



Milling Energy Impacts the Co-Extraction of Globulins, Albumins, and Anti-Nutritional Factors of Peas

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Abstract

Pea protein ingredients are typically produced through alkaline solubilization and isoelectric point precipitation. This work evaluated the influence of milling and the resulting flour particle sizes on the recovery of protein fractions and antinutrient contents via this extraction method. Milling energy inputs of 2.39 to 260 kJ/kg were applied to yellow and green peas. An energy input of 180 kJ/kg yielded a d_{90} of $\sim 2.2 \mu\text{m}$. No further significant difference was found when the input was increased further. Increasing the milling energy input increased the release of globulins, albumins, and phytic acid from both pea cultivars. The protein yield of the globulin-rich fraction increased up to $52.1 \pm 1.3\%$ using yellow peas and up to $54.2 \pm 0.6\%$ using green peas. The resulting protein extracts had protein contents of $77.0 \pm 1.9\%$ and $78.5 \pm 0.9\%$, respectively. Similarly, the protein yield of albumin-rich fractions also significantly increased up to around 17.5% when using both cultivars. The albumin-rich fractions represented the largest mass yielded in the extraction process from both pea types. With increasing milling energy input, phytic acid solubilization increased as well and its yield rose to around 40% in both protein fractions. However, trypsin inhibitor yields were relatively low in them. Overall, a milling energy input at 130 kJ/kg resulted in particle sizes that yielded optimum protein solubilization but simultaneously increased solubilization of phytic acid. This indicates that adjusting the energy input and particle size of the pea protein raw material can customize the composition of the resulting protein ingredient.

Keywords Pea protein isolate · Plant protein · Dry milling · Protein fractionation · Trypsin inhibitor · Phytic acid

Introduction

The use of plant proteins instead of animal proteins has become the leading global response to ensure that the food system remains within the planetary boundaries. Among the different sources of plant protein, peas (*Pisum sativum*) have gained popularity because of their high protein content and quality, low allergenicity, and widespread availability (Guillin et al., 2022; Lam et al., 2018; Lu et al., 2020). Pea proteins possess versatile techno-functional properties, such as gelation, emulsification, and foaming, which makes them a valuable food ingredient that is frequently employed as substitutes for animal proteins (Grossmann, 2023). These pea protein ingredients are typically produced through extraction processes, and wet extraction is one of the predominant

procedures (Lie-Piang et al., 2023). These extraction processes yield different fractions, each with different properties and potential applications, but there is currently limited understanding about how processing impacts the yields of these fractions (R. Kornet et al., 2022; Möller et al., 2021).

The pea wet extraction process follows the alkaline solubilization \rightarrow isoelectric point precipitation route, which ensures high-yield extraction and product purity (Allotey et al., 2022; Boukid et al., 2021). It employs dry milling of the seeds into flour, which is then dispersed in water at alkaline conditions. As alternative, wet milling of hydrated seeds can be applied. After solubilization of proteins, the starch-rich (SRF) fraction is removed as insoluble stream, whereas the pea proteins (globulins and albumins) are recovered as soluble stream or also called protein-rich (PRF) fraction. Non-starch constituents such as cotyledon cell wall fiber are also removed in the SRF. However, different types of carbohydrates can also end up in the PRF (Möller et al., 2021). To purify and concentrate the proteins, an isoelectric point precipitation at pH 4.5 is typically carried out (Lam

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et al., 2018). This primarily causes the globulin fraction to precipitate, while the albumin fraction remains in solution as long as it maintains its native state. Globulins and albumins are categorized according to their solubility in dilute salt solutions and in water, respectively, following the Osborne classification of plant proteins (Osborne, 1907). After separation, this results in two fractions: (i) a globulin-rich protein concentrate/isolate (GRF) and (ii) an albumin-rich side-stream (ARF). Different studies have shown that both fractions possess very different functional properties, which enables tailored applications (C. Kornet et al., 2020; R. Kornet et al., 2022; Yang et al., 2022). GRF, the most valorized product, shows capacity to form self-coacervate structures, stabilize oil–water interfaces, and create solid structures via thermomechanical processing (Grossmann, 2023). In addition, ARF shows high foaming due to its ability to form strong cohesive interfacial layers around air bubbles which GRF is not well suitable for this purpose (Chang et al., 2023; Yang et al., 2022). The use of ARF as a standalone ingredient would therefore contribute to developing a more circular and comprehensive approach to pea protein manufacturing.

Antinutritional factors (ANFs) that are commonly present in pea seeds such as phytic acid and trypsin inhibitors are co-extracted during the pea wet extraction process (Amat et al., 2023; Rivera Del Rio et al., 2022). This may have important implications for the digestibility of the ingredient, because phytic acid reduces bioavailability of minerals such as zinc and iron due to its chelation properties (Millar et al., 2019), whereas trypsin inhibitors inhibit digestive enzymes trypsin and chymotrypsin and consequently protein hydrolysis (Vagadia et al., 2017). As result, minerals and proteins from foods that contain these ANFs often have a lower digestibility. But concerns about ANFs are not limited to nutritional aspects. Phytic acid, for instance, can also negatively affect the techno-functional properties of protein isolates such as gelation, solubility, and emulsification (Amat et al., 2024; Kaspchak et al., 2020; Pedrosa et al., 2020). Protein interactions with phytic acid via hydrogen bonds or electrostatic interactions might interfere with protein structures that are important for their techno-functional properties (Kaspchak et al., 2020). Although concentration of ANFs in pea protein isolates or concentrates (i.e., GRF) is reported in literature, there are still knowledge gaps about the co-extraction of ANFs alongside proteins during the extraction process. Because of their molecular size and solubility properties, we hypothesize that ANFs mostly solubilize in the ARF which might affect its valorization.

The contents of proteins and ANFs in the final products might be affected by the extraction process conditions. Different processing parameters and separation approaches along the wet fractionation route of pea protein have been studied to optimize the production of isolates or concentrates, impacting the generation of side-streams. Examples

of them included the ratio of water to flour (Higa et al., 2022), extraction pH (Cui et al., 2020; Gao et al., 2020), and protein separation by micellar precipitation, dialysis, and ultrafiltration (Boye et al., 2010; Stone et al., 2015; Tanger et al., 2020; Yang et al., 2021). However, the effect of flour particle size after milling on the extraction yields has been not well described. A recent quantitative meta-analysis encompassing over 40 studies examining protein extraction from pulses (including peas) identified challenges in evaluating the effectiveness of milling on protein recovery (Allotey et al., 2022). This was because only a minority of the studies reported on the quality of the flour utilized in their extraction processes. In addition, there is a lack of knowledge about how these different processing parameters affect the solubilization and presence of ANFs in the resulting protein fractions.

Therefore, this study aims to evaluate the influence of milling energy input on peas and the resulting flour particle sizes on the recovery of protein fractions (globulins and albumins) and ANFs (phytic acid and trypsin inhibitors). The main hypothesis is that increasing milling energy decreases the particle size, increases surface area, and increases solubilization of pea proteins and ANFs. Understanding the protein fractionation mechanism will eventually enable more tailored process designs to maximize protein yields and minimize the presence of antinutrients in pea protein ingredients.

Materials and Methods

Materials

Cultivars AAC Delhi and AAC Radius were selected as representative material of peas (*Pisum sativum* L.) with yellow and green cotyledon, respectively. Both cultivars are round, smooth seed phenotypes, and have non-pigmented seed coats. Dehulled seeds were obtained from Columbia Seed Co. Ltd. (Vauxhall, Alberta, Canada). They were planted in May 2022 in the brown chernozemic soil zone in Canada and harvested in August 2022. Bags of 1 kg peas were vacuum sealed and stored at 2 °C until further use. Table 1 shows the components for both cultivars.

All chemicals were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO, USA) or VWR (Radnor, PA, USA). Laemmli sample buffer, 2-mercaptoethanol solution, Tris–glycine-sodium dodecyl sulfate (SDS) buffer, Coomassie brilliant blue R-250, recombinant proteins standard solution, and Mini-PROTEAN TGX 12% precast gels were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

Table 1 Composition of two yellow and green pea cultivars. The protein content was calculated using a nitrogen-to-protein factor of 5.36

	Yellow pea (AAC Delhi)	Green pea (AAC Radius)
Moisture (%)	7.5 ± 0.1	8.1 ± 0.1
Protein (% as is)	18.3 ± 0.1	18.2 ± 0.1
Starch (% as is)	40.2 ± 0.1	39.6 ± 0.1
Phytic acid (mg/g)	8.5 ± 0.2	9.9 ± 0.7
Trypsin inhibitor activity (TIU/mg)	4.0 ± 0.1	3.2 ± 0.1

Flour Production and Flour Characterization

Milling Process

A knife and impact milling system with a temperature-controlled vessel (MultiDrive MI 400, IKA Works, Inc., Wilmington, NC, USA) was used for all milling operations. The jacketed milling vessel was cooled with a refrigerated water bath at 4 °C. This ensured sample temperatures below 40 °C. The mill was operated at 20,000 rpm for all energy inputs, and milling time was varied from 5 to 120 s.

Determination of E_{SME} : The specific milling energy input (E_{SME}) was determined from the reported electrical power uptake of the mill. For each treatment, a 125 g batch of dry peas was ground using nine different time periods from 5 to 120 s. The electrical power uptake was measured using a plug load data logger (HOBO UX120-018, Onset, Bourne, MA, USA), and the energy input was calculated based on the milling time. To account for motor and other losses, a baseline energy uptake was defined as the energy required by the equipment during its operation using only water for the same periods. The E_{SME} (kJ/kg) was calculated using Eq. (1). The milling intensity was categorized as *minimal* (< 5 kJ/kg), *low* (5 to 9.99 kJ/kg), *moderate* (10 to 49.9 kJ/kg), *high* (50 to 149 kJ/kg), and *intense* (≥ 150 kJ/kg).

$$E_{SME} = \frac{E_{input} - E_{baseline}}{m_{peas}} \quad (1)$$

Here, E_{input} (kJ) is the total electrical energy input obtained from the power uptake from the pea milling process, $E_{baseline}$ (kJ) is the total electrical energy input obtained from using only water, and m_{peas} (kg) is the mass of the pea seeds used for milling.

