, currently, about 1 billion individuals are facing food insecurity [1]. Ironically, approximately 1.3 billion tons of food are wasted annually, leading to a carbon footprint of 3.3 billion tons of CO_2 equivalents and the loss of significant quantities of water [2,3]. With a growing population, global food processing systems must expand and adapt as natural resources are becoming more scarce, unless effective mitigation strategies are implemented [4].

Sustainable and cost-effective food production is a crucial challenge in tackling this pressing issue. Both industrial and academic professionals bear significant responsibility in managing and developing innovative methodologies to this emerging trend. Biological conversion, extraction, and purification of valuable compounds from food byproducts are crucial for achieving "zero waste" in food production systems [5]. Utilizing pulses and legumes as a protein source reduces the escalating greenhouse gas emissions associated with animal-based protein sources like meat, dairy based products, and eggs [6]. Moreover, the requirement of water and energy input for the production of plant-based proteins are much lower in comparison with animal-based proteins [7]. In protein production and purification processes, circularity and sustainability are rarely prioritized [8,9].

While the application of purified ingredients is justified in specialised applications, such as infant formula and nutritional beverages, to ensure consistency and predictable processing behaviour, these ingredients are frequently recombined with other purified components in many formulations. A sustainable approach may involve utilizing minimally processed ingredients that

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naturally contain diverse techno-functional biomolecules, including proteins, fibre, and lipids. This strategy presents a promising means to reduce the environmental footprint of food production [10], particularly when derived from food processing side streams, which are generated in substantial quantities. Most recent studies investigating different types of faba bean protein have primarily focused on protein concentrates (~60% protein) [11] or protein isolates (80–92.2% protein) [12,13]. Despite the use of sustainable pulses in industrial processes to generate variety of protein ingredients, that result in significant generation of by-products which containing valuable compounds [14,15]. To achieve a more sustainable food production system, the valorization of processing side streams is crucial, requiring prior assessment of their potential applications in food systems. The recovery and application of such valuable compounds from protein extraction side streams could create novel ingredients with economic opportunities, and applications spanning the food, pharmaceutical, and cosmetic industries. Additionally, repurposing waste into value-added products may generate new revenue streams, contributing to economic growth.

Thus, to establish a circular economy, the utilization of side-stream biomass necessitates exploration of production stages which manage unavoidable food waste and evaluate their potential applications. This article explores the conventional extraction process of isolates from faba beans as well as recovered proteins from side streams following different process conditions. Mass balance was carried out to track protein levels at all stages. The selection and control of extraction conditions was evaluated based on their effectiveness in maximizing protein recovery whilst preserving structural integrity. Extraction efficiency can be evaluated by assessing protein purity and yield, alongside monitoring structural modifications occurring throughout the process.

Materials and Methods

Raw Materials and Chemicals

Faba beans were purchased from Whole Foods Earth (Kent, United Kingdom). NaOH, ((≥ 99.9% pure), β-mercaptoethanol, phosphate-buffered saline (PBS) and HCI were purchased from Sigma-Aldrich (United Kingdom).

Alkaline Isoelectric Precipitation of Faba Bean Protein Isolate

The preparation of faba bean isolate followed the standard protocol described by Sheikh et al., 2023; Shen et al., (2017), with modifications. Whole faba bean grains were dehulled and subsequently milled into flour using a laboratory-scale cyclone mill (Retsch, Twister) equipped with a 0.5mm sieve. The particle size distribution of the milled flour was analysed in replicates using a Mastersizer 3000 laser scattering particle size analyzer (Malvern, UK) with a dry sampling system [18].

Faba bean Protein Isolate (FBPI) was prepared as follows: 50g flour was dissolved in 500mL water (1:10w/v). For alkaline solubilization, the suspension was stirred at 400 rpm using a magnetic stirrer for 20min and adjusted to pH 11 using 1M NaOH. The mixture was centrifuged at 6,000 rpm for 20min at 25°C. After centrifugation, the supernatant was collected and adjusted to pH 4.0, using 1M HCI while stirring for 30 min. Protein isolates were extracted by centrifugation at 6,000rpm for 20min at 25°C. The precipitated isolates were freeze-dried for 48h, and the resulting protein was stored at −20°C. Proteins in side streams from starch fraction and isoelectric precipitation were further isolated using different pH. Detailed process of protein isolation process and conditions is shown in **Figure 1**.

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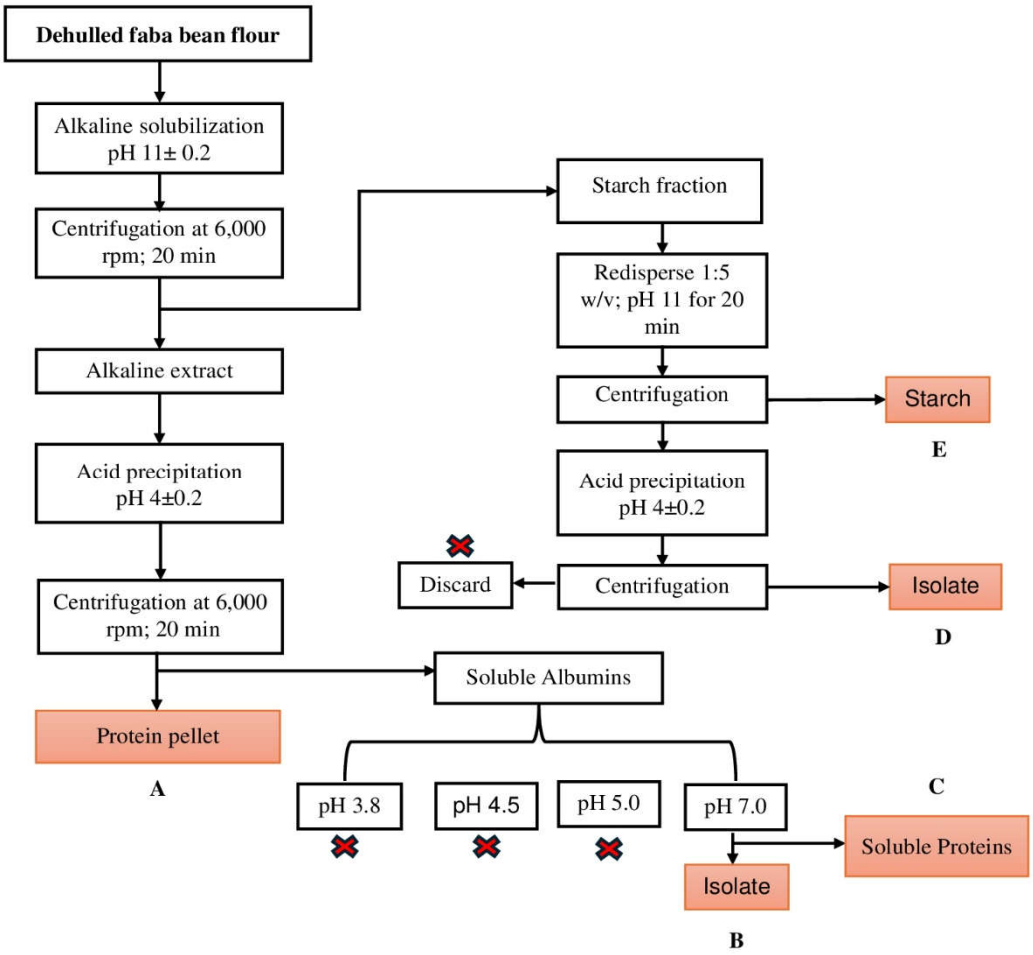

