

Pea and lupin protein ingredients: New insights into endogenous lipids and the key effect of high-pressure homogenization on their aqueous suspensions

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ABSTRACT

The incorporation of plant protein ingredients in foods is a means to promote the transition to vegetable proteins. Pea and lupin meet sustainability demands and their protein ingredients display promising technological properties, yet sometimes poor functionalities. However, the involved mechanisms are still unclear, partly because comprehensive and systematic characterization of those ingredients in terms of composition and physicochemical properties is still lacking. In this work, commercial protein fractions of pea and lupin (one isolate and one concentrate for each) were thoroughly characterized. A high-pressure homogenization (HPH) treatment was applied to their aqueous suspensions (pH 7.0) to improve their dispersibility. Although isolates displayed a higher protein content (up to 72 g/100 g (d.m.) against 39 g/100 g (d.m.) for the concentrates, with respective specific N factors), their solubility (i.e., the proteins remaining in the supernatant after centrifugation) was lower than for the concentrates (15–49 wt% of the total proteins, against 65 wt%). Substantial amounts of endogenous lipids in the powders were measured after chloroform/methanol extraction (3.4–10.3 g/100 g (d.m.)), of which about half were phospholipids. For all ingredients, detailed microscopic investigations (including confocal fluorescence microscopy), and light scattering measurements showed that HPH was useful to break down large powder grains. Thus, it altered the colloidal structures present, released endogenous lipid assemblies and enhanced protein solubility. Those new insights into the non-protein composition of plant protein fractions and their behaviour in aqueous media are key for improving their functionalities and facilitating food products' rational formulation.

1. Introduction

Shifting from animal proteins to plant proteins to stabilise food matrices is currently a great challenge to tackle in food science. Pulses are of high interest from an agronomy perspective, and their high protein content makes them suitable to produce protein ingredients with various functionalities, including emulsifying properties (Boye, Zare, & Pletch, 2010). Yellow pea, a starch-rich seed, and lupin, an oil-rich seed,

are pulses that hold excellent potential regarding those emulsifying properties (Berghout, Boom, & Van Der Goot, 2014; Boye et al., 2010; Geerts, Nikiforidis, van der Goot, & van der Padt, 2017; Karaca, Low, & Nickerson, 2011).

The current pea ingredient market is rising because of the wide range of related applications and growing consumer demand, due to the high protein content (about 14–31 g/100 g (on dry matter, d.m.) (Vogel-sang-O'Dwyer, Zannini, & Arendt, 2021)) and starch content (34–50

Abbreviations: HPH, High-pressure homogenization; LPI, Lupin protein isolate; LPC, Lupin protein concentrate; PPI, Pea protein isolate; PPC, Pea protein concentrate.

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g/100 g (d.m.) (Daba & Morris, 2021)) of the seeds. Lupin does not contain starch (Czubinski, Grygier, & Siger, 2021) but is richer in lipids (5–15 g/100 g (d.m.) (Vogelsang-O'Dwyer et al., 2021) and in dietary fibres, mainly composed of cell wall polysaccharides (14–55 g/100 g (d.m.) (Vogelsang-O'Dwyer et al., 2021)) than pea. Its protein fraction may also overcome pea protein content, as it represents about 32–55 g/100 g (d.m.) of the seed (Vogelsang-O'Dwyer et al., 2021) (Table 1). The proteins in both seeds are mainly composed of globulins, called legumins and vicilin for pea, and conglutins for lupin (Hall, Hillen, & Garden Robinson, 2017). Both plant sources are relevant in terms of local production in Europe and in France, because of their resilient crops (Vogelsang-O'Dwyer et al., 2021; Boukid, Rosell, & Castellari, 2021; Shrestha, Hag, Haritos, & Dhital, 2021).