Flour Particle Size Distribution

Static light scattering was used to obtain the particle size distribution of the obtained flours. An LS 13 320 Laser Diffraction Particle Size Analyzer equipped with a powder

fluidization chamber (Beckman Coulter, Inc., Brea, CA, USA) was used to determine the particle size at which 90% of the particle volume is below or equal to this size (d_{90}) and the surface area mean size ($d_{3,2}$). Surface area values can explain extraction efficiency of pulses proteins (Higa et al., 2022). The obscuration and refractive index were set at 5% and 1.6, respectively. Before the measurement, the flours were sieved using 2 mm and 1 mm mesh sizes to remove excessively large particles that can interfere with the measurement.

Extraction Process Flow

After milling, 62.5 g of flour (all the size fractions) was used for protein extraction using the alkaline solubilization-isoelectric point precipitation method described by Kornet et al. (2020) with some modifications (Fig. 1). Pea flour was dispersed in water at 10% (w/w) and adjusted to pH 8 with 1 M NaOH. The mixture was stirred for 2 h, and its pH was checked every 30 min and adjusted if necessary. The solution was stored for 12 h at 4 °C to ensure complete flour hydration and account for pH buffering. Subsequently, the pH was readjusted and centrifuged at 20,000 g for 30 min at room temperature. The resulting pellet is the start-rich fraction (SRF) and the supernatant is the protein-rich fraction (PRF). The PRF fraction was recovered and acidified to pH 4.5 with 1 M HCl while stirring for 2 h. The mixture's pH was controlled every 30 min and adjusted if necessary. Final centrifugation was applied at 20,000 g for 30 min at room temperature. The pellet and supernatant contain the globulin-rich fraction (GRF) and albumin-rich fraction (ARF), respectively. All fractions were freeze-dried, and the powders were stored at 4 °C in sealed containers until further analysis.

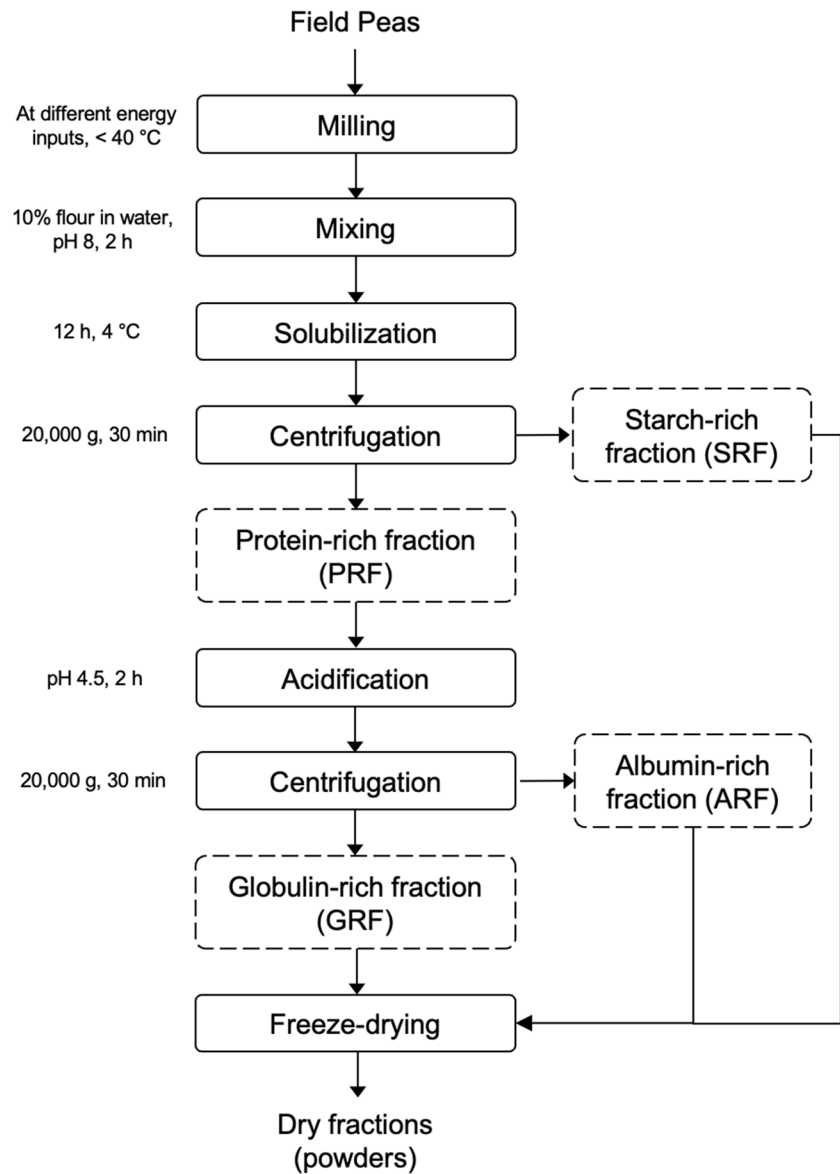
Mass Balance and Yield Calculations

The mass yield of the fractions SRF, PRF, GRF, and ARF before and after freeze-drying were calculated as shown in Eq. (2) for wet mass and Eq. (3) for dry mass. Protein, starch, phytic acid, and trypsin inhibitors yields (%) in the individual fractions were calculated based on how much was recovered from the flour as shown in Eq. (4):

$$Yield_{wet-fraction} = \frac{m_{wet\ fraction}}{m_{initial\ mixture}} \times 100 \quad (2)$$

Here, the $Yield_{wet-fraction}$ (%) is the yield of the fractions SRF, PRF, GRF, or ARF; $m_{wet\ fraction}$ (g) is the mass of the wet fractions; $m_{initial\ mixture}$ (g) is the mass of the pea flour–water mixture.

Fig. 1 Process flow of the pea protein extraction using the alkaline solubilization-isoelectric point precipitation method



$$Yield_{\text{dry-fraction}} = \frac{m_{\text{dry fraction}}}{m_{\text{initial dry flour}}} \times 100 \quad (3)$$

In this equation, the $Yield_{\text{dry-fraction}}$ (%) is the yield of the dry fractions SRF, PRF, GRF, or ARF; $m_{\text{wet fraction}}$ (g) is the mass of the dry fractions; $m_{\text{initial mixture}}$ (g) is the mass of the dry pea flour.

$$Component\ Yield = \frac{c_{\text{fraction}} \times Yield_{\text{dry-fraction}}}{c_{\text{flour}}} \times 100 \quad (4)$$

Here, $Component\ Yield$ (%) is the yield of protein, starch, phytic acid, or trypsin inhibitors in the SRF, PRF, GRF, or ARF; c_{fraction} is the concentration of the component in the dry fraction; c_{flour} is the concentration of the component in the pea flour.

Chemical Compositional Analysis

Protein

The protein content was determined utilizing the Dumas combustion method in a nitrogen analyzer Rapid N exceed (Elementar Analysensysteme GmbH, Langenselbold, Germany) with a combustion temperature of 950 °C. The oxygen dosing flow was set at 225 mL/min. The protein content was calculated using a nitrogen conversion factor of 5.36 (McClements & Grossmann, 2022).

Starch

The starch content or total starch was determined according to AOAC method 996.11 using the Megazyme total starch

assay kit (Neogen, Lansing, MI, USA). The manufacturer's instructions were followed for samples containing resistant starch that is typically found in pea cultivars and isolated pea starches (Chung & Liu, 2012; Zhou et al., 2019).

Phytic Acid

The content of phytic acid was measured using the Megazyme phytic acid/total phosphorus assay kit (Neogen, Lansing, MI, USA) according to the manufacturer's instructions. The assay is designed to extract the phosphorus from phytic acid (IP₆), lower *myo*-inositol phosphate forms (IP₂, IP₃, IP₄, IP₅), and monophosphate esters by phytase and alkaline phosphatase. Sample extraction was carried out overnight at room temperature. The total phytic acid content was calculated assuming that the amount of phosphorous is exclusively released from phytic acid and comprises 28% of phytic acid.

Trypsin Inhibitors

The trypsin inhibitor activity was analyzed as described by Liu (2019) with slight modifications. Samples were extracted with 10 mM NaOH for 3 h. All mixtures were then standardized to the highest pH value of 11.2. The following enzymatic reaction was carried out to achieve 30 to 70% trypsin inhibition. This was achieved by mixing 1 mL of the sample mixture, 2.5 mL of 0.4 mg/mL DL-BAPA (*N*α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride) in 50 mM Tris buffer containing 20 mM CaCl₂ at pH 8.2, and 1 mL of 20 μm/mL trypsin in 1 mM HCl containing 5 mM CaCl₂. After 10 min incubation at 37 °C, the reaction was stopped with 0.5 mL of 30% (v/v) acetic acid. For reference samples, 1 mL of deionized water was used instead of the sample mixture. Reaction blanks for all samples were prepared by adding acetic acid solution before the trypsin solution to inactivate the enzyme. The reaction mixtures were then centrifuged at 1500 g for 5 min and the absorbance of the resulting supernatant was measured at 410 nm. Water was used to blank the spectrophotometer before the measurements. Trypsin inhibitor activity was reported as trypsin inhibitor units (TIU) per mg of sample and was calculated using Eq. (5).

$$TIU = \frac{[(Abs_{Ref.} - Abs_{Ref. Blk}) - (Abs_{Sample} - Abs_{Sample Blk.})]}{c} \times 50 \quad (5)$$

where $Abs_{Ref.}$ is the absorbance of the reference sample (water instead of the sample), $Abs_{Ref. Blk}$ is the absorbance of the reference sample blank (deactivated), Abs_{Sample} is the absorbance of the sample, and $Abs_{Sample Blk}$ is the absorbance of the sample blank (deactivated). c is the concentration of the sample in the sample mixture in mg/mL.

Protein Composition

Qualitative analysis of the protein composition in the flours and the fractions obtained from milling at the lowest and highest energy input was performed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Samples were dispersed in water and mixed with Laemmli sample buffer and 5% 2-mercaptoethanol. Prior, the ARF samples were purified adding four times the sample volume of cold acetone and incubating for 1 h at –20 °C. After centrifugation (15,000 g for 10 min), the pellet was recovered, dried at room temperature for 30 min, and used as is for the dispersion. All dispersions were then incubated at 70 °C for 10 min and centrifuged at 15,000 g for 5 min. An aliquot of the supernatant (60 μg of proteins from flour and SRF, 135 μg of proteins from GRF, and 30 μg of proteins from ARF) and size standard (10–250 kDa) was loaded into the wells of the Mini-PROTEAN TGX 4–20% precast gels. Electrophoresis was then run at 150 V for 1 h in Tris–glycine-SDS buffer in a Mini-PROTEAN Tetra electrophoresis cell (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The gels were stained with Coomassie brilliant blue R-250 for 1 h at constant shaking and decolorized overnight with 27.5% (v/v) methanol and 15% (v/v) acetic acid.

