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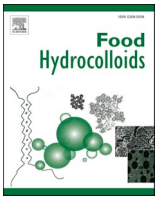

Pre-treatment effects on the composition and functionalities of pigeon pea seed ingredients

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ABSTRACT

Soaking and boiling can improve the digestibility of seeds by reducing anti-nutritional factors and the hard-to-cook phenomenon. The use of pre-treated seeds in protein fractionation is thus of interest for the development of protein-rich ingredients. In this work, soaked and soaked-boiled seeds were applied prior to aqueous fractionation of pigeon pea proteins. Pre-treatments impact the particle size distribution in the flours, with boiling leading to aggregate formation (>1000 μm) due to starch gelatinization. The soaked-boiled flour showed a lower lipid content, higher content in soluble polysaccharides, and loss of proteins with low molecular weight compared to the control. The use of soaked-boiled seeds for protein fractionation reduced trypsin inhibitor activity by 95%, demonstrating a positive effect in improving nutritional quality. Protein fractionation also resulted in a 3-fold accumulation of lipids, and the mannose content, notably, doubled in protein concentrates compared to their flour counterparts. A lower intensity of tryptophan fluorescence emission was observed in protein concentrates, which may be related to chemical degradation or interactions with other molecules. The protein concentrate from soaked-boiled seeds exhibited higher emulsion stabilization properties compared to the others protein concentrates. The decrease in foaming properties caused by the soaking can be attributed to the leaching of minor compounds that also act as foaming agents, such as saponins. This work brings new insights into the effects of soaking and boiling-based pre-treatments on the composition of pulse protein ingredients, and in turn, on their techno-functionalities as exemplified with their emulsifying, foaming and gelling properties.

1. Introduction

Protein transition is a topic widely discussed by government organizations and researchers. Partial replacement of animal-based proteins by plant-based proteins in the diets is among the initiatives to reduce the ecological footprint that seek to ensure greater sustainability for the planet, in addition to achieving food and nutrition security. This transition can be driven by diversifying the protein sources with the inclusion of pulses that are still underused in human nutrition (Bühler, Dekkers, Bruins, & Van Der Goot, 2020; Diedericks, Venema, Mubaiwa, Jideani, & Van der Linden, 2020). Pigeon pea [Cajanus cajan (L.) Millsp.] is a non-conventional crop native to regions of Asia and Africa. This pulse has a high protein content (20–28%), and is rich in minerals such as iron and calcium, and in B-complex vitamins. The easy

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adaptation to warmer and drier environments makes pigeon pea a resilient pulse, which is of great interest for strategies to overcome climate change effects (Locali-Pereira et al., 2023; Sahni, Sharma, & Surasani, 2020; Devindra, Sreenivasa, Bhaskar, & Mulimani, 2012).

Pigeon pea production is currently restricted to some countries in Asia and Africa due to a lack of knowledge about its nutritional and technological potential and relevant aspects to make large-scale production around the world possible. The low digestibility of proteins, presence of antinutritional factors, such as trypsin inhibitors, and the hard-to-cook phenomenon are some aspects that can hamper the consumption of this pulse (Carter & Manthey, 2019). Processing can enhance the use of pulses by reducing the levels of antinutritional factors and by modifying the structures of starch granules and protein bodies, which in turn will modulate their functional properties (Sahni et al., 2020; Sun, Ohanenye, Ahmed, & Udenigwe, 2020). Soaking is a non-thermal treatment that consists of hydrating the seeds; it may be used to reduce the cooking time of pulses and to assist in the removal of hydrophilic antinutritional compounds including tannins, trypsin inhibitors, and raffinose oligosaccharides, that migrate to the aqueous medium by leaching (Carter & Manthey, 2019; Girigowda, Prashanth, & Mulimani, 2005; Yasmin, Zeb, Khalil, Paracha, & Khattak, 2008). Thermal treatments such as boiling are used to eliminate thermolabile antinutrients such as chymotrypsin inhibitors, and improve seed digestibility and softness (Hernández-Infante, Sousa, Montalvo, & Tena, 1998; Onwuka, 2006; Patterson, Curran, & Der, 2017; Wang et al., 2021), in addition to their obvious effect of mitigating microbial contamination issues. (Alfaro-Diaz et al., 2021).

Soaking in combination with boiling has already proved to effectively eliminate antinutritional factors and improve pigeon pea digestibility. For instance, soaking pigeon pea seeds for 12 h followed by boiling for 40 min was sufficient to reduce the levels of tannins, trypsin inhibitors, hemagglutinin and hydrogen cyanide by 80% (Onwuka, 2006). The combination of both treatments was also efficient at removing phytates and compounds responsible for flatulence (Devindra et al., 2012; Igbedioh, Olugbemi, & Akpapunam, 1994). In spite of having a positive impact on the nutritional quality of pigeon pea, the effects of these pre-treatments on the molecular properties of pigeon pea proteins, on the processability of the flours, and on the functionality of the resulting ingredients still need to be clarified. In particular, proteins are responsible for several technological properties of pulse-based ingredients (e.g., emulsifying, foaming, gelling) and their conformation can be partially or fully altered by heat treatments (Espinosa, Guevara-Oquendo, Newkirk, & Yu, 2020). The consequences of these pre-treatments on the other pigeon pea macromolecules (lipids, carbohydrates) have received little attention so far, yet is an important factor when considering ingredient functionality. The aim of this work was therefore to evaluate the impact of soaking and soaking + boiling pre-treatments on the composition, protein conformation and techno-functional properties of pigeon pea protein ingredients.

2. Materials and methods

2.1. Materials

Pigeon pea seeds were purchased from a local supplier (São Paulo, Brazil). Soybean oil was supplied by Cargill (Minas Gerais, Brazil). Sodium hydroxide, hydrochloric acid, sodium phosphate, bovine serum albumin (BSA), trypsin from bovine pancreas, trypsin inhibitor from *Glycine max*, N-a-benzoyl-DL-arginine-p-nitroanilide (BAPNA), and gallic acid standard were purchased from Sigma-Aldrich®. Folin-Ciocalteu reagent was provided by Êxodo (São Paulo, Brazil). Sodium carbonate was purchased from Synth (Sao Paulo, Brazil). Protein assay dye reagent and Coomassie blue were purchased from Bio-Rad (California, USA). Tris-glycine SDS running buffer was purchased from ThermoFisher (Massachusetts, USA). Chloroform, methanol, and n-hexane were purchased from Biosolve Chemicals (Dieuze, France). Standards of α, β, γ and δ-tocopherol were purchased from Calbiochem and the γ-tocotrienol standard was purchased from Cayman Chemical (Ann Arbor, USA). Sodium sulfate anhydrous and acetic acid were purchased from Carlo Erba Reagents (Val de Reuil, France). Sodium chloride was purchased from VWR International (Radnor, USA). Neutral lipid standards DG 1.2 (CAS number 2442-61-7), DG 1.3 (CAS number 2465-32-9), C18:1 (CAS number 112-80-1) and TG 18:1 (CAS number 122 -32-7) were purchased from Sigma Aldrich (St Louis, USA) and MG 18:3 standard was purchased from Nu-Check Prep (Minnesota, USA). The phospholipid standards phosphatidylglycerol (CAS number 383907-64-0), phosphatidylethanolamine (CAS number 97281-40-8), phosphatidylinositol (CAS number 383907-36-6), phosphatidylserine (CAS number 383908-63-2), phosphatidic acid (CAS number 475995-54-1), phosphatidylcholine (CAS number 97281-44-2), sphingomyelin (CAS number 475662-40-9), and lysophosphatidylcholine (CAS number 97281-38-4) were purchased from Avanti (London, UK).

2.2. Seed pre-treatments and flour production

Pigeon pea seeds were manually checked to remove damaged seeds and then subjected to two different physical pre-treatments: soaking, and soaking + boiling. Soaking was performed in distilled water (1:5, w v^{-1}) for 24 h at 25 °C. The soaking liquid was discarded, and part of the soaked seeds was then boiled in distilled water (1:3, w v^{-1}) for 30 min, leading to soaked + boiled seeds. A control consisting of raw seeds (without pre-treatments) was also evaluated. All seeds, including the control, were dried in an oven at 60 °C for approximately 12 h. After drying, the seeds were ground in a disk mill (J.R. Araújo & Cia Ltda., Brazil) at 1730 rpm to produce the flours. Flours produced with raw, soaked-, and soaked-boiled seeds were named FC, FS, and FB, respectively.

The size distribution of the flours was evaluated in a laser diffraction analyzer (Mastersizer 3000, Malvern) in the dry state. It was also assessed using a QICPIC image analyzer (Sympatec, USA) dispersing the flour (50 mg) in 0.5%-ethanol in water solution (1 L), in which the particle-shape images were recorded as well. The flours' morphology was analyzed in a JEOL JSM-7500 F scanning electron microscope (JEOL Ltd., Japan) at 400× magnification by fixing small amounts of powders in SEM stubs and gold coating under vacuum before analysis.

2.3. Protein extraction

Pigeon pea flour was dispersed in distilled water (1:10, w v^{-1}) and the pH was adjusted to 12.0 (using 3 M NaOH) to promote protein solubilization, following the method used by Sun et al. (2020), with modifications. The dispersion was mechanically stirred (MA261, Marconi, Brazil) (1 h/750 rpm), and then centrifuged (10,000 *g*, 30 min, 4 °C). The supernatant was collected, and its pH adjusted to 4.5 (using a 3 M HCl) to induce protein precipitation. A new centrifugation was performed (10,000 *g*, 30 min, 4 °C) and the protein-rich precipitate was collected and redispersed in distilled water, being neutralized to pH 7.0. The pigeon pea protein concentrates (PPC) were frozen, and finally freeze-dried for 72 h. After freeze-drying, the powdered extracts were stored in desiccator containing K_2CO_3 saturated solution (RH = 43.2%) until analysis. The PPC produced with raw, soaked- and soaked-boiled seed flours were named PPC-C, PPC-S and PPC-B, respectively. The macroscopic aspect of the pre-treated seeds, flours, and protein extracts is shown in Supplementary Fig. 1.

2.4. Composition of flours and protein concentrates

2.4.1. Cell wall polysaccharide composition, raffinose family oligosaccharides, and starch content

The total amount of cell wall polysaccharides and their sugar composition were measured by chromatographic and colorimetric methods as previously described, with minor modifications (Le Gall,

Even, & Lahaye, 2016). Briefly, flours and protein concentrates were subjected to hot ethanolic extraction using an automated solvent extractor (ASE™350, Thermo Scientific™ Dionex™; about 500 and 200 mg respectively per cell, at 100 °C with 80% ethanol 2 mL min^{-1} flow mode for 15 min). The insoluble alcoholic fractions were hydrolyzed with sulfuric acid to obtain a mixture of neutral and uronic sugars. The resulting neutral sugars were converted into alditol acetates prior to GC analysis (TraceGOLD™ TG-225 MS GC Column (30 × 0.32 mm ID); TRACE GC Ultra Thermo Scientific™; at 205 °C using H_2 as the carrier gas). Sugar solutions were used as external standards and an inositol solution was used as internal standard for all measurements. The resulting uronic acids were quantified by meta-hydroxydiphenyl (MHDP) colorimetric method. The sugars arising from the cell walls through this procedure are referred to as non-starch polysaccharides (NSP).

