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Dynamic in-vitro system indicates good digestibility characteristics for novel upcycled plant protein; correlation to techno-functional properties

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ABSTRACT

An increasing global population demands the broadening of the plant-based protein portfolio. The high volume of by-products produced across the food industry presents the opportunity to reduce food waste while reclaiming valuable nutrition. The current study examines a novel protein, BRP, extracted from brewers spent grains regarding its techno-functionality and digestibility, in comparison to a variety of proteins including whey, soy, rice and two pea protein sources. Compositional, foaming, emulsifying, structural and rheological properties were examined, while digestion kinetics were determined using the dynamic tiny-TIM in vitro digestion model. BRP performed similarly to whey protein in many respects, demonstrating a high solubility, high nitrogen bioavailability ($>$90%) and comparable N digestion kinetics, however BRP exhibited no rheological changes over heating. The digestible Indispensable amino acid score for BRP was 67% with lysine as the sole limiting amino acid, a higher value than soy, rice and one of the pea proteins investigated.

Industrial relevance: The production of a protein ingredient from a plentiful and otherwise low-value food processing by-product presents an opportunity for an increased shift towards more sustainable protein sources for the food industry. This protein displays enhanced nutritional characteristics when compared to variety of plant protein sources, and promising functional properties such as a high solubility. This study offers an examination of a novel, upcycled protein ingredient as a potential tool for food manufacturers in the shift towards a more sustainable and secure food future.

1. Introduction

A growing global population, against the background of an increasingly unstable food system, presents a considerable challenge for the food industry. As of 2022, progress towards eliminating world hunger and malnutrition has been limited, with ~30% of the global population experiencing food insecurity (FAO, IFAD, UNICEF, WFP, and WHO, 2022). In addition to this, a growing population with finite resources demands the re-thinking of our current food systems. It is evident that the shift towards a more sustainable food system is mandatory, and that this must occur sooner rather than later. One way in which this issue can be addressed is through the increased revalorisation of food processing side-streams (Pereira & Prieto, 2020). The brewing industry in particular produces large volumes of by-products, namely spent grain, spent yeast and rootlets (Jaeger, Arendt, Zannini, & Sahin, 2020; Jaeger, Zannini, Sahin, & Arendt, 2021; Neylon et al., 2020). These by-products have a high nutritional value, particularly with regards to protein and fibre, with recent studies highlighting the potential of these ingredients in bakery applications (Neylon, Arendt, Zannini, & Sahin, 2021; Neylon, Nyhan, Zannini, Sahin, & Arendt, 2023). Further to this, the production of commonly used protein isolates also creates significant volumes of side streams such as starches and fibres, each presenting their own challenges with regards to incorporation into human nutrition. Therefore, the valorisation of existing food processing by-products may be one

way to tackle the move towards more sustainable food systems and increase the variety of food ingredients available to manufacturers.

Functionality is a major factor in determining the success of a commercial ingredient. Generally, food producers will favour a low-cost material that performs according to the needs for specific applications e.g. protein solubility, oil and water binding, emulsion stabilisation and sedimentation behaviour among others all give a practical overview of each protein ingredient and its potential use in product formulation. As the protein transition should not compromise human health, an exploration of protein quality is also essential. It is well known that many plant-based protein sources do not reach the recommended level of all essential amino acids, in particular sulphur containing amino acids in legumes and lysine in cereal-based proteins. It has also been shown that plant-based proteins are generally less digestible than their animal-based counterparts, due to variety of factors including the increased presence of antinutritional compounds. Protein quality can be assessed using a parameter such as the digestible indispensable amino acid score, or DIAAS, where the true bioavailability of amino acids is compared to a reference standard as defined by (FAO, 2013). Protein bioavailability is ideally determined using in-vivo trials in human or animal models; however such studies are invasive, expensive, and require ethical approval. In-vitro systems, such as the tiny-TIM (TNO Gastro- Intestinal Model) system used in the current study, offer accurate and reliable alternatives to animal and human studies (Minekus, 2015).

The current study examines a novel food ingredient isolated from brewers spent grain, comparing it to a variety of protein ingredients currently widely utilised in food manufacturing with regards to protein digestibility and amino acid bio-accessibility while also exploring protein functionality. While studies on protein ingredients of this kind have been limited, particularly regarding digestibility characteristics, recently published data shows that spent grain protein exhibits comparable post-prandial total amino acid uptake to pea protein, with a lower lysine and higher tryptophan level respectively (Ummels et al., 2023). The aim of this study is to provide an overview of how a novel protein isolate may be used in the food industry in place of, or in addition to protein ingredients which are already widely used, providing food manufacturers more opportunity to shift towards a more sustainable food future.

2. Materials and methods

2.1. Materials

The protein isolates used in this study were sourced commercially (Table 1).

2.2. Compositional analysis

Compositional analysis was carried out externally by Chelab S.r.l. (Resana, Italy), using the following methods: Moisture, fat, sugars and ash were quantified using the internal methods reported in (*RAPPORTI ISTISAN,* 1996) based on AOAC and ISO standards; total starch was

Table 1
Summary of commercial protein isolates.

Ingredient Description	Abbreviation	Source
Everpro®- Spent grain (barley-rice) protein isolate	BRP	EverGrain Ingredients, MO, USA
Pisane C9- Pea protein isolate	PPI (P)	Cosucra, Warcoing, Belgium
Provon 190 – Whey Protein Isolate	WPI	Glanbia Nutritionals, Kilkenny, Ireland
Supro 661 G – Soy Protein Isolate	SPI	International Flavours and Fragrances, NY, USA
Brown Rice Protein Isolate	RP	Axiom Foods, CA, USA
Nutralys S85 Plus N – Pea protein isolate	PPI (N)	Roquette Freres, Lestrem, France

determined using the Megazyme kit K-RAPRS (Megazyme, Bray, Ireland); The amino acid composition was determined externally by Chelab S.r.l. (Resana, Italy) using ion chromatography with post-column derivatization with ninhydrin, or HPLC-UV analysis in the case of tryptophan. Protein content was taken as the sum of individual anhydrous amino acid residues.

2.2.1. Calculation of conversion factors

The conversion factor K_p was calculated as defined by Mossé (1990) using the following equation:

$$kp = \frac{\Sigma Ei}{N} \tag{1}$$

Where E_i is the sum of anhydrous amino acid residues and N is the total nitrogen value as determined by the Kjeldahl method.

2.3. Techno-functional properties

2.3.1. pH and total titratable acidity (TTA)

The pH and TTA of each ingredient as dissolved in water was determined using a pH meter (Mettler Toledo, Ohio, United States) as described by Jaeger, Sahin, Nyhan, Zannini, and Arendt (2023) and the results are presented as mL of 0.1 M NaOH/g of sample.

2.3.2. Foaming capacity and foam stability

Foaming properties of samples at an aqueous concentration of 2% *w/w* powder were examined as described in Alonso-Miravalles et al. (2019). Briefly, samples were frothed using an Ultraturrax T10 (IKA Labortechnik, Germany) with S10N-10G dispersing element, at maximum speed for 30 s and the height of the foam phase was measured. Sample expansion was calculated using the following equations:

$$Foaming\ capacity\ (\%) = \left(\frac{Foam\ height\ immediately\ after\ foaming}{Initial\ sample\ height} \right) \times 100 \tag{2}$$

$$Foam\ stability\ (\%) = \left(\frac{Foam\ height\ after\ 1\ hour}{Foam\ height\ immediately\ after\ foaming} \right) \times 100 \tag{3}$$

2.3.3. Protein profile analysis

The protein profile of all samples was determined by SDS – PAGE under reducing conditions using the NuPAGE Bis- Tris mini gels (4–12% 1 mm 12 well) (Invitrogen, Thermo Fisher Scientific, CA, USA). Protein was extracted at 2% (*w*/w) from each sample using an extraction buffer consisting of 5 M urea, 2 M Thiourea, 1 mM EDTA, 2% SDS and 0.1 M Tris base, adjusted to pH 8.8 using 3 M HCL, using 1.75% 2-Mercaptoethanol (BME). Samples were shaken at 500 rpm for 16 h at room temperature followed by centrifugation at 14,680 rpm for 20 min after which an aliquot was mixed with sample buffer and heated at 70 °C for 10 min. Precision plus Protein^{tm} dual xtra Prestained standard (BioRad, CA, USA) ladder with a size range of 2–250 kDa was prepared. 20 μL of diluted ladder and sample were loaded into the gel and ran for 35 min at 200 V constant. Following the completion of electrophoresis, gels were rinsed and fixed (40% methanol, 10% acetic acid) for 30 min while shaking, stained (0.025% Coomassie G250) for 20 min and destained (10% acetic acid) until background colour was removed.

2.3.4. Minimum gelation concentration

Minimum gelation concentration of each protein was determined according to the method of Alonso-Miravalles et al. (2019), with some modifications as described by Jaeger et al. (2023). Briefly, samples (15 mL) were prepared at a variety of concentrations (in the range of 6% to 30%, in 2% intervals). The samples were then adjusted to pH 7 using varying concentrations (0.01 M–2 M) of HCl and NaOH, and hydrated overnight at 4 °C. Samples were heated at 90 °C in a water bath for 30

min, cooled rapidly on ice, and maintained overnight at 4 °C. The samples were then inverted, and the minimum protein concentration at which the dispersion did not flow in <30 s was determined as the minimum gelling concentration.

2.3.5. Rheological characteristics

Rheological tests were carried out using a controlled stress rheometer (MCR301, Anton Paar GmbH, Austria) equipped with a concentric cylinder measuring system (C-CC27-T200/SS, Anton Paar GmbH, Austria) according to the method of Vogelsang-o'Dwyer et al. (2020). Sample concentrations were decided based on the minimum gelling concentrations of each ingredient i.e. PPI (P): 12%, RP: 18%, SPI: 14%, WPI: 10% and PPI(N) 26%. Since no minimum gelling concentrations were determined for BRP, BRP was tested at the minimum gelling concentrations for WPI (10%) and PPI (N) (26%).

