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Characterization of industrial pea canning by-product and its protein concentrate obtained by optimized ultrasound-assisted extraction

Lorenzo Estivi [a,*], Andrea Brandolini [b], Andrea Catalano [a], Roberta Di Prima [c], Alyssa Hidalgo [a]

[a] Department of Food, Environmental and Nutritional Sciences (DeFENS), Università degli Studi di Milano, Via Celoria 2, 20133, Milan, Italy
[b] Council for Agricultural Research and Economics – Research Centre for Animal Production and Aquaculture (CREA-ZA), Viale Piacenza 29, 26900, Lodi, Italy
[c] Casalasco Società Agricola. Stabilimento di Gariga di Podenzano, Via I Maggio 25, 29027, Podenzano, PC, Italy

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ABSTRACT

This work aimed to optimize protein extraction from an industrial pea canning by-product by developing an ultrasound-assisted alkaline solubilization and isoelectric precipitation method. Proximate composition and microbial contamination (total bacterial count, total lactic acid bacteria, Enterobacteriaceae, yeasts and moulds) were assessed in the wet by-product. The protein concentrate obtained, the dry by-product and a commercial pea flour were analysed for protein content, water activity, colour and techno-functional properties. The optimal extraction conditions from the wet by-product were liquid-solid ratio 20 mL/g, pH 11, ultrasound amplitude 80 μm, time 2 × 30 min. The extraction from the dry by-product was not feasible. Sonication reduced extraction time from 4 h (magnetic stirring method) to 1 h and increased three-fold the extraction recovery (from 21.5% to 66.6%). The concentrate had a 74.8 g/100 g DM protein content. The SDS-PAGE revealed protein degradation in some specimens, probably because of fermentation and enzymatic hydrolysis before sampling. However, the microbial counts in the concentrate, mainly related to lactic acid bacteria, were always below the guideline values. Our results demonstrate that the pea canning by-product is a valuable raw material for protein recovery and that the proposed method allows rapid and direct treatment, avoiding drying costs and microbial proliferation.

1. Introduction

Peas (*Pisum sativum* L.) are among the most cultivated legumes worldwide, with a global production of 14.2 million metric tons of dry seeds (18.9% of all pulses, third after beans and chickpeas), and 20.8 million metric tons of fresh legumes (45%, second after beans). Canada, Russia, China and India produce 67% of dry peas, whereas China and India yield 85% of fresh production (FAO, 2022; last accessed on May 6th, 2024). Peas are valuable products for human consumption and occupy a prominent place among vegetables because of their high nutritional value, linked to good protein content (20–25 g/100 g) and health-promoting compounds, such as amylose-rich slowly-digestible starch (39.4–46.2 g/100 g), resistant starch (1.8–7.0 g/100 g), insoluble (19.3–23.1 g/100 g) and soluble fibre (3.9–8.0 g/100 g, mainly pectin), potassium, phosphorus, phenolic compounds and β-carotene (Lam et al., 2018; Wu et al., 2023).

Pea proteins are appreciated for their hypo-allergenicity and nutritional value because their high lysine content makes them complementary to cereal proteins, which are rich in sulphur-containing amino acids (i.e., methionine and cysteine) (Lam et al., 2018; Shanthakumar et al., 2022). As concentrate (>65 g protein/100 g) or isolate (>90 g protein/100 g), pea proteins have been employed in the preparation of baked goods, pasta, meat products, beverages and milk-like drinks (Boukid et al., 2021; Boye et al., 2010). However, the typical beany off-flavour associated with pulses hinders the diffusion of their protein extracts.

Besides nutritional enrichment and animal protein substitution, pea proteins provide viscoelasticity, missing in gluten-free foods for celiacs (Boukid et al., 2021) and, thanks to their interfacial properties, are used as emulsifiers, thickeners, fat binders and gelling or bulking agents in food formulations. Such properties can be further improved by different techniques including high-pressure treatment, ultrasonication, hydrolysis, glycation, acylation, deamidation and enzymatic cross-linking (Shanthakumar et al., 2022). Protein modification usually leads to conformational changes and, thus, better adsorption at the interface of food dispersions: when unfolding occurs, surface hydrophobicity

* Corresponding author.
E-mail address: lorenzo.estivi@unimi.it (L. Estivi).

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increases, enhancing the protein ability to stabilize foams and emulsions (Xiong et al., 2018). In addition, pea proteins in heterogenous mixtures are exploited as encapsulating material and in the manufacturing of edible films (Boukid et al., 2021; Shanthakumar et al., 2022).

Industrial processing of legumes discards from 5% to 25% of the harvested material as a waste, containing pods, hulls, leaves, stems, and broken, dark, or stained seeds (Tassoni et al., 2020). These residues are rich in proteins, dietary fibre, polyphenols and other molecules (e.g., peptides) with antioxidant, antimicrobial and other beneficial activities (Belghith-Fendri et al., 2016, 2022; Dueñas et al., 2004; Mateos-Aparicio et al., 2010). Currently, legume waste is mainly used for biogas production or livestock feeding, but there is a growing interest in its upcycling for human consumption, especially by protein recovery. Dry extraction methods are scarcely applicable for this purpose, thus the waste is more often re-processed by wet extraction, frequently exploiting alkaline solubilization followed by isoelectric precipitation (Kamani et al., 2023; Tassoni et al., 2020); however, this technique leads to loss of solubility and worsening of technological properties, due to denaturation and formation of insoluble aggregates (Boukid et al., 2021; Vogelsang-O'Dwyer et al., 2020). The ultrasound technology has gained interest in the food industry for its versatility, safety and low energy requirements; in fact, coupled with wet extraction increases recovery and shorten treatment time (Estivi et al., 2022; Tassoni et al., 2020). Although ultrasonication has been previously studied for protein extraction from pea flour (F. Wang et al., 2020) and pea pods (Karabulut et al., 2023), to the best of our knowledge this is the first attempt involving an industrially generated pea by-product as raw material. Therefore, the aims of this work were to characterize the industrial pea canning by-product collected over different years, to optimize an ultrasound-assisted method of alkaline extraction and isoelectric precipitation of protein, and to verify the quality of the protein concentrate thus obtained.