Statistical Analysis

All experiments were performed at least three times and measurements were carried out at least in duplicate. Average and standard deviations were calculated with Excel (Microsoft Inc., Redmond, USA). The effect of milling energy input on flour particle size was analyzed using *t*-test (two-tailed distribution) to identify whether an increment in the energy input had a significant effect on the particle size. Prior, *F*-test was used to test for equal variances. The effect of milling and cultivar on the extraction efficiency (yields and composition) was completed using analysis of variance (ANOVA) followed by Tukey's post hoc test. OriginPro (OriginLab Corp., Northampton, Massachusetts, USA) was used to apply all statistical tests. Significant difference was defined as $p < 0.05$.

Results and Discussion

Effect of Milling Energy Input on Flour Particle Size

The milling energy input is positively correlated with a decrease in flour particle size (Palavecino et al., 2019). This first part of the study investigated the impact of the energy input on the achievable flour particle size of yellow and

green peas. To reveal the particle sizes at different energy inputs and find the minimum attainable particle size, the energy input was varied from $E_{SME} = 2.39$ to 260 kJ/kg.

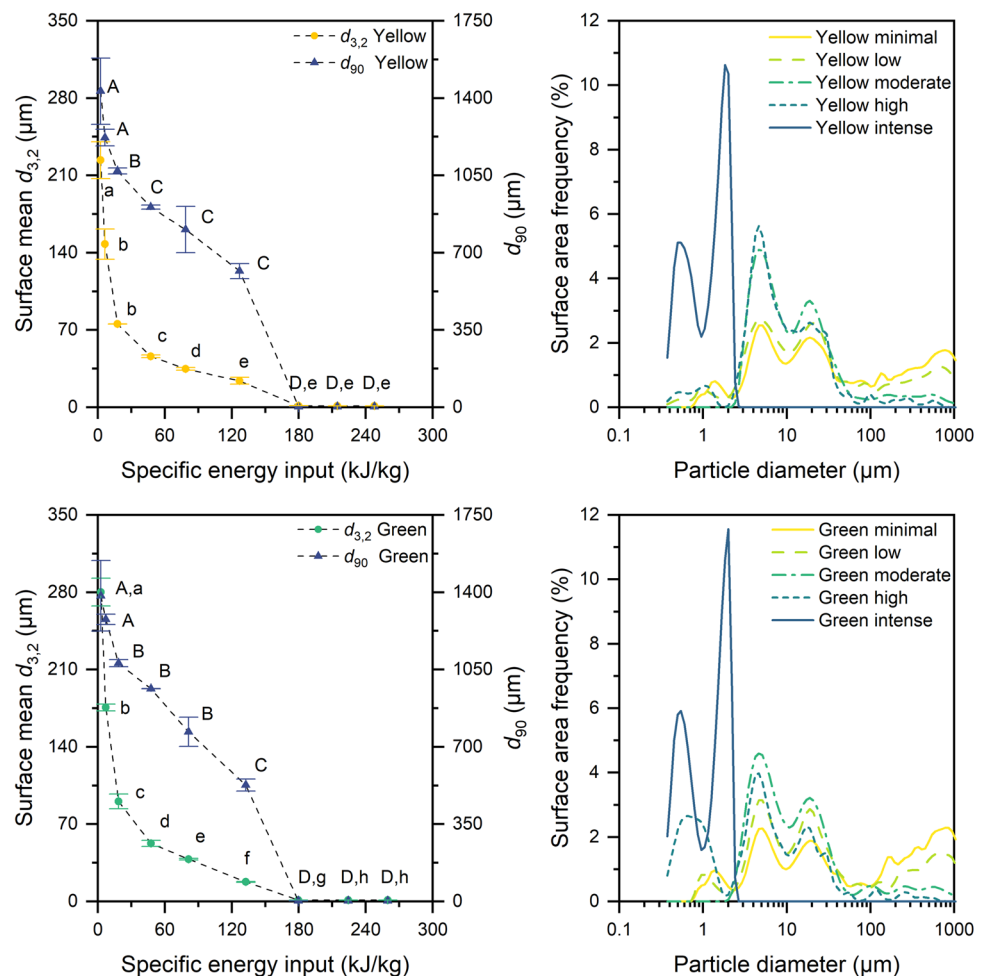
Overall, only slight differences in particle size were found between the two cultivars tested at the different energy inputs. Milling reduced the particle sizes of yellow and green peas similarly at different energy inputs. A minimal milling process at around 2 kJ/kg for yellow and green peas resulted in very coarse flours with ~75% (w/w) of particles being > 2 mm and ~10% (w/w) of particles being < 1 mm in both cultivars. Increasing the milling energy to a low input (around 6.5 kJ/kg) increased the < 1 mm fraction to ~38% (w/w). After a moderate input (47 kJ/kg), more than 90% (w/w) of particles of yellow and green pea flours were < 1 mm. Increasing the energy input further decreased the particle size of all samples < 1 mm when the energy input exceeded around 80 kJ/kg. Figure 2 shows the resulting $d_{3,2}$ and d_{90} for the 1 mm fraction from both cultivars.

Milling further at an intense energy input (180 kJ/kg for both cultivars) significantly reduced the d_{90} to 2.24 ± 0.01 μm and 2.27 ± 0.01 μm for yellow and green pea flours, respectively. At this energy input, the $d_{3,2}$ was 1.31 ± 0.01

μm for both cultivars. No significant difference in the d_{90} was found in the flours milled at higher energy inputs for each cultivar. For this reason, the energy input range of 2.39 to 180 kJ/kg was chosen for all further experiments. Moreover, Fig. 2 also includes the surface area distribution of the < 1 mm fractions of flours after minimal, low, moderate, high, and intense milling for yellow and green flours. The particle size distributions of the flours from both cultivars were similar at the chosen energy inputs. After moderate and high milling inputs, the flours had a multimodal distribution for surface area with two peaks at 5 and 20 μm . After intense milling input, the flours had a clear bimodal distribution with peaks with maxima at 0.5 and 2 μm .

The structure of pea cotyledon consists of storage cells of 100 to 150 μm where starch granules of 5 to 30 μm and protein bodies < 10 μm are embedded (Jiang et al., 2021; C. Kornet et al., 2020; Monnet et al., 2019; Pelgrom et al., 2013). It can be expected that these storage cells are disrupted during the milling operation. Depending on the milling energy input applied, starch granules can be exposed but remain within the cells, released as a whole, or fragmented (*i.e.*, particles < 30 μm). In contrast, protein bodies

Fig. 2 Particle size analysis of yellow and green pea flours after milling at different energy inputs. Different letters denote significant differences ($p < 0.05$) among $d_{3,2}$ (lower case) and d_{90} (upper case) values. Surface area frequency is shown for the < 1 mm fraction of flours milled under minimal (2.39 kJ/kg for yellow and 2.74 kJ/kg for green), low (6.39 kJ/kg for yellow and 7.03 kJ/kg for green), moderate (47.4 kJ/kg for yellow and 47.6 kJ/kg for green), high (127 kJ/kg for yellow and 133 kJ/kg for green), and intense (180 kJ/kg for both cultivars) milling conditions



can be present either attached to the surface of the granule, absorbed in vacuoles, or free in the form of aggregates (Dashek & Clore, 2017; Möller et al., 2021; Monnet et al., 2019). Hence, the different flours obtained in this study from both cultivars likely contained a mixture of these elements. After minimal milling, flours would most likely contain large fragments of cotyledon ($\sim 90\%$ w/w of the flour > 1 mm), partially disrupted storage cells (< 100 μm), and minimal fractions of released starch granules (< 30 μm). Increasing to moderate and high energy inputs, the disrupted storage cells would then be reduced further into starch granules with attached protein bodies (< 30 μm). Free forms of proteins and damaged starch granules therefore most likely compose the peaks at 5 and 20 μm (Fig. 2). Finally, after an intense milling input, flours would contain mostly small damaged starch granules and partially disrupted and free protein bodies composing the peaks with maxima at 0.5 and 2 μm .

Effect of Particle Size on Mass and Protein Yields

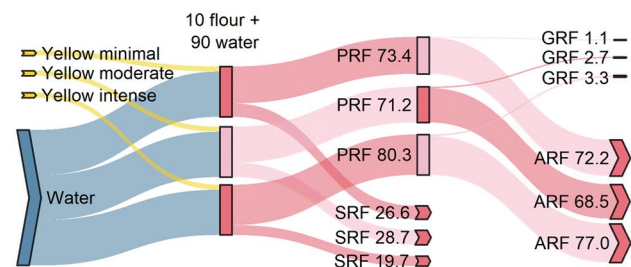
The protein extraction was carried out by wet fractionation through alkaline solubilization-isoelectric point precipitation using flours of peas milled at minimal, low, moderate, high, and intense milling energy inputs. Wet mass balance, dry mass (powder) balance, and protein yield of the fractionations were calculated and are illustrated in Fig. 3 for yellow pea flours and in Fig. 4 for green pea flours.

Wet and Dry Mass Balances

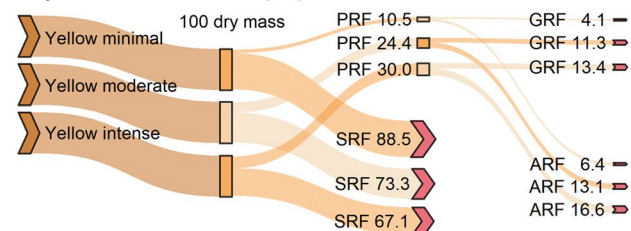
The wet balance shows that the alkaline solubilization-isoelectric point precipitation route generates large amounts of side-streams, *i.e.*, the ARF. The SRF can be considered a side-stream of protein extraction, but it is used to produce pea starches. In general, when yellow and green pea flours were subjected to intense milling that resulted in the smallest particle size ($d_{3,2} = \sim 1.3$ μm and $d_{90} = \sim 2.2$ μm in both cultivars), the ARFs represented 77% of the initial wet mass ($77.0 \pm 1.1\%$ for yellow and $76.4 \pm 2.5\%$ for green) and the SRFs represented 68% of the initial dry mass ($67.1 \pm 6.0\%$ for yellow and $68.2 \pm 3.7\%$ for green). In comparison, the final product of the protein extraction process (GRF), represented only about 3% of the total wet mass ($3.3 \pm 0.1\%$ for yellow and $3.3 \pm 0.2\%$ for green) and 13% of the dry mass under the same conditions ($13.4 \pm 0.3\%$ for yellow and $13.7 \pm 1.1\%$ for green).