2.3.6. Sulfhydryl groups

Exposed, free and total sulfhydryl (SH) groups were determined using Ellman's reagent [5,5′-dithio-bis-(2-nitrobenzoic acid)] according to the method of Alonso-Miravalles et al. (2019) with some adjustments. Protein samples of 75 mg were extracted overnight at 4 °C in 10 mL Tris-Glycine buffer at pH 8 consisting of 0.1 M Tris, 0.1 M glycine and 4 mM EDTA for exposed SH groups with the addition of 8 M Urea to Tris-Glycine buffer for free and total SH groups. To determine exposed and free SH groups, 80 μL of Ellman's reagent was added to 2.5 mL of diluted sample, including a reagent blank. Sample blanks were prepared by replacing Ellman's reagent with the respective Tris-Glycine buffer. Samples were developed at room temperature for 15 min, centrifuged at 4000 rpm for 15 min at 22 °C and absorbance was measured on a spectrophotometer at 412 nm. For total SH groups, 4 mL Tris-Glycine buffer with urea and 50 μL BME was added to 1 mL diluted protein sample and shaken at 500 rpm at room temperature for one hour. This was followed by the addition of 10 mL 12% trichloroacetic acid (TCA) to the sample solutions and a one hour shaking period. To remove the BME the supernatant was discarded, and the pellet resuspended twice in 12% TCA followed by centrifugation at 4893 *xg* for 15 min at 22 °C. The pellet was resuspended in 10 mL Tris-Glycine buffer containing 8 M urea and total SH content was determined following the same procedure as exposed and free SH groups, instead using half the volume of Ellman's reagent and double the volume of diluted sample with no centrifugation step before reading the absorbance. The content of SH groups was calculated as follows:

$$\mu M \frac{SH}{g} protein = \frac{(\,(\,A\,412 - A\,412\,a - A\,412\,b)*1000000/\varepsilon\,)}{C} \quad (4)$$

Where A_{412} is the absorbance at 412 nm, A_{412a} and A_{412b} are the absorbance values for the blank and reagent blank, respectively, ε is the extinction coefficient which was taken as 13,600 $M^{-1}\,cm^{-1}$ (Shimada & Claude Cheftel, 1988) and C is the protein concentration in mg/mL of the diluted sample.

2.3.7. Surface hydrophobicity

Surface hydrophobicity was measuring using 1-anilinonaphthalene-8-sulphonate as a fluorescent marker, as described in literature (Li et al., 2018; Vogelsang et al., 2022). Sample dispersions were prepared and analysed according to the method described by Alonso-Miravalles et al. (2019). Briefly, protein dispersions were serially diluted with 10 mM phosphate buffer (pH 7) in the range of 0.0006–0.015% (*w/v*). Then, 8-Anilino-1-naphthalenesulfonic acid ammonium salt (ANS)(10 μL; 8.0 mM in 0.1 M phosphate buffer, pH 7) was mixed with 2 mL of diluted sample and stored in the dark for 15 min at room temperature. Fluorescence was measured (λ excitation 390 nm, λ emission 470 nm) and corrected using blanks, sample dilutions measured without the addition of ANS. The results are presented as the slopes (R2 ≥ 0.98) of the absorbance versus protein concentration (% w/v).

2.3.8. Water and oil holding capacity

Water holding (WHC) and oil holding (OHC) capacity, was determined using the method described by Boye, Aksay, Roufik, Ribéreau, Mondor, and Farnworth (2010), with slight modifications. Briefly, 1 g of ingredient was mixed with 6 g of either deionised water or sunflower oil. The sample mixtures were allowed stand for 1 h for sufficient uptake of water or oil following centrifugation at 4000 rcf at 20 °C for 30 min. The supernatant was then carefully removed before being inverted for 30 min to allow sufficient drainage. WHC/OHC (%) was calculated using the following formula:

$$\frac{WHC}{OHC}(\%) = \frac{(Weight\ of\ tube + pellet) - (Weight\ of\ empty\ tube)}{Weight\ of\ ingredient\ input} \times 100\%$$

(5)

2.3.9. Protein solubility

Protein solubility was determined at various pH values according to Alonso-Miravalles et al. (2019) with modifications. Sample dispersions of 1% (*w/w*) protein were prepared in distilled water followed by pH adjustment to pH 3, 5 or 7 using varying concentrations (0.01–1 M) HCl and NaOH. The dispersions were shaken at 500 rpm overnight at 4 °C. The pH was readjusted if necessary and then centrifuged at 4000 rpm for 30 min at 20 °C. The protein content in the supernatant was measured using the Kjeldahl method AACC Method 46–12 (AACC International, 2011) and calculated based on nitrogen to protein Kp conversion factors for each individual ingredient. Protein solubility was expressed as the % of protein remaining in the supernatant.

2.3.10. Emulsifying characteristics

Emulsion stability was measured as described by Vogelsang O'Dwyer, Sahin, and Arendt (2021) using an analytical centrifuge (Lumisizer, GmbH, Berlin, Germany), with parameters as defined by Jaeger et al. (2023). Emulsions of 1% (*w/v*) protein were prepared with ultrapure water with the addition of 10% sunflower oil. Results are given as separation rate (%/min) of individual emulsions and light transmission profiles.

2.3.11. Colour

The Commission Internationale de L'Eclairage (XYZ) values were measured to obtain the Hunter colour system CIE (L* a* b*) values of each ingredient as described by Jaeger et al. (2023). The whiteness index (WI) was calculated according to the following equation (Boeck et al., 2022):

$$WI = 100 - \sqrt{\left((100 - L^*)^2 + a^{*2} + b^{*2}\right)} \quad (6)$$

2.3.12. Ultrastructure

Scanning electron microscopy (SEM) was used to examine the ultrastructure of all samples according to the method reported by Atzler, Sahin, Gallagher, Zannini, and Arendt (2021). The following settings were applied for the analysis: 5 kV voltage, 20 mm working distance and a magnification factor of 1000.

2.4. Digestion kinetics

2.4.1. In-vitro digestion simulation

Adult gastrointestinal conditions were simulated using the tiny-TIM model (Havenaar et al., 2013, 2016), an in-vitro model with the ability to assess true ileal digestibility as well as the bioaccessibility of nitrogen and amino acids. This was completed externally by The TIM Company and their analytical lab partners Ducares-Triskelion and BioAnalytiX.

The tiny-TIM model consists of a gastric compartment and one small intestinal compartment connected by a peristaltic valve (pyloric sphincter). It simulates very closely the successive dynamic conditions in the upper gastrointestinal tract, such as body temperature, pH curves, concentrations of electrolytes, digestive enzymes. The enzymes and bile

used were sourced from Sigma-Aldrich NV (Zwijndrecht, The Netherlands). Specifically, α-amylase from *Bacillus* sp., pepsin from porcine gastric mucosa, lipase from *Rhizopus oryzae*, and porcine bile were utilised in this study. The set-points for gastrointestinal simulation were controlled and monitored by specific computer programs. During the experiments, the digested and dissolved molecules were dialyzed from the intestinal lumen through a semipermeable membrane unit (Fresenius FX-5 dialyser, Fresenius Medical Care, Bad Homburg, Germany) with a cutoff of 5–7 kDa connected to the small-intestinal compartment. This allowed sampling during the digestion process and the assessment of the bioaccessible fraction in the dialysate. The dialysate represents the bioaccessible fraction of the digestate, determined by the ability of the nutrients to pass from the lumen by a semi-permeable membrane unit.

Total gastric intake was 150 g, with 7 g of sample per run in combination with water, simulated saliva and gastric start residue. For the blank experiments, 15 g of citrate buffer was included to replace the sample. The conditions simulated were the standard post-meal conditions of an average, healthy human adult (Table 2). A total of 10 samples were collected per tiny-TIM run: 7 dialysate samples collected in 15-,30-, and 60-min aliquots (i.e., at 0–15 min, 15–30 min, 30–60 min, 60–120 min, 120–180 min, 180–240 min, 240–300 min), 1 gastric residue (including rinse) sample, 1 intestinal residue (including rinse), and a pooled dialysate sample (0–300 min). The digestion of each sample and the blank was performed in duplicate, resulting in 14 individual tiny-TIM runs.

2.4.2. Analysis of dialysate samples

Each of the collected samples, as well as the initial isolates, were analysed for total nitrogen concentration using the Kjeldahl method (ISO 5983-2). The initial protein isolates and the final pooled dialysate (0–300 min) were analysed for their content of 9 essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) and 2 conditionally essential amino acids (tyrosine and cysteine) using LC-MS/MS, and UPLC in the case of tryptophan, for calculation of digestible indispensable amino acid score (DIAAS). This was performed after hydrolysis with 6 M HCl, and Ba(OH)$_2$ in the case of tryptophan. For the determination of cystine and methionine the procedure described is proceeded by oxidation with performic acid for 16–20 h. Alpha-amino nitrogen was determined by UV spectroscopy at 405 nm, using 2,4,6- trinitrobenzen-sulphonic acid (TNBS) as a derivatising agent.

True ileal digestibility is calculated by correcting the absolute amount of nitrogen or individual amino acid recovered from the TIM samples for the absolute amount of the blank samples, relative to the exogenous input:

$$True\ ileal\ digestibility\ (\%) = \frac{\sum A\ dialysate - \sum A\ blank}{A\ exogenous\ input}.100 \quad (7)$$

The digestible indispensable amino acid (DIAA) reference ratio of the essential amino acids was calculated using the sum of each amino acid content in the pooled fraction (0–300 min), and the digestible indispensable amino acid score (DIAAS) was calculated from the lowest

DIAA, according to the adult essential amino acid reference patterns (FAO, 2013).

$$DIAAS\ (\%) = \frac{limiting\ DIAA\ [mg]\ in\ 1\ g\ test\ protein}{limiting\ DIAA\ [mg]\ in\ 1\ g\ reference\ protein}.100 \quad (8)$$

2.5. Statistical analysis

All analyses were completed in triplicate unless stated otherwise. Results were tested for normality and a one-way ANOVA with post hoc Tukey test ($p < 0.05$) was performed using IBM SPSS version 26 (Armonk, NY, USA). When equal variances were not assumed, a correction using the Welch test and Games Howell post hoc test ($p < 0.05$) was applied. When data were not normally distributed a non-parametric Kruskal Wallis test ($p < 0.05$) was performed. Principal component analysis (PCA) was performed using Origin Pro 2023b (MA, USA), where surface hydrophobicity was excluded due to missing values.