2. Materials and methods

2.1. Materials

The by-products of a canned green pea (*Pisum sativum* L.) production line were sampled at the Casalasco Società Agricola S. p.A. plant in Gariga di Podenzano (PC, Italy). The amount of waste was computed as the percentage of raw material discarded, based on a six-year history (2014–2016; 2020–2022). The samples, collected from 2013 to 2022, were characterized for their chemical composition (n = 10) and microbial contamination (n = 5). The by-products collected on three different days in 2021 were stored at −20 °C and then either thawed at 4 °C for 16 h (wet by-product sample) or dried at 55 °C for 24 h (dry by-product sample) in a Venticell 55 oven (MMM Medcenter Einrichtungen Gmbh, Planegg, Germany). The analyses were performed on the dried by-product, the protein concentrate obtained by an optimized ultrasound-assisted extraction from the thawed by-product, and a pea flour purchased from a local store (NaturaSì, Milan, Italy).

2.2. Optimization of the ultrasound-assisted protein extraction method

The proteins were extracted by solubilization in alkalized water and subsequent isoelectric precipitation. The samples were suspended in distilled water according to the selected extraction ratio (from 10 to 20 mL/g, varying according to the experiment), considering their moisture content. The by-product mixture was homogenized using an immersion blender (Hr1611/00, Philips, Amsterdam, Netherlands) for two 30 s cycles with a 30 s of rest in-between; the pH was adjusted with 1 mol/L NaOH under constant stirring. Ultrasonication (experimental method) or magnetic stirring (control method) were used during the extraction. The ultrasonication was achieved with an Up400St homogenizer (Hielscher, Teltow Germany) mounting a 14 mm diameter titanium probe, set up with on/off cycles of 5/5 s. The temperature was maintained between 25 °C and 30 °C with an icy water bath. The samples were transferred into a 400 mL polyethylene bottle and centrifuged at 10800 *g* for 10 min with a Sorvall® RC-5B Plus Superspeed Centrifuge (Thermo Fisher Scientific, Waltham, MA, USA). The sediment was extracted a second time, the supernatants were pooled, adjusted to pH 4.5 with 1 mol/L HCl and centrifuged as detailed above. The sediment, corresponding to the protein extract, was dried at 55 °C for 24 h to obtain the protein concentrate sample.

The ultrasonication extraction conditions were optimized in a series of preliminary experiments considering as response variable the extraction recovery (%), calculated using Equation (1):

$$Recovery\ (\%) = \frac{weight_{concentrate}\ (g) \times N_{concentrate}\ (g/g)}{weight_{by-product}\ (g) \times N_{by-product}\ (g/g)} \times 100 \qquad \text{(Eq. 1)}$$

where *N* is the total nitrogen content.

The preliminary *Experiments 1* and *2* were conducted using commercial pea flour, while the *Experiments 3*, *4* and *5* were performed using the thawed pea by-product.

Experiment 1. A fractional factorial design 2^{4-1} with three central points (Supplementary Table 1) was performed to study the effects of liquid:solid ratio (10–20 mL/g), pH (8–11), amplitude (30–50 μm) and time (5–15 min) on extraction recovery. The choice of the factors and their levels was based on a previous investigation (F. Wang et al., 2020).

Experiment 2. A full factorial design 2^2 with three central points, replicated twice by assigning a block to each repetition (Supplementary Table 2), was applied to study the effect of amplitude (60–80 μm) and time (10–20 min) at a liquid:solid ratio of 20 mL/g and pH 11.

Experiment 3. A full factorial design 2^2 with three central points (Supplementary Table 3), was performed considering as independent variables amplitude (40–80 μm) and time (5–15 min), at a liquid:solid ratio of 20 mL/g and pH 11.

Experiment 4. The extraction time was set up by applying the steepest ascent method (Montgomery, 2019); four runs at a constant amplitude of 80 μm were performed between 18.75 and 30.00 min, with 3.75 min increments for each run (Supplementary Table 4). The trials were performed in duplicate.

Experiment 5. A rotatable Central Composite Design (CCD) with two factors and four central points (Supplementary Table 5) was applied to optimize amplitude (70–90 μm) and time (25–35 min) conditions.

Finally, a comparison of the recovery and protein content achieved by the traditional magnetic stirring method (ratio = 20 mL/g, pH = 11, time = 2 × 2 h) and by the optimized ultrasound-assisted extraction method (ratio = 20 mL/g, pH = 11, amplitude = 80 μm, and time = 2 × 30 min) was performed.