For the determination of raffinose family oligosaccharides (RFOs), approximately 15 mg of flour or protein concentrate were mixed in absolute ethanol containing an internal standard (IS, melezitiose), incubated (15 min at 75 °C), and evaporated under dry air at 40 °C to obtain RFO extract (Gangola, Jaiswal, Khedikar, & Chibbar, 2014). Then, the resulting pellet was redispersed in ultrapure water, vortexed, and centrifuged to collect the supernatant. After filtration, the samples were injected into an HPAEC-PAD (ICS-6000 Thermo Scientific™, column PA1, flow rate 0.25 mL/min, 30 °C, elution gradient with 50–500 mM NaOH for 40 min). For quantification, external standards of saccharose, raffinose, stachyose and verbascose, mixed in solution with melezitiose (IS) were used.

Total starch content was determined after sample amylolysis using two commercial enzymes (α-amylase from *Bacillus licheniformis* and amyloglucosidase from *Aspergillus niger*; E-BLAAM and E-AMGDF, Megazyme) followed by HPAEC-PAD analyzes (ICS-6000 Thermo Scientific™) (Le Gall et al., 2016).

The total carbohydrate content was expressed by the sum of starch, non-starch polysaccharides (NSP), raffinose family oligosaccharides (RFO), and saccharose contents.

2.4.2. Total lipids, neutral lipids, phospholipids, and tocopherol content: extraction, identification, and quantification

Total lipids were extracted using a method described by Bligh and Dyer (1959) and Folch, Lees, and Sloane Stanley (1956), with adaptations. First, 1 g of sample was weighed and hydrated in 6 mL of 50 mM pH 8.0 phosphate buffer overnight at 4 °C under stirring. Then, 50 mL of chloroform/methanol solution (2:1 v v^{-1}) were added. The mixture was stirred and filtrated under vacuum to recover the liquid phase. The powder was then recovered and the procedure was repeated twice. The filtrate was transferred into a separating funnel and 35 mL of NaCl solution (0.73% w/v) were added, mixed, and let to decant overnight at 4 °C. The lower phase was recovered after filtration on anhydrous sodium sulfate and glass wool, and the solvent was evaporated under vacuum (R-100, Rotavapor, Büchi, France), and then under nitrogen (N-Evap 111, Organomation, USA). After evaporation, the round flasks containing the lipid extract were weighed, and the total lipid content was calculated based on the mass recovered. The lipid extract was then collected in chloroform and stored in an amber flask at −80 °C until further analyses.

For quantification and identification of neutral lipids and phospholipids, a U-HPLC (Ultimate 3000 RSLC, Thermo Scientific™, France) equipped with an evaporative light scattering detector (ELSD, Sedex 85) was used (Keuleyan et al., 2023); prior to injection, the total lipid concentration was adjusted to 0.5 and 2.0 mg of lipids/mL of solvent, respectively. The amount of each compound was calculated using a calibration curve made with commercial standards of DG 1.2, 18:1, DG 1.3, 18:1, C18:1, TG 18:1 and MG 18:3, for neutral lipids; and phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidic acid, phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine, for phospholipids.

To investigate the tocopherol content and composition, aliquots of dried lipid extracts were solubilized in hexane to reach a final concentration of 10 mg of lipids/mL and injected in U-HPLC (Ultimate 3000 RSLC, Thermo ScientificTM, France) equipped with a RS-fluorescence detector (Thermo ScientificTM, France) (Keuleyan et al., 2023). The excitation and emission wavelengths of the fluorescence detector were 295 and 330 nm, respectively. An external calibration curve of α, β, γ and δ-tocopherol was used.

2.4.3. Protein content: quantification and identification

The total nitrogen content of the flours and protein concentrates was determined by the Dumas combustion analysis method (Elementar, Langenselbold, Germany) using a nitrogen-to-protein conversion factor of 5.7 (Alamanou & Doxastakis, 1997).

Protein identification was carried out by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing and reducing conditions with 2-β-mercaptoethanol (5%, v v^{-1}), following the method described by Lawrence and Besir (2009). Samples were dispersed in Laemmli buffer (2 mg of protein/mL buffer) and heated at 95 °C for 5 min. Then, 10 μL aliquots of the protein dispersions and molecular weight marker standard were deposited onto gradient polyacrylamide gels (4–12%, Bolt™ Bis-Tris Plus, ThermoFisher), and subjected to a 100 V-current, immersed in Tris-glycine SDS running buffer (Novex™) with constant voltage of 150 V. After the run, the gel was placed in a sensitization solution (2% phosphoric acid, 50% ethanol, v v^{-1}) for 1 h under agitation. Then, the gel was rinsed (2% phosphoric acid solution, v v^{-1}) for 40 min under agitation, transferred to a sensitization solution (17% ethanol, 2% orthophosphoric acid and 15% ammonium sulfate, v v^{-1}), and stained with Coomassie Brilliant Blue after 20 min. After complete staining, the gel was rinsed with distilled water and scanned.

2.4.4. Trypsin inhibitor activity (TIA)

The TIA of flour and protein concentrates was determined using the AOCS method (AOCS Official Method Ba 12a-2020, 2020), which determines the presence of tryptic inhibitors by a colorimetric reaction in the presence of trypsin and a substrate (Nα-benzoyl-DL-arginine 4-nitroanilide hydrochloride, BAPNA) and which was optimized and described by Liu (2021) with slight modifications. Inhibitors were extracted from 300 mg of sample in 15 mL of 10 mM NaOH, sonicated for 3 min, stirred for 3 h at 600 rpm and finally centrifuged at 20,000 *g* for 5 min at room temperature. Supernatants were diluted with distilled water so that 60 μL caused 30–70 % trypsin inhibition (linearity range). Extractions were performed in triplicates. The TIA assay was conducted in 96-well microplates. First, 60 μL of a dilute extract was mixing with 150 μL of BAPNA (0.4 mg/mL) prewarmed at 37 °C. Then, 60 μL trypsin solution (20 μg mL^{-1}) was added to start the colorimetric reaction. The microplate was immediately place in an oven at 37 °C. Exactly 10 min later, 30 μL 30% (v v^{-1}) acid acetic were added to stop the reaction. A blank sample of each extract was prepared by adding acetic acid before trypsin solution to prevent the reaction. Concurrently, the reaction was run in the absence of inhibitors using the same procedure but by replacing the sample extract with an equal amount of water. Absorbance was measured at 410 nm using a microplate reader (Varioskan Lux, Thermo Fisher, USA). The ΔA_{410}, percentage of trypsin inhibition (%TIn) and TIA calculations were performed using equations (1)–(3) below (Liu, 2021):

$$\Delta A_{410} = (A_{410R}\text{-}A_{410RB}) - (A_{410S} - A_{410SB}) \quad (1)$$

$$\%TIn = [\Delta A_{410}/ (A_{410R}\text{-}A_{410RB})] \text{ x } 100 \quad (2)$$

$$TIA = (\%TIn \text{ x the amount of trypsin } (\mu g)) \text{ /mg of sample (or mg of protein)} \quad (3)$$

where ΔA_{410} is the absorbance at 410 nm corrected for the difference between the absence and presence of sample extract, A_{410R} is the reference reading, A_{410RB} is the reference blank, A_{410S} is the sample

reading, A_{410SB} is the sample blank, %TIn is the percentage of trypsin inhibition, and TIA is the trypsin inhibitor activity.

2.4.5. Total soluble phenolic compounds (TSPC)

The content in TSPC was determined by the Folin-Ciocalteu method, according to Mba, Kwofie, and Ngadi (2019), with modifications. About 1 g of sample was diluted in 20 mL of 70% methanol solution (v v^{-1}), stirred for 1 h, and centrifuged (4000 *g*, 10 min). After centrifugation, 400-μL aliquots of the supernatant were collected and diluted in 600 μL of distilled water. Then, 1 mL of Folin-Ciocalteu reagent, previously diluted in distilled water (1:4, v:v), 2 mL of 20% Na_2CO_3 solution (w/v), and 1 mL of distilled water were added. The mixture was kept in the dark for 30 min for complete reaction and the absorbance was read in a spectrophotometer at 750 nm. Gallic acid was used to construct the standard curve (0–50 μg mL^{-1}) and TSPC was expressed as mg of gallic acid equivalent (GAE) g^{-1}.

2.4.6. Moisture and ash contents

Moisture and ash contents were determined gravimetrically by drying in an oven at 105 °C until constant weight, and by incinerating in a muffle oven at 550 °C, respectively (AOAC, 1990).

2.5. Characterization of endogenous fluorophores

The occurrence, fluorescence patterns and intensity of the fluorophores endogenously present in the flours and protein concentrates were evaluated by conducting three-dimensional fluorescence measurements (excitation-emission fluorescence matrices, 3D-EEFM) using a spectrofluorimeter (FP-8550, Jasco, Japan). Fluorescence measurements were performed at excitation wavelengths range from 240 to 480 nm, and emission wavelengths range from 280 to 520 nm. A constant slit of 1 nm and a bandwidth of 2.5 nm were defined for both excitation and emission. The samples were evaluated in their solid (powders) and liquid (dispersions) states, to assess the influence of a solvent on the behavior of the endogenous fluorophores. For dry state, samples were evaluated using a powder cell and performing the measurements in front-surface mode; and in the liquid state using a quartz cuvette and performing the measurements in right-angle mode. The liquid dispersions were prepared by dispersing the powders in a 50 mM pH 7.0 phosphate buffer (20 mg/mL of buffer) and centrifuging (10,000 *g*, 10 min, 20 °C). The supernatants were diluted to reach an absorbance at 280 nm equal to 0.075, which is suited to right-angle measurements.

2.6. Protein conformation and functional properties of pigeon pea protein concentrates

2.6.1. Intrinsic fluorescence spectroscopy

The intrinsic fluorescence emission spectra of pigeon pea proteins were obtained using an ISS PC1 steady-state spectrofluorimeter (Champaign, IL, USA) at 25 °C (Caruso et al., 2016). Protein samples were diluted in 50 mM pH 7.0 phosphate buffer at a concentration of 0.15 mg/mL. The excitation wavelength was set at 280 nm and the emission spectra were recorded from 300 to 520 nm. A constant slit of 2 nm was defined for both excitation and emission. A 10 × 10 mm quartz cuvette was used for measurements, which were conducted in right-angle mode.

2.6.2. Protein solubility and electrostatic charge

Protein solubility was evaluated at pHs ranging from 2.0 to 12.0 using the Bradford method (Bradford, 1976). First, 100 mg of sample were diluted in 10 mL of distilled water at different pHs. The mixtures were vortexed and centrifuged (1000 rpm, 25 min, 25 °C). A 100 μL aliquot of the supernatant was collected from each tube and diluted in 1900 μL of distilled water. In a microplate, 10 μL of each solution were mixed with 200 μL of Coomassie dye reagent (G-250, Bio-Rad) previously diluted in distilled water (1:4, v v^{-1}). The plate was incubated for 5 min and the absorbance was read at 595 nm. Protein concentration was determined by constructing a standard curve of BSA at concentrations ranging from 0 to 0.5 mg/mL. Protein solubility (%) was determined as the ratio between the protein content in the supernatant and the protein content in the initial mass of sample.

The zeta potential of protein dispersions at different pHs (2.0–12.0) was determined in a Zetasizer Nano Series equipment (Malvern, UK) (Sun et al., 2020). Protein dispersions were prepared in the same manner as described for the protein solubility analysis. Each sample was analyzed in triplicate at 25 °C and the refraction indices used were 1.33 (for water) and 1.45 (for protein dispersions). For each reading, eleven repetitions were performed by the equipment.