To reach protein-rich ingredients from these seeds, proteins must be separated from the other polymers (oil, starch, cell wall polysaccharides, among others) via *ad hoc* processing. Wet fractionation consists in an alkaline extraction of an aqueous suspension of flour, followed by a precipitation at the isoelectric point of the proteins (Taherian, Mondor, & Lamarche, 2015). The protein-rich fraction is then dried (in industry, by spray drying) to obtain an isolate with high protein content (between 70 and 90 g/100 g proteins) (Tabatabaei, Konakbayeva, Rajabzadeh, & Legge, 2019). When dry fractionation is applied, the protein bodies are separated from the other structures of the grain without adding water, often via the exploitation of the difference in densities of flour particles containing different compounds. The obtained ingredient contains about 40–60 g/100 g of proteins and is then called a concentrate (Schutyser & van der Goot, 2011). There are major differences in the physico-chemical conditions involved during those processes, which therefore leads to ingredients with contrasted chemical and colloidal states of proteins. Indeed, wet fractionated ingredients display high protein purity, but it seems to be at the expense of their functionality (Van Der Goot et al., 2016) which is damaged through the process because of protein denaturation (Wagner, Sorgentini, & Anon, 2000) and aggregation (Chen et al., 2019). Alternatively, dry fractionation is highlighted for its assets in terms of environmental impact and allows for preserving the native state of proteins (Geerts, Nikiforidis, et al., 2017). Yet, it leads to a less pure ingredient as compared to an isolate (Berghout, Pelgrom, Schutyser, Boom, & Van Der Goot, 2015). Being aware of these features and advantages/disadvantages is important as both fractionation routes lead to protein-enriched ingredients that are commercially available, and largely used both in academic research and at the industrial level to formulate model or real food systems, such as emulsions.

Plant protein-stabilized emulsions have been the matter of increasing research, especially for the past few years. We conducted an analysis of the scientific literature (40 research articles) (Fig. 1) to establish the state of the art regarding the research on the emulsifying properties of plant protein ingredients obtained through various fractionation routes. First, it revealed that many authors focused on plant protein ingredients processed at the lab scale (22 articles), others used commercial references for their studies (15 articles), and a few confronted their lab-made ingredient against a commercial reference (3 articles). Although lab-made samples hold the advantage to allow for a good control over the preparation process, they do not reflect the chemical and colloidal state of proteins in commercial isolates which are actually used in the food industry, as the latter encounter processing steps that are usually not included at the lab scale (e.g., thermal treatment, spray-drying). Yet,

such steps are critical for the final properties and functionalities of the ingredients. Moreover, our literature analysis highlighted that a comprehensive and systematic characterization of the ingredient composition was generally not conducted (Fig. 1). This is somewhat striking, especially for mildly fractionated ingredients that contain around half of non-proteinaceous components, as mentioned earlier. Besides, the protein content is usually assessed with a nitrogen-to-protein conversion factor (N) of 6.25, which is the most commonly used for commercial purposes as prevailed by both the European regulation 1169–2011 and the Codex Standard (Codex-Stan 174–1989). Nevertheless, N = 6.25 was established on two main assumptions: the nitrogen content of proteins is 16 g/100 g, and the samples do not contain non-protein nitrogen. Those arguments for N = 6.25 have been under hot debate for plant protein samples, as the actual conversion factor for such proteins is often substantially lower (Mariotti, Tomé, & Mirand, 2008; FAO/WHO, 2019; Mossé, 1990; Krul, 2019). This gap of knowledge on the in-depth composition of plant-based protein ingredients implies that the role of non-protein components on the overall properties in food structuring may have been largely overlooked (Funke, Boom, & Weiss, 2022).