2.3. Analyses

2.3.1. Chemical composition

The moisture content was determined gravimetrically according to the 925.10 official method (AOAC, 1990). The water activity (a_w) was measured with an Aqua Lab Series 3 TE instrument (Decagon Devices, Inc., Pullman, WA, USA). Total nitrogen content was determined by the Kjeldahl method 920.87, and protein content was computed as N x 6.25; lipid content was assessed by the Soxhlet method 920.39C; total crude fibre by method 962.09; ash by method 923.03 (AOAC, 1990); starch by ISO method 10520:1997 (ISO, 1997) and total carbohydrates by difference. The results are expressed as g/100 g dry matter (DM).

2.3.2. Colour

The colour was evaluated in triplicate in the CIElab *L* a* b** colour space using a Chroma Meter CR-II tristimulus colorimeter (Minolta Camera Co., Osaka, Japan) with a C standard illuminant.

2.3.3. Techno-functional properties

Water absorption capacity (WHC), oil absorption capacity (OHC), least gelling concentration (LGC), foam capacity (FC), foam stability (FS) and emulsifying capacity (EC) were appraised as described by F. Wang et al. (2020).

2.3.4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The glutenin fraction, extracted according to Singh et al. (1991), was fingerprinted by discontinuous SDS-PAGE electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS), following Morel (1994). A protein marker (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with molecular weight bands ranging from 116 kDa to 14.4 kDa was used as a reference.

2.3.5. Microbiological analyses

The microbiological analyses were performed by plate count. The total bacterial count (TBC) was determined according to the ISO 4833–1:2013 method (ISO, 2013); Enterobacteriaceae according to the ISO 21528–2:2017 method (ISO, 2017); the mesophilic lactic acid bacteria according to the ISO 15214:1998 method (ISO, 1998); total moulds and yeasts according to the ISO 21527–2:2008 method (ISO, 2008). The plates containing 10–200 colonies were scored, recording at least two values for each replicate. The analyses were performed on three independent replicates and the results are expressed as the decimal logarithm of colony forming units per gram of sample ($\log_{10}$ CFU/g).

2.4. Statistical analysis

After verifying the normal distribution of the data, the recoveries obtained in *Experiment 4* and the characteristics of commercial pea flour, dried pea by-product and protein concentrate were evaluated by one-way Analysis of Variance (ANOVA). When significant differences ($p \leq 0.05$) were found, Fisher's Least Significant Difference (LSD) was calculated at a 95% significance level. To compare the performance of the traditional magnetic stirring method and the optimized ultrasound-assisted extraction method, the Student's t-test was applied. All analyses were performed using the statistical programme STATGRAPHICS® Centurion XVI (Statgraphics Technologies, Inc., The Plains, USA). Mean, standard deviation and coefficient of variation (CV, expressed in %) were computed using the programme Excel 2016 (Microsoft®, Redmond, USA). The statistical processing of the experimental designs was performed with the Design Expert 10 software (StatEase, Minneapolis, Minnesota).

3. Results and discussion

3.1. By-product characterization

The waste recorded by the industry over six years corresponded to 7.6% ± 2.5% (CV = 33%) of the input. Fig. 1 presents the chemical composition (Fig. 1A) and microbial contamination (Fig. 1B) of the canned peas by-product across different years. The moisture content was characterized by the lowest variability (CV = 6.9%), ranging from 76.5 to 90.7 g/100 g. The proximate composition, expressed on dry matter

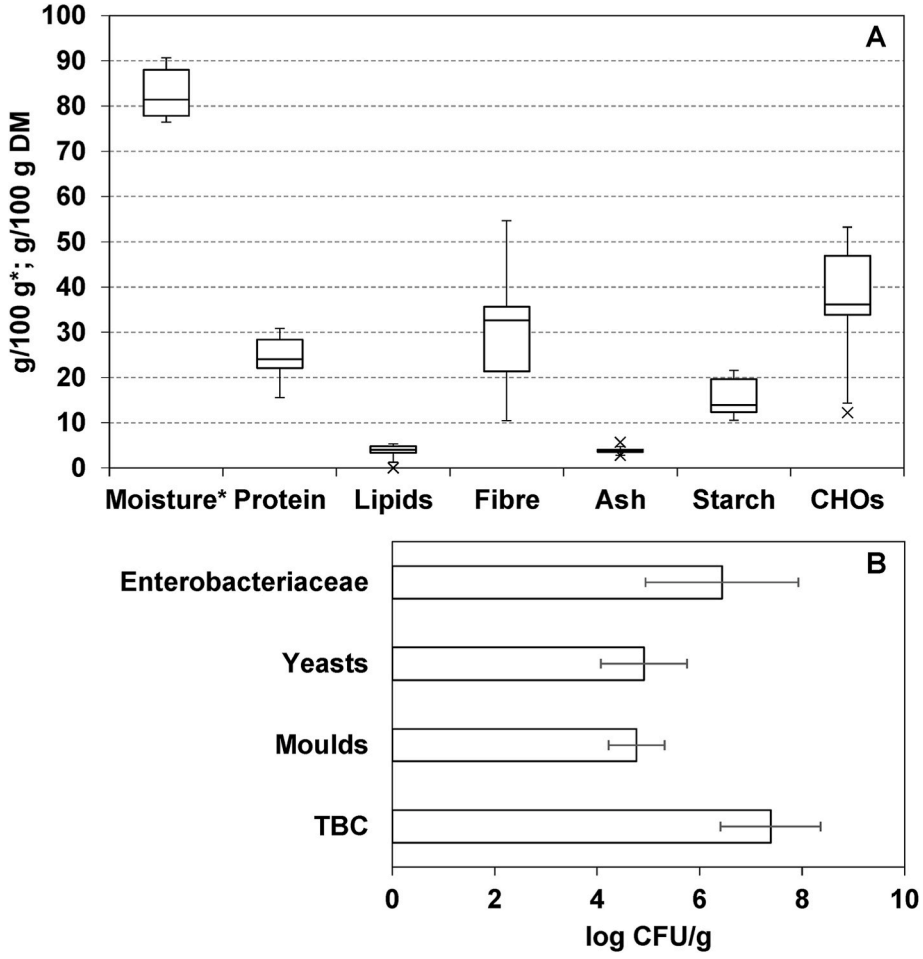

Fig. 1. Chemical composition (A; g/100 g DM, except moisture, g/100 g) and microbial count (B; log CFU/g) of industrial pea-canning by-product collected from 2013 to 2022. TBC, Total bacterial count.