2.6.3. Water and oil holding capacities (WHC and OHC)

The water holding capacity (WHC) and oil holding capacity (OHC) of the protein concentrates were determined according to Zhao, Shen, Wu, Zhang, and Xu (2020), with modifications. About 1 g of sample was weighed in a polypropylene centrifuge tube and 20 mL of distilled water or soybean oil were added. The mixture was vortexed and allowed to stand for 30 min. Then, the tubes were centrifuged (4500 rpm, 10 min, 25 °C) and the supernatants were discarded. The tubes were inverted to drain any remaining liquid. Then, the samples were weighed again to calculate WHC and OHC by Equation (4):

$$WHC\ or\ OHC\ \left(g\ g^{-1}\right) = \frac{m_2 - m_1}{m_1} \tag{4}$$

in which m_2 is the mass of sample + water or oil after centrifugation and m_1 is the initial mass of sample.

2.6.4. Emulsifying properties

To determine the emulsifying activity index (EAI) and emulsion stability index (ESI), protein dispersions (0.5 %, w w^{-1}) were prepared and the pH was adjusted to 7.0 (using 0.1 M NaOH and HCl solution). Then, 1.5 mL of soybean oil were added to 4.5 mL of protein dispersion and homogenized (20,000 rpm, 1 min) (T-25 Ultra-Turrax®, IKA, Germany). An aliquot (25 μL) of the produced emulsion was diluted in 5 mL of 0.1% SDS (sodium dodecyl sulfate) solution. The absorbance of the diluted emulsion was determined by spectrophotometry (UV-M51, BEL Engineering, Italy) at 500 nm. The EAI and ESI were calculated according to Equations (5) and (6) (Tontul, Kasimoglu, Asik, Atbakan, & Topuz, 2018).

$$EAI\ \left(m^2 / g\right) = \frac{2\ \mathrm{x}\ 2,303\ \mathrm{x}\ A_0\ \mathrm{x}\ DF}{C\ \mathrm{x}\ \varphi\ \mathrm{x}\ 10000}\ \mathrm{x}\ 100 \tag{5}$$

$$ESI\ (min) = \frac{A_0}{A_0 - A_{10}} x\ 10 \tag{6}$$

in which A_0 is the absorbance of the diluted emulsion after production, DF is the dilution factor, C is the weight of protein per volume of emulsion (g/mL), φ is the volume fraction of the soybean oil in the emulsion and A_{10} is the absorbance of the diluted emulsion 10 min after production.

2.6.5. Foaming properties

The foaming capacity was determined by adding 1.5 g of protein concentrates to 50 mL of distilled water (pH adjusted to 7.0), followed by stirring (20,000 rpm, 2 min) (T-25 Ultra-Turrax®, IKA, Germany) (Vinayashree & Vasu, 2021). The foam produced was added to a graduated cylinder and the volume was measured (t = 0 min). For foam stability, the foam volume was measured after 120 min of storage. Foaming capacity and foam stability were calculated using Equations (7) and (8)

$$Foaming\ capacity\ (\%) = \frac{V_0 - V_i}{V_i}\ \mathrm{x}\ 100 \tag{7}$$

$$Foam\ stability\ (\%) = \frac{V_{120} - V_i}{V_i} \text{ x } 100 \quad (8)$$

in which V_i, V_0, V_{120} are the volumes before stirring, after stirring and 120 min after stirring, respectively.

2.6.6. Gelling properties

The least gelation concentration (LGC) was evaluated according to Sahni et al. (2020). Protein suspensions in distilled water (pH = 7.0) were prepared at different concentrations (2–20 % w w^{-1}). The suspensions were vortexed for 30 s, placed in a boiling water-bath for 1 h, cooled in running water, and stored at refrigeration temperature for 24 h. After this period, the tubes containing the suspensions were inverted. The LGC was determined as the lowest concentration necessary for the gel not to flow after the tube inversion.

2.7. Statistical analysis

Data were analyzed using the OriginPro 2016 and XLSTAT software. Variance analysis (ANOVA) followed by Tukey post hoc test was applied to determine the difference between samples at a 5% significance level. The experiments were carried out in three independent replicates. The factors were sample type (flours and protein concentrate) and pretreatments (control, soaking and soaking + boiling). Data were expressed as mean ± standard deviation. Significant differences between samples are indicated with different uppercase or lowercase letters.

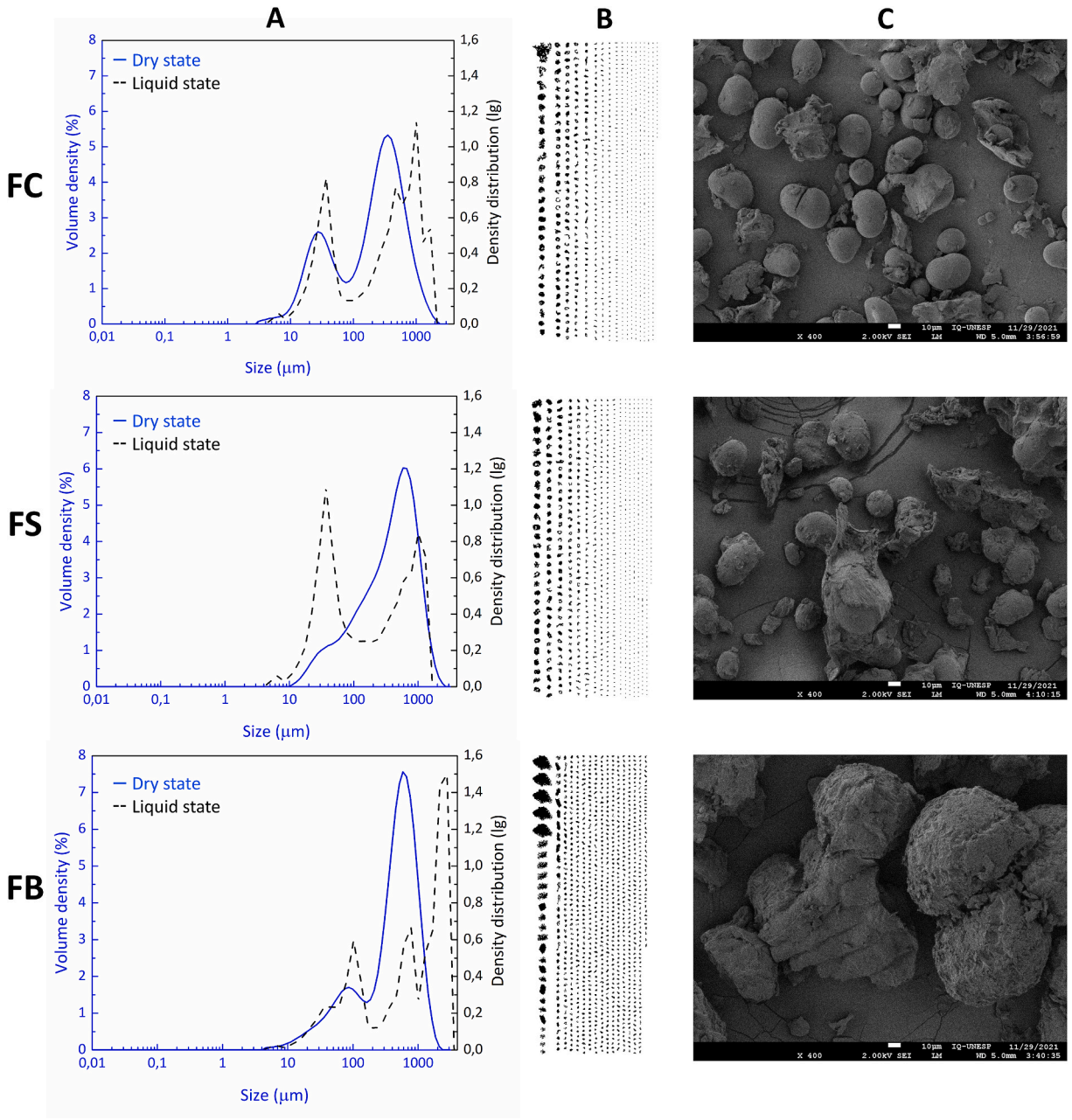

Fig. 1. Size distribution **(a)**, particle morphology **(b)** and scanning electron micrographics (SEM) **(c)** of pigeon pea flours. Size distribution measurements were carried out on samples in the dry state (solid blue line) and on aqueous dispersions containing 0.5% ethanol (dashed black line). FC: flour produced with raw seeds; FS: flour produced with soaked seeds; FB: flour produced with soaked + boiled seeds.

3. Results and discussion

3.1. Flour morphology and size distribution

Soaking is a widely used process, both in domestic cooking and on the industrial scale, to improve the digestibility of pulse seeds. This pre-treatment has the main objective of hydrating the seeds, and in particular biopolymers constituents such as proteins, starch and pectin. This, in turn, facilitates subsequent cooking and is thus very helpful in reducing the hard-to-cook phenomenon often reported in underused pulses. Boiling makes pre-soaked seeds softer and more palatable, which are important attributes for their consumption. Heating results in protein denaturation, starch gelatinization, and pectin solubilization, mechanisms responsible for seed softening (Chigwedere, Njoroge, Van Loey, & Hendrickx, 2019). The impact of these phenomena appears in the size distribution, particle shape and morphology of the flours (Fig. 1 and Supplementary Table 1).

The flour produced with raw seeds (control, FC) presented a bimodal size distribution in the dry state (blue solid line), with a small population between 10 and 100 μm and a larger population with size >100 μm (Fig. 1a). The soaked seeds generated a flour (FS) with a broader distribution between 100 and 1000 μm and only a shoulder between 10 and 100 μm, whereas soaking + boiling (FB) caused the size distribution shifting to the right, resulting in a well-defined peak between 200 and 2000 μm and suggesting the formation of aggregates as a consequence of boiling. The size distribution measured in the flour suspensions (black dotted lines) partly overlapped with the distribution in dry state, with predominantly bimodal distributions for FC and FS, and trimodal for FB. A decrease in the smaller size range was observed in the dry state measurement for FS (Fig. 1a). As suggested by the SEM images, the starch granules in FS show a tendency to form aggregates, since we cannot see as many individual granules as in FC. Soaking may induce a change in the matrix around the starch granules, leading to agglomeration, which in turn leads to a shift towards larger size. When the FS sample was evaluated in the liquid state, however, we can see this peak again, more pronounced, probably due to the solubilization of the matrix between the starch granules.

All the samples had a major population with size >100 μm, of which the relative importance increases with applying pre-treatments. Starch granules generally have a size ranging from 1 to 110 μm and an oval or spherical shape (Hoover, 2001), like the particles that appear in great number in FC (Fig. 1c) and that probably correspond to the population observed in the size region between 1 and 100 μm. The particle shape distribution and SEM images (Fig. 1b and c) of flours FS and FB corroborate these results. The particle shape distribution shows the incidence of a greater number of smaller and more uniform particles and few larger and uneven particles in FC and FS, whereas in FB there was a greater incidence of larger and uneven particles (Fig. 1b.), which contributes to the shift of particle size distribution for sizes >1000 μm.

The SEM images (Fig. 1c) shows the effect of the seeds pre-treatments in the flour morphology, such as the presence of native starch granules in non-treated flour (FC); starch granules with a more wrinkled surface due to hydration after soaking (FS); and larger structures formed after gelatinization of starch granules by boiling (FB) (Ma et al., 2021). Also, compact particles could be seen in FB, which may be directly related to the gelatinization of starch granules under high moisture and high temperature conditions. During thermal treatments, moisture content plays an important role in the morphological changes of starch granules, so that a higher moisture content can lead to a more compact starch structure (Li et al., 2021; Zhang et al., 2022). The starch content of the samples is further discussed in the following section, along with the composition of the pigeon pea ingredients.