Next to the presence of non-proteinaceous components, another factor affecting the functional properties of plant proteins is their solubility and their molecular/colloidal state in aqueous media (native, partially denatured, denatured, aggregated). In this research, protein solubility is regarded as the amount of proteins that does not sediment during centrifugation in given conditions (Kornet, 2021; Tanger, Mertens, & Kulozik, 2022). This implies that oligomers, small protein aggregates (sometimes referred to as 'soluble aggregates') and protein fragments can be found in the supernatant after centrifugation, when large insoluble aggregates or particles would remain in the pellet (Hinderink, 2021). When protein ingredients are targeted to formulate emulsions, high pressure homogenization (HPH) is often used to obtain small oil droplets. This process consists of creating intense shear rates by pushing a coarse emulsion made of oil and protein solution (or suspension) through a narrow gap (Melchior, Moretton, Calligaris, Manzocco, & Nicoli, 2022). It may therefore also impact the colloidal state of proteins, when present as large enough supramolecular structures, which is thus highly relevant for most plant protein ingredients (Bouaouina, Desrumaux, Loisel, & Legrand, 2006; Yang, Liu, Zeng, & Chen, 2018). Considering the major differences expected in the colloidal structure of the protein ingredients according to their fractionation route, and the fact that those aqueous suspensions are to be processed by HPH during emulsification, it appears paramount to understand how such a process can alter the structures present in the ingredients. Some authors reported an increase in protein "solubility" for pea protein isolates following a HPH treatment (Burger, Singh, Mayfield, Baumert, & Zhang, 2021; Lan Luo, Cheng, Zhang, & Yang, 2022; Lijuan Luo, Cheng, et al., 2022; Melchior et al., 2022; Moll, Salminen, Schmitt, & Weiss, 2021) sometimes up to 86 wt% (Moll et al., 2021) or to 95 wt% (Luo, Cheng, et al., 2022). Yet, knowledge is still lacking regarding the morphological impacts of these treatments on the suspensions, and regarding the potential modifications induced by HPH on the distribution of the non-proteinaceous components (notably lipids, cell wall polysaccharides and starch) between the structures and compartments present.

The aim of this work was to characterize the fine chemical composition of the protein and non-proteinaceous fractions of pea and lupin ingredients obtained by various fractionation processes. Commercial ingredients were selected as they are both largely used in academic research in food science, and of high relevance to real-life applications. We also aimed to understand the structural organization and morphology of their aqueous suspensions, and how these properties are affected by a HPH treatment, notably regarding the so-called 'soluble' protein fraction.

Table 1

Mean composition of pea and lupin seeds (Daba & Morris, 2021; Vogelsang-O'Dwyer et al., 2021). Data are presented as "min (g/100 g d.m.) – max (g/100 g d.m.)" of the reported values.

	Proteins	Lipids	Carbohydrates	Starch	Fibres	Ash
Pea	14–40	1–4	55–72	35–50	20	3–4
Lupin	32–55	5–15	28–40	–	14–39	3–5