(DM), varied broadly for lipids and fibre (CV = 43%), ranging from 0.0 to 5.3 g/100 g DM and from 10.5 to 54.6 g/100 g DM, respectively, due to the different proportion of pods, leaves, hulls and seeds. The protein and ash contents ranged with a CV = 20%, from 15.5 to 30.9 g/100 g DM and from 2.8 to 5.7 g/100 g DM, respectively. These values agree with the composition reported by Belghith-Fendri et al. (2016) and Mateos-Aparicio et al. (2010) for pea pods and by Hall et al. (2017) for pea cotyledons.

The starch content varied between 10.5 and 21.6 g/100 g DM (CV = 28%). The great variability observed can be attributed to multiple factors that influenced the by-product composition over ten years, including different suppliers (and thus regions of origin), edaphic and climatic conditions, sampling points and methods, and microbial contamination or degradation before sampling.

The microbial contamination presented a wide fluctuation among the different samplings: the total bacterial count varied from 6.3 to 8.4 log CFU/g, the Enterobacteriaceae from 5.4 to 7.5 log CFU/g, the yeasts from 3.7 to 5.7 log CFU/g and the moulds from 4.4 to 5.7 log CFU/g; *Salmonella* was always absent.

3.2. Optimization of the protein extraction process

3.2.1. Preliminary trials on commercial pea flour

The first extraction trial (*Experiment 1*), performed using commercial pea flour and following a fractional factorial design 2^{4-1} with three central points (Supplementary Table 1), showed that only the liquid: solid ratio had a significant ($p \leq 0.01$) effect on the recovery (Supplementary Table 6). The pH *p*-value (0.067) was just above the 0.05 threshold, suggesting the inclusion in the model of this parameter to verify its effect on the response variable (i.e., recovery). The chosen model was significant ($p \leq 0.001$) while the curvature was not significant ($p > 0.05$) and presented the following coded equation: Recovery = 62.1 + 2.2 liquid:solid ratio + 1.3 pH. As clearly displayed in the contour plot (Fig. 2), greater recoveries were obtained with higher ratios and pH. Thus, for the subsequent trials the extraction ratio was set at 20 mL/g and pH = 11. Diluting solids in a higher amount of solvent increases the concentration gradient, thus the mass of the extract, until further dilution results in negligible improvements. In this case, ratios higher than 20 mL/g were considered unfeasible, because they would have reduced the amount of material treated. Furthermore, a trial performed at a 20 mL/g with pH values increasing from 8 to 11 confirmed the pH = 11 as the best choice.

Ultrasonication improves the extraction of various analytes, including protein, from food matrices (Estivi et al., 2022). Therefore, amplitude and time were furtherly studied in *Experiment 2* according to a replicated full factorial 2^2 with three central points (Supplementary Table 2) exploring higher condition levels. As reported in Supplementary Table 6, the amplitude was the only significant factor, with the following coded equation: Recovery = 66.30 + 0.95 amplitude.

3.2.2. Fine tuning trials on by-product

Initially, protein extraction from the dry by-product was attempted. However, as soon as the sonication started, the dry by-product formed a viscous mixture with water, preventing it from flowing and causing hot spots (>60 °C). This might be related to gelation or swelling of damaged starch or starch pre-gelatinized during drying. Although the drying temperature (55 °C) corresponded only to the pea starch gelatinization onset (53.6–59.5 °C) (Sun et al., 2020; S. Wang & Copeland, 2012), the treatment lasted 24 h in the presence of an excess of water during the early stages. The starch gelatinization extent is temperature-dependent (Lund & Lorenz, 1984), but partially occurs over a long period even when the temperature is maintained around the onset (Pielichowski et al., 1998; Sablani et al., 2007). The gelatinization follows a first order kinetics, and the reaction rate depends on the temperature according to the Arrhenius equation (Sablani et al., 2007); in addition, an alkaline pH facilitates starch swelling (Lam et al., 2018). As the fluid viscosity increases, the ultrasonic homogenizer needs to impart greater power to the probe to keep the amplitude constant. Therefore, a modest initial amount of swollen starch may have had an autocatalytic effect: greater viscosity corresponds to higher power, dissipated as heat, and consequently more gelatinization. This behaviour prevented a steady temperature control, necessary to limit the thermal denaturation of the proteins, as well as its reliable measurement, thus thwarting correct recording of the experimental conditions. Increase of liquid:solid ratio and off-period of sonication cycles, and reduction of amplitude and coolant temperature were all unsuccessful, hence it was decided to treat the thawed by-product.