3.2. Pigeon pea ingredients composition

As a preliminary characterization of the FTIR spectra of pigeon pea protein concentrates revealed the significant contribution of other macronutrients such as lipids (3000-2800 cm^{-1}) and carbohydrates (1200-900 cm^{-1}) (Andrade et al., 2019; Gholizadeh et al., 2014) (Supplementary Fig. 2), the composition of these components in both flours and protein concentrates was characterized. In the following sections the impact of seed pre-treatments on major (polysaccharides, lipids, and proteins) and minor compounds (trypsin inhibitor activity, total soluble phenolic compounds, raffinose family oligosaccharides (RFOs), ashes) of pigeon pea ingredients are presented.

3.2.1. Carbohydrate composition

Carbohydrates are the largest constituents of pigeon pea flour, corresponding to approximately 60 wt % of its total composition, regardless of the pre-treatments used (Table 1). Of this total content in flours, around 54–62% corresponds to starch, 27–29% corresponds to non-starch polysaccharides (NPS), around 7–11% correspond to raffinose family oligosaccharides (RFO), and around 4–6% correspond to sucrose (Table 2).

Regarding the composition in non-starch polysaccharides (NSP), soaking + boiling pre-treatment decreased the levels of uronic acid and glucose residues from 2.8 to 8.4 g 100 g^{-1} to 1.8 and 7.9 g 100 g^{-1}, respectively. This could be attributed to a solubilization of some cell wall polysaccharides in the cooking water. The soaking + boiling pre-treatment also affects the soluble polysaccharide content: FB showed an increase in uronic acids suggesting an increased content of pectin as compared to FC and FS. Also, the combined pre-treatment seems to have completely solubilized the uronic acid content in flour. The increase in the soluble NSP content can be attributed to a modification of the structure of homogalacturonans during boiling, reducing the hard-to-cook phenomenon present in these seeds (Chigwedere et al., 2019; Mubaiwa, Fogliano, Chidewe, & Linnemann, 2017).

The content of RFOs in pulses varies from 2 to 10 g 100 g^{-1} (Guillon & Champ, 2002). In this work, the RFOs corresponded to around 7.3 g 100 g^{-1} for FC, whereas in FS and FB this value was reduced by around 25 and 37%, respectively. Soaking leads to a reduction in RFOs levels probably through the leaching of raffinose, stachyose and verbascose into the soaking water. During boiling, RFOs can be reduced by heat-induced hydrolysis of oligosaccharides to disaccharides and monosaccharides (Onigbinde & Akinyele, 1983). Unlike other seeds such as chickpeas, lupin, and lima beans that present only traces of verbascose, pigeon pea is a species that presents high levels of this oligosaccharide, as discussed by Raja, Agasimani, Varadharajan, and Ram (2016). According to the authors, the reduction of these sugars through seed pre-treatments is beneficial for better digestibility of pigeon pea, as the consumption of RFOs is associated with flatulence problems.

Sucrose is the only disaccharide present in pigeon pea, except for some cultivars that may have small amounts of maltose (Raja et al., 2016). The sucrose content was 4 g 100 g^{-1} for FC and decreased by about 48% with soaking, reaching 2.1 g 100 g^{-1} for FS. However, the sucrose content in FB showed an increase of approximately 10% compared to FS, possibly resulting from the hydrolysis of oligosaccharides to sucrose.

The protein fractionation process resulted in a large decrease in carbohydrate content as compared to flours: 9–11 g 100 g^{-1} for PPC-C and PPC-S and about 15 g 100 g^{-1} for PPC-B (Table 1). Mannose and galactose residues were the most abundant NSP residues in the protein concentrates (about 1 g 100 g^{-1} each), and their levels were not affected by pre-treatments ($p > 0.05$). Mannose residues had their content doubled in relation to the levels presented in flour counterparts, whereas the proteins were 3-fold concentrated in the fractionation process. The high mannose content in protein concentrates is surprising and appears not to be related to the pre-treatments used. The presence of mannose can be attributed to the potential existence of (galacto) (gluco)mannanes in protein concentrates but also to high mannose *N*-glycans of *N*-glycoproteins. It is known that soybean *β*-conglycinin, for example, is likely

Table 1
Proximate composition, total soluble phenolic compounds (TSPC) and trypsin inhibitors activity (TIA) of pigeon pea flours and protein concentrates.

Sample	Proximate composition						TSPC	TIA	
	Moisture (g 100 g^{-1})	Protein (g 100 g^{-1}, d.b)	Lipid (g 100 g^{-1}, d.b)	Carbohydrate (g 100 g^{-1}, d.b)	Ash (g 100 g^{-1}, d.b)	Total amount of characterized compounds (g 100 g^{-1}, d.b)	mg GAE g^{-1}	μg TId mg^{-1} sample	μg TId mg^{-1} protein
Flours									
FC	9.0 ± 0.0^{b}	19.5 ± 0.2^{a}	4.0 ± 0.2^{a}	64.6 ± 0.7^{a}	4.3 ± 0.0^{a}	92.4 ± 0.7^{a}	7.2 ± 0.0^{a}	8.3 ± 0.2^{a}	42.6 ± 0.8^{a}
FS	10.0 ± 0.3^{a}	21.5 ± 0.8^{a}	3.1 ± 0.1^{b}	61.1 ± 1.1^{b}	4.1 ± 0.0^{b}	90.3 ± 2.0^{a}	6.0 ± 0.1^{b}	5.7 ± 0.3^{b}	26.1 ± 1.1^{b}
FB	7.8 ± 0.1^{c}	20.2 ± 0.7^{a}	2.8 ± 0.0^{c}	64.5 ± 1.6^{a}	2.9 ± 0.0^{c}	90.4 ± 1.5^{a}	3.1 ± 0.0^{c}	1.2 ± 0.0^{c}	5.8 ± 0.2^{c}
Protein concentrates									
PPC-C	8.3 ± 0.0^{c}	63.7 ± 0.3^{b}	12.6 ± 0.4^{a}	11.1 ± 0.0^{b}	7.5 ± 0.0^{a}	94.9 ± 0.6^{a}	5.6 ± 0.1^{a}	11.5 ± 0.5^{a}	18.0 ± 0.8^{a}
PPC-S	10.1 ± 0.0^{a}	68.3 ± 0.5^{a}	9.3 ± 0.0^{b}	9.1 ± 0.4^{c}	7.2 ± 0.3^{a}	93.9 ± 0.9^{a}	5.7 ± 0.0^{a}	6.2 ± 0.3^{b}	9.0 ± 0.4^{b}
PPC-B	9.3 ± 0.0^{b}	62.2 ± 0.5^{c}	4.0 ± 0.1^{c}	15.4 ± 0.2^{a}	7.5 ± 0.0^{a}	89.1 ± 0.7^{b}	5.7 ± 0.1^{a}	0.6 ± 0.0^{c}	1.0 ± 0.0^{c}

Different letters in the same column (for flours and protein concentrates) indicate statistical difference ($p < 0.05$). TId: trypsin inhibited; FC: flour produced with raw seeds; FS: flour produced with soaked seeds; FB: flour produced with soaked-boiled seeds; PPC-C: protein concentrate produced with FC; PPC-S: protein concentrate produced with FS; PPC-B: protein concentrate produced with FB.

Table 2
Carbohydrates composition in pigeon pea flours and protein concentrates.

	NSP composition (g 100 g^{-1}, d.b)									RFO composition (g 100 g^{-1}, d.b)				Sucrose (g 100 g^{-1}, d.b)	Starch (g 100 g^{-1}, d.b)
	Rha	Fuc	Ara	Xyl	Man	Gal	UA	Glc	Total	Raf	Sta	Ver	Total		
Total content															
FC	0.3 ± 0.0^{a}	0.0 ± 0.0^{a}	2.9 ± 0.1^{a}	2.6 ± 0.5^{a}	0.4 ± 0.0^{a}	1.3 ± 0.1^{a}	2.8 ± 0.1^{a}	8.4 ± 0.8^{a}	18.7 ± 0.5^{a}	1.0 ± 0.1^{a}	2.6 ± 0.1^{a}	3.7 ± 0.1^{a}	7.3 ± 0.2^{a}	4.0 ± 0.2^{a}	34.7 ± 0.4^{b}
FS	0.3 ± 0.0^{a}	0.1 ± 0.0^{a}	3.4 ± 0.3^{a}	2.9 ± 0.6^{a}	0.4 ± 0.0^{a}	1.5 ± 0.1^{a}	2.3 ± 0.1^{b}	7.1 ± 0.2^{a}	18.0 ± 1.2^{a}	0.5 ± 0.0^{b}	1.2 ± 0.0^{c}	3.8 ± 0.1^{a}	5.5 ± 0.1^{b}	2.1 ± 0.0^{c}	35.6 ± 0.2^{b}
FB	0.4 ± 0.0^{a}	0.0 ± 0.0^{a}	3.0 ± 0.2^{a}	2.2 ± 0.5^{a}	0.4 ± 0.0^{a}	1.6 ± 0.1^{a}	1.8 ± 0.1^{c}	7.9 ± 0.4^{b}	17.3 ± 0.6^{b}	0.5 ± 0.0^{b}	1.7 ± 0.0^{b}	2.4 ± 0.0^{b}	4.6 ± 0.1^{c}	2.3 ± 0.0^{b}	40.3 ± 1.9^{a}
Soluble content															
FC	n.d	n.d	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.7 ± 0.0^{b}	1.5 ± 0.0^{b}	0.2 ± 0.0^{a}	2.7 ± 0.0^{b}	n.a	n.a	n.a	n.a	n.a	n.a
FS	n.d	n.d	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.8 ± 0.1^{b}	1.4 ± 0.0^{b}	0.2 ± 0.0^{a}	2.6 ± 0.1^{b}	n.a	n.a	n.a	n.a	n.a	n.a
FB	n.d	n.d	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	1.1 ± 0.0^{a}	2.1 ± 0.1^{a}	0.2 ± 0.0^{a}	3.7 ± 0.1^{a}	n.a	n.a	n.a	n.a	n.a	n.a
Total content															
PPC-C	n.d	n.d	0.6 ± 0.0^{a}	0.2 ± 0.0^{a}	1.0 ± 0.0^{a}	1.0 ± 0.1^{a}	0.8 ± 0.0^{a}	n.d	3.6 ± 0.1^{a}	0.2 ± 0.0^{a}	1.2 ± 0.1^{a}	1.4 ± 0.0^{a}	2.8 ± 0.1^{a}	1.8 ± 0.0^{a}	2.8 ± 0.1^{a}
PPC-S	n.d	n.d	0.4 ± 0.1^{b}	n.d	0.9 ± 0.1^{a}	0.8 ± 0.1^{a}	0.6 ± 0.0^{b}	n.d	2.8 ± 0.2^{b}	0.2 ± 0.0^{a}	0.9 ± 0.0^{b}	1.1 ± 0.0^{b}	2.1 ± 0.1^{b}	1.3 ± 0.0^{b}	2.8 ± 0.1^{a}
PPC-B	n.d	n.d	0.6 ± 0.0^{a}	0.1 ± 0.0^{b}	1.1 ± 0.1^{a}	0.8 ± 0.0^{a}	0.8 ± 0.0^{a}	n.d	3.4 ± 0.1^{a}	0.1 ± 0.0^{a}	0.7 ± 0.0^{c}	1.0 ± 0.0^{b}	1.7 ± 0.1^{c}	0.9 ± 0.0^{c}	9.4 ± 0.2^{b}

Different letters in the same column (for flours and protein concentrates) indicate statistical difference ($p < 0.05$). FC: flour produced with raw seeds; FS: flour produced with soaked seeds; FB: flour produced with soaked + boiled seeds; PPC-C: protein concentrate produced with FC; PPC-S: protein concentrate produced with FS; PPC-B: protein concentrate produced with FB. Rha, Fuc, Ara, Xyl, Man, Gal, UA, Glc, NSP, Suc, Raf, Sta, Ver, and RFO are rhamnose, fucose, arabinose, xylose, mannose, galactose, uronic acid, glucose, non-starch polysaccharides, sucrose, raffinose, stachyose, verbascose, and raffinose family oligosaccharides, respectively. Values bellow the detection limit are expressed as n.d and measures not performed are expressed as n.a.

to be glycosylated, with mannose being one of the main sugars involved in this process (Thanh & Shibasaki, 1976). Soybean β-conglycinin has some homology with pigeon pea β-conglycinin, according to a study by Krishnan, Natarajan, Oehrle, Garrett, and Darwish (2017), so that the glycosylation of the main proteins in pigeon pea may occur in a similar way to that in soybean. However, more studies must be carried out to understand this possible phenomenon.