Figure 1. Flow chart for recovering protein isolate from faba bean flour.

Protein Content and Extraction Yield

Protein content of the extracted protein isolates and side stream was determined using the Elementar Dumas system (Elemental, UK, Ltd). A factor of 6.25 was used for the conversion of nitrogen content into protein content. The extraction yield was calculated by dividing the weight of the protein isolate obtained by the initial weight of measured faba bean flour, as given in Equation (1).

Extraction yield (%) = $\frac{mi}{ms}$ x 100 % (Eq.1)

Protein yield (%) == $\frac{mi\ x\ pi}{ms\ x\ ps}$ x 100 % (Eq.2)

The mass of the protein product and starting flour samples are denoted by *mi* and *ms*, respectively, while protein content of protein product, and flour is represented by *pi* and *ps* respectively.

The mass of the protein product and supernatant was determined after the centrifugation step, following extraction this was to identify which step proteins are lost and recovered. The protein content of the supernatant and the pellet was measured. The protein of the faba bean flour is either extracted into the supernatant or stays in the plant cell and lost in the pellet. If extraction is incomplete, residual proteins are found in the discarded supernatant or starch pellet.

As shown in Figure 1., the recovered proteins are represented by B, C, D and E (loss to starch fraction) in all extraction methods. Thus, the protein in the pellet recovered or lost during each step is shown in equation (3).

L or R = $mi\ x\ pi$ (Eq.3)

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The loss (L) or recovered (R) protein in percentage relative to the amount of protein in the faba bean flour was calculated according to equation (4)

L or R (%) == $\frac{mi\ x\ pi}{ms\ x\ ps}\ x\ 100\ \%$ (Eq.4)

A mass balance was conducted from the protein content and protein recovery data for all different fractions as follows:

$m_F p_F = m_A p_A + m_B p_B + m_C p_C + m_D p_D + m_E p_E$

The sum of all fractions should equate 100%.

Qualitative Analysis of Proteins Using Electrophoresis (SDS-PAGE)

As per Laemmli, (1970), with some modifications, electrophoresis was carried out on SDS-PAGE in a reducing solution of β-mercaptoethanol. 50mg protein powders were dissolved in 10mL of PBS buffer (0.01M, pH 7) and shaken at 200rpm for 2hr at room temperature. 10µL protein solutions were dissolved, vortexed with 10µL loading buffer (reducing solution containing 10% 2-mercaptoethanol). The samples were heated for 4min at 95°C, cooled, and centrifuged at 13300xg for 3min. An aliquot was injected into the pocket of the Bio-Rad 4% acrylamide stacking gel and 20% acrylamide was used to separate the Precast Gels. DC separation at a current of 25mA was performed for one hour at a voltage of 200V for 35mins. SDS-PAGE pre-stained ladder ranging from 260–8kDa was used as standard marker. The gel was rinsed with water and stained sequentially with Coomassie blue and imperial stain. The destained gel was scanned using the Gel analyse software (Nugenius, United Kingdom).

Protein Oil and Water Holding Capacity

Oil and water holding capacities were determined according to the method by Yang et al., (2023) with modification. Faba bean protein isolate (1.0g) was dispersed in 40mL of distilled water for water holding capacity (WHC) and rapeseed oil for oil holding capacity (OHC). The mixtures were vortexed for 1 min on maximum speed (2,500rpm) before standing for 6hr at room temperature (20–23°C). Samples were then centrifuged at 3000 x g for 30min at 20°C.

WHC/OHC = $\frac{W0-W1}{W3}$

Where $W0$ is the mass of the tube and protein isolate and absorbed water or oil; $W1$ is the mass of the tube and protein isolate while $W3$ is the mass of faba bean protein.

Fourier-Transform Infrared Spectroscopy Analysis (FTIR)

The Fourier-transform infrared (FTIR) study was carried out using an Attenuated total reflectance (ATR)-FTIR spectrophotometer (Spectrum 100 PerkinElmer, USA). Prior to the experiment, the absorbance spectrum of the air was recorded and automatically removed from the sample spectra. Spectroscopic studies were carried out with freeze-dried faba bean protein isolates in the range 4000–650cm^{-1} at a resolution of 4cm^{-1} with 16 scans.

Results and Discussion

Extraction Yield

Extraction efficiency can be determined by tracking protein purity and yield while structural changes were also observed along the extraction process. The first processing step in the fractionation process was grinding. This step is essential, producing fractured cells which liberate the starch granules and protein bodies. **Figure 2** and **Table 1** shows faba bean flour ground to a mesh cut off size of 0.5mm. The smaller classes may be associated with mainly protein bodies.