In *Experiment 3* the by-product was extracted by varying the amplitude and time factors according to a full factorial design 2^2 with three central points (Supplementary Table 3). As shown in Supplementary Table 7, the amplitude was the only significant factor ($p = 0.025$); however, the time was included in the model because its *p*-value (0.059) was just above the 0.05 significance level. The model was significant ($p \leq 0.05$), but the curvature was also significant ($p = 0.02$). The adjusted r^2 was 0.74 and the coded equation was: Recovery = 47.51 + 7.22 amplitude +5.42 time. The response maximization direction is evident in the contour plot shown in Fig. 2.

In *Experiment 4* the factors conditions were explored following the

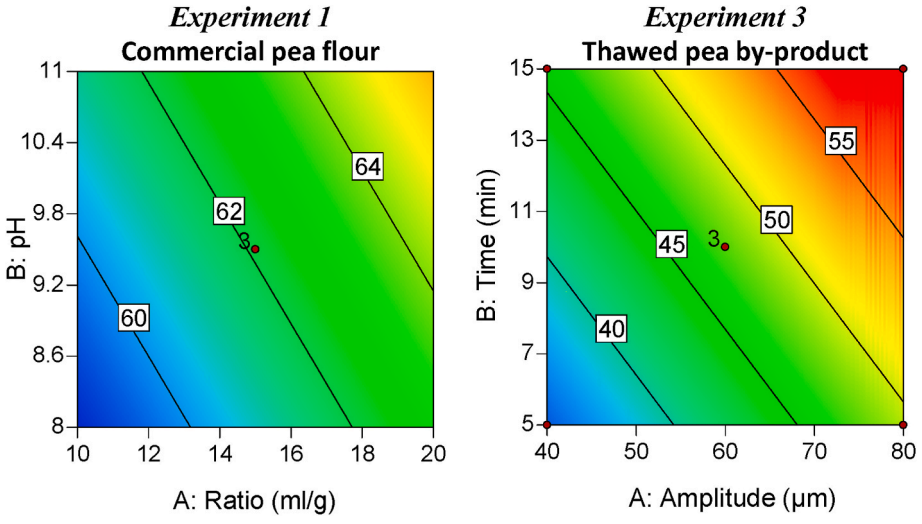

Fig. 2. Contour plots of the experiments performed for the protein extraction by ultrasound-assisted method.

steepest ascent path (Montgomery, 2019). The ratio of *Experiment 3* equation coefficients suggested a 0.75 incremental step for treatment time and 1.00 incremental step for amplitude. However, increasing the amplitude would have brought the ultrasound probe to its operating limits in just two steps, also making it impossible to control the temperature. Therefore, the amplitude was kept constant at the highest previously explored value (80 μm) and only the treatment time was increased. The results (Supplementary Table 4) evidenced higher extraction recovery after 26.25 min of treatment. This experiment identified a new region, probably close to the process optimum, which was further investigated with a Response Surface design by means of the CCD depicted in Supplementary Table 5. The new model was not significant ($p > 0.05$; Supplementary Table 7) but confirmed that the highest recoveries were obtained in a wide region whose central conditions were amplitude = 80 μm and time = 30 min. In this region, factor modifications led to negligible changes in recovery, therefore the central point was considered as the optimum: ratio = 20 mL/g, pH = 11, amplitude = 80 μm and time = 2 × 30 min.

Table 1 reports the recovery and protein content in the extracts obtained from thawed pea-by product by the magnetic stirring and by the optimized ultrasound-assisted method. The new method achieved a 66.6% recovery rate, over three times higher than the 21.5% recovery rate of traditional magnetic stirring. Additionally, it significantly reduced treatment time from 4 h to one. Protein recovery from the by-product employing ultrasonication was comparable or inferior to those previously reported for pea flour extraction (62.6–76.7%) by Stone et al. (2015), using magnetic stirring, and by F. Wang et al. (2020) (82.6% with ultrasonication and 60% with magnetic stirring). However, lower recoveries were reported for protein alkaline extraction from pea pods with (21.1%) and without (16.2%) ultrasound pre-treatment (Karabulut et al., 2023). Probably, the protein nitrogen was already present as soluble peptides due to pre-sampling fermentation and thus was lost during the wet extraction, leading to scarcer recovery. In addition, the particles dimension, coarser in the by-product than in the pea flour, and the presence of other components (e.g., cellulose, pectin and other polysaccharides) likely hindered protein diffusion in the solvent. An improvement in protein extraction by ultrasonication is reported for several vegetable matrices, including almond production residues (+480%), peanut flour (+136%), soy flour (+39%), sunflower seed cake (+317%), rice bran (+134%), rice syrup production residue (+100%) and quinoa flour (+140%) (Jahan et al., 2022; Li et al., 2017; Quintero-Quiroz et al., 2022). Furthermore, a considerable reduction in treatment time was generally observed. The ultrasonication advantage in the extraction processes is universally attributed to cavitation, which interrupts cellular compartments facilitating the release of solutes and renews the boundary layer of solvent maintaining a high gradient of concentration (Estivi et al., 2022).