Protein fractionation also affected the RFO composition of protein concentrates, which decreased even further compared to the original flours. PPC-C, PPC-S and PPC-B presented values of 2.8, 2.1, and 1.7 g 100 g^{-1} of total RFOs, respectively, and all concentrates had a reduction of around 63% in relation to their flour counterparts. This significant reduction highlights the cumulative effect of seed pre-treatments with aqueous fractionation of proteins in improving the digestibility of pigeon pea-based ingredients. It has been reported that in processes to produce globulin-rich extracts, the protein precipitation step at acidic pH generates a side stream that is rich in water-soluble proteins, such as albumins, and sugars (Yang, Eikelboom, van der Linden, de Vries, & Venema, 2022). It is possible that this step is essential to further reduce the levels of RFOs and sucrose in protein concentrates.

Finally, of the total carbohydrates in protein concentrates, around 25–31% correspond to the starch fraction for PPC-C and PPC-S (Table 2). For the protein concentrate produced with soaked-boiled seeds, however, a greater accumulation of starches was observed (corresponding to

61% of the total carbohydrates in PPC-B). This can be explained by the starch gelatinization that occurs during boiling, leading to greater release and solubilization of amylose and amylopectin (Watson & Johnson, 1965).

3.2.2. Lipid composition

The total lipid content in pigeon pea flour was reduced by soaking and boiling pre-treatments (Table 3). While FC presented a total lipid content of 4 g 100 g^{-1}, FS and FB showed values of 3.1 g 100 g^{-1} and 2.8 g 100 g^{-1}, respectively.

Neutral lipids corresponded to 50% of the total lipids in FC, whereas FS and FB had 45 and 71% of neutral lipids in their composition, respectively. All flours showed a predominance of triglycerides (1–1.8 g 100 g^{-1}) and the same level of free fatty acids (0.2 g 100 g^{-1}). However, only samples subjected to pre-treatments showed detectable levels of monoglycerides, which is an indication of triglyceride hydrolysis during pre-treatments (Abdel-Rahman, El-Fishawy, El-Geddawy, Kurz, & El-Rify, 2007). The total phospholipid content was around twice higher in FB, compared to FC and FS. This may seem surprising, since it is conceptually not possible that additional lipids would be incorporated in the seeds (and resulting flour) upon soaking + boiling. A possible explanation is that this pre-treatment would improve the extractability of lipids, in particular polar lipids, which also implies that extraction might not have been complete for FC and FS, although conducted with the recommended Folch extraction. Comparable analyses are hardly available in literature; one of the only references found is the work of Liukkonen, Montfoort, and Laakso (1992), who observed a moderate increase in the phospholipid content for flour of oat soaked-steamed-grains, compared to a reference with non-steamed grains. Regarding tocopherols, only γ-tocopherol was identified in the samples (Table 3). The high content of γ-tocopherol observed for FC (877 μg g^{-1} lipids) shows that pigeon pea is a good source of tocopherol, as well as several Canadian and Indian pulses, as demonstrated by Padhi, Liu, Hernandez, Tsao, and Ramdath (2017) and Krishnaa, Prabhakara, and Aitzetmüllerb (1997). Soaking alone considerably decrease the γ-tocopherol contents to 304 μg g^{-1} lipids, indicating that processes that do not use heat can also result in significant losses of such lipophilic compounds. Tocopherol losses were even greater when pre-treatment was combined with boiling (around 218 μg g^{-1} lipids). The degradation of tocopherols during processing has been reported for various ingredients and food products, and is most likely due to the capacity of these molecules to act as chain-breaking antioxidants in the presence of free radicals, notably when heat treatments (e.g., steaming) are applied in the presence of air (Balakrishnan & Schneider, 2023; Hidalgo, Brandolini, & Gazza, 2008; Ottaway, 2010).

The protein fractionation process resulted in the accumulation of lipids, which presented values of 12.6, 9.3 and 4.0 g 100 g^{-1} for PPC-C, PPC-S and PPC-B, respectively (Table 3). These values shows that the lipids initially present in raw-seeds and soaked-seeds flours were 3-fold concentrated for PPC-C and PPC-S. The soaking + boiling pre-treatment, however, resulted in only a 1.4-fold concentration of lipids in PPC-B, compared to its flour counterpart. It is important to highlight that although pigeon pea is a pulse with a low lipid content (<5% of total lipids in seeds) the fractionation process resulted in a significant concentration of the endogenous lipids, which was recently reported in other pulse protein ingredients (Grasberger et al., 2024; Keuleyan et al., 2023). This points out to a possible genericity of this lipid accumulation effect, but the exact mechanisms involved still need to be unraveled. Our results therefore confirm that investigating the mechanisms behind protein-lipid interactions during the fractionation of plant-based proteins is of great interest.

The neutral lipid content ranged from 43 to 51% of the total lipid composition of the protein concentrates. All protein concentrates showed a predominance of triglycerides, whereas monoglycerides were identified only for PPC-C and PPC-S. However, the level of free fatty acids doubled for PPC-B, when compared to its flour counterpart. This increase is a primary response to the occurrence of lipolysis reactions, and the wet fractionation process can intensify this phenomenon due to the homogenization step (Liukkonen et al., 1992). Soaking + boiling followed by proteins fractionation resulted in a complete loss in the phospholipid fraction in PPC-B (values below the detection limit). PPC-C and PPC-S presented values of 1.7 and 1.3 g 100 g-1 of total phospholipids, respectively, agreeing with the values reported by Keuleyan et al. (2023) for lupin protein concentrate and isolate (1.4–1.9 g 100 g-1) but lower than the values reported for pea protein concentrate and isolate (2.3–5.4 g 100 g-1). Thus, compared to the starting flours, phospholipids accumulated in the concentrates for the control and soaked samples, but were fully eliminated when the seeds were initially subjected to soaking + boiling. The latter effect could potentially be due to the physical release of phospholipid-rich structures, which would then be eliminated upon the processing to yield the concentrate. The exact mechanisms are still to be unraveled, and certainly deserve attention as polar lipids are important drivers for the sensory and functional attributes of plant protein ingredients. Among the different types of phospholipids, only

Table 3
Neutral lipids, phospholipids and tocopherols content of pigeon pea flours and protein concentrates.

	Total lipids (g 100 g^{-1}, d.b)	Neutral lipids (g 100 g^{-1}, d.b)				Phospholipids (g 100 g^{-1}, d.b)					Tocopherols (μg g^{-1} lipids)
		TG	MG	FFA	**Total**	PE	PI	PA	PC	**Total**	γ-tocopherol
Flours											
FC	**4.0 ± 0.2^{a}**	1.8 ± 0.1^{a}	n.i	0.2 ± 0.0^{a}	**2.0 ± 0.1^{a}**	n.d	0.3 ± 0.0^{a}	n.d	n.d	**0.3 ± 0.0^{b}**	877.0 ± 215.0^{a}
FS	**3.1 ± 0.1^{b}**	1.0 ± 0.0^{b}	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	**1.4 ± 0.1^{b}**	n.i	0.1 ± 0.0^{b}	0.1 ± 0.0	n.d	**0.3 ± 0.0^{b}**	304.0 ± 70.6^{b}
FB	**2.8 ± 0.0^{c}**	1.6 ± 0.1^{a}	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	**2.0 ± 0.0^{a}**	0.2 ± 0.0	n.i	n.i	0.4 ± 0.0	**0.6 ± 0.0^{a}**	218.6 ± 15.0^{b}
Protein concentrates											
PPC-C	**12.6 ± 0.4^{a}**	6.4 ± 0.4^{a}	n.i	n.d	**6.4 ± 0.2^{a}**	n.d	0.8 ± 0.0^{a}	n.i	0.9 ± 0.0^{a}	**1.7 ± 0.1^{a}**	1274.5 ± 142.6^{a}
PPC-S	**9.3 ± 0.0^{b}**	3.4 ± 0.3^{b}	0.6 ± 0.0^{a}	n.d	**4.0 ± 0.3^{b}**	n.d	0.7 ± 0.0^{a}	n.d	0.6 ± 0.0^{b}	**1.3 ± 0.0^{b}**	377.5 ± 22.5^{b}
PPC-B	**4.0 ± 0.1^{c}**	1.1 ± 0.1^{c}	0.2 ± 0.0^{b}	0.4 ± 0.0	**1.7 ± 0.1^{c}**	n.i	n.d	n.i	n.d	n.d	167.1 ± 3.5^{c}

Different letters in the same column (for flours and protein concentrates) indicate statistical difference ($p < 0.05$). FC: flour produced with raw seeds; FS: flour produced with soaked seeds; FB: flour produced with soaked-boiled seeds; PPC-C: protein concentrate produced with FC; PPC-S: protein concentrate produced with FS; PPC-B: protein concentrate produced with FB.TG: triglycerides; MG: monoglycerides; FFA: free fatty acids; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PC: phosphatidylcholine; PA: phosphatidic acid. Values bellow the detection limit are expressed as n.d and compounds not identified are expressed as n.i.

phosphatidylinositol and phosphatidylcholine were identified in pigeon pea protein concentrates. Like in flours, γ-tocopherol contents were the highest for PPC-C (1274 μg g-1 lipids), decreasing to around 378 and 167 μg g-1 lipids for PPC-S and PPC-B, respectively. This confirms the effect of soaking and boiling on tocopherol chemical degradation, as mentioned earlier.

3.2.3. Protein composition

The protein content of flours was comprised between 19.5 and 21.5%, (Table 1), not being affected by the applied pre-treatments ($p > 0.05$). Corzo-Ríos et al. (2022) and Danhassan, Salihu, and Inuwa (2018) also did not observe changes in the protein content of seeds subjected to similar processes.

The SDS-PAGE analysis (Fig. 2) showed three main protein bands in all the samples: two between 37 and 50 kDa and one between 50 and 75 kDa. These bands may correspond to 7S-vicilin subunits, the main protein of pigeon pea seeds (about 60% of their composition) (Sun et al., 2020). FC presented more bands <37 kDa than FS and FB, which could be seen mainly in reducing conditions. Seed pre-treatments can lead to the loss of low molecular weight proteins. In work carried out by Sun et al. (2020), protein bands with molecular weight <25 kDa completely disappeared in pigeon pea protein extracts produced with microwave-cooked seeds.