3. Results

3.1. Compositional analysis

The compositional data of the protein isolates examined in this study are displayed in Table 3. BRP contained a high level of protein (79.29 g/100 g) when compared to the other plant-based samples (69.87–71.42 g/100 g), second only to WPI protein (90.42 g/ 100 g) and similar to SPI (76.19 g/100 g). BRP and WPI contained only trace levels of fat, while both of the pea proteins tested contained a higher amount with 8–9%. Examining carbohydrate composition, all samples contained minimal amounts of starch, with the exception of RP, although levels were still low. BRP contained a small amount of glucose and traces of fructose. RP and PPI (N) also contained glucose, but at lower levels. The sugar present in the highest amount throughout all the ingredients was sucrose in RP. Sucrose was also present in PPI (P), SPI, and PPI (N) but at significantly lower levels. The primary sugar present in WPI was lactose, with none of the other screened sugars detected. Maltose was only present in small amounts in RP and PPI (N). Among the six samples, BRP, PPI (P) and PPI (N) contained similar levels of ash (~5%), while the other samples contained 2.5–3%. All ingredients contained similar levels of sodium (1–1.5%) with the exception of WPI protein with a lower level (0.29 g/100 g).

3.2. Physical and techno-functional properties

3.2.1. pH and total titratable acidity (TTA)

The pH and TTA are presented in Table 4. All pH values are within the range of 6.31–8.06 with WPI and RP exhibiting the lowest and highest pH values, respectively. BRP and WPI displayed similar TTA values at 2.44 mL and 2.39 mL of 0.1 M NaOH/g. RP presented the lowest TTA result at 0.25 mL 0.1 M NaOH/g, followed by PPI (P), SPI and PPI (N) respectively.

3.2.2. Foaming properties

Foaming capacity and foam stability of all the protein ingredients are presented in Table 4. WPI and BRP displayed high foaming capacities (144% and 139%, respectively), and low foam stabilities of 41% and 45%, respectively. Both pea derived proteins, PPI (P) and PPI (N), showed similar foaming behaviour with low foaming capacities (36.13–40.15%) and relatively high foam stabilities (88.89–98.04%). SPI also follows this trend with a foaming capacity of 41% and a stability of 99%. RP falls between these two extremes with a foaming capacity of 91% but possesses the lowest foam stability of 39%.

3.2.3. Surface hydrophobicity

The surface hydrophobicity of the samples is also presented in

Table 2
Overview of parameters utilised in the TinyTIM in-vitro gastrointestinal model.

Stomach	
Intake (total)	150 g
Gastric half emptying time (t1/2)	50 min
Gastric pH	6.5 to 2.0 in 90 min
Enzymes	Amylase, lipase, pepsin
Small Intestine	
Small intestinal pH	6.5
Bile Secretion	
Pancreatic enzymes secretion	Containing trypsin, amylase, lipase, protease
Temperature	37 ± 1 °C
Experiment duration	5 h

Table 3

Compositional analysis of all protein isolates, expressed as g/100 g as mean ± standard deviation on "as-is" and dry matter basis. <LoQ indicates that the obtained result was below level of quantification for the method of analysis, *Protein value calculated using individual amino acid residues; SD calculated using Gaussian error propagation.

	BRP		PPI (P)		RP		SPI		WPI		PPI (N)	
	g/100 g as-is	g/100 g DM	g/100 g as-is	g/100 g DM	g/100 g as-is	g/100 g DM	g/100 g as-is	g/100 g DM	g/100 g as-is	g/100 g DM	g/100 g as-is	g/100 g DM
Moisture	7.23 ± 0.26		5.19 ± 0.19		3.81 ± 0.14		5.21 ± 0.19		3.80 ± 0.14		7.26 ± 0.26	
Protein	79.29 ± 2.45	85.47 ± 2.64	69.87 ± 2.07	73.69 ± 2.18	71.42 ± 2.03	74.25 ± 2.11	76.19 ± 2.23	80.38 ± 2.35	90.42 ± 2.71	93.99 ± 2.82	69.63 ± 2.07	75.08 ± 2.23
K_P	6.19		5.78		5.85		5.82		6.65		5.93	
Fat	0.07 ± 0.03	0.08 ± 0.04	8.92 ± 0.54	9.41 ± 0.57	1.95 ± 0.12	2.03 ± 0.13	3.47 ± 0.21	3.66 ± 0.22	<LoQ	<LoQ	7.99 ± 0.48	8.62 ± 0.52
Carbohydrate												
Starch	<LoQ	<LoQ	<LoQ	<LoQ	3.98 ± 0.11	4.13 ± 0.11	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ
Sugars												
Glucose	0.436 ± 0.033	0.470 ± 0.036	<LoQ	<LoQ	0.143 ± 0.013	0.149 ± 0.014	<LoQ	<LoQ	<LoQ	<LoQ	0.030 ± 0.007	0.032 ± 0.008
Fructose	0.088 ± 0.009	0.095 ± 0.009	<LoQ	<LoQ	0.045 ± 0.007	0.046 ± 0.008	<LoQ	<LoQ	<LoQ	<LoQ	0.084 ± 0.009	0.091 ± 0.009
Lactose	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	<LoQ	0.247 ± 0.033	0.257 ± 0.034	<LoQ	
Sucrose	<LoQ	<LoQ	0.270 ± 0.027	0.285 ± 0.028	4.900 ± 0.480	5.094 ± 0.499	0.214 ± 0.022	0.226 ± 0.023	<LoQ	<LoQ	0.192 ± 0.020	0.207 ± 0.022
Maltose	<LoQ	<LoQ	<LoQ	<LoQ	0.202 ± 0.031	0.210 ± 0.032	<LoQ	<LoQ	<LoQ	<LoQ	0.123 ± 0.020	0.133 ± 0.022
Ash	5.04 ± 0.34	5.43 ± 0.37	5.00 ± 0.34	5.27 ± 0.36	2.76 ± 0.19	2.87 ± 0.20	3.46 ± 0.23	3.65 ± 0.24	2.68 ± 0.18	2.80 ± 0.19	4.94 ± 0.33	5.33 ± 0.36
Sodium	1.31 ± 0.24	1.41 ± 0.26	1.58 ± 0.29	1.67 ± 0.31	1.06 ± 0.20	1.10 ± 0.21	1.02 ± 0.19	1.08 ± 0.20	0.29 ± 0.05	0.31 ± 0.06	1.30 ± 0.24	1.40 ± 0.26

Table 4

Functional and physical properties of protein isolates. Results are expressed as Mean ± standard deviation. Results contained in the same row with the same letter marking do not differ significantly.

	BRP	PPI (P)	RP	SPI	WPI	PPI (N)
pH	7.40 ± 0.01 [a]	7.50 ± 0.02 [b]	8.06 ± 0.01 [e]	6.83 ± 0.02 [d]	6.31 ± 0.02 [c]	7.08 ± 0.02 [f]
TTA (mL 0.1 M NaOH/g)	2.44 ± 0.03 [a]	0.81 ± 0.01 [b]	0.25 ± 0.01 [e]	1.74 ± 0.25 [d]	2.39 ± 0.01 [c]	2.12 ± 0.01 [f]
Foaming Capacity (%)	138.79 ± 3.06 [a]	36.13 ± 3.18 [b]	91.01 ± 7.79 [c]	41.21 ± 1.84 [b]	143.94 ± 3.47 [a]	40.15 ± 1.31 [b]
Foaming Stability (%)	44.60 ± 1.10 [a]	98.04 ± 3.40 [b]	38.83 ± 2.10 [a]	99.89 ± 5.72 [b]	41.08 ± 2.32 [a]	88.89 ± 9.62 [b]
Surface hydrophobicity (AU)	n/a	6816.33 ± 548 [a]	6040.86 ± 384 [a]	6434.33 ± 418 [a]	1516.33 ± 151 [b]	7951.00 ± 387 [c]
Minimum gelling concentration (%)	n/a	12	18	14	10	26
Water holding capacity (%)	n/a	394.16 ± 6.38 [a]	201.98 ± 2.05 [c]	310.67 ± 3.49 [b]	n/a	98.17 ± 1.42 [d]
Oil holding capacity (%)	236.65 ± 1.46 [a]	79.40 ± 7.80 [b]	86.98 ± 1.18 [bd]	84.83 ± 0.80 [bd]	23.20 ± 1.60 [c]	90.80 ± 2.37 [d]
Separation rate	2.86 ± 0.11 [a]	0.97 ± 0.02 [b]	2.43 ± 0.25 [d]	1.82 ± 0.05 [c]	2.87 ± 0.11 [a]	0.90 ± 0.01 [e]
Whiteness index (%)	58.17 ± 0.92	67.26 ± 0.46	59.71 ± 1.16	77.75 ± 1.21	87.67 ± 1.00	56.81 ± 0.99
Exposed SH groups (μM/g protein)	0.41 ± 0.02 [a]	0.07 ± 0.05 [b]	1.69 ± 0.03 [e]	3.63 ± 0.13 [d]	16.60 ± 0.02 [c]	11.14 ± 0.13 [f]
Free SH groups (μM/g protein)	0.17 ± 0.01 [a]	0.67 ± 0.12 [a]	1.25 ± 0.09 [c]	3.47 ± 0.58 [c]	21.66 ± 0.21 [b]	10.93 ± 0.25 [d]
Total SH groups (μM/g protein)	4.17 ± 1.04 [a]	76.29 ± 1.49 [be]	81.37 ± 4.00 [e]	71.29 ± 2.59 [bd]	93.15 ± 2.10 [c]	66.26 ± 4.79 [d]
Protein solubility at pH 3 (%)	91.05 ± 4.51 [a]	13.05 ± 0.75 [b]	11.88 ± 0.25 [b]	23.58 ± 1.15 [c]	94.73 ± 1.25 [a]	29.22 ± 0.75 [d]
Protein solubility at pH 5 (%)	91.47 ± 0.50 [a]	4.56 ± 0.08 [b]	8.63 ± 0.50 [c]	4.62 ± 0.10 [b]	86.16 ± 1.97 [a]	27.57 ± 1.28 [d]
Protein solubility at pH 7 (%)	95.16 ± 1.84 [a]	30.64 ± 1.37 [b]	19.33 ± 0.99 [c]	30.89 ± 0.94 [b]	97.82 ± 0.45 [a]	44.89 ± 0.76 [d]

Table 4. PPI (P), RP and SPI exhibited similar results (6040–6816 A.U.) whereas PPI (N) had the highest hydrophobicity of all ingredients with a value of 7951. In contrast to this, WPI exhibited the lowest hydrophobicity with a value of just 1516 A.U. It was not possible to determine a result for BRP.