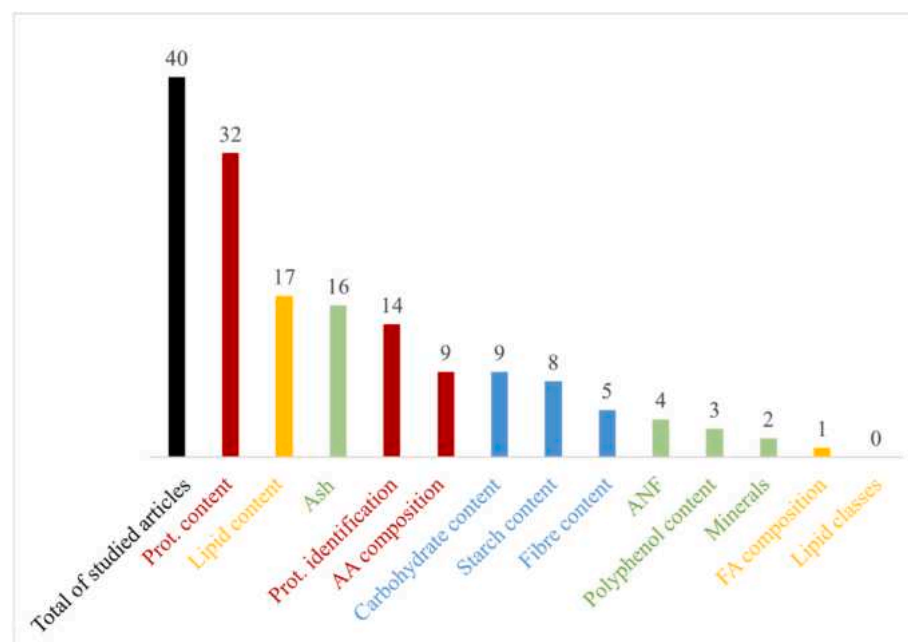


Fig. 1. Literature cross-study analysis of 40 articles investigating the functional (mainly emulsifying) properties and composition of plant-based protein ingredients, wet- or dry fractionated. The bars indicate the number of the articles investigating the respective components. Among the articles, 26 used wet fractionated protein powders, 10 used dry fractionated protein powders, and 4 used ingredients obtained with a mild wet process. The plant matrices studied were pea (23), lupin (10), faba bean (2), lentil (2), quinoa (1), rapeseed (2), bean (2) and soy (4). FA: fatty acid; ANF: anti-nutritional factors. Red: protein components; Yellow: lipid components; Blue: polysaccharide components; Green: other components. The full list of articles considered for this analysis is given in [Supplementary information S1](#).

2. Materials and methods

2.1. Samples and reagents

Commercial samples differing by their fractionation process and protein content (isolates and concentrates) were kindly provided by pea and lupin ingredients suppliers ([Table 2](#)). The ingredients provided by both lupin protein suppliers come from a common variety, blue lupin (*Lupinus angustifolius*). According to the suppliers, the pea protein concentrate was not heat treated, whereas the lupin protein concentrate was. The aqueous suspensions of the protein ingredients were prepared in a 10-mM phosphate buffer containing 90 mM NaCl, adjusted at pH = 7.0. Sodium phosphate dibasic heptahydrate (CAS number: 7782-85-6), sodium phosphate monobasic (13472-35-0), heptadecanoic acid (C17:0) (506-12-7), boron trifluoride-methanol (375-57-9), Nile Red (7385-67-3) and lugol solution for microscopy (62650-1L-F) were from Sigma-Aldrich (St Louis, USA). Alexa 488 was from Invitrogen, Thermo Fisher Scientific (Waltham, USA). Standards of α , β , γ and δ -tocopherol were from Calbiochem Item from Sigma, and that of γ -tocotrienol was from Cayman Chemical (Ann Arbor, USA). Sodium chloride (7647-14-5) was from VWR International (Radnor, USA), and β -mercaptoethanol and dimethylformamide were from Merck (Darmstadt, Germany). Chloroform, methanol, acetone and n-hexane were from Biosolve Chemicals (Dieuze, France). Sodium sulfate anhydrous (7757-82-6), toluene, cyclohexane and diethyl ether were from Carlo Erba Reagents (Val de Reuil, France). All the chemicals were of analytical grade, and ultrapure water was used for all the experiments.

Table 2
Sample description and protein content as provided by the suppliers.

Supplier	Commercial name	Abbreviation
Roquette Frères (Lestrem, France)	Pea protein isolate – Nutralys S85F	PPI
Elementa (Saint-Nolff, France)	Pea protein concentrate	PPC
Prolupin GmbH (Grimmen, Germany)	Lupin protein isolate	LPI
Inveja (Haute-Goulaine, France)	Lupin protein concentrate - Fralucon	LPC

2.2. Compositional analyses

2.2.1. Protein quantification

The dry matter content of the samples was measured by overnight drying in an oven at 105 °C (Mettler U-15, Schwabach, Germany) (method reference ISO 24557:2009). The nitrogen content of the powders was determined with the Dumas combustion analysis method (Elementar, Langensfeld, Germany) (method reference ISO/TS 16634-2:2009). Different nitrogen-to-protein conversion factors were applied and discussed. First, a specific factor resulting from the actual amino acid analysis of each of our samples (conducted by an external lab by UHPLC-MS) was used. Accordingly, N conversion factors of 5.66, 5.57, 5.46 and 5.39 were calculated for PPI, PPC, LPI and LPC (respectively) (see details in [Supplementary info 2](#)). Then, for comparison, factors of 5.7 ([Alamanou & Doxastakis, 1997](#)) and 6.25 were also used, as they are commonly used in literature or for commercial applications. The results are expressed on a dry basis, as mean \pm standard deviation (S.D.) of three independent measurements.