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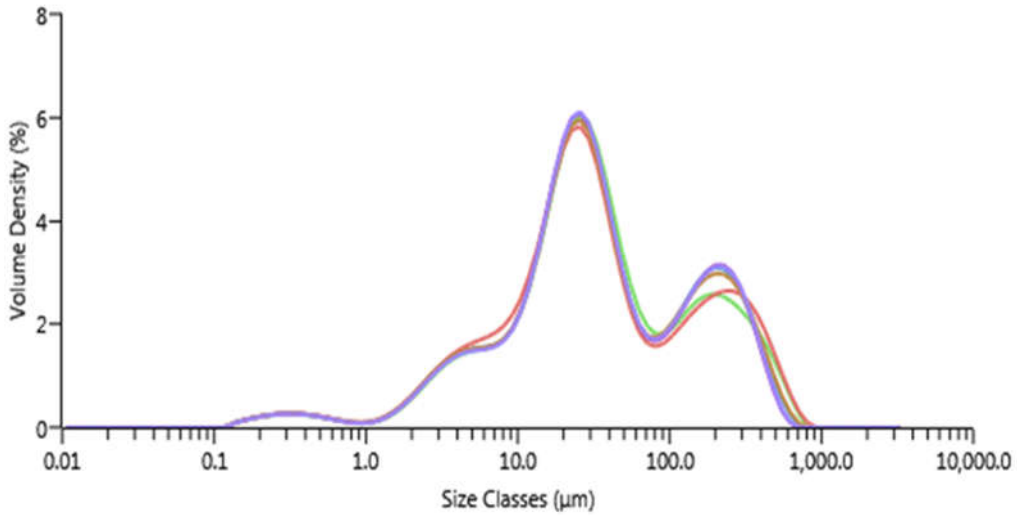

Figure 2. Shows the particle size distribution of faba bean flour (n = 4).

Table 1. Particle size of faba bean flour used during protein fractionation.

Particle size of flour		
Dx [10]	4.68 ± 0.1	µm
Dx [50]	29.4 ± 0.54	µm
Dx [90]	243.4 ± 2.61	µm
Span: (D90-D10)/D50	8.12	-

The effect of each purification step on the composition was studied in the milled faba bean protein extraction process. The first step was grinding of faba beans into flour. This flour was considered the initial starting point with a protein content of 27.29%. The first functional fraction was labelled as fraction A from the usual extraction process and resulted in an extraction yield, protein yield and protein content of 16.41±0.50%, 53.22±0.30% and 90.16±0.25% respectively. Fraction B and C were obtained from soluble albumins mostly discarded during protein isolation. Fraction B was obtained by simple precipitation at pH 7 of the soluble albumin solution (pH 3.8, 4.5 and 5.0 were investigated, however no extract was obtained) and subsequent centrifugation to obtain protein pellets, while the remaining supernatant was labelled as fraction C (soluble proteins). Fraction B resulted in an extraction yield, protein yield and protein content of 1.06±0.32%, 3.41±0.43% and 89.60±0.10% respectively. Fraction C after freeze drying showed an extraction yield, protein yield and protein content of 7.45±0.37%, 9.29±0.40% and 34.63±0.12% as shown in **Figure 3**. Fraction D and E were obtained from starch residues which are usually discarded after alkaline solubilization and centrifugation. Starch pellets were redispersed in distilled water, and pH adjusted to 11. This was stirred and centrifuged to obtain a supernatant and starch pellet (E). The supernatant was adjusted to pH 4 to precipitate residual proteins represented as fraction D. The supernatant was then discarded. The step-by-step extraction and recovery process is fully illustrated in **Figure 1.** Fraction D showed that extraction yield, protein yield, and protein content of 1.39±0.37%, 4.01±0.52%, and 80.44±0.28% respectively. Refined starch fraction (E) showed extraction yield, and protein content of 40.13±0.43% and 2.75±0.15%. The fat content of faba bean flour used in the study was less than 2% and it is assumed that fractions contain a negligible amount of oil [21]. The protein purity of faba bean isolate (A), labeled fractions B, C, and D is consistent with that reported in the literature using a similar extraction process [22,23].

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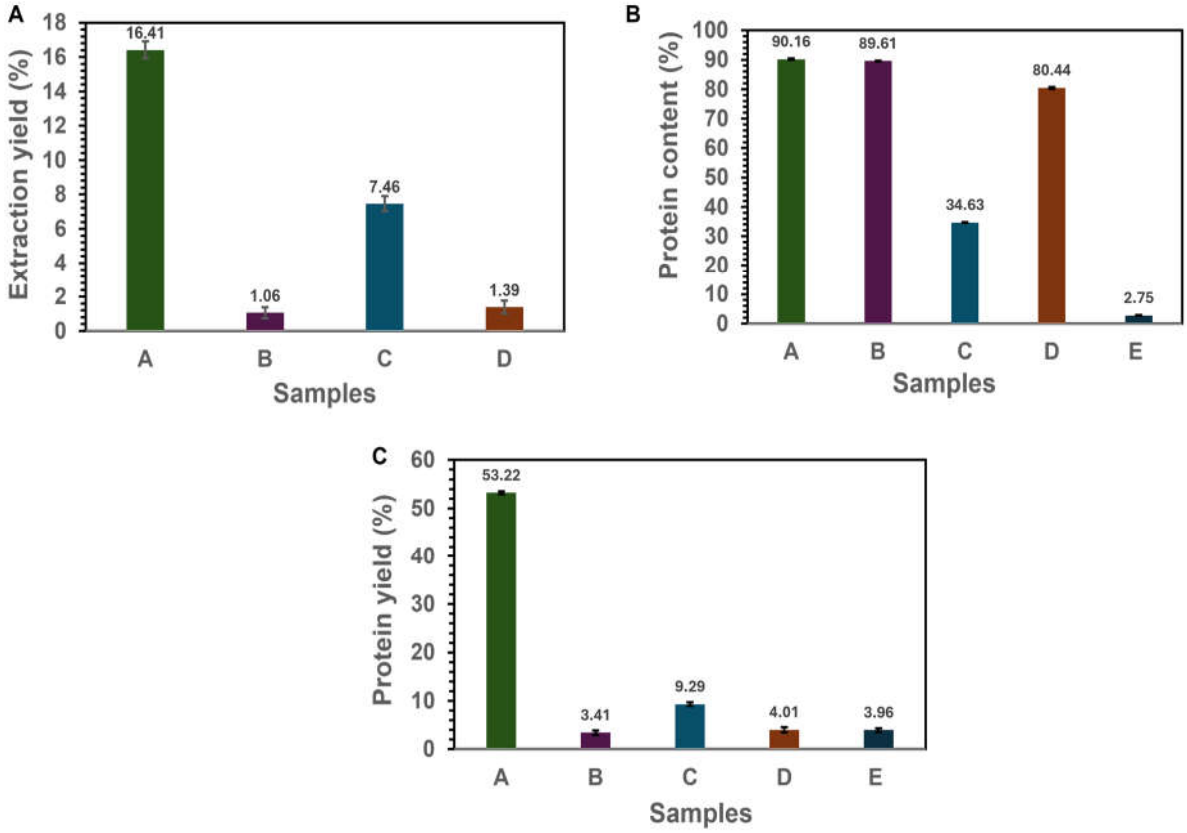

Figure 3. Extraction yield, protein yield and content during extraction and yield of the four different side-streams and resulting isolates (n=3).**Mass balance for different steps of Protein extraction.**

$m_f p_f = m_A p_A + m_B p_B + m_C p_C + m_D p_D + m_E p_E + L_p$

(50 x 0.2779) = (8.2023 x 0.9016) + (0.52825 x 0.896) + (3.72695 x 0.3463) + (0.6932 x 0.8044) + (20.0626 x 0.0275) + L_p

13. 895 =10.2681 + Lp

Lp = 3.63 g

Thus, the general loss to the extraction process was 3.63 g representing 26 % which may be attributable to discarded supernatant.