3.2.4. Water and oil holding capacity

As shown in Table 4, water holding capacity could not be determined for BRP and WPI due to their high solubility in water. PPI (P) showed the highest WHC at 394.16% followed by SPI, RP and PPI (N) at 310.67%, 201.98% and 98.17%, respectively. BRP had a significantly higher OHC than the other samples at 236.65%. PPI (N), RP, SPI and PPI (P) had OHC values of 90.8%, 86.98%, 84.83% and 79.40%, respectively, while WPI showed the lowest ability to absorb oil at 23.20%.

3.2.5. Emulsifying characteristics

The separation rates of all emulsion dispersions are shown in Table 4. WPI emulsion exhibited a separation rate of 2.87%/min, whereas PPI (N) emulsion separated more slowly at 0.9%/min. BRP emulsion also separated quickly with a separation rate of 2.86%/min, similarly to WPI. SPI and RP emulsions showed a separation rate of 1.82 and 2.43%/min, respectively. PPI (P) and PPI (N) both showed lower separation rates, indicating better emulsifying ability. Fig. 1 displays the light transmission curve of each sample, alongside sample cuvettes. Little to no sedimentation was observable in BRP (Fig. 1A) and WPI (Fig. 1E) while significant sedimentation can be seen in PPI (P) (Fig. 1B), RP (Fig. 1C), SPI (Fig. 1D) and PPI (N) (Fig. 1F).

3.2.6. Colour

Whiteness index (WI) of the ingredients is shown in Table 4. WPI displayed the highest WI (87.7 ± 1) whereas PPI (N) had the lowest
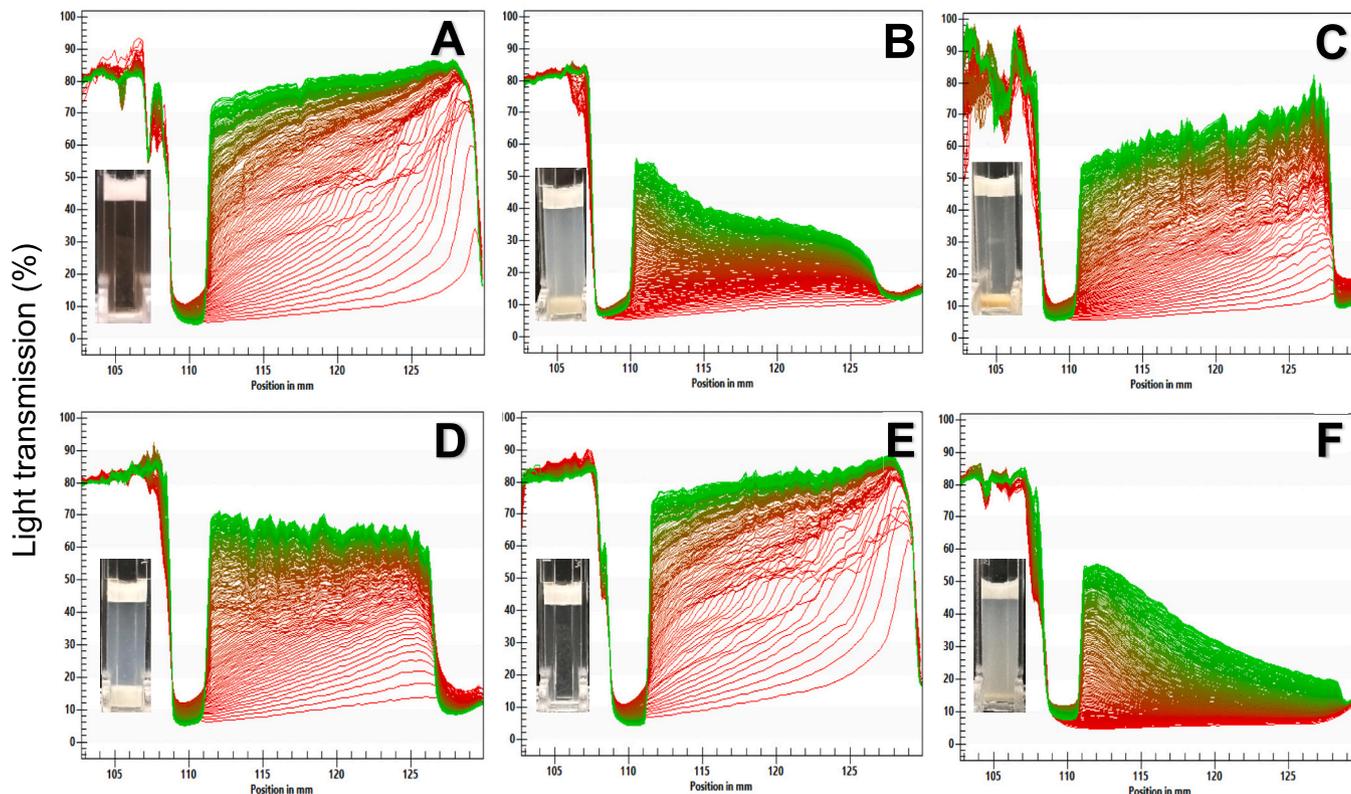
Fig. 1. Light transmission profiles of (A) BRP, (B) PPI (P), (C) RP, (D) SPI, (E) WPI and (F) PPI (N) emulsion as a function of time.

(56.8 ± 1). A pure white colour is represented by 100%. The whiteness index of the other ingredients was within the range of 56.81–87.67%.

3.2.7. Sulfhydryl groups

Exposed, free and total SH groups are shown in Table 4. WPI exhibited the highest concentration of exposed, free, and total SH groups

at 16.60 μM/g, 21.66 μM/g, and 93.15 μM/g of protein, respectively. BRP obtained the lowest concentration of free SH groups at 0.17 μM/g and total SH groups at 4.17 μM/g protein, while PPI (P) showed the lowest concentration of exposed at 0.07 μM/g. After WPI, total SH groups were found to be higher in RP (81.37 μM/g), followed by PPI (P) (76.29 μM/g) and SPI (71.29 μM/g) which gave similar values, and
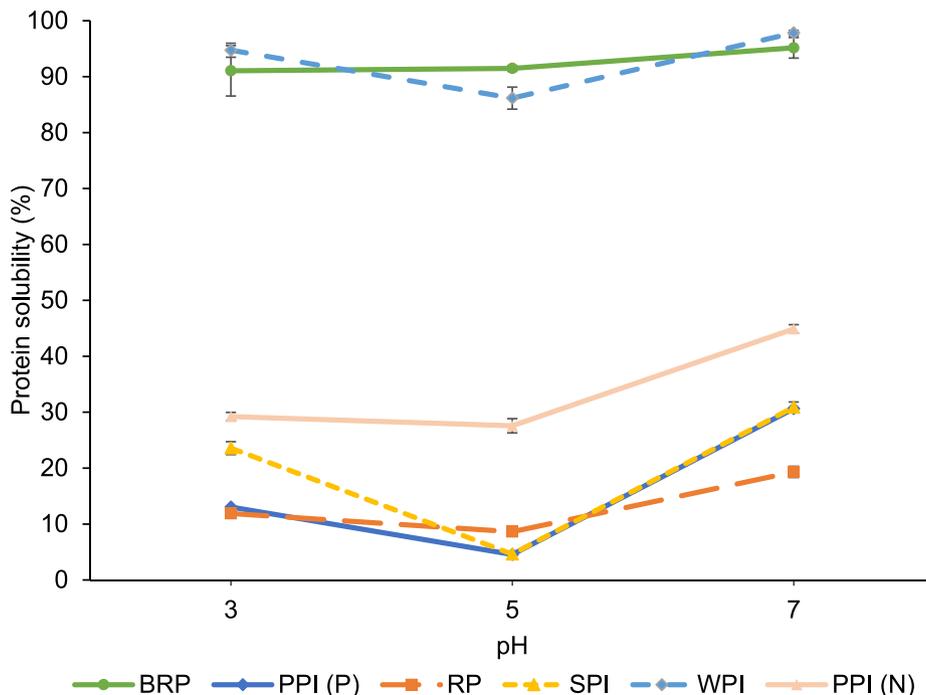

Fig. 2. Protein solubility of protein ingredients at pH 3, pH 5, and pH 7, expressed as % protein in supernatant over initial protein concentration.

lastly PPI (N) at 66.26 μM/g protein, respectively.

3.2.8. Protein solubility

Fig. 2 represents the protein solubility of all ingredients at pH 3, pH 5 and pH 7. BRP and WPI displayed consistently high solubility values across the tested pH range. At pH 7, WPI demonstrated the highest solubility (97.82%), followed by BRP with 95.16%. The lowest solubility for all ingredients was found at pH 5 with the exception of BRP at pH 3. PPI (P) and SPI showed similar results at pH 5, with solubility values of 4.56% and 4.62%, respectively.