When increasing the energy milling input from minimal to intense, wet mass yield of SRF decreased using yellow and green pea flours. Conversely, the recovery of GRF and ARF increased in the fractionation process from both flours due to decreased particle sizes and increased surface area after milling. Higher milling input enhances the extraction of PRF. Differences between balances show that the SRF

Wet mass balance (%)



Dry mass balance (%)



Protein yield (%)

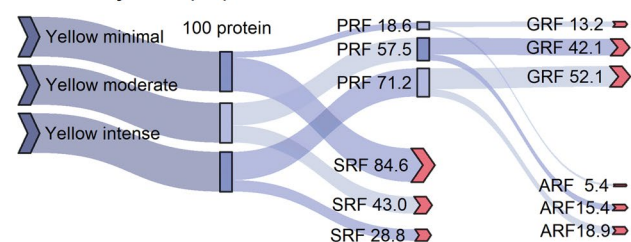


Fig. 3 Wet and dry mass balance as well as protein yield along the fractionation process from yellow pea flours after minimal (2.39 kJ/kg), moderate (47.4 kJ/kg), and intense (180 kJ/kg) milling conditions. PRF: protein-rich fraction; SRF: starch-rich fraction; GRF: globulin-rich fraction; ARF: albumin-rich fraction. The total ranged from $100 \pm 0.2\%$ to $100 \pm 0.3\%$ for the wet mass balance, $97 \pm 6.4\%$ to $101 \pm 3.1\%$ for the dry mass balance, and $99 \pm 7.7\%$ to $103 \pm 2.5\%$ for protein yield

contains a high solid content whereas the ARF contains large amount of water. Further downstream operations of ARF therefore require the capacity to process large volumes.

Protein Yields

Starch Fraction. As shown in Figs. 3 and 4, the SRF mass decreased when increasing the energy input. This is explained by an increase in protein recovery in the GRF and ARF, which was a result of enhanced protein extraction from the SRF. More protein is being solubilized from the flours milled at higher energy inputs showing that larger surface area increases extraction efficiency. When increasing the energy milling input from minimal to intense, the protein content in SRF significantly decreased from $18.9 \pm 0.5\%$ to $8.5 \pm 0.1\%$ using yellow pea flours and from $19.4 \pm 0.4\%$ to

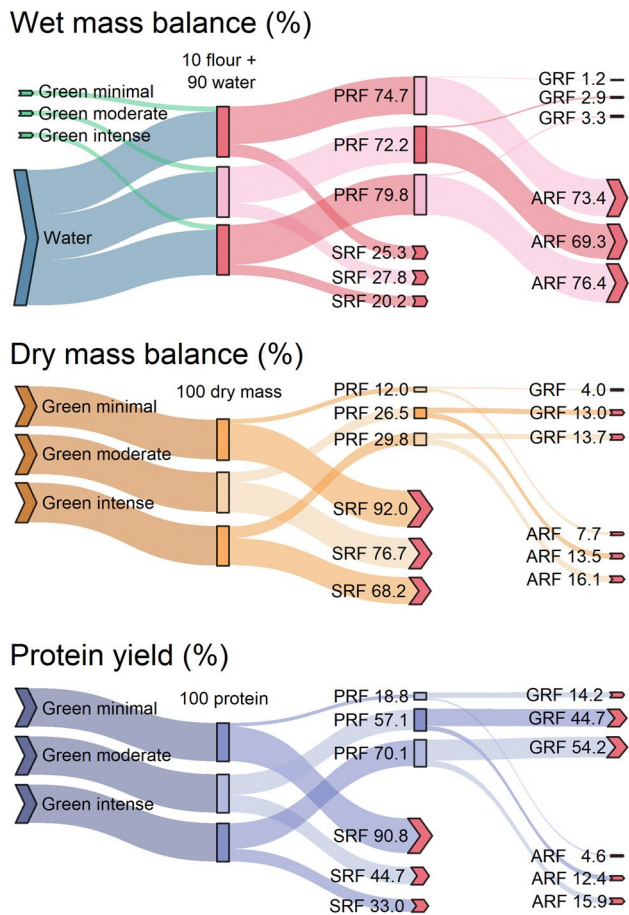


Fig. 4 Mass balance of wet and dry mass and protein yield along the fractionation process from green flours after minimal (2.74 kJ/kg), moderate (47.6 kJ/kg), and intense (180 kJ/kg) milling conditions. PRF: protein-rich fraction; SRF: starch-rich fraction; GRF: globulin-rich fraction; ARF: albumin-rich fraction. The total ranged from $100 \pm 0.1\%$ to $100 \pm 0.3\%$ for the wet mass balance, $97 \pm 2.1\%$ to $105 \pm 3.2\%$ for the dry mass balance, and $102 \pm 2.1\%$ to $109 \pm 1.6\%$ for protein yield

$9.5 \pm 1.0\%$ using green pea flours. However, no significant increases in extraction yields from the SRF were found anymore for high and intense milling for both cultivars.

The particle sizes in flours after intense milling ($d_{3,2} = \sim 1.3 \mu\text{m}$ and $d_{90} = \sim 2.2 \mu\text{m}$ in both cultivars) should have sufficed to expose protein bodies ($< 10 \mu\text{m}$) from the storage cells. Yet $\sim 30\%$ of the protein was retained in the SRF even for the smallest particle sizes obtained under intense milling. Kornet et al. (2020) showed that smaller aggregates of protein bodies attach to the starch granules and become insoluble, which means they do not solubilize into the PRF and remain in the SRF. This effect might be induced by the milling process that may promote electrostatic attraction between protein bodies and cell wall fragments or starch granules (Möller et al., 2021). Electrostatic attraction of particles occurs when they become charged through electron

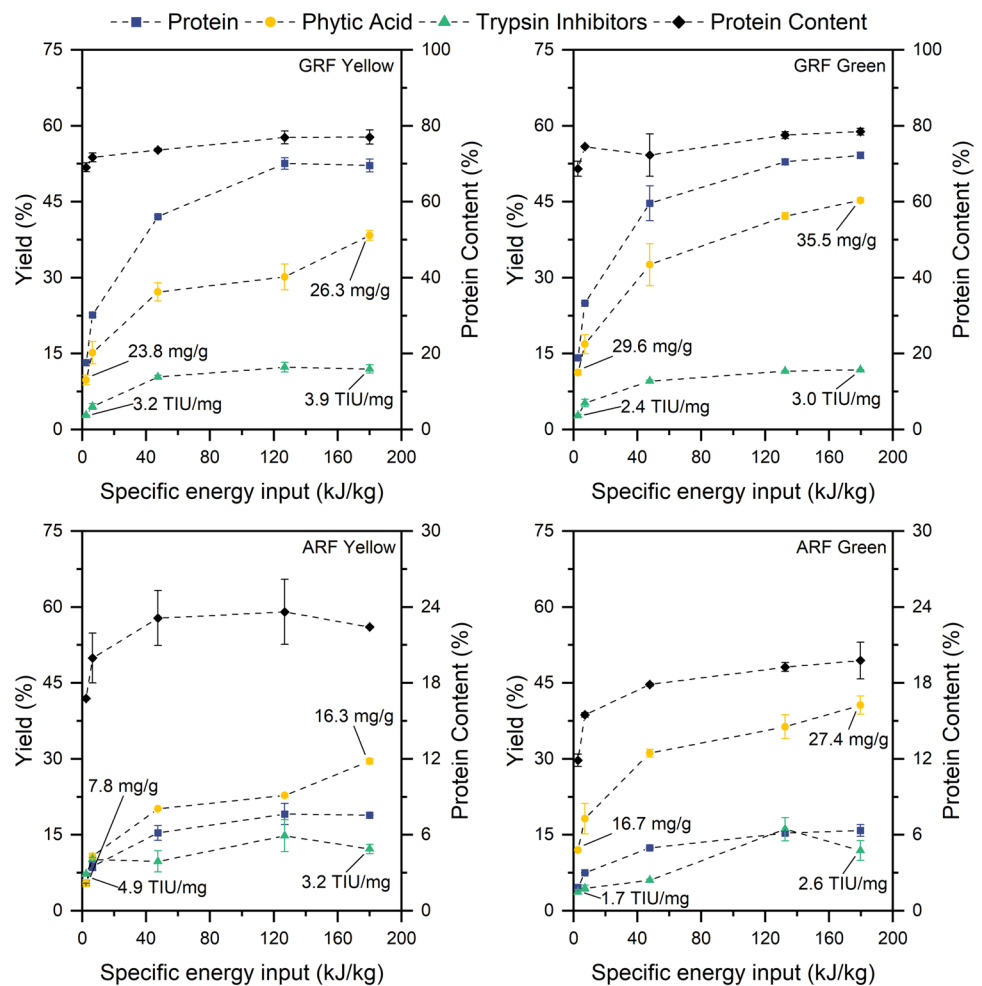
transfer by contacting each other (contact electrification) or by impacting on the milling vessel (Hemery et al., 2009). In general, protein fractions are charged to a much higher extent than starch fraction after milling (Assatory et al., 2019). Perhaps, the solubilization conditions used during the extraction were insufficient to overcome the strength of these interactions.

Globulin Fraction. As the milling energy input increased from minimal to intense, the protein yield of the GRF significantly increased using yellow and green pea flours as shown in Fig. 5. Higher milling energy inputs reduced particle sizes and increased surface area of flour particles which enhanced the extraction of pea proteins. No significant differences in the yields and contents of protein were found in the fractions from flours obtained with high milling and intense milling inputs for each cultivar and between them. This indicates that there is an optimum particle size at which maximum globulin solubilization is achieved and decreasing the particle size further does not increase the solubilization. In this study, the optimum particle size was $d_{90} = 617 \pm 34 \mu\text{m}$ ($d_{3,2} = 24.0 \pm 3.1 \mu\text{m}$) for yellow peas and $d_{90} = 527 \pm 27 \mu\text{m}$ ($d_{3,2} = 17.7 \pm 0.4 \mu\text{m}$) for green peas. In addition, this study also found that among all milling conditions, 71 to 73% of the solubilized proteins in PRF from yellow pea flours ended up in GRF, whereas slightly higher recoveries were found from green pea flours with 75 to 78% of the solubilized proteins in the PRF ended up in GRF.