Regarding the low molecular weight proteins that can be lost by soaking and boiling treatments, trypsin inhibitors are of special interest, since their removal can improve the digestibility of these seeds. Krishnan et al. (2017) identified Kunitz trypsin inhibitors (KTI) in pigeon pea seeds with a molecular weight of 21 kDa. A protein band close to 21 kDa can be observed in FC (orange triangle symbol, under reduced conditions), being less visible in the other samples. The possible loss of KTI during the pre-treatment could directly affect the trypsin inhibitor activity (TIA) of the samples and will be further discussed in the next section.

The presence of a protein band close to 18 kDa (yellow triangle symbol, under not-reduced conditions) in FC could suggest the presence of lectins in the samples. Pigeon pea lectins have two polypeptide chains joined only by non-covalent and non-disulfide bonds and their presence in the seeds was reported in study of Siddiqui, Hasan, and Salahuddin (1995).

The extraction process increased the protein content of powders from 62.2 to 68.3% (Table 1). In SDS-PAGE, the identified bands are in the region of 7S-vicilin subunits (Sun et al., 2020). Thus, the extraction process eliminated proteins with molecular weight >75 kDa and reduced the amount of proteins with molecular weight <37 kDa.

3.2.4. Minor compounds: trypsin inhibitor activity (TIA), total soluble phenolic compounds (TSPC), and ash content

Trypsin inhibitors, phenolic compounds and minerals may be leached and/or degraded during soaking and boiling pre-treatments (Chigwedere et al., 2019), so we probed their content (Table 1).

The TIA was 8.3, 5.7 and 1.2 μg TId mg^{-1} sample for FC, FS and FB, respectively. The soaking pre-treatment reduced TIA by 31%, whereas boiling treatment led to a reduction of about 86% for pigeon pea flour. As the protein content is similar for all samples, the same reduction percentages are observed if we consider the TIA results expressed in mg of protein (Table 1). The reduction of TIA is a good indicator of the efficiency of the processes used to improve the digestibility of pigeon pea flours.

The protein concentrates showed values of 11.5, 6.2 and 0.6 μg TId mg^{-1} for PPC-C, PPC-S, and PPC-B, respectively (Table 1). PPC-C increased TIA by approximately 38% over its flour counterpart, close to values reported by Avilés-Gaxiola, Chuck-Hernández, and Serna Saldívar (2018) for soybean protein concentrate (increase of 32% in TIA compared to the initial activity in soybean flour). For PPC-S, however, TIA only increased around 9% in relation to FS, whereas PPC-B presented half of the TIA value determined for FB.

Legume trypsin inhibitors are classified into two families according to their molecular weight: Bowman-Birk (BBTIs) of 8 kDa and Kunitz (KTIs) of 20 kDa (Avilés-Gaxiola et al., 2018). Low molecular weight proteins (<37 kDa) showed less visible bands in the electrophoresis

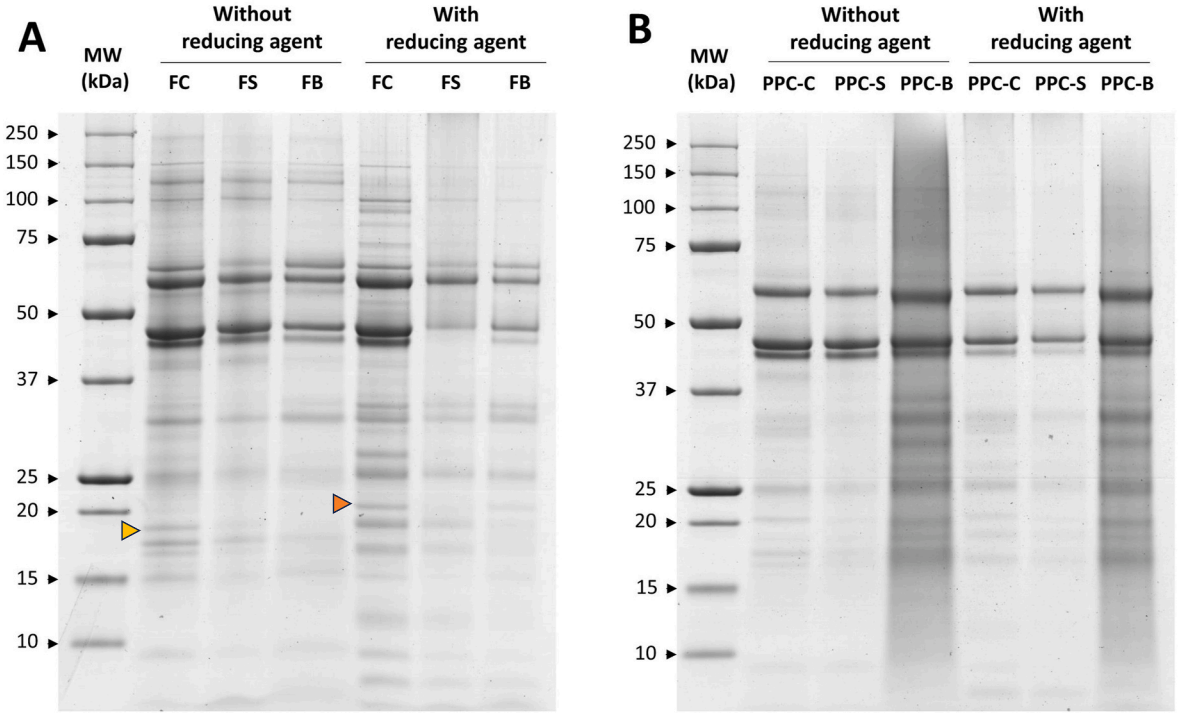

Fig. 2. SDS-PAGE of pigeon pea flours **(a)** and protein concentrates **(b)** with or without the presence of the reducing agent 2-mercaptoethanol (10%, v v^{-1}). FC: flour produced with raw seeds; FS: flour produced with soaked seeds; FB: flour produced with soaked + boiled seeds; PPC-C: protein concentrate produced with FC; PPC-S: protein concentrate produced with FS; PPC-B: protein concentrate produced with FB.

analysis (Fig. 2) even for the sample subjected only to the soaking, suggesting leaching of these proteins. A band below 10 kDa, maybe related to BBTIs, is visible in the flour produced with raw seeds, but not for the pre-treated flours. The same can be observed for the bands around 20 kDa, that can be associated with KTIs.

The results show that soaking pigeon pea seeds for 24 h followed by boiling for 30 min is enough to reduce the TIA by almost 86% in flours. An additive effect of the protein fractionation process was also observed, so that the combination of soaking + boiling pre-treatments followed by proteins aqueous fractionation can reduce the TIA by 95%. There is a clear positive effect in combining seed soaking + boiling pretreatments with the protein fractionation method adopted to mitigate the activity of trypsin inhibitors in protein concentrates.

The FC sample had the highest TSPC content (7.2 mg GAE/g), which is in the range of values reported for dried beans (Yang, Gan, Ge, Zhang, & Corke, 2018). Soaking reduced the content of TSPC by 16%, whereas boiling treatment led to further reduction (57%) of TSPC content in flour (Table 1). This may result from the leaching of phenolic compounds during soaking, as well as from degradation of these compounds by high temperature during the boiling treatment. Leaching of phenolic compounds during soaking depends on the rate of water absorption, coat permeability, hilum size and cotyledon composition. Soaking conducted for long hours (up to 16 h) considerably reduces the content of native phenolic compounds in pulses (Mba et al., 2019), and this loss can be intensified during seed boiling (Yang et al., 2018). According to Fleuriet and Macheix (2003), phenolic acids can undergo oxidative processes during boiling, in addition to the release of free acids from conjugated forms and the formation of complex structures of phenolic compounds with other molecules, such as proteins. The reduction in TSPC levels observed for the samples has a positive impact on the digestibility of pigeon pea ingredients, since some phenolic compounds can form complexes with proteins, making them less soluble, in addition to potentially inhibiting the action of digestive enzymes (Chigwedere et al., 2019).

Regarding protein concentrates, all samples had the same TSPC content, around 5.7 mg GAE/g, this suggests that the process has little effect on the solubility of the phenolic compounds. It is important to highlight that although our results indicate the same TSPC content for all protein concentrates, their phenolic composition may change due to the different pre-treatments. Future studies should investigate the impact of pre-treatments on individual phenolic compounds. Also, the method used in our work only probed soluble phenolic compounds, so we do not have information about phenolic compounds that are chemically bound to proteins. The dark brown color of protein concentrates (Supplementary Fig. 1) suggests the presence and/or modification of phenolic compounds during protein extraction at alkaline pH (Potin, Lubbers, Husson, & Saurel, 2019). The alkaline conditions of the plant-based protein fractionation process cause rapid oxidation of phenolic compounds, which bind to protein side chains (Rashwan, Osman, Abdelshafy, Mo, & Chen, 2023). Protein-polyphenol interactions can also alter the sensory characteristics and digestibility of protein concentrates (Rashwan et al., 2023).

Finally, the ash content decreased in pre-treated flours (approximately 33% for flours produced with soaked-boiled seeds), suggesting mineral leaching during seed hydration and boiling. Conversely, protein concentrates had an ash content of around 7.5 g 100 g^{-1}, showing no differences between samples ($p > 0.05$).

3.3. Endogenous fluorophores in flours and protein concentrates

In addition to the biochemical analysis of flours and concentrates, the exploration of intrinsic fluorescence may help in identifying additional minor compounds and/or provide a way to investigate chemical modifications in relation to the process (Locquet, Aït-Kaddour, &