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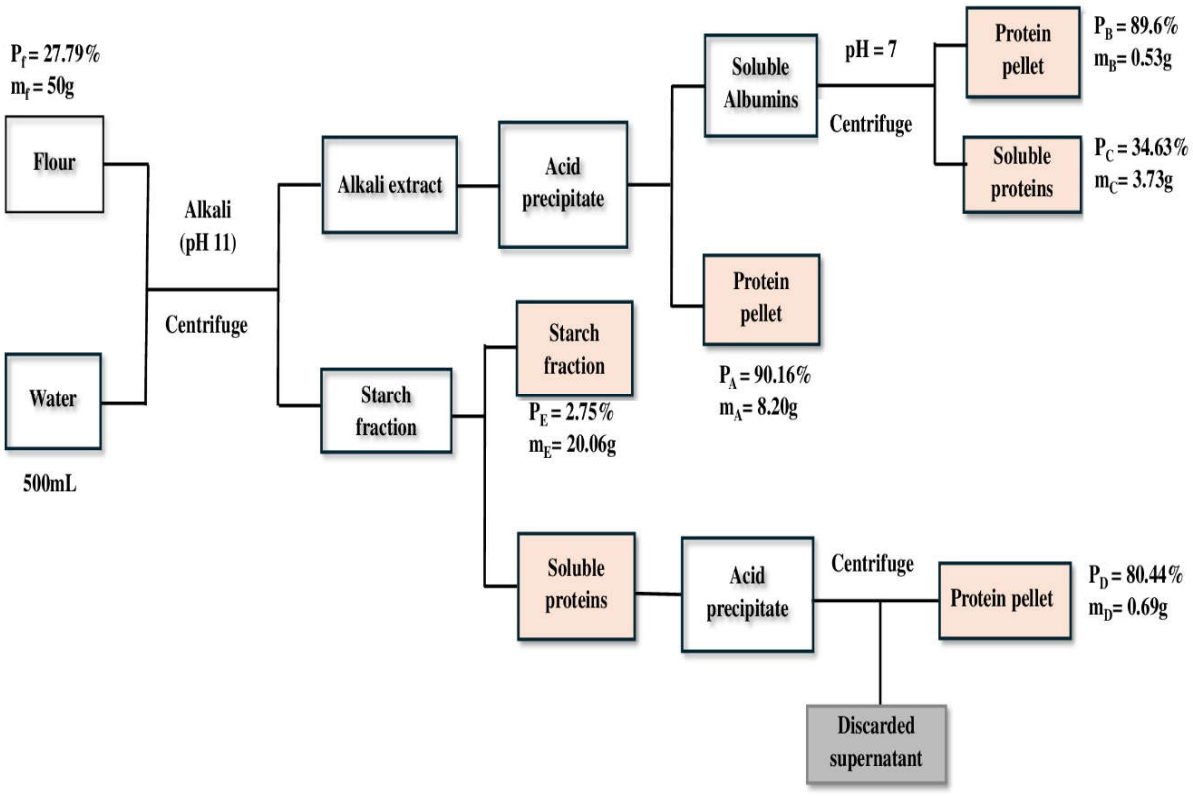

Figure 4. Schematic representations of the faba bean protein isolation process showing mass balance and protein balance.

Qualitative Analysis of Proteins Using Electrophoresis (SDS-PAGE)

The primary structure of FBPI and the side stream recovered were analyzed by the patterns of reducing SDS-PAGE (**Figure 5**). Bands of the different proteins generated(A-D) showed different protein profiles. A similar SDS PAGE profile was observed between A and D which is not surprising since D represents the remaining globulin fraction extracted from discarded starch fraction. Protein bands ranging from 260–8 kDa were observed in A and D. Both A & D were extracted using Ip of 4.0 thus mostly composed of globulin fraction, hence the similarity in SDS band. Major bands were observed at 15, 30-38, 50, 60-70, 90, and ~ 125 kDa for samples A and D. Smearing bands were also observed from 260–125 kDa. Samples B and C represent albumin-soluble fractions that are usually discarded after protein precipitation. From **Figure 5.** major differences in protein profile were observed between fractions B and C despite being from the same albumin solutions. Sample B showed a protein band from 8~90 kDa while sample C showed a major band at only ~8 and 90 kDa. Additionally, minor bands were observed between 70-80 kDa and 30 kDa. Sample B contained a high protein content as compared to sample C which may indicate the presence of major protein bands. Comparing all fractions, globulin fraction (A and D) and albumin fraction (B and C) showed differences in protein profile, however, albumin fraction B showed some similarity in bands to that of globulin fractions. Interestingly, there was a band observed between 25–30 kDa in fraction B which was not found in any other fraction. Under the reducing conditions of SDS-PAGE, vicilin was found in its dissociated subunits of ~33–35 kDa (α and β) and 47–50 kDa (α, β, and γ), while legumin was observed in its acidic (α: 40 kDa) and basic (β: 20 kDa) subunits [24]. The observed band of ~70 kDa was presumed to correspond to convicilin [25]. Bands around 14 kDa have been reported to be a mixture of albumins and prolamins [26]. Overall, globulin fractions (A and D) showed a different profile compared to albumin fractions (B and C).