Results in the GRFs from yellow and green pea flours milled at high energy input were similar to previous studies (Adebiyi & Aluko, 2011; Boye et al., 2010; Chang et al., 2022; Karaca et al., 2011; C. Kornet et al., 2020; R. Kornet et al., 2022; Lam et al., 2017; Stone et al., 2015). They reported protein contents in protein isolates (*i.e.*, the GRF) from 70 to 82% (nitrogen-to-protein conversion of 5.36 as used in our study) and protein yields from 44 to 68% (protein extracted in the dry powder extracted from the protein in the flour). Also, our results were in the range of the protein contents of five commercial pea protein products (48 to 79%) reported in Burger et al. (2022). The broad range of their protein contents, however, suggests that different protein extraction methods or downstream processes were applied in their production.

Nevertheless, limited studies reported the particle size of the flours used for the extraction process and its effect on the wet fractionation of peas. Higa et al. (2022) concluded that pulses flours with smaller particle sizes ($d_{4,3} = 63$ to $142 \mu\text{m}$ and $d_{90} = 195$ to $318 \mu\text{m}$) had higher extraction and protein yields in their protein concentrates than the flours with larger particles ($d_{4,3} = 312$ to $413 \mu\text{m}$ and $d_{90} = 752$ to $945 \mu\text{m}$), which is in line with our results. In the GRF from yellow peas, the protein yield and content were 65.2% and 79.5%, respectively, from flour with small particles ($d_{4,3} = 86 \mu\text{m}$ and $d_{90} = 249 \mu\text{m}$). The protein yield was higher than the

Fig. 5 Yields of protein, phytic acid, and trypsin inhibitors in the globulin-rich fraction (GRF, top) and albumin-rich fraction (ARF, bottom) from yellow and green pea flours milled at different specific energy inputs. The resulting content of each component is also shown for the fractions from flours milled at the lowest and highest energy input



values reported in this study from flours with smaller particles. Lam et al. (2017) also reported higher protein yields from six different yellow and green pea cultivars. Using flours refined through a 1 mm screen, they obtained protein yields from 64.5 to 67.5% but no significant differences were found between cultivars. Like Higa et al. (2022), both used defatted flour and flour dispersion at pH 9 to 9.5 which differed from the conditions used in the present study, *i.e.*, whole flour with extraction at pH 8.

Gao et al. (2020) reported a significant increase of protein yields at higher alkaline conditions (49.2% at pH 8.5, 52.4% at pH 9.0, and 57.6% at pH 9.5) but lower yields compared to previously reported values. Hence, our data need to be interpreted with caution and it could be that also other effects beyond pH value during the extraction step play an important role here, especially because the globulins typically show no considerable increase in solubility above pH 8 (Grossmann, 2023; Kimura et al., 2008). Moreover, the protein content in the GRF is not affected by the pH, as demonstrated by Cui et al. (2020) who studied protein extraction in different yellow pea cultivars from pH 8.5 to 10. We hypothesize that the adhesion mechanism between

protein bodies and starch granules previously explained was the main factor that limited the solubilization of proteins, but more studies will be needed to confirm this.

Albumin Fraction. In the ARF, protein yield increments were also observed as shown in Fig. 5. When increasing the energy milling input from minimal to intense, the protein yield in ARF significantly increased using yellow and green pea flours due to increased surface area. No significant differences in the protein yields were found using yellow pea flours milled at moderate, high, and intense energy inputs. Under these conditions, the protein content of the ARFs was around 23% and showed no significant differences between them. Hence, the optimum particle size to obtain ARF from yellow pea flours was $d_{90} = 906 \pm 9 \mu\text{m}$ ($d_{3,2} = 46.2 \pm 1.4 \mu\text{m}$). In the ARFs from green pea flours, no significant differences in protein yields were found when the milling energy input was increased from high to intense. The protein contents of the ARFs under these conditions were around 19% and showed no significant differences between them. For green pea flours, the optimum particle size to obtain ARF was $d_{90} = 527 \pm 27 \mu\text{m}$ ($d_{3,2} = 17.7 \pm 0.4 \mu\text{m}$). When comparing between cultivars, no significant differences in

the protein yield and content were observed from both optimum conditions.

Protein contents in the ARF were previously reported as well (nitrogen-to-protein conversion of 5.36 as used in our study). For instance, Kornet et al. (2020) studied yellow pea flours (80 μm) and obtained ARF with protein contents of 19.8% and protein yield of 17%. In a later study, Kornet et al. (2022) showed higher protein content in the ARF (48.9%) and lower protein yield (12.3%) from flours from yellow peas (80 μm). This was explained due to the use of diafiltration as an additional downstream operation. Likewise, Yang et al. (2022) obtained an ARF with 49% protein content (12.3%) after diafiltration.

Protein Profile of the Fractions

Under reducing conditions in SDS-PAGE, we performed a qualitative analysis of the proteins in yellow and green flours as well as the fractions (SRF, GRF, and ARF) obtained from the extraction process after minimal and intense milling. As shown in Fig. 6, yellow and pea flours contain different globulin and albumin proteins, with legumin and vicilin being the most dominant protein fractions. Based on the band intensity, no considerable differences in the content of legumin and vicilin among different untreated green and yellow pea flours were identified (Choi et al., 2022; Lam et al., 2017). After the wet fractionation method to produce protein isolates, the presence of the pea proteins overall was similar in the fractions, except in ARF, after minimal and intense milling. In ARF, a broader range of proteins was observed after intense milling. Among the fractions, the profile of

ARF differed from the GRF which showed similar ranges of proteins with the SRF and pea flours.

The GRF was comprised of a broad range of pea proteins: the 11S fraction (legumin) and the 7S fraction (vicilin and convicilin) of the globulin component and lipoxygenases, lectins, pea albumin 1 (PA1), and pea albumin 2 (PA2) of the albumin component. Conversely, the ARF mainly had ~ 30 kDa vicilin, legumin β -subunit, lectins, PA1, and PA2 which overall had lower MWs. The protein profile of ARF also included lipoxygenases that have higher MW (100 kDa). Dziuba et al. (2014) reported pea albumin component with high MW proteins (~ 50 to 110 kDa) which might include pea albumin large (PMA-L) fraction and pea albumin small (PMA-S) fractions. The presence of some globulins in the ARF can be also possible with differences in the solubility at the isoelectric point of 4.5. Further studies are needed to characterize the protein composition of ARF. Overall, similar differences between GRF and ARF were also reported in previous studies (Chang et al., 2023; Moller et al., 2022).

Starch Yields

In general, we found that the majority of the starch from flours ended up in the SRF ($87.8 \pm 6.3\%$ to $95.4 \pm 1.4\%$). No significant differences in the starch yields were found. When increasing the energy input, the starch content of SRF increased from $46.9 \pm 0.7\%$ to $55.6 \pm 0.2\%$ using yellow pea flour and from $42.7 \pm 3.6\%$ to $56.3 \pm 2.0\%$ starch using green pea flour. However, changes in the starch content were not significant among different milling intensity conditions and between cultivars. In the GRF and ARF, less than 2% of starch yield was obtained for each fraction independently of

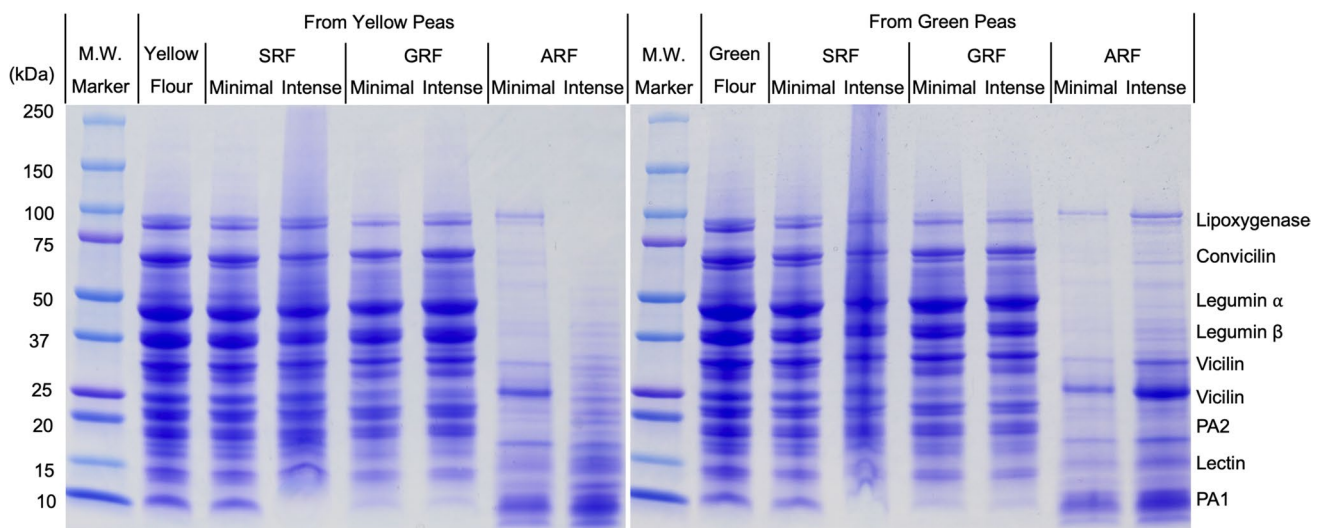


Fig. 6 Protein profile visualized by reducing SDS-PAGE of yellow and green pea flours and SRF, GRF, and ARF from the protein extraction process after using minimal (2.39 kJ/kg for yellow and

2.74 kJ/kg for green) and intense (180 kJ/kg for both cultivars) milling conditions. SRF: starch-rich fraction; GRF: globulin-rich fraction; ARF: albumin-rich fraction

the energy input. Using yellow pea flours, the starch content of GRF decreased from $5.2 \pm 0.1\%$ to $2.4 \pm 0.1\%$; and using green pea flours, it decreased from $5.4 \pm 0.6\%$ to $2.2 \pm 0.2\%$. In the ARF, the starch content increased with increasing milling intensity from $1.7 \pm 0.4\%$ to $4.7 \pm 0.3\%$ for yellow pea flour and from $2.2 \pm 0.1\%$ to $3.7 \pm 0.6\%$ for green pea flour. This might be due to the solubilization of starches due to the disruption of starch granules at higher milling intensities.