3.2.9. Protein profile

Fig. 3 shows the electrophoretogram of all protein samples. Minimal bands are present in BRP, with the exception of a faint band at ~37 kDa. However, there is an increase in density of small peptides towards the lower molecular weights (<10 kDa), suggesting extensive protein hydrolysis, indicated by a deeper blue colour in this region. Both pea protein isolates exhibit similar band profiles, with some evidence of hydrolysis apparent in PPI (N) with increased density at lower molecular weights. Several bands present in PPI (P) are either less defined or absent in PPI (N). Similarly to PPI (P) and PPI (N), SPI displays significant bands at ~70 kDa, ~ 30 kDa and ~ 20 kDa. However, there is also a smaller band present at ~12 kDa. RP shows two significant bands at ~12–15 kDa while WPI exhibits well resolved bands at ~75 kDa and ~ 60 kDa, as well as bands between 15 and 20 kDa and at ~14 kDa. Significant protein aggregation is also visible in PPI (P), RP and SPI, with a lesser degree in PPI (N), as an accumulation in the gel well.

3.2.10. Viscoelastic behaviour

Fig. 4 shows the rheological behaviour of the protein isolates over a heating and cooling cycle. G', the storage modulus, represents the elastic (solid-like) response of a material while G", the loss modulus, represents the viscous (or liquid-like) response. BRP displayed unmeasurably low G' and G" values without change over the testing period. In pretrials, BRP was tested for gelling behaviour at concentrations up to 30% *w*/w.
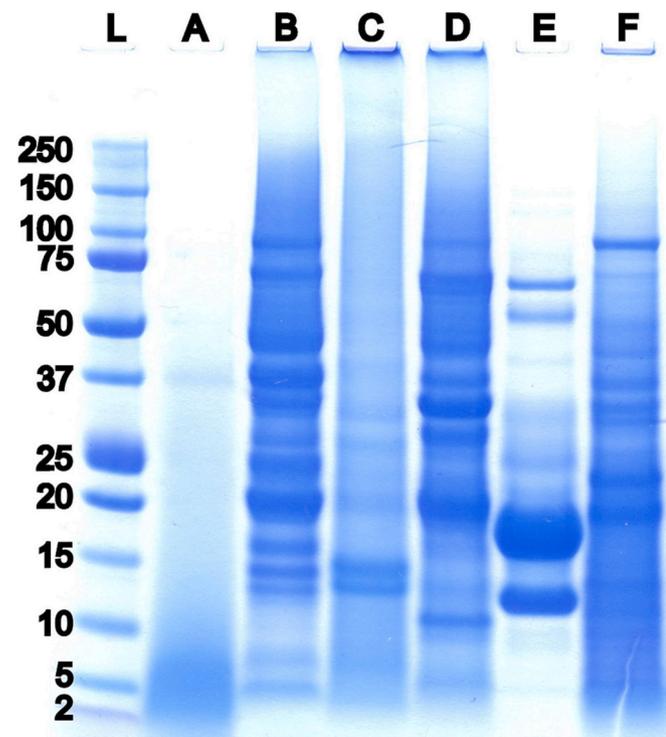

Fig. 3. SDS-PAGE electrophoretogram of BRP (A), PPI (P) (B), RP (C), SPI (D), WPI (E), and PPI (N) (F) in addition to reference ladder (L).

PPI (P) and SPI displayed similar behaviour over the cycle, with G' dominating over the length of the profile. Starting with a relatively high G', this then decreased significantly during heating followed by an increase upon cooling. RP followed a different pattern, with G' slowly increasing during heating, and further strengthening during cooling. WPI displayed the strongest gel forming behaviour, with G' gradually increasing during heating with a notable increase at the initiation of cooling. The crossover point of a material is described as the point at which G' crosses over G", indicating a dominance of elastic behaviour, also called "gel-point" (Shevkani, Singh, Chen, & Kaur, 2019). For WPI, this occurred at 32 min, when the sample had reached 83.5 °C. PPI (N) displayed vastly different rheological behaviour to PPI (P), under the applied conditions. In contrast, G" initially dominated, with the gel point occurring at 16 min, at a temperature of 47.5 °C. In this instance, PPI (N) had a profile most similar to that of WPI, with a gradual increase in G' during heating with a significant strengthening during cooling to give similar final G' values.

3.2.11. Ultrastructure

The ultrastructure of all protein ingredient powders was examined using scanning electron microscopy (SEM) at 1000 x magnification and is shown in Fig. 5. Each of the protein ingredients differ in morphology. BRP can be seen to have irregular shaped particles in globular forms in a variety of sizes with evidence of surface interruptions. PPI (P) presents larger spherical particles with dimple surfaces. WPI displaces smoother and rounder globular particles in various sizes, some with slight dents. SPI displays a range of different shapes and sizes of irregular particles. RP shows shrunken and wrinkled structures while PPI (N) displays irregular shapes and uneven surfaces.

3.3. In-vitro digestion model

3.3.1. True ileal digestibility

Cumulative true ileal digestibility for all protein ingredients is presented in Fig. 6. True ileal digestibility, as analysed in using the dynamic TIM model, represents the bioavailability of nitrogen (N) over time and allow the calculation of a single 'digestibility' value. As can be seen in Fig. 6, in the first 15 min of digestion, all samples, with the exception of PPI (N), have similar amounts of available N. This trend continues through to 30 min, with available N in PPI (N) lagging behind. Moving ahead to the 60-min timepoint, EP, PPI (P) and WPI begin to show increased N bioavailability. This trend becomes more pronounced throughout the remaining timepoints during digestion. From T120 onwards, BRP, PPI (P), WPI and PPI (N) show similar N bioavailability while digestion appears slower in RP and SPI. True ileal digestibility over the entire digestion period (0–300 min) is displayed in Table 5. PPI (P) displays the highest N digestibility (98.6%) followed closely by WPI (94.9%), PPI (N) (93.6%) and BRP (90.1%). Values are significantly lower for RP and SPI, 71.9% and 79.6% N bioavailability, respectively.

3.3.2. Protein quality – digestible indispensable amino acid score (DIAAS)

The digestible indispensable amino acid scores (DIAAS) for each protein ingredient are displayed in Table 5, alongside the corresponding limiting amino acid. DIAAS values were calculated according to Eq. (8), using true ileal bio-accessibility data for each individual essential amino acid (available as supplementary file) and reference values for an adult population. Due to the expected application of BRP in food for those above the age of 3 and the applied digestion parameters, adult DIAAS are seen as the most relevant for discussion. It can be concluded that RP and SPI contained the lowest levels of bio-accessible essential amino acids, which corresponds to both of these ingredients having low overall N digestibility, and low adult DIAAS of 38% in both cases. PPI (P), although having the highest N digestibility, is limited by the sulphur containing amino acids, giving a low DIAAS of 40%. The remaining protein ingredients display higher values, with BRP and PPI (N) displaying DIAAS of 67% and 68% respectively, the highest of the plant-
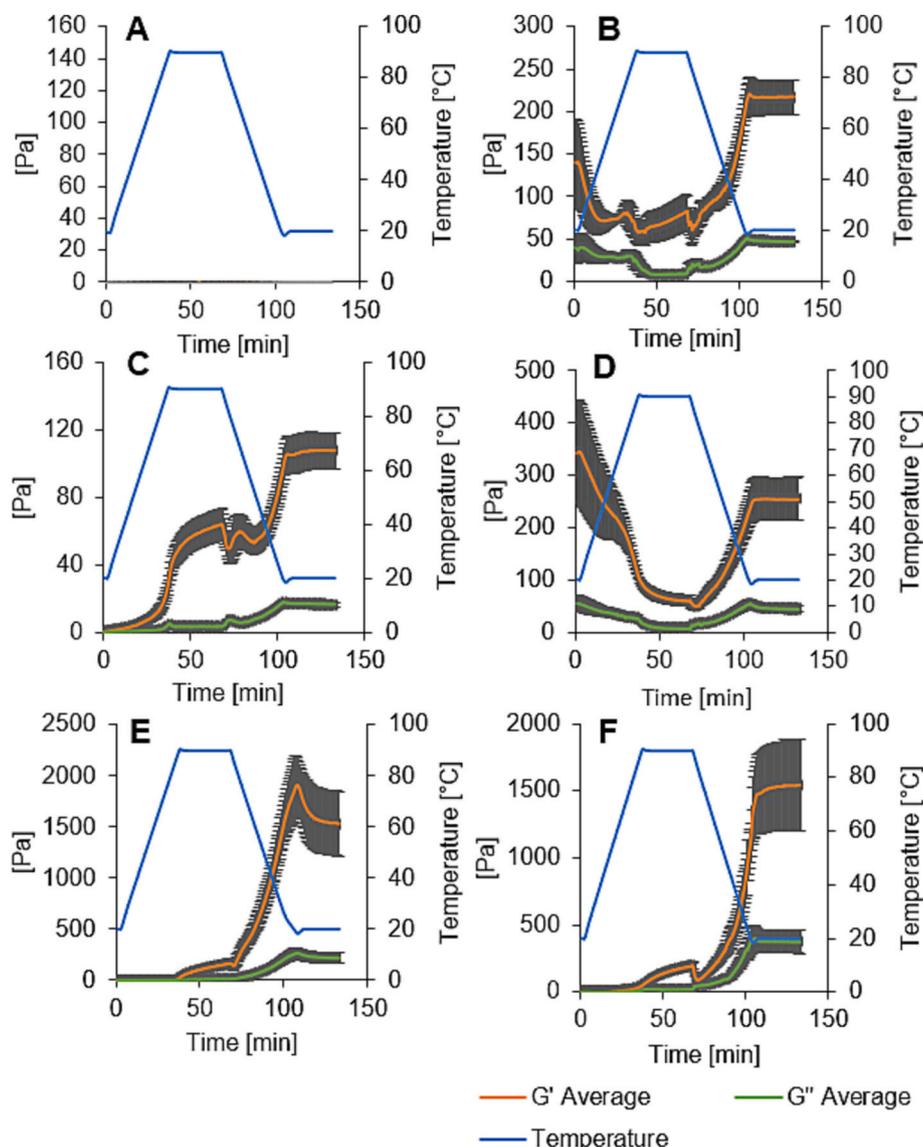
Fig. 4. - Rheological behaviour of BRP (A), PPI (P) (B), RP (C), SPI (D), WPI (E) and PPI (N) (F) during a heating and cooling cycle, including a 90 °C hold. Data is the averaged result of triplicate analysis, with standard deviation displayed in grey.

based protein sources examined in this study. WPI protein presents the highest DIAAS of 90%, with the limiting amino acid being histidine.