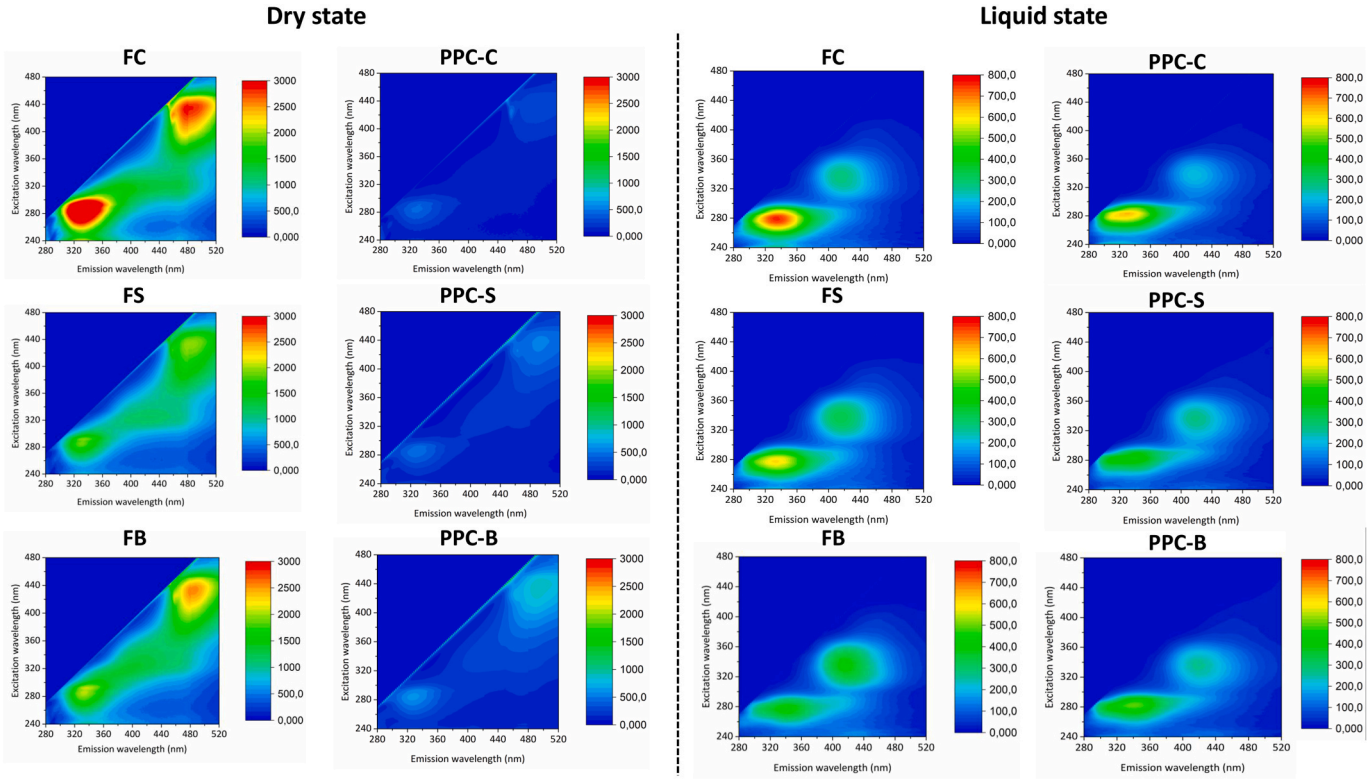

Fig. 3. Three-dimensional excitation-emission fluorescence matrices (3D-EEFM) of pigeon pea flours and protein concentrates analyzed in dry and liquid state. FC: pigeon pea flour produced with raw seeds (control); FS: pigeon pea flour produced with soaked seeds; FB: pigeon pea flour produced with soaked + boiled seeds. PPC-C: protein concentrate produced with raw seeds flour (control); PPC-S: protein concentrate produced with soaked-seeds flour; PPC-B: protein concentrate produced with soaked + boiled seeds flour.

Cordella, 2018). The three-dimensional excitation-emission fluorescence matrices (3D-EEFM) (Fig. 3) showed two distinct regions for pigeon pea flours and protein concentrates when evaluated either in the solid or liquid state: one corresponding to emission of proteins (λ_{Ex} = 270–290 nm, λ_{Em} = 300–360 nm corresponding mainly to the tryptophan/tyrosine emission), and another region referring to the fluorescence emission of non-protein compounds ($\lambda_{Ex} \approx$ 400–440 nm, λ_{Em} = 450–500 nm for solid state and $\lambda_{Ex} \approx$ 300–340 nm λ_{Em} = 440–480 nm for liquid state).

The different configuration adopted for each measurement (front-surface vs. right-angle mode) does not allow a direct comparison between the results obtained in dry and liquid state in relation to the observed fluorescence intensity. However, the use of both methods allows to see the influence of the presence of a solvent on the configuration of the endogenous fluorophores present in the samples.

A lower intensity of tryptophan fluorescence emission region was observed in the pre-treated flours, both in dry and liquid state. This decrease in tryptophan fluorescence may be related to chemical degradation and/or complexation with other molecules during pre-treatments. The second region ($\lambda_{Ex} \approx$ 430 nm for solid state) could be seen in the flours, having a higher intensity in FC and decreasing in FB and FS, respectively. The analysis of the suspensions also showed a second category of fluorophores with $\lambda_{Ex} \approx$ 340 nm, implying that the solubilization of the samples in buffer may cause a large shift in the excitation wavelength from 430 nm to 340 nm. Another hypothesis is that a less visible peak in the dry state samples ($\lambda_{Ex} \approx$ 300–340 nm λ_{Em} = 440–480 nm) may correspond to the second region observed in the liquid state samples, but more intense. For protein concentrates, the fluorescence intensity of this second region, both in solid and liquid state, increased with applying pre-treatments, in the order PPC-C < PPC-S < PPC-B. This region is associated with the fluorescence emission of several phenolic compounds (Locquet et al., 2018).

Since the concentration of samples in liquid state was standardized (absorbance of all samples ≈ 0.075), the same fluorescence intensity was expected for protein concentrates and flours. However, a slightly lower fluorescence intensity was observed for protein concentrates, suggesting possible degradation of tryptophan (Lakowicz, 2006) with the fractionation process. Even so, in the liquid state we did not observe the almost complete extinction of the fluorescence intensity that was observed for protein concentrates when measured in the dry state. This almost complete extinction of fluorescence intensity may be related to the darker color of these powders. The presence of dark and/or highly light-absorbing compounds can lead to fluorescence extinction (Muller, Milori, Déléris, Steyer, & Dudal, 2011). The evaluation of food powders by 3D-EEFM is still scarce in the literature, and we did not find studies that evaluated brownish or darkish food ingredients. Yet, some studies were conducted to evaluate fluorescent compounds in soils in dry and liquid states. Mounier, Nicolodelli, Redon, and Milori (2017) also did not observe fluorescence for samples of a humic substance in solid state, but samples showed fluorescence when evaluated in solutions. Nakaya et al. (2020) studied the use of Al_2O_3 as a white diluent for soils to improve solid-state fluorescence measurements.

3.4. Protein concentrates obtained from soaked and soaked-boiled pigeon pea seeds: conformation and functionality

3.4.1. Protein conformation

Protein extraction from pre-treated flours allowed investigating aspects related to the conformation and functionality of pigeon pea proteins. The process yield (about 13%, w w^{-1}) and protein recovery (about 20%, w w^{-1}) were not affected by seed pre-treatments and their values are shown in Supplementary Table 2.

The deconvolution of the amide I region in the FT-IR spectrum (1600–1700 cm^{-1}) showed differences in the composition of the secondary structures of PPC-B, compared to the other two concentrates. The boiling treatment resulted in a decrease in β-sheet and α-helix structures and in an increase in disordered structures (random coil) (Supplementary Fig. 3), which is an indication of higher molecular flexibility and chain unfolding provided by the heat treatment (Sun et al., 2020). On the other hand, the secondary structures of proteins were similar in PPC-C and PPC-S, with the β-sheet being the most prevalent structure (46–47%), followed by the α-helix (18–20%), turn (15–16%), random coil (10–12%) and β-antiparallel (9%).

The tryptophan emission peaks for PPC-C and PPC-S had intensity maxima at 338 and 341 nm, respectively, whereas the peak of PPC-B had a lower intensity and a shift of the peak maximum to 357 nm (Fig. 4a) ($p < 0.05$). They could indicate an unfolding of the protein chains (Vivian & Callis, 2001). A second peak was observed for the three samples (from 402 to 408 nm), which may be indicative of the presence of phenolic compounds (Yan et al., 2021).

3.4.2. Solubility and electrostatic charge

Solubility is a key parameter for the performance of proteins functional properties such as emulsifying, foaming and gelling agents in food systems. In this work, the solubility of protein concentrates was defined as the amount of soluble proteins in the supernatant of a suspension after centrifugation under predefined conditions. The solubility at different pHs presented the characteristic U-shape curve of proteins (Fig. 4b), in which higher solubility values are observed at acid and alkaline extremes and lower solubility values are observed in regions close to the isoelectric point of globulins (pH 4–6) (Gravel & Doyen, 2023). PPC-C showed a higher protein solubility at pH 2.0 when compared to PPC-S and PPC-B ($p < 0.05$). Thermal processes can alter protein solubility due to denaturation that results in exposure of hydrophobic groups and formation of aggregates/precipitates. Irreversible changes in the structure of proteins can occur in processes that involve temperatures higher than the denaturation temperature of these proteins (Bühler et al., 2020). This is the case of the present work, in which the thermal process involved temperatures (100 °C) higher than the denaturation temperatures reported for pigeon pea proteins (90.4–96.7 °C) (Sosa, Chaves, Quiroga, & Avanza, 2021). This may be an indication that irreversible changes were caused in the structure of the proteins, decreasing their solubility. Although PPC-S did not show evidence of changes in its conformation, its solubility was also affected.

At the alkaline extreme (pH = 12.0), PPC-B showed a higher solubility compared to the other protein concentrates, whereas PPC-S showed higher protein solubility at intermediate pHs. The causes of these differences in solubility are unclear. Pre-treatments that involve the use of heat can affect solubility in different ways. Wang et al. (2021) reported that the solubility of quinoa protein isolate pre-treated by boiling and microwaves had a solubility equivalent to that of the untreated protein isolate, while other heat treatment methods such as steaming and baking reduced its solubility.

The zeta potential of pigeon pea protein concentrates ranged from 22.3 mV to −32.5 mV (Fig. 4c). These values agree with the surface charge of several protein isolates as evaluated by Ge et al. (2021), such as protein isolates from pea, mung bean, black bean, kidney bean, cowpea, soybean, chickpea, etc. No difference was observed between treatments ($p < 0.05$), except for pH 3.0. Sun et al. (2020) also did not observe differences in the surface charge of non-treated vs microwave-heated pigeon pea proteins. All samples showed lower solubility and zeta potential close to zero between pH 3.0 and 4.0, comprising the region of the isoelectric point of the extracted proteins.

3.4.3. Functional properties

The way water interacts with protein ingredients can determine the sensory and storage properties of various food products (Alfaro-Diaz et al., 2021). Determining the water holding capacity (WHC) is a way of evaluating how much water certain mass of protein ingredient can absorb. A high WHC is important for developing structured products and for reducing moisture losses in packaged products (Ge et al., 2021; Bühler et al., 2020). Soaking + boiling pre-treatment increased the WHC

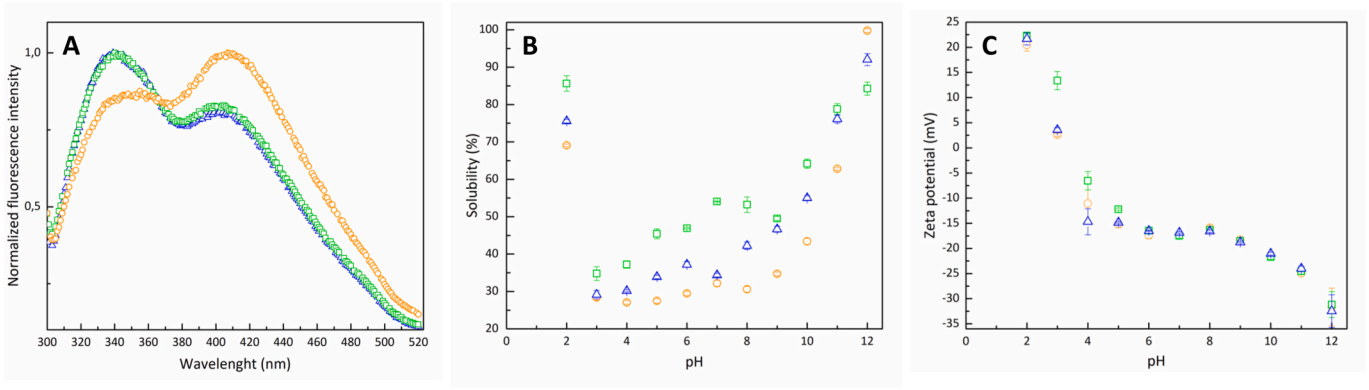

Fig. 4. Intrinsic fluorescence spectra (λ_{Ex} = 280 nm) **(a)**, solubility **(b)** and zeta potential **(c)** of pigeon pea protein concentrates. Green square symbols: protein concentrate produced with raw seeds (PPC-C); blue triangle symbols: protein concentrate produced with soaked seeds (PPC–S); orange circle symbols: protein concentrate produced with soaked + boiled seeds (PPC–B).

of PPC-B (3.7 g g^{-1}) when compared to PPC-C and PPC-S (3.1–3.3 g g^{-1}) ($p < 0.05$) (Fig. 5a). The increase in WHC of PPC-B may be due to the partial denaturation of proteins caused by heating, thus exposing the peptide bonds and polar side chains (Arise, Aliyu, & Ajidagba, 2020), and the higher starch content presented by this sample (Table 2), since starch may have an additive effect with proteins on ingredient hydration.