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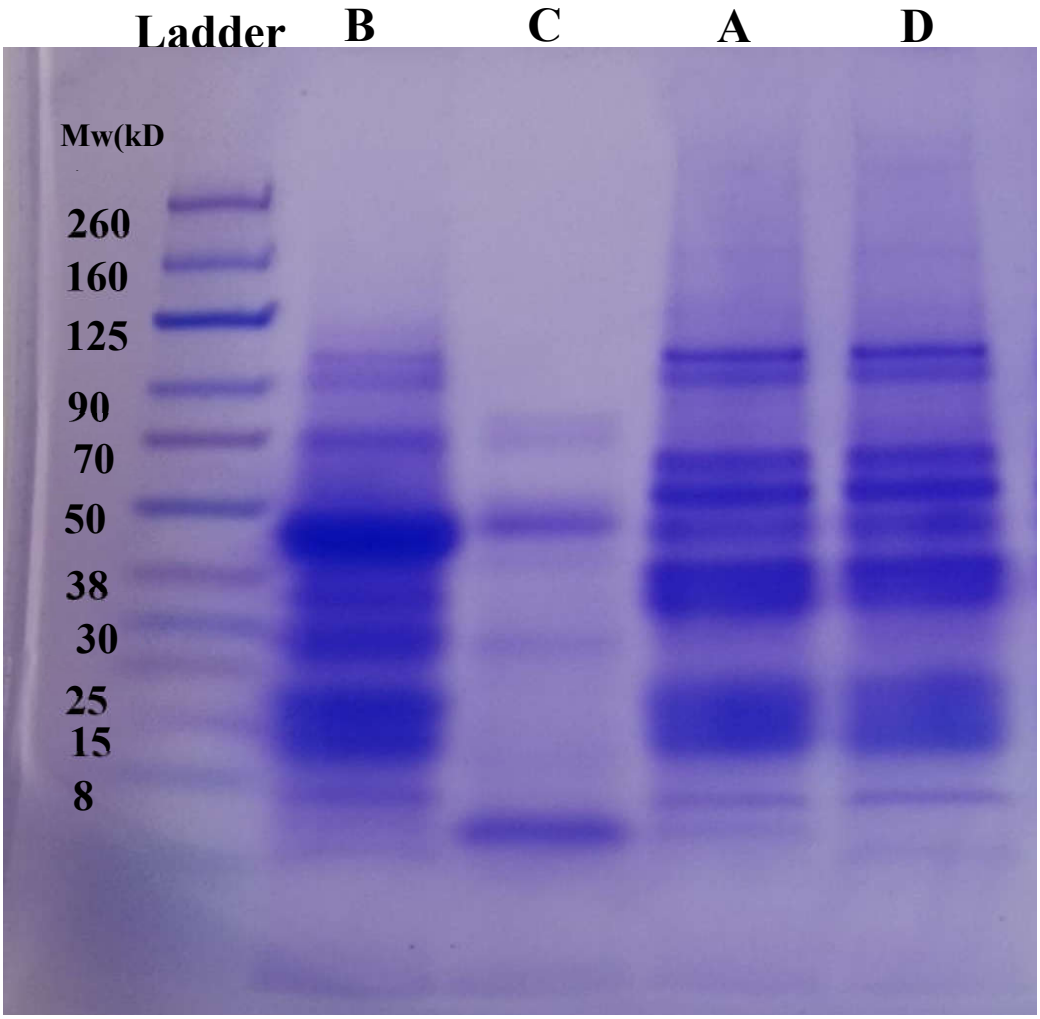

Figure 5. SDS-PAGE gel visualization of the different protein fractions under reducing conditions.

Functional Properties

Water and Oil Holding Capacity

Water holding capacity (WHC) is defined as the quantity of water that can be absorbed per gram of protein. This is the affinity of water to bind to the proteins via electrostatic interactions and are related to protein structure and the hydrophilic groups readily present to interact with water while oil holding capacity (OHC) represents the quantity of oil that can bind 1g of protein [27]. Both WHC and OHC are affected by several factors such as surface hydrophobicity, protein composition, particle size and processing conditions [12]. Understanding the water and oil holding capacities of ingredients is particularly important in developing novel food products such as plant-based meat analogues, egg, and yogurt alternatives. The ability to retain these fluids plays a crucial role in developing the desired juiciness, while preventing liquid separation, which could negatively impact visual and sensory appeal [28,29]. The OHC of protein ingredients is of great interest for food applications, as it is reflected in the emulsifying capacity, which is relevant for products such as mayonnaise. From **Figure 6.A**, major differences in WHC were observed among all of the different fractions. Faba bean flour showed low WHC compared to protein samples except for C. The highest water absorption of samples A and B as well as sample D could be attributed to the high amounts of protein content. However, sample C showed a negative effect on WHC and therefore is not suitable for food applications due to its high solubility in water. Comparable OHC results were evident as shown in **Figure 6.B.** The highest OHC was observed in starch fraction E (1.8±0.04 g/g) and protein fraction D (1.6± 0.12g/g). Protein isolates A, B, and C showed OHC values of 1.44±0.01, 1.40±0.01, and

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1.22±0.10 g/g respectively. Raw faba bean flour was observed to have an OHC of 1.29±0.25 g/g. This indicates the possibility of incorporating these side streams usually regarded as waste in different food systems to achieve a circular economy.

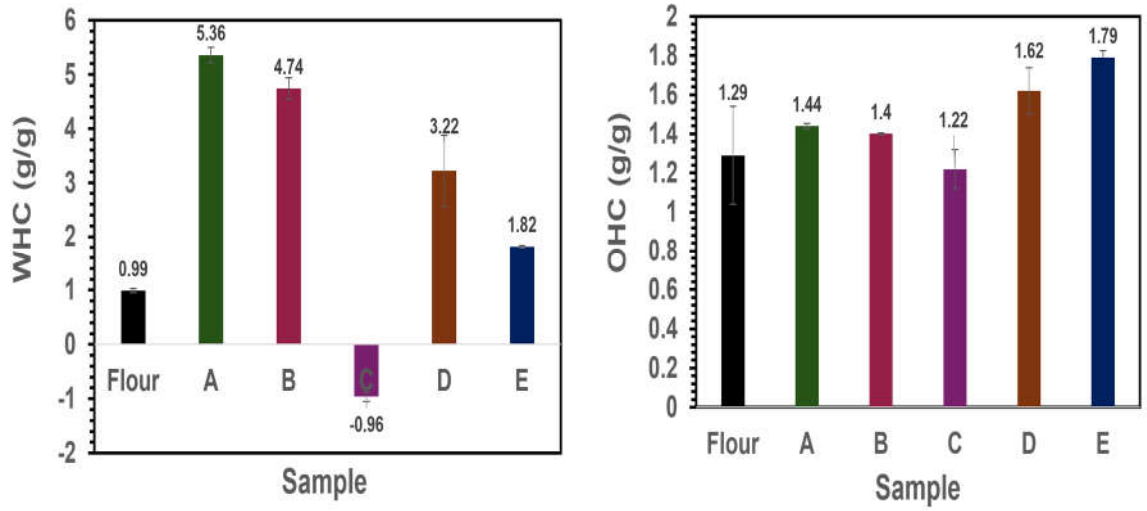

Figure 6. Water (A) and oil (B) holding capacity of different protein fractions from faba bean flour.