Effect of Particle Size on Antinutritional Factor Extraction

Phytic Acid

Initially, the content of phytic acid in the untreated flours was measured. A phytic acid content of 8.5 ± 0.2 mg/g in yellow pea flour and 9.9 ± 0.7 mg/g in green pea flour was found as shown in Table 1. Although a higher content of phytic acid was identified in green peas, there is no correlation between cultivars with different cotyledon colors and phytic acid content (Amarakoon et al., 2012). In general, our values were slightly higher than previous data reported in literature that ranged from 4.5 to 8.3 mg/g (Amarakoon et al., 2012; Gultekin Subasi et al., 2024; Millar et al., 2019; Wang et al., 2008). These studies concluded that phytic acid in yellow and green peas depends on the cultivar and its origin.

In the SRF, similar to the protein yield results, the content of phytic acid significantly decreased in the fractions when increasing the milling energy. Its phytic acid content decreased from 8.5 ± 0.1 mg/g ($82.4 \pm 1.3\%$ yield) to 3.4 ± 0.1 mg/g ($25.1 \pm 0.7\%$ yield) in the fractions from yellow pea flours when increasing the milling from minimal to intense energy input. Likewise, the content decreased from 8.1 ± 0.1 mg/g ($70.2 \pm 0.3\%$ yield) to 1.9 ± 0.2 mg/g ($12.4 \pm 1.5\%$ yield) in the fractions from green pea flours after a similar increase in milling energy conditions. Hence, the reduction of phytic acid in the SRF indicated that it was released alongside the proteins from the flour at pH 8. The inherent association between protein and phytic acid has been previously reported (Chigwedere et al., 2023; Fredrikson et al., 2001), and it is known that at pH > 6, phytic acid is soluble due to interactions with proteins and multivalent cations from minerals (Amat et al., 2024; Cheryan & Rackis, 1980).

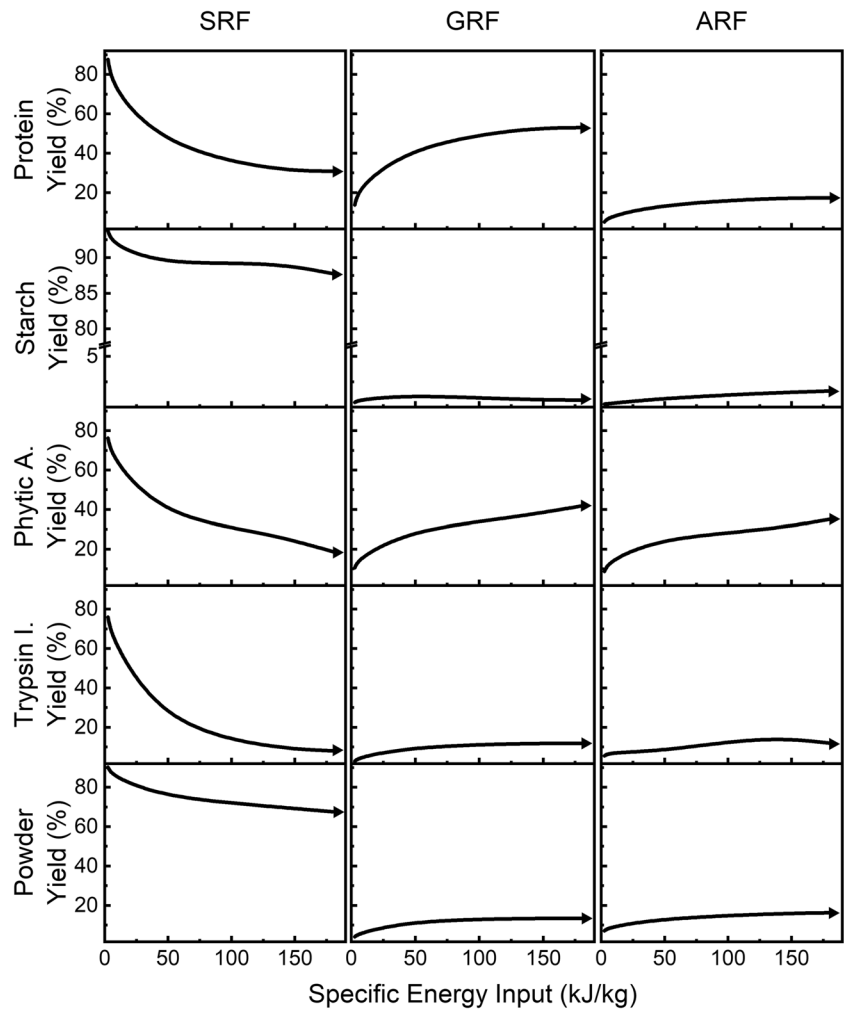
Figure 5 shows the contents and yields of phytic acid in the GRF and ARF obtained from flours milled at increasing energy inputs. In the GRF, the phytic acid yield significantly increased when increasing the milling energy input from minimal to intense in both cultivars. However, no significant differences were found in the phytic acid contents of GRF when increasing the energy inputs within each cultivar. When comparing yellow and green flour, it was

revealed that the phytic acid contents in the GRFs from green pea flours milled at intense and high energy inputs (33.5 ± 0.5 mg/g and 35.5 ± 0.4 mg/g, respectively) were significantly higher than the phytic acid contents among all the GRFs from yellow pea flours. No significant differences were found between the GRFs from green pea flours milled at low and moderate energy inputs (27.3 ± 3.0 mg/g and 28.5 ± 3.6 mg/g, respectively) and all the GRFs from yellow pea flours. These values were in a similar range than previously reported, which ranged from 9.6 to 25.5 mg/g (Chigwedere et al., 2023; Gultekin Subasi et al., 2024; Pedrosa et al., 2020). Phytic acid yields in the protein fractions, however, were lower than expected. In acidic conditions, phytic acid has a strong phytate-protein interaction which explains that 60 to 70% of the initial phytic acid from soybean ended up in the protein isolates prepared by isoelectric precipitation (Cheryan & Rackis, 1980). Differences in the protein profile, pH, and ionic strength in the protein fractions might explain the differences (Amat et al., 2023).

In the ARF, the yield and content of phytic acid significantly increased in both cultivars when increasing the milling energy input from minimal to intense. The ARF from intense energy input in yellow flour had significantly higher concentration of phytic acid (16.3 ± 0.3 mg/g) than the ARFs from moderate and intense input (14.1 ± 0.1 mg/g and 13.1 ± 0.2 mg/g, respectively). No significant differences were found, however, in the phytic acid contents of ARFs from green pea flours milled at moderate, high, and intense energy inputs (24.3 ± 0.6 mg/g to 27.4 ± 1.2 mg/g). These fractions had significantly higher contents of phytic acid than the ARFs from all yellow pea flours evaluated.

In conclusion, the phytic acid content in the ARF was affected by the cultivar and energy input. To produce an ARF with lower phytic acid content can be tailored by particle size and cultivar without neglecting its protein content. In this study, the optimum condition (i.e., maximum protein content and minimum phytic acid) was the use of yellow pea flours with $d_{90} = 617 \pm 34$ μm ($d_{3,2} = 24.0 \pm 3.1$ μm). Conversely, in the GRF, its content was not affected by energy input but by cultivar. The use of yellow pea flours produced GRF with lower phytic acid. Moreover, evaluating the mineral composition of the ARFs and GRFs, especially zinc and iron, would help to better evaluate the nutritional risk of the presence of phytic acid. Higher concentrations of these minerals can overcome the interference by phytic acid during mineral absorption in the digestive tract (Amarakoon et al., 2012; Auer et al., 2024; Millar et al., 2019). Moreover, membrane filtration or micellar precipitation can help to reduce the phytic acid content in the GRF and ARF (Fredrikson et al., 2001; Taherian et al., 2011; Tanger et al., 2020).

Fig. 7 Yield trends of protein, starch, phytic acid, trypsin inhibitors, and powder (dry fraction) in the starch-rich fraction (SRF, left), globulin-rich fraction (GRF, middle), and albumin-rich fraction (ARF, right) from yellow and green peas



Trypsin Inhibitors

Yellow and green pea flours had an initial inhibitor content of 4.0 ± 0.1 TIU/mg and 3.2 ± 0.1 TIU/mg, respectively, as shown in Table 1. Like phytic acid, the content of trypsin inhibitors in peas depends on the cultivar and origin rather than the cotyledon color. Among different cultivars, trypsin inhibitor content can vary between 1.3 and 5.5 TIU/mg (Fenn et al., 2022; Millar et al., 2019; Reinkensmeier et al., 2015; Wang et al., 2008). In this work, yellow pea flour contained higher trypsin inhibitors than green pea flour.

In the SRF, the trypsin inhibitor activity significantly decreased in the fractions with increasing milling energy input from minimal to intense. Its trypsin inhibitor activity decreased from 3.6 ± 0.3 TIU/mg ($73.2 \pm 5.2\%$ yield) to 0.4 ± 0.1 TIU/mg ($6.9 \pm 1.0\%$ yield) in the fractions from yellow pea flours. Likewise, it decreased from 3.0 ± 0.1 TIU/mg ($78.9 \pm 3.7\%$ yield) to 0.5 ± 0.1 TIU/mg ($9.5 \pm 0.4\%$ yield) in the fractions from green pea flours under similar conditions. As observed with other fractions, a threshold was identified and no significant difference in the inhibitor

activity in the SRF was observed when the milling energy intensity was increased from high to intense milling inputs for each cultivar and between them.

The reduction of trypsin inhibitors in the SRF with a decrease in particle size indicated that it was released alongside the proteins during solubilization at pH 8. A larger surface area of pea flours increased extraction efficiency of trypsin inhibitors. However, the measured trypsin inhibitor yield in the PRF was only around 30% from both studied yellow and green flours milled at high and intense energy input (which had the highest protein yields). In the GRF and ARF, as shown in Fig. 5, the trypsin inhibitor yield was only 12% in both fractions from yellow and green pea flours milled at intense energy input. Hence, it seems that the extraction procedure has influenced the trypsin inhibitor activity, highlighting the necessity for further investigation to pinpoint the precise mechanism involved.

No significant differences in the trypsin inhibitors were found in the GRF obtained from flours milled at increasing energy inputs and in the ARF obtained from the same flours. Milling energy input was not a factor that affected

the content of trypsin inhibitors in both fractions. However, differences between cultivars were observed. When comparing both cultivars, the trypsin inhibitors in the GRFs from yellow pea flours milled under moderate, high, and intense input (3.9 ± 0.3 TIU/mg to 4.0 ± 0.3 TIU/mg) were significantly higher than their content in the fractions from all green flours studied. Moreover, no significant differences in the trypsin inhibitors were found in the ARF from green peas milled at high and intense input (2.6 ± 0.4 TIU/mg and 3.6 ± 0.5 TIU/mg, respectively) and the fractions from all the yellow pea flours studied.