2.2.2. Protein identification

The identification of the proteins was achieved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in reducing conditions. Aqueous suspensions (total, supernatants, treated and non-treated by HPH) were normalized at 2 mg proteins/mL and mixed with 2x Laemmli sample buffer (50% v/v) (Bio-Rad, USA) with β -mercaptoethanol (5% v/v) and boiled in water for 5 min ([Lawrence & Besir, 2009](#)). Then, 10 μ L sample were injected in pre-cast gradient polyacrylamide gels (8–16% Mini-PROTEAN TGX, 10 \times 30 μ L well combs, Bio-Rad, USA), and two wells were used for a molecular weight standard from Euromedex (14.4–116.0 kDa, ref. #06U-0511). The electrophoresis was run for 45 min at 50 mA (Mini Protean Tetra-System, Bio-Rad, USA). Then the gels were rinsed four times with ultrapure water, coloured with Coomassie brilliant blue ([Lawrence & Besir, 2009](#)) for 2 h, rinsed, and scanned (Image Scanner III, GE Healthcare, USA). Molecular weight determination was performed thanks to Multigauge software (version 3.0, Fujifilm).

2.2.3. Lipid extraction

Lipids were extracted from protein powders by following an adapted methodology from Folch et al. and Bligh and Dyer ([Bligh & Dyer, 1959](#);

Folch, Lees, & Sloane Stanley, 1956). After the hydration of 1 g powder in 6 mL phosphate buffer overnight at 4 °C under magnetic stirring, 50 mL of chloroform/methanol extraction solvent (2:1 v/v) were added. The mixture was stirred for 30 min and filtrated under vacuum to retrieve the liquid phase. The powder was then recovered and the procedure was repeated twice with 40 mL of extraction solvent. The filtrate was then transferred into a separating funnel with 35 mL of a solution of NaCl (0.73% w/v), mixed, and let to decant overnight at 4 °C. Then the bottom organic phase (chloroform phase) was recovered after filtration on anhydrous sodium sulfate and glass wool, and the solvent was evaporated under vacuum in a water bath at 40 °C (R-100, Rotavapor, Büchi, France), and then under nitrogen (N-Evap 111, Organomation, USA). Lipid extractions were carried out in five independent experiments, and the results were expressed on dry basis as mean \pm S.D.

2.2.4. Lipid class quantification and identification

Before further analysis, total lipid extracts were fractionated on silica cartridges (SPE, Sep-Pak 500 mg, Waters) (Juaneda & Rocquelin, 1985). The extracts were washed with chloroform to collect neutral lipids, then with acetone to collect glycolipids and lipophilic pigments, and finally with methanol to collect phospholipids. Each collected fraction was then dried under vacuum and/or under nitrogen and re-solubilized in chloroform to reach a concentration of 0.5 or 1 mg/mL. The fractions solubilized in chloroform were separately analyzed by U-HPLC (Ultimate 3000 RSLC, Dionex, France) equipped with an evaporative light scattering detector (ELSD, Sedex 85) to quantify the different lipid classes. The amount of each compound was calculated thanks to a calibration curve made with commercial standards (see [Supplementary info 3](#) for commercial standards references). As lipid fractionation required 70 mg of lipids, the lipid class analysis was carried out on lipid extracts pooled from independent triplicates.

2.2.5. Fatty acid composition

The lipid extracts obtained before and after lipid fractionation were methylated according to the method described by Morrison and Smith (1964). After adding the lipid extracts with an internal standard of C17:0, and drying under nitrogen, the samples were solubilized in 1 mL of toluene mixed with 1 mL of boron fluoride-methanol (14% in methanol) to catalyze the reaction and heated at 100 °C for 45 min in a dry bath (Fisher Bioblock Scientific, Illkirch, France). After cooling, the addition of 1 mL of cyclohexane and 0.5 mL of water induced phase separation, and the upper phase containing the methylated fatty acids was collected and injected in gas chromatography (GC Clarus 690, Perkin Elmer). For certain samples, a centrifugation at 1800g (tanta-mount to 3000 rpm) for 5 min was applied to enhance phase separation. The analyses were carried out in five independent measurements.