The digestible indispensable amino acid (DIAA) ratios of all samples are presented in Fig. 7. A 'good-quality' source is indicated by exceeding the threshold line at 0.75, as recommended in the FAO report on dietary protein quality (2013). A DIAA of >1 indicates a sufficient supply of the given amino acid. Regarding BRP, the DIAA exceeds 1 for all essential amino acids with the exception of the limiting amino acid lysine. This is similar for WPI and PPI (N), where the only essential amino acid not meeting the requirement of the reference pattern is the first limiting amino acid; histidine in the case of WPI, and the sulphur containing amino acids and tryptophan for PPI (N). However, in WPI histidine still falls above 0.75. At the other end of the spectrum, RP and SPI exhibit a DIAA below 1 for almost all essential amino acids, with many under 0.75. PPI (P) lies between these two extremes with only tryptophan falling short of the 0.75 benchmark, in addition to the limiting amino acids.

3.4. Principal component analysis

Principal component analysis was performed to identify correlations

within the obtained data (Fig. 8). PPI (P) and SPI are particularly clustered together. DIAAS is significantly positively correlated with protein solubility at all pH values (pH 3; *p*-value: 0.03, r-value: 0.91, pH 5; p-value: 0.01, r-value: 0.95, pH 7; p-value: 0.02, r-value: 0.94), as well as exposed (p-value: 0.00, r -value: 0.98) and free -SH-groups (p-value: 0.002, r -value: 0.98). Foaming capacity was found to be significantly positively correlated with separation rate (p-value: 0.03, r-value: 0.91), whereas it was positively correlated but not significantly to protein solubility at all pH values (pH 3; p-value: 0.11, r-value: 0.79, pH 5; p-value: 0.09, r-value: 0.81, pH 7; p-value: 0.17, r-value: 0.72).

4. Discussion

This study examines the nutritional and functional properties of six protein isolates; five from well-known and extensively studied sources (whey, pea, rice, soy), and one from a novel source, brewers spent grain. As expected of isolates, all ingredients contained a high level of protein. The accurate measurement of protein in foods is of crucial importance regarding determining ingredient value, as well as for the formulation of foods, particularly those intended for nutritionally vulnerable subgroups such as infants and the elderly. In an industrial setting, protein
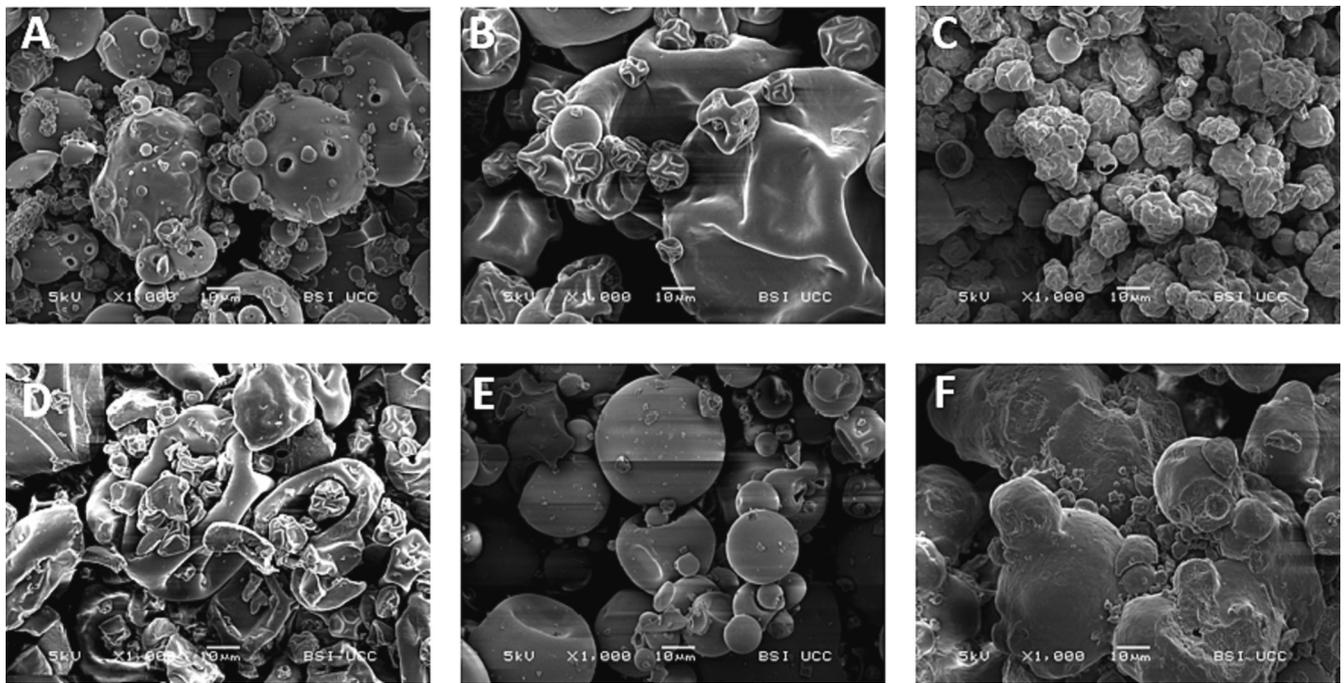
Fig. 5. Scanning electron microscope (SEM) imaging of BRP (A), PPI (P) (B), RP (C), SPI (D), WPI (E) and PPI (N) (F) at 1000× magnification.
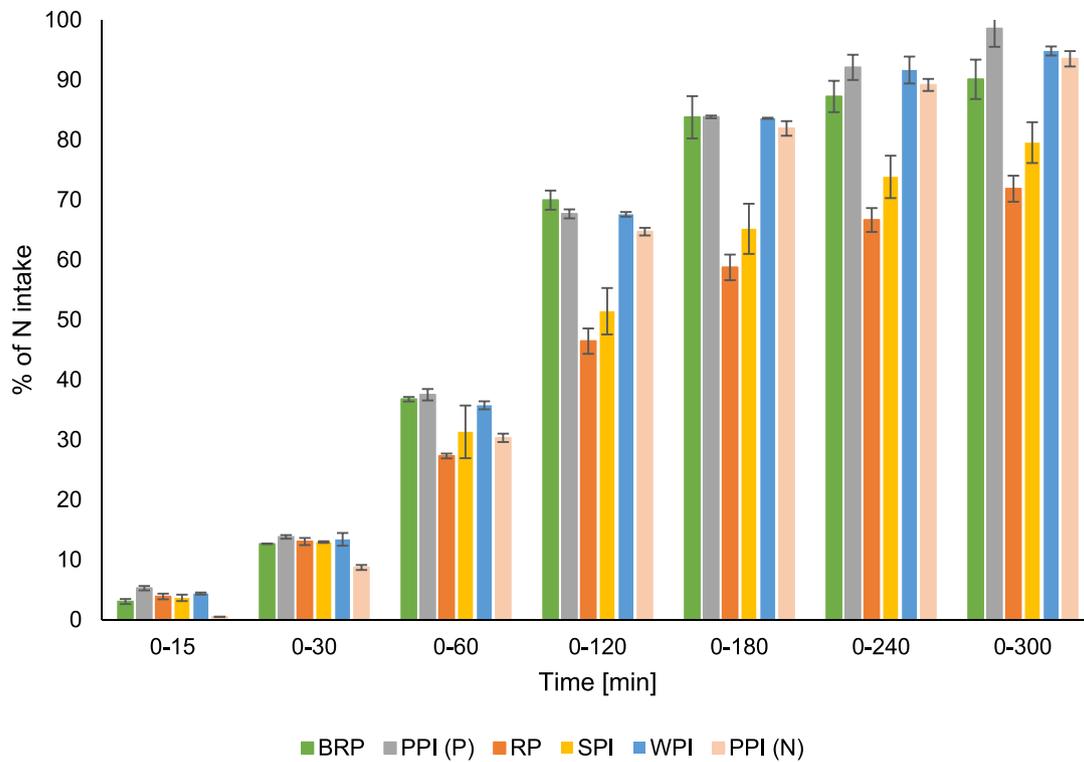

Fig. 6. Cumulative true ileal digestibility of nitrogen (blank corrected) for all test products of time during 5 h in tiny-TIMsg, expressed as the % of the exogenous nitrogen intake (corrected for nitrogen in gastric residue).

Table 5
The true ileal bio-accessible nitrogen digestibility and digestible indispensable amino acid score (DIAAS) calculated as described in Eq. 8, with limiting AA(s) indicated.