The protein content of the isolates produced from pea flour by alkaline extraction can reach 90 g/100 g (Boye et al., 2010). F. Wang et al. (2020) reported values of 81.5 and 87.5 g/100 g DM in isolates produced with agitation and ultrasonication, respectively. The highest protein concentration (Table 1) was observed in the extract obtained with magnetic stirring (91.5 g/100 g DM), while the optimized ultrasound method generated a less pure product (74.9 g/100 g DM). Karabulut et al. (2023) observed the same tendency in pea pods isolates, as they determined 54.3 g protein/100 g with ultrasound pre-treatment and 63.7 g/100 g without it, ascribing that to co-extraction of water-soluble polysaccharides coming from the cell-wall. According to Prestes Fallavena et al., 2022 and Thirunavookarasu et al. (2022) evidence has been gathered on protein glycation mediated by high-power ultrasound and on the formation of complexes between denatured proteins and sugars, oligosaccharides or polysaccharides, including pectin. Protein-polysaccharide complexes are stabilized by electrostatic, hydrophobic interactions, van der Waals forces and hydrogen bonding (Thirunavookarasu et al., 2022), thus it can be inferred that the polysaccharides from the abundant soluble fibre of the pods formed soluble complexes with the proteins, reducing the purity of the concentrate. In fact, the sonication conditions, more drastic than those used by F. Wang et al. (2020), the higher soluble fibre, especially the pectic polysaccharides from pods and hulls (Belghith-Fendri et al., 2018), and the presence of lectins, carbohydrates-binding proteins (Shi et al., 2018), could explain the lower purity of the extract. Additionally, the drying treatment could have reinforced or formed new protein-polysaccharides conjugates by the Maillard reaction, even at relatively low temperature (i.e., 60 °C) (Tamnak et al., 2016), while the ultrasonication increases protein unfolding, promoting glycation (Ma et al., 2020). Finally, the high pH may have facilitated the contamination of the extract with swollen starch (Lam et al., 2018).

Table 1
Recovery and protein content (mean ± standard deviation) in the extracts obtained from thawed pea by-product by magnetic stirring or by the optimized ultrasound-assisted method.

	Treatment time	Recovery (%)	Protein (g/100 g DM)
Magnetic stirring	2 × 2 h	$21.5^{b} \pm 0.9$	$91.5^{a} \pm 0.3$
Ultrasonication	2 × 30 min	$66.6^{a} \pm 1.6$	$74.9^{b} \pm 0.3$

Different letters indicate significant differences between values according to the *t*-test ($p \leq 0.05$).

Dry fractionation of legumes desiccated in the field is more economical and sustainable to operate than wet extraction, although the protein fraction thus separated has a concentration between 49% and 73%, lower than that achieved from alkaline extraction (Fernando, 2021). However, the concentrate and the other co-products (starchy and fibrous fractions) are obtained directly in the dehydrated form, facilitating their conservation. Conversely, the by-product evaluated in this research is conveyed to the collection point by hydraulic transport, before being drained by compression in a rotating drum. Therefore, to scale-up the process it should be treated as soon as collected, avoiding the drying energy costs.

3.3. Pea products characterization

The characteristics of commercial flour, dry by-product and protein concentrate are reported in Table 2. Protein content was significantly higher in the concentrate (74.9 ± 0.3 g/100 g DM) than in the commercial flour and the by-product (24.7–25.1 g/100 g DM). Water activity was low in all samples, although the by-product had the lowest one, probably due to the water-binding capacity of its higher fibre content (36.1 g/100 g DM). The brighter green colour of the pea flour (Table 2 and Fig. 3) is probably attributable to the drying conditions in the field (ambient temperature) *versus* those in the oven (55 °C) employed for the dry by-product and protein concentrate. In fact, the transition to a darker tinge can be a consequence of chlorophyll degradation into grey/brown pheophorbide or pheophytin (Zielinska et al., 2013). All samples showed an important yellow component (b^*), with the highest value in the by-product.

Significant differences in techno-functional properties, except emulsifying capacity, were detected. Generally, the wet extraction appeared detrimental for all the interfacial properties, probably due to formation of poorly soluble aggregates (Fernando, 2021; Vogelsang-O'Dwyer et al., 2020) in the concentrate that prevailed over ultrasound-induced increase in surface hydrophobicity (Estivi et al., 2022; Xiong et al., 2018). The concentrate WHC (2.13 g H_2O/g) was similar to those (2.06 and 2.4–2.6 g H_2O/g) reported for pea protein isolates by Kumar et al. (2022) and Stone et al. (2015), respectively, although higher values (3.2 and 3.0–4.2 g H_2O/g) were described by Sareen et al. (2023) and F. Wang et al. (2020). The OHC (1.68 g oil/g) was like those (1.72 g and 1.46 g oil/g) reported by Kumar et al. (2022) and Sareen et al. (2023). The by-product showed the greatest WHC and OHC, due to the higher content in dietary fibre. In fact, hemicellulose and pectin are hydrophilic and correlated to WHC, while cellulose also

Table 2
Protein content, colour, technological and functional properties, and microbial count (mean ± standard deviation) of commercial pea flour, dry pea by-product and protein concentrate.