2.2.6. Antioxidant investigation

The lipid extracts were adjusted to a concentration of 10 mg/mL in hexane, and were injected in U-HPLC (Ultimate 3000 RSLC, Dionex, France) equipped with a RS-fluorescence detector (Dionex, France) to investigate the tocopherol content and composition, using an external calibration curve of α , β , γ and δ -tocopherol. Experiments were carried out on three to five independent lipid extracts, and the results are expressed as mean \pm S.D. In addition, UV-visible spectra (UV-1800, Shimadzu) of the lipid extracts solubilized in diethyl ether (standardized at 2.5 mg of lipids/mL) were measured between 200 and 700 nm (steps of 2 nm, speed medium of 360 nm/min) to investigate the presence of lipophilic pigments, in independent duplicates, and one representative curve is given in [Supplementary info 4](#).

2.2.7. Cell wall polysaccharides and starch composition

Total cell wall polysaccharides were measured as previously described with minor modifications (Lahaye, Falourd, Laillet, & Le Gall, 2020). All protein powders were subjected to hot ethanol extraction in order to remove small soluble oligosaccharides potentially present in the

fractions and inactivate enzymes. This extraction ensures the measurement of cell wall polysaccharides only. Then, 5 mg of protein powders or the corresponding alcohol-insoluble fractions were acid-hydrolyzed with sulfuric acid, and the resulting neutral sugars were derivated into alditol-acetates and analyzed on a TG-225 GC column (30 \times 0.32 mm ID) using a TRACE™ Ultra Gas Chromatograph (Thermo Scientific™; temperature 205 °C, carrier gas H₂). Uronic acids were quantified according to the *meta*-hydroxydiphenyl method (MHDP) method. All measurements were performed in triplicates using a standard sugars' solution and inositol as an internal standard for calibration, and are expressed as mean \pm S.D.

Starch measurement was performed on pea protein powders after amylolysis using a commercial thermostable amylase from *Bacillus* sp. and a commercial amyloglucosidase from *Aspergillus niger* (Megazyme) and HPAEC analyses (ICS-6000 Thermo Scientific™) as previously described (Le Gall, Even, & Lahaye, 2016).

2.2.8. Ash content

Ashes were quantified according to the methodology from AOAC Official Method 942.05. About 5 g powder were weighed in porcelain crucible, and let to carbonize successively 1 h at 150 °C and 1 h at 350 °C, and finally overnight at 550 °C using a muffle furnace (MF4 Hermann Moritz Régulateur 2068; France). The ash content was determined after weighing the remaining minerals after carbonization and cooling down.

2.3. High pressure homogenization treatment

The aqueous suspensions of the protein powders (130 mL) were hydrated overnight at 1 g of proteins/100g phosphate buffer. High-pressure homogenization of these suspensions was run (Panda plus 1000, GEA Niro Soavi, Italy) for 3 min recirculation (average of 7 cycles) at 300 bars. A preliminary screening of homogenization pressures was performed (3 min at 0; 50; 100; 200; 300; 400 and 500 bars, successively) for each aqueous suspension (results not shown). A stable particle size was reached at 300 bars, which was therefore the pressure selected for the homogenization of suspensions in the rest of the study. Independent triplicates were run.

2.4. Solubility measurements

As explained above, there is no consensus in literature regarding the definition of protein solubility; in practice, it is generally regarded as the amount of proteins that does not sediment during centrifugation in given conditions. Here, the determination of the solubility was based on the measurement of nitrogen content (expressed in mg/mL) using Dumas method (Rapid MAX N exceed, Elementar) analysis. The hydrated aqueous suspensions, subjected to a homogenization treatment or not, were centrifuged (20 000 g; 30 min; 4 °C) (Sigma 4K15, Thermofisher) in 2-mL tubes. The supernatant was collected after cautiously taking away the upper creamed phase with a glass pipette. Both the total aqueous suspension and the supernatant were used for the total nitrogen measurement. Experiments were carried out in independent triplicates, and results are given as mean \pm S.D. Solubility was given as follows (Equation (1)):

$$\text{Solubility (wt.\%)} = \frac{\text{Nitrogen content}_{\text{supernatant}}}{\text{Nitrogen content}_{\text{total suspension}}} * 100 \quad (\text{Eq. 1})$$

The absorbance of the supernatants of the suspensions (prepared at 1 g proteins/100 g buffer) was also measured by spectrophotometry (UV-1800, Shimadzu) between 200 and 600 nm. The samples were diluted 20 times (v/v) in phosphate buffer (pH = 7.0) prior to measurements. One representative spectrum over independent triplicates is provided for each sample. The results are given and discussed in [Supplementary info 5](#).