ATR-FTIR Spectroscopy

Average spectra were acquired which show the characteristic band distribution of different fractions (A-E) (**Figure 7.**). In general, high absorbance was observed in the regions 1500 –1700 cm^{-1} (Amide I and II region) [30] and 2700–3500cm^{-1} (lipid and carbohydrate regions) [31] , moderate absorbance at 1200–950 cm^{-1} (carbohydrate band) [32] and relatively low average absorbance at 950–700cm^{-1} and 1200-950cm^{-1} (**Figure 7.A**). All spectra of fractions A-E showed major peaks in the Amide I, II, III, A, and B. Similar peaks were also observed in the starch fractions (fraction E) though it had relatively low protein content compared to the other protein fractions. The modes most widely used in protein structural studies are amide I, amide II, and amide III (**Figure 7.B**). Major differences among the fractions can be observed in the protein amide regions and fingerprint region 1200-700cm^{-1}. Starch fraction E from **Figure 7.B** showed a poor absorption peak in this region due to relatively low protein content. However, observing the fingerprint region 1200–700cm^{-1}, starch fraction E showed relatively high absorption peaks in the region 1200–950cm^{-1}. Similar high peaks were observed for peak Fraction B-D, though fraction A was low due to high protein content. Closely observing the fingerprint region 1200-700cm^{-1} clearly shows similar peaks and intensity between fractions C and E. This may be due to low protein content and possibly the presence of carbohydrates and lipids in these two fractions. Fractions A, B, and D had similar peaks in the fingerprint regions mainly due to high protein content and less amount of residual compounds. When looking at the carbohydrate region for the different extraction fractions from 1150–1000cm^{-1} (**Figure 7.C**), Fractions A and B showed low levels of absorbance, but they were more distinct for fractions C, D, and E. This region may be useful for differentiating these fractions. Conformational differences in both protein regions and fingerprint regions could be attributed to changes in micro-environment [33] of extracted fractions and different protein components.

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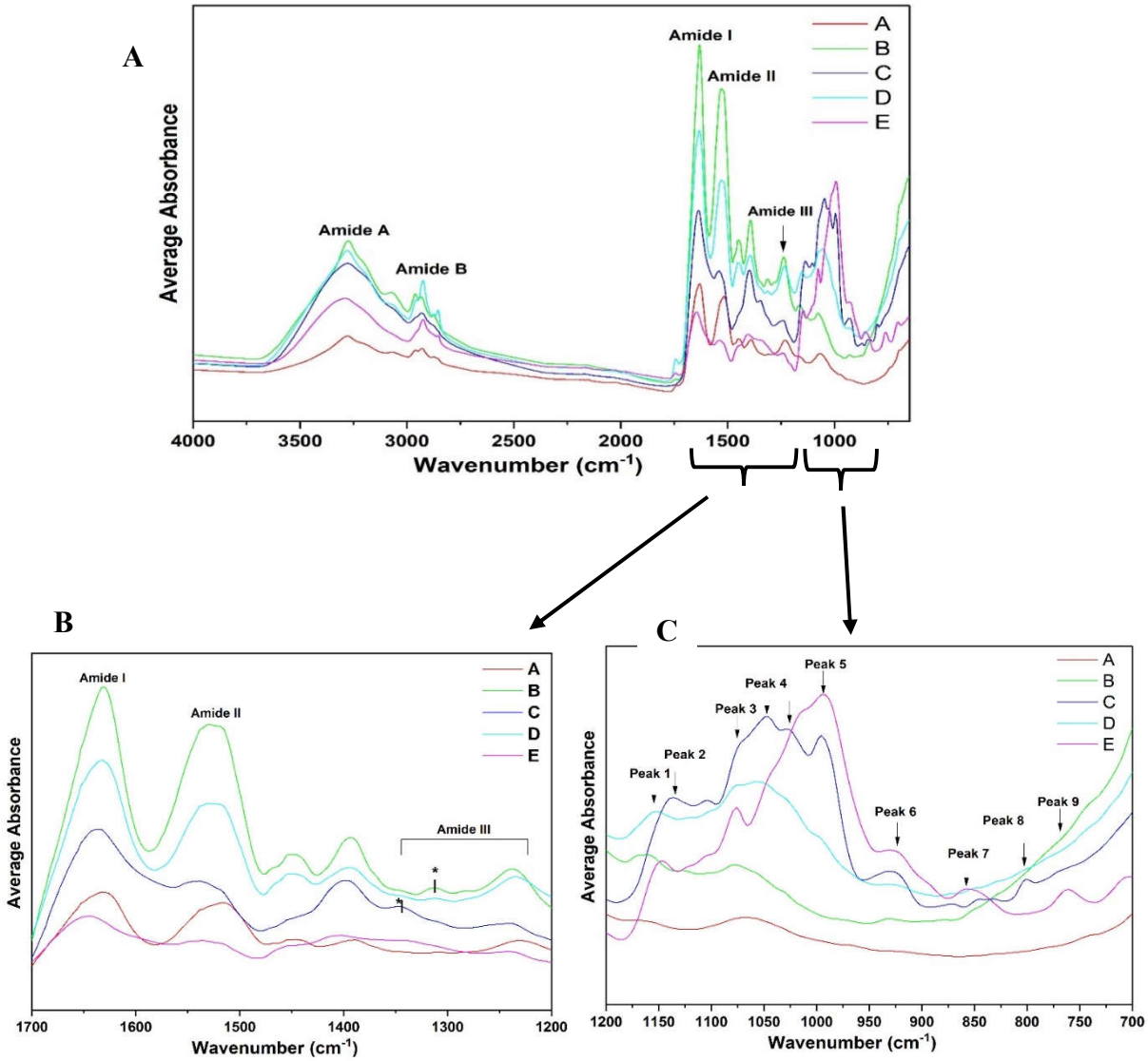

Figure 7. FTIR spectra of protein samples extracted from faba beans and their side-streams (a) original spectra (n=3) (b) Amide I-III region 1700–1200cm $^{-1}$ (c) fingerprint region 1200-700cm $^{-1}$.