Earlier studies reported varying trypsin inhibitor activities in protein isolates from different pulses using the wet fractionation method by alkaline solubilization-isoelectric point precipitation. Trypsin inhibitor activities in protein isolates (*i.e.*, GRF) derived from different peas ranged from 0.13 to 3.7 TIU/mg (Cipollone & Tironi, 2020; Fernández-Quintela et al., 1997; Pedrosa et al., 2020). However, no data has been previously published that reported the trypsin inhibitor activity in the ARF.

Conclusions

Increasing the energy input during milling of peas resulted in a decrease in particle size and increase in surface area. Smaller particle sizes (*i.e.*, larger surface area) enhanced co-extraction efficiency of proteins and ANFs from pea flours during the alkaline extraction-isoelectric point precipitation process. Higher protein contents, but also higher ANFs, were recovered in the final protein products (*i.e.*, GRF and ARF) at higher milling input as shown in Fig. 7. Most proteins ended up in the GRF which has been traditionally the final product of the extraction process. However, the ARF was the largest fraction by wet mass, which resulted in considerable amounts of protein that can be recovered from this fraction (5 to 19%). A milling energy input of around 130 kJ/kg ($d_{90} \sim 570$ μm and $d_{3,2} \sim 20$ μm) might suffice to prepare yellow or green pea flours to obtain maximum protein yields in the GRF and ARF. With this condition, to produce 1 kg of dry GRF with a protein content of 77%, approximately 10 kg of yellow or green peas would be required; and to produce 1 kg of dry ARF with a protein content of 22%, approximately 30 kg of yellow peas or 36 kg of green peas would be required. No practical differences would exist in the content of starch, phytic acid, and trypsin inhibitors in the final products when the energy input is increased beyond 130 kJ/kg. Thus, milling intensity has a considerable influence on total and protein yields, which is important to design optimized plant protein extraction processes.

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Author Contribution Galo Chuchuca Moran: conceptualization, methodology, formal analysis, investigation, visualization, writing—original draft; Lutz Grossmann: conceptualization, supervision, writing—review and editing, resources and funding acquisition.

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Data Availability No datasets were generated or analyzed during the current study.

Declarations

Competing Interests The authors declare no competing interests.

References

- Adebiyi, A. P., & Aluko, R. E. (2011). Functional properties of protein fractions obtained from commercial yellow field pea (*Pisum sativum* L.) seed protein isolate. *Food Chemistry*, 128(4), 902–908. <https://doi.org/10.1016/j.foodchem.2011.03.116>
- Allotey, D. K., Kwofie, E. M., Adewale, P., Lam, E., & Ngadi, M. (2022). A meta-analysis of pulse-protein extraction technologies: Impact on recovery and purity. *Journal of Food Engineering*, 327, 111048. <https://doi.org/10.1016/j.jfoodeng.2022.111048>
- Amarakoon, D., Thavarajah, D., McPhee, K., & Thavarajah, P. (2012). Iron-, zinc-, and magnesium-rich field peas (*Pisum sativum* L.) with naturally low phytic acid: A potential food-based solution to global micronutrient malnutrition. *Journal of Food Composition and Analysis*, 27(1), 8–13. <https://doi.org/10.1016/j.jfca.2012.05.007>
- Amat, T., Assifaoui, A., Buczkowski, J., Silva, J. V. C., Schmitt, C., & Saurel, R. (2024). Interplay between soluble and insoluble protein/calcium/phytic acid complexes in dispersions of faba bean and pea protein concentrates around neutral pH. *Food Hydrocolloids*, 147, 109273. <https://doi.org/10.1016/j.foodhyd.2023.109273>
- Amat, T., Assifaoui, A., Schmitt, C., & Saurel, R. (2023). Importance of binary and ternary complex formation on the functional and nutritional properties of legume proteins in presence of phytic acid and calcium. *Critical Reviews in Food Science and Nutrition*, 63(33), 12036–12058. <https://doi.org/10.1080/10408398.2022.2098247>
- Assatory, A., Vitelli, M., Rajabzadeh, A. R., & Legge, R. L. (2019). Dry fractionation methods for plant protein, starch and fiber enrichment: A review. *Trends in Food Science & Technology*, 86, 340–351. <https://doi.org/10.1016/j.tifs.2019.02.006>
- Auer, J., Alminger, M., Marinea, M., Johansson, M., Zamaratskaia, G., Höggberg, A., & Langton, M. (2024). Assessing the digestibility and estimated bioavailability/ bioaccessibility of plant-based proteins and minerals from soy, pea, and faba bean ingredients. *LWT*, 115893. <https://doi.org/10.1016/j.lwt.2024.115893>
- Boukid, F., Rosell, C. M., & Castellari, M. (2021). Pea protein ingredients: A mainstream ingredient to (re)formulate innovative foods and beverages. *Trends in Food Science & Technology*, 110, 729–742. <https://doi.org/10.1016/j.tifs.2021.02.040>
- Boye, J. I., Aksay, S., Roufik, S., Ribéreau, S., Mondor, M., Farnworth, E., & Rajamohamed, S. H. (2010). Comparison of the functional properties of pea, chickpea and lentil protein concentrates processed using ultrafiltration and isoelectric precipitation

- techniques. *Food Research International*, 43(2), 537–546. <https://doi.org/10.1016/j.foodres.2009.07.021>
- Burger, T. G., Singh, I., Mayfield, C., Baumert, J. L., & Zhang, Y. (2022). Comparison of physicochemical and emulsifying properties of commercial pea protein powders. *Journal of the Science of Food and Agriculture*, 102(6), 2506–2514. <https://doi.org/10.1002/jsfa.11592>
- Chang, L., Lan, Y., Bandillo, N., Ohm, J.-B., Chen, B., & Rao, J. (2022). Plant proteins from green pea and chickpea: Extraction, fractionation, structural characterization and functional properties. *Food Hydrocolloids*, 123, 107165. <https://doi.org/10.1016/j.foodhyd.2021.107165>
- Chang, L., Lan, Y., Chen, B., & Rao, J. (2023). Interfacial, and emulsifying properties nexus of green pea protein fractions: Impact of pH and salt. *Food Hydrocolloids*, 140, 108652. <https://doi.org/10.1016/j.foodhyd.2023.108652>
- Cheryan, M., & Rackis, J. J. (1980). Phytic acid interactions in food systems. *Critical Reviews in Food Science and Nutrition*, 13(4), 297–335. <https://doi.org/10.1080/10408398009527293>
- Chigwedere, C. M., Stone, A., Konieczny, D., Lindsay, D., Huang, S., Glahn, R., House, J. D., Warkentin, T. D., & Nickerson, M. (2023). Examination of the functional properties, protein quality, and iron bioavailability of low-phytate pea protein ingredients. *European Food Research and Technology*, 249(6), 1517–1529. <https://doi.org/10.1007/s00217-023-04232-x>
- Choi, H., Taghvaei, M., Smith, B., & Ganjyal, G. M. (2022). Biochemical analysis of protein compositions among pea (*Pisum sativum*) cultivars grown in the northwest USA. *ACS Food Science & Technology*, 2(7), 1067–1076. <https://doi.org/10.1021/acscfoodscitech.1c00460>
- Chung, H.-J., & Liu, Q. (2012). Physicochemical properties and in vitro digestibility of flour and starch from pea (*Pisum sativum* L.) cultivars. *International Journal of Biological Macromolecules*, 50(1), 131–137. <https://doi.org/10.1016/j.ijbiomac.2011.10.004>
- Cipollone, M. A., & Tironi, V. A. (2020). Yellow pea flour and protein isolate as sources of antioxidant peptides after simulated gastrointestinal digestion. *Legume Science*, 2(4), e59. <https://doi.org/10.1002/leg3.59>
- Cui, L., Bandillo, N., Wang, Y., Ohm, J.-B., Chen, B., & Rao, J. (2020). Functionality and structure of yellow pea protein isolate as affected by cultivars and extraction pH. *Food Hydrocolloids*, 108, 106008. <https://doi.org/10.1016/j.foodhyd.2020.106008>
- Dashek, W. V., & Clore, A. M. (2017). Vacuoles and protein bodies. In W. V. Dashek & G. S. Miglani (Eds.), *Plant Cells and their Organelles* (1st ed., pp. 351–370). Wiley. <https://doi.org/10.1002/9781118924846.ch12>
- Dziuba, J., Szerszunowicz, I., Nałęcz, D., & Dziuba, M. (2014). Proteomic analysis of albumin and globulin fractions of pea (*Pisum sativum* L.) seeds. *Acta Scientiarum Polonorum Technologia Alimentaria*, 13(2), 181–190. <https://doi.org/10.17306/J.AFS.2014.2.7>
- Fenn, D., Wang, N., & Maximiuk, L. (2022). Physicochemical, anti-nutritional, and functional properties of air-classified protein concentrates from commercially grown Canadian yellow pea (*Pisum sativum*) varieties with variable protein levels. *Cereal Chemistry*, 99(1), 157–168. <https://doi.org/10.1002/cche.10506>
- Fernández-Quintela, A., Macarulla, M. T., del Barrio, A. S., & Martínez, J. A. (1997). Composition and functional properties of protein isolates obtained from commercial legumes grown in northern Spain. *Plant Foods for Human Nutrition*, 51(4), 331–341. <https://doi.org/10.1023/A:1007936930354>
- Fredrikson, M., Biot, P., Alminger, M. L., Carlsson, N.-G., & Sandberg, A.-S. (2001). Production process for high-quality pea-protein isolate with low content of oligosaccharides and phytate. *Journal of Agricultural and Food Chemistry*, 49(3), 1208–1212. <https://doi.org/10.1021/jf000708x>
- Gao, Z., Shen, P., Lan, Y., Cui, L., Ohm, J.-B., Chen, B., & Rao, J. (2020). Effect of alkaline extraction pH on structure properties, solubility, and beany flavor of yellow pea protein isolate. *Food Research International*, 131, 109045. <https://doi.org/10.1016/j.foodres.2020.109045>
- Grossmann, L. (2023). Structural properties of pea proteins (*Pisum sativum*) for sustainable food matrices. *Critical Reviews in Food Science and Nutrition*, 1–21. <https://doi.org/10.1080/10408398.2023.2199338>
- Guillin, F. M., Gaudichon, C., Guérin-Deremaux, L., Lefranc-Millot, C., Airinei, G., Khodorova, N., Benamouzig, R., Pomport, P.-H., Martin, J., & Calvez, J. (2022). Real ileal amino acid digestibility of pea protein compared to casein in healthy humans: A randomized trial. *The American Journal of Clinical Nutrition*, 115(2), 353–363. <https://doi.org/10.1093/ajcn/nqab354>
- Gultekin Subasi, B., Forghani, B., & Abdollahi, M. (2024). Exploring Swedish pea varieties suitable for protein isolation, focusing on antinutrients and off-flavors. *Journal of Food Composition and Analysis*, 128, 105988. <https://doi.org/10.1016/j.jfca.2024.105988>
- Hemery, Y., Rouau, X., Dragan, C., Bilici, M., Belega, R., & Dascalescu, L. (2009). Electrostatic properties of wheat bran and its constitutive layers: Influence of particle size, composition, and moisture content. *Journal of Food Engineering*, 93(1), 114–124. <https://doi.org/10.1016/j.jfoodeng.2009.01.003>
- Higa, F. A., Boyd, L., Sopiwnyk, E., & Nickerson, M. T. (2022). Effect of particle size, flour:Water ratio and type of pulse on the physicochemical and functional properties of wet protein extraction. *Cereal Chemistry*, 99(5), 1049–1062. <https://doi.org/10.1002/cche.10552>
- Jiang, H., Gu, Z., Manthey, F., Chen, B., & Rao, J. (2021). Comparison of the proximate compositions, nutritional minerals, pasting properties, and aroma differences of flours from selected yellow pea cultivars grown across the Northern Great Plains. *ACS Food Science & Technology*, 1(9), 1529–1537. <https://doi.org/10.1021/acscfoodscitech.1c00149>
- Karaca, A. C., Low, N., & Nickerson, M. (2011). Emulsifying properties of chickpea, faba bean, lentil and pea proteins produced by isoelectric precipitation and salt extraction. *Food Research International*, 44(9), 2742–2750. <https://doi.org/10.1016/j.foodres.2011.06.012>
- Kaspchak, E., Silveira, J. L. M., Igarashi-Mafra, L., & Mafra, M. R. (2020). Effect of antinutrients on heat-set gelation of soy, pea, and rice protein isolates. *Journal of Food Science and Technology*, 57(11), 4201–4210. <https://doi.org/10.1007/s13197-020-04458-7>
- Kimura, A., Fukuda, T., Zhang, M., Motoyama, S., Maruyama, N., & Utsumi, S. (2008). Comparison of physicochemical properties of 7S and 11S globulins from pea, fava bean, cowpea, and french bean with those of soybean—French bean 7S globulin exhibits excellent properties. *Journal of Agricultural and Food Chemistry*, 56(21), 10273–10279. <https://doi.org/10.1021/jf801721b>
- Kornet, C., Venema, P., Nijse, J., Van Der Linden, E., Van Der Goot, A. J., & Meinders, M. (2020). Yellow pea aqueous fractionation increases the specific volume fraction and viscosity of its dispersions. *Food Hydrocolloids*, 99, 105332. <https://doi.org/10.1016/j.foodhyd.2019.105332>
- Kornet, R., Yang, J., Venema, P., Van Der Linden, E., & Sagis, L. M. C. (2022). Optimizing pea protein fractionation to yield protein fractions with a high foaming and emulsifying capacity. *Food Hydrocolloids*, 126, 107456. <https://doi.org/10.1016/j.foodhyd.2021.107456>
- Lam, A. C. Y., Can Karaca, A., Tyler, R. T., & Nickerson, M. T. (2018). Pea protein isolates: Structure, extraction, and functionality. *Food*