Parameters	Commercial flour	Dry by-product	Protein concentrate
Protein (g/100 g DM)	$25.09^{b} \pm 0.08$	$24.71^{b} \pm 0.71$	$74.86^{a} \pm 0.32$
Colour coordinates			
L^*	$86.50^{a} \pm 0.30$	$54.47^{b} \pm 0.83$	$40.43^{c} \pm 0.35$
a^*	$-9.63^{b} \pm 0.21$	$-4.10^{a} \pm 0.46$	$-3.50^{a} \pm 0.30$
b^*	$16.90^{b} \pm 0.35$	$19.17^{a} \pm 0.50$	$15.43^{c} \pm 0.55$
Technological and functional properties			
a_w	$0.530^{a} \pm 0.004$	$0.320^{c} \pm 0.002$	$0.510^{b} \pm 0.002$
Water holding capacity (g H_2O/g)	$1.23^{c} \pm 0.34$	$4.19^{a} \pm 0.28$	$2.13^{b} \pm 0.22$
Oil holding capacity (g oil/g)	$1.57^{b} \pm 0.08$	$2.24^{a} \pm 0.16$	$1.68^{b} \pm 0.22$
Least gelling concentration (g/100 mL)	10	15	10
Foaming capacity (%)	$69.18^{a} \pm 1.39$	$21.59^{c} \pm 1.36$	$28.46^{b} \pm 1.39$
Foam stability (%)	$49.55^{a} \pm 0.01$	$15.21^{b} \pm 2.08$	$6.38^{c} \pm 1.39$
Emulsifying capacity (%)	63.26 ± 1.15	62.20 ± 1.28	60.89 ± 1.07
Microbial counts ($\log_{10}$ CFU/g)			
Total bacterial count	–	–	5.46 ± 0.17
Total lactic acid bacteria	–	–	5.38 ± 0.12
Enterobacteriaceae	–	–	2.54 ± 0.11
Yeasts and moulds	–	–	3.32 ± 0.15

Different letters indicate significant differences among values in the same row according to the LSD test ($p \leq 0.05$).

retains oil due to a certain hydrophobicity (He et al., 2023). Pea flour and protein concentrate showed the same LGC (i.e., least gelling concentration; 10 g/100 mL), as the lack of starch was probably compensated by the higher protein amount, while the by-product formed a strong gel only above 15 g/100 mL. The concentrate LGC was slightly lower, thus better, than those (11–13 and 12 g/100 mL) determined by F. Wang et al. (2020) and Kumar et al. (2022), suggesting that the hypothesized aggregates became soluble in the presence of heat. The protein concentrate displayed some foam capacity (28%) but poor stability (6%), both inferior to those of the flour. Foam-forming proteins have, ideally, low molecular weight, high surface hydrophobicity, good solubility, low net charge and proneness to denaturation (Barac et al., 2010). Poor foaming properties were previously detected in pea protein isolate, especially at pH 4.5 (Kumar et al., 2022; Sareen et al., 2023). As the pH increases to 7 the proteins net charge increases as well, improving unfolding, flexibility and, therefore, foaming (Barac et al., 2010). Furthermore, the SDS-PAGE (Fig. 4) highlighted the presence of albumins only in the commercial flour. Albumins are rapidly adsorbed at the air-water interface and have strong protein-protein interactions (Kornet et al., 2022) resulting in a stiff and dense interfacial layer and contributing to the greater foaming capacity and stability of the flour (Barac et al., 2010).

3.4. SDS-PAGE

The SDS-PAGE gel extraction and separation were performed to detect possible effects of the different treatments on pea proteins. The fingerprint patterns of glutenin extracts of commercial flour, dry by-product and protein concentrate are shown in Fig. 4. The concentrate and the dry by-product showed overall the same protein pattern of the commercial flour. The presence of convicilin and legumin was detected near the 66.2 kDa band, while vicilin and legumin α were found at 45 kDa and 35 kDa, respectively. Finally, at approximately 25 kDa the presence of legumin β was observed. Albumin, the thin band around 14.4 kDa (Chang et al., 2022; Gao et al., 2020), was found only in the commercial flour sample. However, smeared bands in the dry by-product and concentrate lanes were also evident (Fig. 4) and are proof of protein degradation, probably because of the wet by-product fermentation before collection, with consequent enzymatic hydrolysis. In fact, the processing waste was very humid and was exposed to

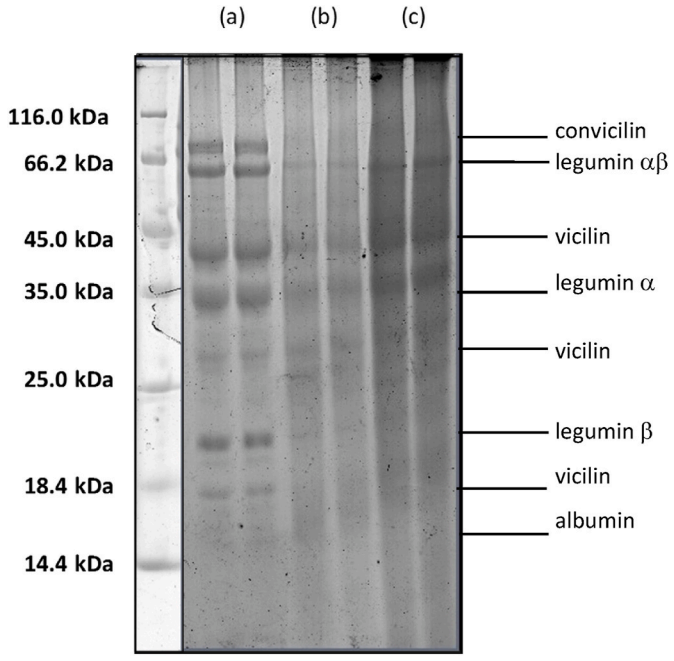

Fig. 4. Electrophoretic profiles and molecular weight distribution of glutenin extracts from pea commercial flour (a), dry pea by-product (b) and pea protein concentrate (c). The first lane on the left displays the weight markers.