2.5. Microstructure and physical organisation

2.5.1. Optical microscopy

Optical microscopy images were recorded between slides and cover-glass using a Zeiss Axioscope2 (Göttingen, Germany) at 10X and 40X magnification, in bright field or DIC mode. The PPC suspension was also observed after staining with lugol to highlight starch by adding 7.5% (v/v) of the solution on the sample placed on the microscopy slide.

2.5.2. Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) was performed with an inverted Nikon A1R microscope (Nikon, France) on homogenized and non-homogenized aqueous suspensions of the protein powders. Proteins and lipids were stained by adding respectively 5 μ L Alexa 488 (0.4 μ g/ μ L in methanol) and 2.5 μ L Nile Red (0.5 μ g/ μ L in dimethylformamide), to 1 mL of aqueous suspension. Alexa 488 and Nile Red have a maximum excitation wavelength of 490 nm and 552 nm respectively, and a maximum emission wavelength of 525 nm and 636 nm, respectively. The wavelengths of the scanning lasers were 488 nm and 560 nm. The microscope magnification lengths used were 10X (Plan APO 10X), 20X (Plan APO 20X) and 40X (Plan APO 40X water immersion lens), and image zoom (2X) was used when necessary. Images were recorded between slides and cover-glass, processed with the software NIS-Element (Nikon) and further treated with the FIJI software (ImageJ).

2.5.3. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) was performed on the powders pasted on an adhesive slide, and observed under vacuum with an environmental scanning electron microscope (ESEM, FEI Quanta 200 FEG, The Netherlands).

2.5.4. Particle size analyses on dry powders and aqueous suspensions

The particle size distribution was studied both in dry state (the powdered samples as received) and in the full aqueous suspensions with a laser particle size granulometer, equipped with the Dry Powder System Unit (LS 13 320 XR, Beckman Coulter, California, USA), and a particle size analyser (LA-960, Horiba Scientific, Jobin Yvon, France), respectively. Analyses are respectively based on Fraunhofer diffraction and on the Mie theory. A few drops of the aqueous suspensions, either treated by high-pressure homogenization or not, were sampled under stirring and put in the dispersion unit filled with ultrapure water until about 10% of light obscuration was reached. The refraction indices used were 1.33 (for water) and 1.45 (for the dispersed phase). The average particle and droplet sizes are given as the volume moment mean diameter ($d_{4,3}$) (μ m). Analysis were carried out in independent triplicates, and one

representative distribution curve is provided in the results.

2.5.5. Dynamic light scattering analysis

The size distribution of the 'soluble aggregates' remaining in the supernatants of the aqueous suspensions, treated by HPH or not, was assessed by dynamic light scattering (DLS) using a Zetasizer Nano Series (Malvern Instruments, UK). The samples were diluted 11 times in phosphate buffer. The dispersant was water (refractive index 1.33). The refractive index used for the dispersed phase was 1.45. Three measurements of 120 s each were performed at an angle of 173°. The PPC and LPC samples had to be filtrated on 0.45- μ m cellulose Whatman filters (Chromafil Xtra RC45/25, Macherey-Nagel, Düren, Germany) to eliminate large particles and ensure a correct measurement. Independent duplicates were performed.

2.6. Statistical analysis

Data were analyzed with the software XLSTAT (Version 2023; Addinsoft, Paris, France) via variance analysis (ANOVA) and a Tukey post-hoc test. Significances were given by *p*-values <0.0001 and homogeneous groups were identified by different small letters on the graphs. The factors were the plant nature (either pea or lupin) and the type of ingredient (either isolate or concentrate) for the following variables: contents of dry matter, proteins (calculated with the specific N:P), lipids, fatty acids, ashes, polysaccharides and tocopherols. The statistical significance of the treatment of the suspension (either treated by HPH, or not) on the soluble protein content was also assessed.