Qualitative Analysis of ATR-FTIR Spectra: Amide I Region

For the structural characterization of these intermediate steps in the extraction process, ATR-FTIR spectroscopy was employed. Given the sensitivity of infrared absorption to protein conformation, the amide regions of the infrared spectra serve as a valuable tool for both qualitative and quantitative assessment of protein secondary structures. To fully exploit the potential of ATR-FTIR spectra, each amide region was individually analysed for all obtained fractions.

Analysis of all spectra indicates notable absorption at higher wavenumbers, particularly in the Amide I region (1600–1700cm^{-1}), which exhibits the highest sensitivity to conformational changes among all amide regions. In contrast, variations in the adjacent Amide II and III regions appear to be less influenced by secondary structure content. The Amide I region primarily originates from C=O stretching vibrations and out-of-phase CN stretching vibrations within the polypeptide backbone [34,35]. Upon protein extraction, due to differences in protein content and secondary structure changes, spectral differences are very pronounced. **Figure 8** shows the overlaid average spectra of protein fraction A and other recovered fractions B-E in the amide I region. The spectra of fractions A, B, C and D display prominent peaks around 1640 and 1620 cm^{-1} while a minor peak was observed between 1660–1640 cm^{-1}. Observing the Amide, I region, each fraction spectra induced spectral changes. There is a shift in the peak absorption maxima from 1640–1620cm^{-1}. The average magnitude of the absorption of fraction A was lower compared to fractions B, C, and D. Generally, shifts in

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Amide I spectra region and differences in magnitude of absorption could be attributed to the fact that fraction may be mainly composed of globulin fractions while the fractions B, C and D may be composed of albumin fractions.

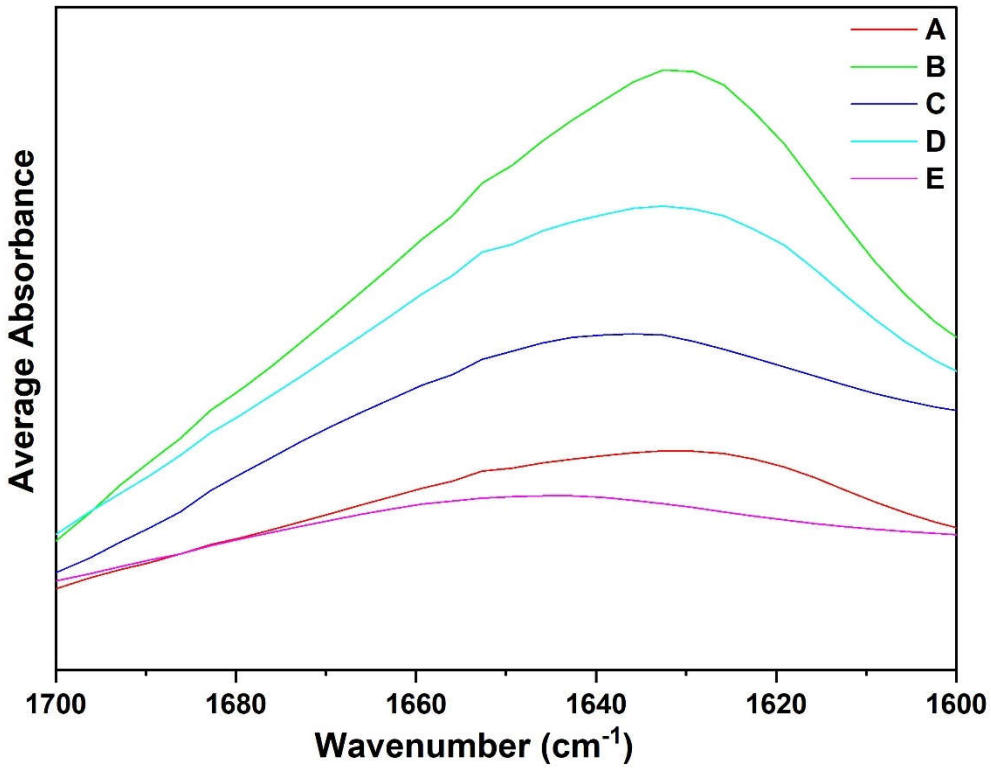

Figure 8. Amide I region of faba bean isolate (A) and different side streams (B-E) (n=3).

Qualitative Analysis of FTIR Spectra: Amide II Region

Regardless of complete perturbations in the Amide I region, faba bean fractions spectra in the Amide II conformational changes in the tertiary structure, suggesting that several amide NH groups are involved in strong hydrogen bonds and/or are buried within the hydrophobic protein core [36]. When comparing the different fractions, absorption maxima were observed around 1560–1500cm^{-1} for all samples except Fraction C and E which were mostly composed of starch and low protein. Observing fractions, A, B, and D which had high protein content shows major differences in the absorption maximum at this spectral region. Differences must be attributed to high globulin content in fraction A compared to the other fractions which may be mainly composed of albumin or other compounds. The results suggest that fractions A-D contained secondary structures with amide NH bond groups that are involved in stronger hydrogen bonds. Differences in spectra of all fractions reflect conformational variations in the tertiary structure among the samples.show major spectra differences from 1500–1600cm^{-1} due to differences in protein content and composition. The Amide II region, which primarily results from NH in-plane bending and CN stretching vibrations, exhibits significantly lower sensitivity to specific secondary structures compared to the Amide I region [2,37]. Due to its low sensitivity to secondary structure variations, the Amide II region is well-suited as an internal reference for comparing Amide I band intensities across different samples [38,39].