On the other hand, the oil holding capacity (OHC) was the only evaluated functional property that was not affected by either treatment. Wang et al. (2021) also did not observe the impact of boiling treatment on OHC, which ranged from 2.4 to 2.8 g g^{-1} for heat-treated or non-heat-treated quinoa protein concentrates. This is worth noticing, considering the variation in lipid content presented by the samples. These results suggest that the oil holding capacity is not necessarily linked to the amount of endogenous lipids in the ingredients.

The emulsifying properties of a protein ingredient are affected by several factors, such as: pH, surface charge, solubility, hydrophobicity, molecular flexibility, conformation state and protein composition (Ge et al., 2021). Among the indicators that can be used to describe the emulsifying properties are the emulsifying activity index (EAI) and the

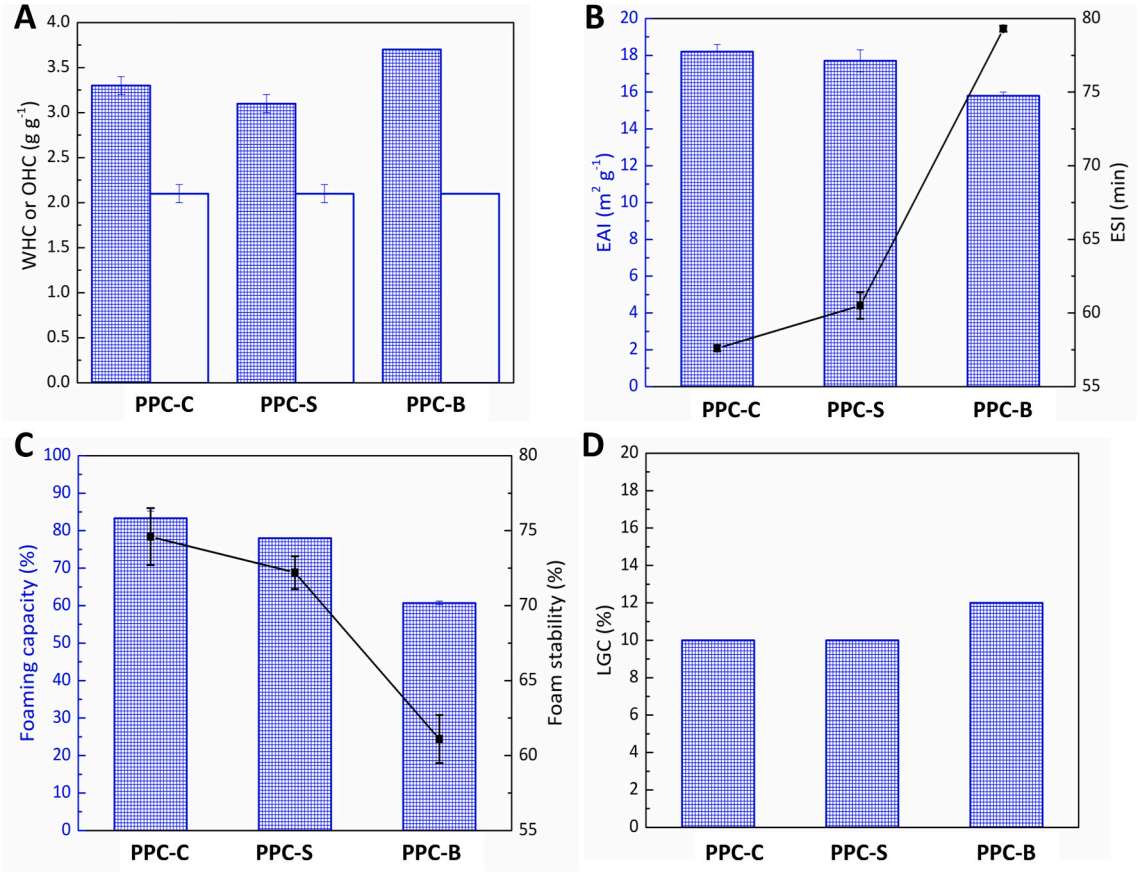

Fig. 5. Functional properties of pigeon pea protein concentrates. **(a)** Water holding capacity (WHC, blue bar) and oil holding capacity (OHC, white bar); **(b)** emulsifying activity index (EAI, blue bar) and emulsion stability index (ESI, black line); **(c)** foaming capacity (FC, blue bar) and foam stability (FS, solid black line); **(d)** Least gelation concentration (LGC). PPC-C: protein concentrate produced with FC; PPC-S: protein concentrate produced with FS; PPC-B: protein concentrate produced with FB.

emulsion stability index (ESI). EAI is based on the turbidity measurement of a diluted emulsion, providing an estimate of the stabilized interfacial area per unit weight of protein ($m^2\ g^{-1}$). The ESI measures the stability of the same diluted emulsion over a certain time, expressed in min (Karaca, Low, & Nickerson, 2011). PPC-B showed lower EAI (15.8 $m^2\ g^{-1}$) when compared to samples PPC-C and PPC-S, which showed values of 18.2 and 17.7 $m^2\ g^{-1}$, respectively ($p < 0.05$). A low emulsification and gel formation capacity of PPC-B can be explained by its lower solubility and conformational changes (Fig. 4). Phospholipids are compounds that also may play an important role in the emulsifying capacity of food ingredients. Phospholipids with a high PC content, for example, can contribute to oil droplet stabilization (Xia et al., 2022) and, of the pigeon pea protein concentrates evaluated, PPC-B was the only one that did not present significant amounts of phospholipids. The absence of phospholipids in PPC-B may also have contributed to the lower EAI observed for this sample compared to the others (Fig. 5b), highlighting the influence that lipids may have on the functional properties of these ingredients (Vondel, Janssen, Wouters, & Delcour, 2023).

It is interesting to note that, in spite of the fact that the boiling treatment decreased the emulsifying capacity of the pigeon pea protein concentrate, it had a positive impact on emulsion stability. The ESI of PPC-B (79.3 min) was higher than that of PPC-S (60.5 min) and PPC-C (57.6 min) ($p < 0.05$). Cai et al. (2020) observed similar ESI values for soy protein isolate and okara protein concentrate, which ranged from 54 to 61 min. The positive effect on emulsion stability caused by the boiling treatment, however, can be explained by stronger protein-protein interactions resulting from thermal processing, thus preventing coalescence of the droplets over time (Iyenagbe, Malomo, Idowu, Badejo, & Fagbemi, 2017). The higher starch content presented by PPC-B may have also contributed to this result. The presence of polysaccharides such as cellulose, pectin and starches can affect the stability of the emulsion through interactions with proteins, modifying the viscosity of the interfacial film and reducing the size of the droplets, delaying or hindering destabilization mechanisms of emulsified systems (Albano, Cavallieri, & Nicoletti, 2019; Locali-Pereira, Guazi, Conti-Silva, & Nicoletti, 2021).

The foaming capacity was higher for PPC-C (83.3%), whereas the PPC-S and PPC-B showing reductions of 6.4 and 27.1% of this value, respectively (Fig. 5c). Regarding foaming stability, PPC-C and PPC-S showed similar values (74.6 and 72.2%, respectively), which were higher than the observed for PPC-B (61.1%) ($p < 0.05$). Arise et al. (2020) also observed poor foaming properties for bambara protein isolate extracted from boiled-seeds. The decrease in foaming properties caused by the soaking treatment can be explained by the leaching of minor compounds that act in foam formation and stabilization. Saponins, for example, which are excellent foaming agents, are present in pigeon pea seeds in amounts that can vary from 2164 to 3494 mg/100 g (Singh, Singh, Singh, & Kaur, 2017) and their losses after soaking can reach 8%, whereas losses after cooking rise up to 66% (Duhan, Khetarpaul, & Bishnoi, 2001).

Protein concentrates produced from raw and soaked seeds presented LGC of 10 %, while the protein concentrate produced from soaked-boiled seeds presented LGC of 12 % ($p < 0.05$). (Fig. 5d). Wang et al. (2021) also obtained higher LGC (16%) for quinoa protein isolate extracted from boiled seeds, in relation to quinoa protein isolate extracted from raw seeds (12%).

4. Conclusions

Our study delved into the effects of soaking and boiling pre-treatments on pigeon pea seeds and their subsequent ingredients flour and protein concentrates. These pre-treatments modified the microstructure and size distribution within the resulting flours, primarily driven by solids leaching processes, starch gelatinization, and protein denaturation. The carbohydrate composition showed a greater accumulation of starch in protein concentrates produced with soaked-boiled seed flours and all protein concentrates showed mannose residues from oligo and/or polysaccharides. The total lipid content decreased in pre-treated flours, whereas the protein concentrates showed a significant accumulation of lipids. In addition, the use of soaked-boiled seeds for protein fractionation resulted in the total elimination of phospholipids in protein concentrates, which can affect the functionality of these ingredients due to the well-known action of these lipids in stabilizing emulsions and foams, for example. We also demonstrated remarkable efficiency in reducing trypsin inhibitor activity and raffinose family oligosaccharides using flour from soaked-boiled seeds for protein fractionation processes, a relevant aspect for pulse ingredients digestibility. Regarding functional properties, soaking reduced the foaming capacity of protein concentrates, possibly due to the elimination by leaching of compounds that have foaming ability, such as saponins. Since soaking can be used as a preliminary step in various pre-treatments, including dehulling, germination, and fermentation, these results are relevant to design several processes. On the other hand, boiling showed to be a favorable process for enhancing water holding capacity and emulsion stability in protein concentrates, thus offering advantageous prospects for food product applications. Molecular structural analysis indicated that boiling induced a degree of protein unfolding, that may explain the changes observed in its functional properties. Our results encourage the use of pre-treated seeds to produce protein concentrates due to the advantages provided for better digestion of these ingredients, such as reduction of anti-nutritional factors. However, the impacts on functionality are aspects that must be taken into consideration, depending on the intended application of the protein concentrates.

CRediT authorship contribution statement

Adilson Roberto Locali-Pereira: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Ícaro Putinhon Caruso:** Writing – review & editing, Methodology. **Hanitra Rabesona:** Writing – review & editing, Methodology. **Sophie Laurent:** Writing – review & editing, Methodology, Investigation. **Anne Meynier:** Writing – review & editing, Methodology. **Alice Kermarrec:** Investigation. **Lucie Birault:** Investigation. **Audrey Geairon:** Writing – review & editing, Methodology, Investigation. **Sophie Le Gall:** Writing – review & editing, Methodology, Investigation. **Loric Thoulouze:** Methodology, Investigation. **Véronique Solé-Jamault:** Writing – review & editing. **Claire Berton-Carabin:** Writing – review & editing, Supervision, Conceptualization. **Adeline Boire:** Writing – review & editing, Supervision, Conceptualization. **Vânia Regina Nicoletti:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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inrae.fr/bibs_eng/, UR1268 BIA, IBiSA, Phenome-Emphasis-FR ANR-11-INBS- 0012, PROBE infrastructure, Biogenouest).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2024.109923.

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