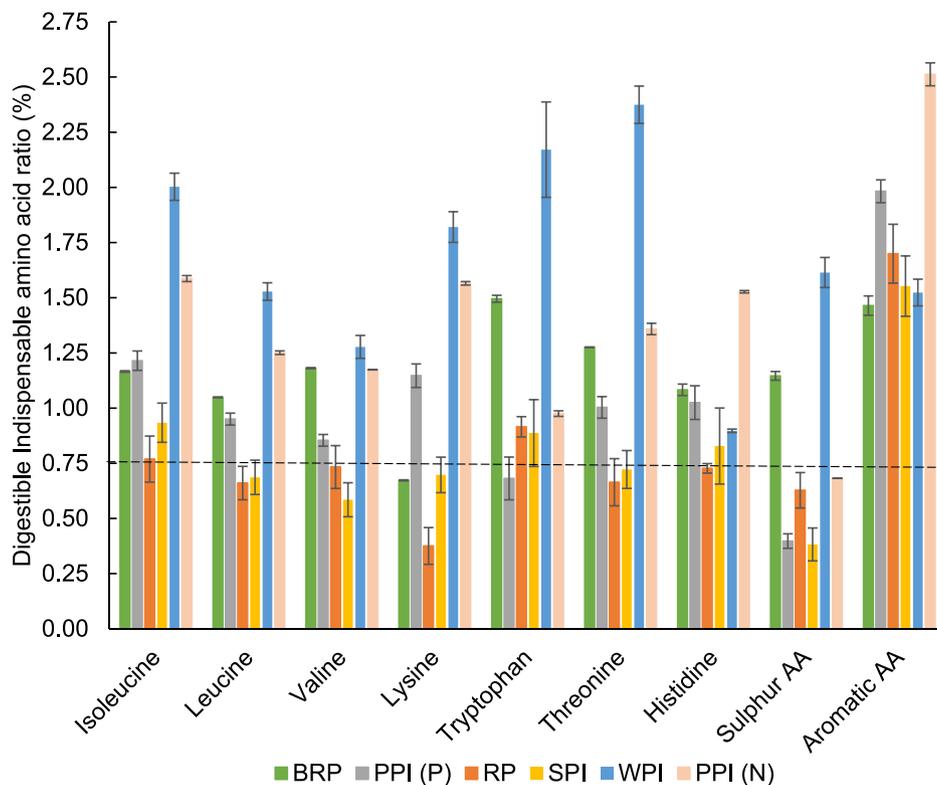|		BRP	PPI (P)	RP	SPI	WPI	PPI (N)
Digestibility (% bio-accessible N)		90.1 ± 2.3	98.6 ± 2.2	71.9 ± 1.5	79.6 ± 2.4	94.9 ± 0.5	93.6 ± 0.9
Adult (> 3 yrs)	DIAAS (%)	67.3 ± 0.23	39.8 ± 3.28	37.5 ± 8.34	38.3 ± 7.45	89.7 ± 0.78	68.2 ± 0.05
	Limiting AA	Lysine	Sulphur AA	Lysine	Sulphur AA	Histidine	Sulphur AA

Fig. 7. DIAA reference ratio over 5 h of digestion in tiny-TIM, line at 0.75 and indicates a 'good' content of the essential amino acid while great while >1 indicates a 'high-quality' protein. Calculated using >3 yrs. reference values (FAO, 2013).
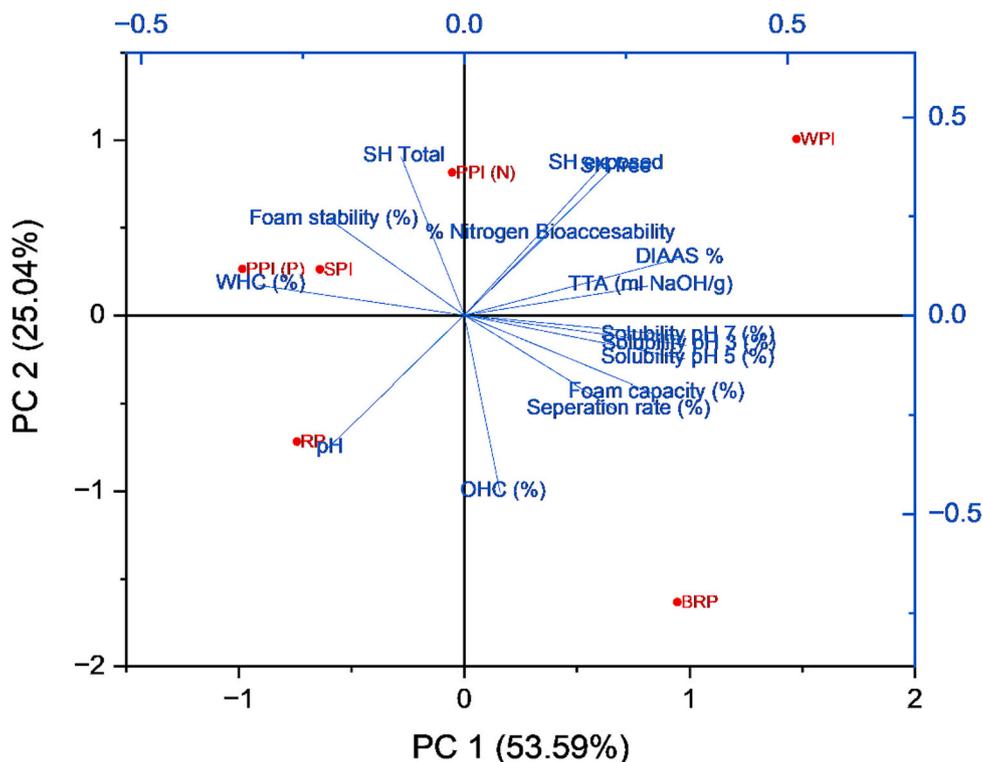

Fig. 8. Principal Component Analysis of techno-functional and digestion properties of protein ingredients.

is usually determined indirectly by initial nitrogen quantification followed by protein calculation using a conversion factor. While acceptable and generally used as standard, these methods are prone to protein overestimation due to the inclusion of non-protein nitrogen as well as the use of the common conversion factor 6.25 for many proteins. Nitrogen content of proteins alters drastically based on protein source, and

can even vary greatly within the same sources, depending on a wide variety of external factors. There also arises the issue of novel protein sources, such as BRP, where the suitable conversion factor is unknown, and research in this area is limited. In this study, protein content was determined by amino acid analysis as recommended by the FAO (FAO, 2003), and these values, alongside N as determined by the Kjeldahl method, were then used to calculate the conversion factor K_p (Mossé, 1990). For all except WPI, the calculated conversion factors are lower than the standard conversion factor of 6.25. Interestingly, for BRP the conversion factor is higher than for the other plant proteins. In addition, the conversion factor for BRP exceed that of its individual components, which can be found in literature as 5.5 and 5.17–5.26 for barley and rice respectively (Fujihara, Sasaki, Aoyagi, & Sugahara, 2008; Mossé, 1990).

Fat levels are low in all samples, with the exception of PPI (P) and PPI (N). Higher levels of lipids can negatively affect the foaming behaviour of proteins (Lam, Can Karaca, Tyler, & Nickerson, 2018), which can be observed in this study, with both pea proteins having the lowest foaming capacity. Differences in composition, including carbohydrates, ash, and sodium contents, can likely be attributed to differences in production processes, such as enzymatic treatments, filtrations, and precipitation steps. In particular, RP contains higher levels of starch and sucrose when compared to the other analysed ingredients. This may be due to reduced use of starch solubilising enzymes such as gluco-amylases and α-amylases during the extraction process (Jayaprakash, Bains, Chawla, Fogarasi, & Fogarasi, 2022), although the exact process is unknown.

Protein solubility is a key factor in protein digestion, as an increase in protein aggregation can inhibit proteolysis (Loveday, 2022). BRP displays a very high solubility ($>91\%$) across the pH range tested, as was also observed in a previous study (Jaeger et al., 2023). This could be due to the enzymatic hydrolysis treatment (Nyhan, Sahin, Schmitz, Siegel, & Arendt, 2023), as is also indicated by abundance of low MW peptides in SDS PAGE electrophoresis. In fact, the only visible band for BRP is at ~39 kDa, potentially identifiable as Protein Z, a major barley albumin known to be resistant to enzymatic degradation (Jaeger et al., 2021). Enzyme hydrolysis may also be responsible for the increased solubility of PPI (N) over PPI (P). Protein solubility was also high for WPI over the pH range, although there was a decrease at pH 5. This slight decrease may be due to β-lactoglobulin or α-lactalbumin, the major proteins in whey and the most abundant bands in Fig. 3, having an isoelectric point of ~ pH 5 (McSweeney & Fox, 2009; O'Mahony & Fox, 2014). A similar trend was observed for several of the other proteins, particularly SPI, where solubility reduced at pH 5, the isoelectric point lying at approximately pH 4–5 (Karaca, Low, & Nickerson, 2011; Lam et al., 2018; Liu, Elmer, Low, & Nickerson, 2010). PCA indicated that protein solubility is positively correlated with DIAAS, foaming capacity and separation rate.

Surface hydrophobicity can be linked to protein solubility, and therefore protein digestibility. A high degree of surface hydrophobicity can promote protein-protein interactions, increasing the tendency of a material to aggregate and potentially inhibit solubility (Carbonaro, Maselli, & Nucara, 2012; Shevkani et al., 2019). Inversely, a lower surface hydrophobicity promotes protein-water interaction, promoting solubility. Although it was not possible to obtain a valid result for BRP regarding hydrophobicity, from the obtained data it can be hypothesised that the surface hydrophobicity is negligible, likely due to the lack of suitable 8-anilino-1-naphthalenesulfonic acid (ANS) binding sites (Cardamone & Puritt, 1992), potentially as a result of protein degradation as was discussed in a previous study (Jaeger et al., 2023). A hydrophilic nature would promote protein solubility, as can be observed with BRP and WPI. Increased solubility may, in turn, enhance proteolytic access and therefore lead to increased digestibility. RP and SPI both have a relatively high surface hydrophobicity, low protein solubility and exhibit the slowest N digestibility, the lowest overall N digestibility and a low DIAAS.

Protein solubility also significantly influences techno-functional properties, in particular foaming and emulsifying characteristics. These properties rely greatly on the ability of a protein to be active on the border between the protein and the associated water or oil. In some instances, an increased surface hydrophobicity and increased fat content, as observed for PPI (P) and PPI (N), can indicate improved surfactant properties. However, a review by Van Vliet et al. (2002) outlines the relevance of processes involving pH change or protein conformational change on the interfacial behaviour of proteins (Van Vliet et al., 2002) and many descriptions of this effect in literature are contradictory (Lam et al., 2018). In the current study, it was observed through PCA that increased protein solubility was related to increased foaming capacity and a quicker emulsion separation rate. A higher content of fat present in the initial pea protein powders may also indicate an increased ability of the proteins to bind fat molecules. Therefore, for applications such as milk-style beverages which require emulsifying power the pea protein sources appear to be the most appropriate while BRP and WPI may require additional emulsifying agents. However, BRP and WPI, having a higher protein solubility than the other samples in this study, exhibit a minimal degree of sedimentation under accelerated stability analysis which is a desirable trait in beverage applications.