The spectral overlay for the amide II region, from 1600–1500cm^{-1}, has been provided for the different fractions extracted (A-E) (**Figure 9.**). Intensity changes in the amide II bands can reflect

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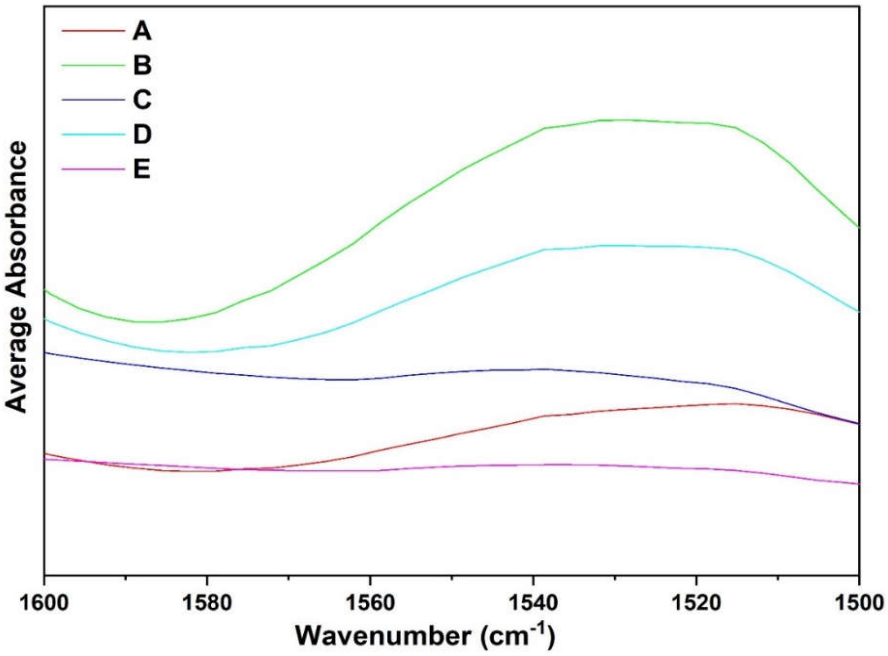

Figure 9. Amide II region of faba bean isolate (A) and different side streams (B-E) (n=3).

Qualitative Analysis of ATR-FTIR Spectra: Amide III and A-B Region

The Amide III region is generally considered a less sensitive region within protein infrared spectra. Its bands primarily originate from NH bending and CN stretching vibrations, which exhibit some degree of conformation dependence [34]. Structural modifications influenced by variations in extraction conditions, protein content, and chemical composition also led to notable changes in this region.

The spectra shown in **Figure 10** were analysed based on the Amide III band position ranges, as extensively studied by Cai et al., (1999). The Amide I region (1700–1600 cm^{-1}) is widely used due to its strong signal; however, it has limitations, including water interference, an unstructured spectral contour, and overlapping bands from various secondary structures. In contrast, the Amide III region (1300–1200 cm^{-1}), though weaker in intensity, is free from these limitations [41]. In recent years, several researchers have used amide III region to determine protein structures [33,42]. Observing **Figure 10.A**, the Amide III spectra can differentiate between all the different fractions. A major peak was found in the region 1260 and 1225cm^{-1}, however, this was less pronounced in fraction C and E due to their comparatively low protein content compared to fractions A, B, and D. Additionally, fraction A showed a lower absorption rate maxima compared to B and D. **Figure 10.B** shows differences in Amide A and B regions of the different fractions obtained.

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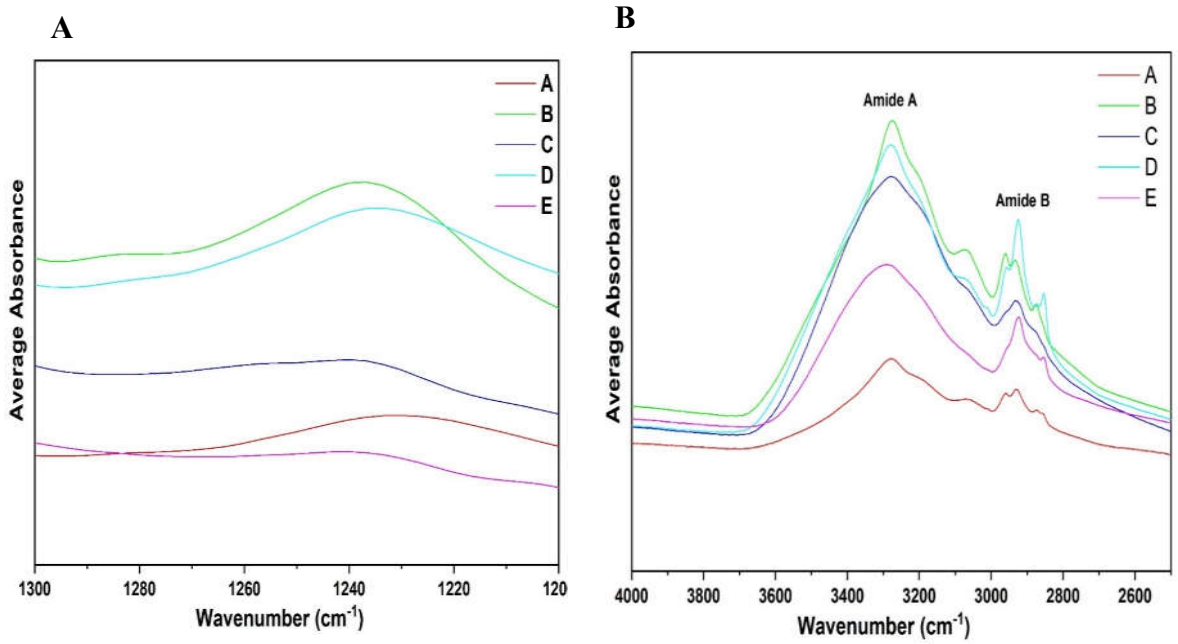

Figure 10. Amide III region of different extracted fractions (A) and (B) Amide A-B regions (n=3).

Conclusion

A sustainable food production system requires valorisation of side streams which have potential for different applications in the food, pharmaceutical, and cosmetic industries. Large scale of production of protein ingredients generates a significant volume of such by-products. In this study, we explored the maximisation of the traditional alkaline-isoelectric process to obtain valuable side streams which are usually discarded. The results show the possibility of recovering lost proteins with a reasonable amount of protein content using different process conditions. Additionally, ATR-FTIR was applied to monitor discrete compositional changes of each fraction that was obtained. Structural differences were observed in the Amide I, II, III, A, and B regions between fractions. SDS-PAGE analysis revealed, different protein profile bands for all protein fractions. Differences in functional properties such as water and oil holding capacity were observed for the individual fractions' indicative of the usefulness of specific side streams for specific food applications.

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**.**

Authorship contribution statement: Abraham Badjona: Investigation, Writing – review & editing, Data curation, Formal analysis, Methodology, Writing – original draft. **Robert Bradshaw:** Methodology, Supervision, Writing – original draft, Writing – review & editing. **Caroline Millman:** Supervision, Writing – review & editing. **Martin Howarth:** Supervision, Writing – review & editing. **Bipro Dubey:** Conceptualization, Data curation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Data Availability Statement: The data generated during the current study are available upon reasonable request.

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