Fig. 3. Images of the commercial flour (left), the dry by-product (centre), and the protein concentrate obtained by the ultrasound-assisted method (right) at the optimized conditions (liquid-solid ratio 20 mL/g, pH 11, amplitude 80 μm, time 2 × 30 min).

summer temperatures for an unknown time before sampling. Schlegel et al. (2021) observed that the combination of fermentation and enzymatic hydrolysis of lupin protein isolate resulted in the breakdown of large polypeptides into shorter low molecular weight peptides. Furthermore, Lee et al. (2018) noted that the formation or disappearance of protein bands within an SDS-PAGE was clearly affected by the duration of the fermentation: up to 9 h no changes were noticed, indicating that the protein subunits were still intact, but from 12 h the intensity of the 7S band and the 11S globulins decreased and new bands with lower molecular weight appeared; after 18 h, the 7S globulin and the 11S globulin acid subunit largely disappeared, while the base subunit of 11S globulin was much weaker. Smeared bands were previously observed for a pea protein isolate that was conjugated with pectin (Tamnak et al., 2016), due to the wide range of molecular weights in the resulting glycated proteins. This complementary explanation better matches with the protein concentrate, because the by-product was dried before milling and therefore contact between proteins and polysaccharides was less likely. Smeared bands in pea pods isolates, more evident in those obtained with alkaline solubilization, were also reported by Karabulut et al. (2023).

3.5. Microbiological analysis of protein concentrate

Microbiological counts on the protein concentrate are reported in Table 2. In the absence of specific microbiological criteria concerning protein isolates, a comparison may be attempted with reasonably similar products (i.e., dried vegetables and cereal flours). Micro-organisms commonly isolated from dried vegetables include lactic acid bacteria, *Enterococcus faecalis,* staphylococci, *Bacillus* spp. spores, yeasts, and moulds (*Penicillium* and *Aspergillus* spp.) (ICMSF, 2005). The total mesophile bacterial count of the concentrate (2.9×10^5 CFU/g) was lower than the reference values proposed by the Fédération du Commerce et de la Distribution (FCD, 2023) for wholemeal flours (5×10^5 CFU/g) and by Gilbert et al. (2000) in various ready-to-eat foods including dried vegetables (10^6 CFU/g), whereas 10^4 CFU/g has been reported as the guide value for commercial vegetable protein ingredients (D'Agostina et al., 2005). Previously, TBC ranging from 10^1 to 1.5×10^4 CFU/g were reported among 35 different commercial protein isolates (26 from pea, 7 from faba bean, 1 from mung bean and 1 from chickpea) (Kyrylenko et al., 2023), whereas 1.9×10^7 CFU/g count was determined in lupin protein isolate (Melde et al., 2016). In the protein concentrate, the greatest contribution to the TBC came from presumptive lactic acid bacteria (2.4×10^5 CFU/g). The Enterobacteriaceae (3.5×10^2 CFU/g) did not exceed the safety criteria for dried vegetables (10^4 CFU/g) reported by Gilbert et al. (2000) or the hygienic criteria in the processing of cereals (10^2–10^3 CFU/g) stated by ICMSF (2011). The sum of yeasts and moulds (2.1×10^3 CFU/g) was lower than the moulds limit specified by the FCD (2023). *Salmonella* was absent, as it was in the by-product. The native by-product presented an average TBC of 2.4×10^7 CFU/g, but the extraction significantly reduced microbiological contamination. In fact, sonication has a sanitizing effect because of the mechanical damage on cells (Bhargava et al., 2021); additionally, the first centrifugation precipitated cells and non-protein pellets, further reducing the number of bacteria in the solubilized protein extract. Finally, as previously discussed for other parameters, the microbiological quality of the extract could be dramatically improved by in-line processing of the by-product or by the adoption of a hygienic design at the collection point to minimize cross-contamination from pre-fermented residues.

4. Conclusions

The ultrasonication increased three-fold the extraction recovery of protein (from 21.5% to 66.6%) and reduced the treatment time from 4 to 1 h compared to the traditional method. The protein concentrate obtained under the optimal conditions presented a 74.9 g/100 g DM protein content and a gelling capacity equal to pea flour. Protein degradation, probably due to fermentation and enzymatic hydrolysis occurred before sampling, was observed in the by-product and concentrate. Total bacterial count, Enterobacteriaceae and moulds in the concentrate were always below the guideline values. The pea canning by-product stands out as a valuable raw material for protein recovery. Due to logistical and construction characteristics of the processing plant, the by-product had a high moisture content at the collection point. Therefore, for a scale-up of the extraction process a direct treatment of the by-product, avoiding drying costs, would be advantageous. Furthermore, a direct and rapid processing would prevent or limit the fermentation phenomena which partially compromise quality of the concentrate and protein recovery.

In this study the protein concentrate was oven-dried, possibly triggering the formation of insoluble aggregates; therefore, milder drying treatments should be tested and their influence on protein digestibility and technological behaviour monitored. Our research demonstrated the feasibility of protein recovery from pea by-product, but we did not assess the economic affordability of the process. It must also be acknowledged that the effluent will not be significantly reduced after extraction. Other emerging food processing technologies (e.g., microwave, pulsed electric fields, high-pressure, membrane filtration), could be coupled with ultrasound to promote protein solubilization or isolation. Finally, future research should investigate how the integration of protein concentrate from pea by-product may influence food formulations, especially in terms of safety and consumer acceptability.

CRediT authorship contribution statement

Lorenzo Estivi: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Andrea Brandolini:** Writing – review & editing, Writing – original draft, Investigation. **Andrea Catalano:** Writing – review & editing, Investigation, Formal analysis. **Roberta Di Prima:** Writing – review & editing, Resources, Data curation. **Alyssa Hidalgo:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization.

Declaration of competing interest

The 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.lwt.2024.116659.

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