This study also examined the rheological properties of the chosen protein isolates over a heating and cooling cycle. This gives beneficial information regarding the implementation of such ingredients in food applications, where changes in structure during heating may be desired or are to be avoided. Gel strength can be influenced by protein concentration and the degree of protein denaturation, as well as the concentration of sulfhydryl groups in a protein matrix, where di-sulfhydryl crosslinking is one of the major factors responsible for stabilising gel matrices (Havea, Watkinson, & Kuhn-Sherlock, 2009; Zayas, 1997). Whey protein in particular is known for forming a strong, irreversible gel on heating due to the denaturation of whey proteins, mainly β-lactoglobulin, and the formation of di-sulphide linkages and other interactions. In this study, the final G' values of each protein correspond to the concentration of exposed and free sulfhydryl groups, with those with a higher concentration displaying a higher overall G' at the end of the heating and cooling cycle. The examined rheological properties can also be linked to the WHC, with PPI (P) and SPI exhibiting the highest WHC and also the highest initial G'. This thickening effect, in the absence of heating, may be useful in product formulations requiring thickening or cold-set gels. Alternatively, RP, WPI and PPI (N) show varying degrees of thickening on heating and may be suitable for use in sauces and soups, meat and cheese alternatives, and pudding type desserts. In addition, RP was the only ingredient to contain significant amounts of starch which may also influence the rheological properties. However, further studies into gel characteristics such as elasticity and brittleness would be beneficial.

The surface morphology of the protein, as observed using scanning electron microscopy imaging, displays a variety of particle shapes and sizes. PPI (P) and SPI appear to be similar, with a collection of dimpled globular individual bodies, whereas PPI (N) appears as a more homogenous structure, potentially as a result of protein hydrolysis as seen in Fig. 3. Interestingly, BRP has a different appearance than observed in a previous study (Jaeger et al., 2023), with the additional presence of small holes in the particles. This variation is likely due to batch or processing parameter variation. The presence of these small holes may be evidence of enzymatic hydrolysis treatment (Nyhan et al., 2023), as supported by the absence of protein bands in Fig. 3 and the high protein solubility.

Literature shows that plant proteins generally have a lower protein digestibility rate than animal-derived proteins, that is, because they tend to be more resistant to proteolysis (Bessada, Barreira, & Oliveira, 2019; Kaur et al., 2022; Mariotti, 2017). This can be observed in the current study, particularly with regards to RP and SPI. Protein digestion can be influenced by a variety of factors including integral protein structure and the presence of cell walls or membranes, processing parameters, and the presence of anti-nutritional compounds (Becker & Yu, 2013; Sá, Moreno, & Carciofi, 2019)The structure of the food matrix will greatly affect both digestion kinetics and overall digestibility. Particularly in

plant-based sources, proteins may be shielded from digestive enzymes by the presence of a poorly permeable cell wall or membrane (Becker & Yu, 2013; Sá et al., 2019), although in protein isolates this effect is likely minimal due to increased ingredient purity as a result of the isolation process. What is more probable is that digestion is inhibited by protein structure or protein aggregation therefore limiting enzymatic access and influencing protein solubility. β-sheet structures are the major protein conformation type present in legume proteins and play a major role in decreasing protein digestibility due to an inhibition of protease activity (Carbonaro et al., 2012; Carbonaro, Maselli, & Nucara, 2015; Guo et al., 2019). In addition, pH changes during digestion may also affect protein aggregation and therefore digestion kinetics (Zhang & Vardhanabhuti, 2014). Grains and legumes are known to contain significant levels of anti-nutritional compounds such as phytic acid, trypsin inhibitors, tannins and lectins (Bajpai, Sharma, & Gupta, 2005; Kaushal, Kumar, & Sharma, 2012; Kumar et al., 2022) which may also be contributing to this lowered level of protein digestion. It may be of interest in future studies to examine BRP for these compounds.

Aside from RP and SPI, the other plant proteins in this study have a high N digestibility (>90%), regardless of their source. This may be due to certain processing parameters during isolation that alter protein structure or reduce the effect of antinutritional factors, through protein denaturation or degradation, respectively. Simple thermal processes such as cooking, autoclaving, or microwaving have been shown to increase protein digestibility and reduce the impact of anti-nutritional factors (Boye, Wijesinha-bettoni, & Burlingame, 2012; Park, Kim, & Baik, 2010; Vagadia, Vanga, Singh, Gariepy, & Raghavan, 2018). Potentially, chemical processes such as glycosylation may also affect digestibility. Glycosylation is an early stage step in the Maillard reaction sequence causing the covalent conjugation of a free carbonyl group and the amine group of a protein, peptide or amino acid, and may induce structural changes, or unfolding, that has been described as increasing protein digestibility (Kaur et al., 2022) as well as altering functional and allergenic characteristics (Kutzli, Weiss, & Gibis, 2021; Shao, Zhang, Zhu, Liu, & Cai, 2020).

However, when considering protein quality in foods, N digestibility is only one of multiple factors to consider. DIAAS (Digestible Indispensable Amino Acid Score) is the successor to PDCAAS (Protein Digestibility Corrected Amino Acid Score) and is the preferred method for determining protein quality, with regards to the bioavailability of the essential amino acids (FAO, 2013). In a recent study by Herreman, Nommensen, Pennings, and Laus (2020), the DIAAS of common plant and animal based proteins were compiled and compared using datasets collected from literature, specifically using pig models, giving a comprehensive overview of the average DIAAS ranges for various protein sources (Herreman et al., 2020). These largely agree with the findings observed in this study with similar values for rice, whey, and pea-based proteins and corresponding first limiting amino acids for all sources. In addition, in-vivo studies completed in a more recent pig (Bailey, Fanelli, & Stein, 2023) and rat (Rutherfurd, Fanning, Miller, & Moughan, 2015) models also show similar DIAAS values for rice, pea and whey. However, the DIAAS for SPI and PPI (P) are significantly lower than what is described in literature (Bailey et al., 2023; van den Berg, Mes, Mensink, & Wanders, 2022). In a review examining the DIAAS of a wide variety of soy products by van den Berg, the mean adult DIAAS for soy is given as 84.5 ± 11.4, and Herreman et al. propose an average DIAAS >100 for soy protein. This significant gap could be due to treatments applied to this specific soy protein ingredient during processing (eg alkaline or heat treatment) leading to protein aggregation or structural change as described previously, or might be a result of differences in the innate protein structure due to cultivar or growing conditions (Gilani & Sepehr, 2003; Kaur, Singh, Kaur, Yadav, & Singh, 2021; Vasconcelos et al., 2010). This is further backed by the overall low (<50%) bioavailability of the majority of amino acids in SPI, and a low N digestibility. The same can be said of PPI (P). In addition, differences in methods of determination are likely also be contributing to this effect. In

this particular study, it can also be noted that the isolates with a low DIAAS also exhibited a lower protein solubility and increased protein aggregation, potentially inhibiting digestion. Interestingly, this in not true for PPI (P) which shows a high N digestibility. PCA also shows this, with DIAAS being positively correlated to protein solubility as well as N bio-accessibility. Although minimal studies exist on the digestion characteristics of BRP, it is interesting to note that the DIAAS obtained for BRP exceed those of its components, barley and rice, that can be observed in literature where the DIAAS values range from 40 to 56% (Cervantes-pahm, Liu, & Stein, 2014; Fu, Gao, & Li, 2023; Nosworthy & House, 2017)and 37–52% (Bailey et al., 2023; Hertzler, Lieblein-bo, Weiler, & Allgeier, 2020; Rutherfurd et al., 2015), respectively. While Lysine is the limiting amino acid for both protein sources, this increase is likely due to BRP being a refined and hydrolysed food ingredient.

A recently published study examines the amino acid uptake behaviour of BRP in 12 participants (Ummels et al., 2023). The estimated total amino acid uptake for BRP was 69% and 87% as a percentage of whey and pea protein PPI (N), respectively. This trend is similar to those observed in the present study, specifically regarding N % digestibility and DIAAS values, with WPI displaying the highest digestibility and DIAAS, followed by PPI (N) and BRP respectively.

5. Conclusion

This study outlines the digestibility and functional behaviour of five widely used protein isolates and introduces a novel protein isolate extracted from brewers spent grain. This new protein displayed a high protein solubility, similar to WPI, as well as a high N digestibility (> 90%), and foam capacity. However, over heating BRP alone displayed no rheological changes. BRP may be useful in beverage applications where pasteurisation or similar treatments are required, without changes in product stability. As previously seen in literature, this study shows that many of the plant-based proteins were nutritionally inferior to whey, with PPI (P), RP and SPI displaying low DIAAS vales (< 40%). However, regarding BRP, the DIAA for all essential amino acids was >1 with the exception of lysine giving a DIAAS of 67%. In combination with a N digestibility >90%, the data shows that BSG protein is a nutritious protein. The combination of BRP with other protein sources, e.g. pea protein isolate as examined in this study, may be the solution, altering both the nutritional and functional characteristics depending on the specific requirements of the food product. The implementation of such proteins in different food matrices likely affects protein digestibility and so further study in this area is required. However, the commercial availability of such a protein ingredient gives food manufacturers the ability to begin the shift towards a circular food system and is an important step in the journey towards an increasingly sustainable food future.

CRediT authorship contribution statement

Alice Jaeger: Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Niamh Ahern:** Writing – original draft, Investigation, Formal analysis. **Aylin W. Sahin:** Writing – review & editing, Supervision, Conceptualization. **Laura Nyhan:** Writing – review & editing, Project administration. **Jurriaan J. Mes:** Writing – review & editing, Data curation. **Claire van der Aa:** Writing – review & editing, Resources. **Ioannis Vrasidas:** Writing – review & editing, Formal analysis, Data curation. **Elke K. Arendt:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

Claire van der Aa is employed by EverGrain Ingredients International, all other authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2024.103571.

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