- Reviews International*, 34(2), 126–147. <https://doi.org/10.1080/87559129.2016.1242135>
- Lam, A. C. Y., Warkentin, T. D., Tyler, R. T., & Nickerson, M. T. (2017). Physicochemical and functional properties of protein isolates obtained from several pea cultivars. *Cereal Chemistry*, 94(1), 89–97. <https://doi.org/10.1094/CCHEM-04-16-0097-FI>
- Lie-Piang, A., Yang, J., Schutyser, M. A. I., Nikiforidis, C. V., & Boom, R. M. (2023). Mild fractionation for more sustainable food ingredients. *Annual Review of Food Science and Technology*, 14(1), 473–493. <https://doi.org/10.1146/annurev-food-060721-024052>
- Liu, K. (2019). Soybean trypsin inhibitor assay: Further improvement of the standard method approved and reapproved by American Oil Chemists' Society and American Association of Cereal Chemists International. *Journal of the American Oil Chemists' Society*, 96(6), 635–645. <https://doi.org/10.1002/aocs.12205>
- Lu, Z. X., He, J. F., Zhang, Y. C., & Bing, D. J. (2020). Composition, physicochemical properties of pea protein and its application in functional foods. *Critical Reviews in Food Science and Nutrition*, 60(15), 2593–2605. <https://doi.org/10.1080/10408398.2019.1651248>
- McClements, D. J., & Grossmann, L. (2022). *Next-generation plant-based foods: Design, production, and properties* (1st ed.). Springer Cham. <https://doi.org/10.1007/978-3-030-96764-2#bibliographic-information>
- Millar, K. A., Gallagher, E., Burke, R., McCarthy, S., & Barry-Ryan, C. (2019). Proximate composition and anti-nutritional factors of fava-bean (*Vicia faba*), green-pea and yellow-pea (*Pisum sativum*) flour. *Journal of Food Composition and Analysis*, 82, 103233. <https://doi.org/10.1016/j.jfca.2019.103233>
- Möller, A. C., Van Der Padt, A., & Van Der Goot, A. J. (2021). From raw material to mildly refined ingredient – Linking structure to composition to understand fractionation processes. *Journal of Food Engineering*, 291, 110321. <https://doi.org/10.1016/j.jfoodeng.2020.110321>
- Moller, A. C., Van Der Padt, A., & Van Der Goot, A. J. (2022). Influence of the fractionation method on the protein composition and functional properties. *Innovative Food Science & Emerging Technologies*, 81, 103144. <https://doi.org/10.1016/j.ifset.2022.103144>
- Monnet, A., Eurieult, A., Berland, S., Almeida, G., Jeuffroy, M., & Michon, C. (2019). Damaged starch in pea versus wheat flours: Fragmentation behavior and contribution of fine and coarse fractions. *Cereal Chemistry*, 96(3), 465–477. <https://doi.org/10.1002/cche.10146>
- Osborne, T. B. (1907). *The proteins of the wheat kernel*. Carnegie Institution. <https://archive.org/details/cu31924024559167>. Accessed 20 Jul 2024
- Palavecino, P. M., Penci, M. C., & Ribotta, P. D. (2019). Effect of planetary ball milling on physicochemical and morphological properties of sorghum flour. *Journal of Food Engineering*, 262, 22–28. <https://doi.org/10.1016/j.jfoodeng.2019.05.007>
- Pedrosa, M. M., Varela, A., Domínguez-Timón, F., Tovar, C. A., Moreno, H. M., Borderías, A. J., & Díaz, M. T. (2020). Comparison of bioactive compounds content and techno-functional properties of pea and bean flours and their protein isolates. *Plant Foods for Human Nutrition*, 75(4), 642–650. <https://doi.org/10.1007/s11130-020-00866-4>
- Pelgrom, P. J. M., Vissers, A. M., Boom, R. M., & Schutyser, M. A. I. (2013). Dry fractionation for production of functional pea protein concentrates. *Food Research International*, 53(1), 232–239. <https://doi.org/10.1016/j.foodres.2013.05.004>
- Reinkensmeier, A., Bußler, S., Schlüter, O., Rohn, S., & Rawel, H. M. (2015). Characterization of individual proteins in pea protein isolates and air classified samples. *Food Research International*, 76, 160–167. <https://doi.org/10.1016/j.foodres.2015.05.009>
- Rivera Del Rio, A., Möller, A. C., Boom, R. M., & Janssen, A. E. M. (2022). In vitro gastro-small intestinal digestion of conventional and mildly processed pea protein ingredients. *Food Chemistry*, 387, 132894. <https://doi.org/10.1016/j.foodchem.2022.132894>
- Stone, A. K., Karalash, A., Tyler, R. T., Warkentin, T. D., & Nickerson, M. T. (2015). Functional attributes of pea protein isolates prepared using different extraction methods and cultivars. *Food Research International*, 76, 31–38. <https://doi.org/10.1016/j.foodres.2014.11.017>
- Taherian, A. R., Mondor, M., Labranche, J., Drolet, H., Ippersiel, D., & Lamarche, F. (2011). Comparative study of functional properties of commercial and membrane processed yellow pea protein isolates. *Food Research International*, 44(8), 2505–2514. <https://doi.org/10.1016/j.foodres.2011.01.030>
- Tanger, C., Engel, J., & Kulozik, U. (2020). Influence of extraction conditions on the conformational alteration of pea protein extracted from pea flour. *Food Hydrocolloids*, 107, 105949. <https://doi.org/10.1016/j.foodhyd.2020.105949>
- Vagadia, B. H., Vanga, S. K., & Raghavan, V. (2017). Inactivation methods of soybean trypsin inhibitor – A review. *Trends in Food Science & Technology*, 64, 115–125. <https://doi.org/10.1016/j.tifs.2017.02.003>
- Wang, N., Hatcher, D. W., & Gawalko, E. J. (2008). Effect of variety and processing on nutrients and certain anti-nutrients in field peas (*Pisum sativum*). *Food Chemistry*, 111(1), 132–138. <https://doi.org/10.1016/j.foodchem.2008.03.047>
- Yang, J., Kornet, R., Diedericks, C. F., Yang, Q., Berton-Carabin, C. C., Nikiforidis, C. V., Venema, P., Van Der Linden, E., & Sagis, L. M. C. (2022). Rethinking plant protein extraction: Albumin—From side stream to an excellent foaming ingredient. *Food Structure*, 31, 100254. <https://doi.org/10.1016/j.foostr.2022.100254>
- Yang, J., Zamani, S., Liang, L., & Chen, L. (2021). Extraction methods significantly impact pea protein composition, structure and gelling properties. *Food Hydrocolloids*, 117, 106678. <https://doi.org/10.1016/j.foodhyd.2021.106678>
- Zhou, D., Ma, Z., Xu, J., Li, X., & Hu, X. (2019). Resistant starch isolated from enzymatic, physical, and acid treated pea starch: Preparation, structural characteristics, and in vitro bile acid capacity. *LWT*, 116, 108541. <https://doi.org/10.1016/j.lwt.2019.108541>

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