3. Results and discussion

3.1. Composition of the samples

3.1.1. Protein content and composition

The protein content of the samples is given in Table 3. Protein isolates displayed between 66 and 72 g/100 g (d.m.) of proteins, against 39 g/100 g (d.m.) for the protein concentrates. To obtain those results, the nitrogen content obtained by Dumas method was multiplied with a nitrogen-to protein conversion factor (N:P) specific for each sample: 5.66 for PPI, 5.57 for PPC, 5.46 for LPI and 5.39 for LPC. Indeed, as commercial plant protein ingredients result from the pool of several crops, we specifically calculated the N:P based on both the amino acid composition of the protein ingredients and the amount of non-protein nitrogen in the samples (details of the calculation are given in Supplementary info 2). Those resulting specific factors are relevant with the accepted N:P factor range for those plant sources (Doxastakis, 2000;

Table 3

Proximate composition of the samples. The protein content was calculated with a nitrogen-to-protein conversion factor (N) specific for each sample, as herein indicated. The results for each compound are given as mean \pm SD of three to five independent replicates. The fatty acid (FA) composition (percentage of total FA) results from the analysis of the full lipid extracts. Among pea fractions, starch was only detected in the PPC sample. Results are expressed in g/100 g of total powder for water content, and in g/100 g of dry matter (d.m.) for the other components. Letters from a to d indicate significant differences between the samples for each analyzed parameter (*p*-values <0.0001).

	PPI	PPC	LPI	LPC
Water (g/100 g)	6.18 \pm 0.06 ^c	9.30 \pm 0.21 ^b	4.75 \pm 0.11 ^d	9.90 \pm 0.08 ^a
Proteins (g/100 g d.m.)	65.7 \pm 0.4 ^b	39.4 \pm 0.6 ^c	71.9 \pm 0.8 ^a	38.9 \pm 0.2 ^c
N factor	N = 5.66	N = 5.57	N = 5.46	N = 5.39
Lipids (g/100 g d.m.)	10.3 \pm 0.4 ^a	4.9 \pm 0.3 ^c	3.4 \pm 0.4 ^d	8.9 \pm 0.4 ^b
Saturated FA	17.5 \pm 0.2 ^d	19.1 \pm 0.7 ^c	26.9 \pm 0.2 ^a	22.9 \pm 2.6 ^b
Monounsaturated FA	25.1 \pm 0.4 ^d	28.1 \pm 0.8 ^c	42.0 \pm 0.3 ^a	36.1 \pm 3.4 ^b
FA (ω6)	43.4 \pm 0.5 ^a	37.6 \pm 1.1 ^b	23.9 \pm 0.1 ^d	27.9 \pm 5.1 ^c
FA (ω3)	7.8 \pm 0.1 ^a	5.9 \pm 0.3 ^b	1.3 \pm 0.1 ^d	2.8 \pm 1.4 ^c
Polyunsaturated FA	51.2 \pm 0.5 ^a	43.5 \pm 1.4 ^b	25.2 \pm 0.1 ^d	30.7 \pm 6.5 ^c
(ω6)/(ω3)	5.2 \pm 0.1 ^c	5.8 \pm 0.2 ^c	17.3 \pm 0.8 ^a	10.8 \pm 4.6 ^b
Cell wall polysaccharides (g/100 g d.m.)	2.6 \pm 0.4 ^c	15.3 \pm 0.6 ^a	3.7 \pm 0.2 ^b	14.9 \pm 0.3 ^a
Starch (g/100 g d.m.)	-	5.2 \pm 0.1	-	-
Ashes (g/100 g d.m.)	3.47 \pm 0.02 ^c	5.36 \pm 0.03 ^a	4.91 \pm 0.13 ^b	3.39 \pm 0.02 ^d
Total amount of characterized compounds (g/100 g d.m.)	82.07	70.16	83.91	66.09

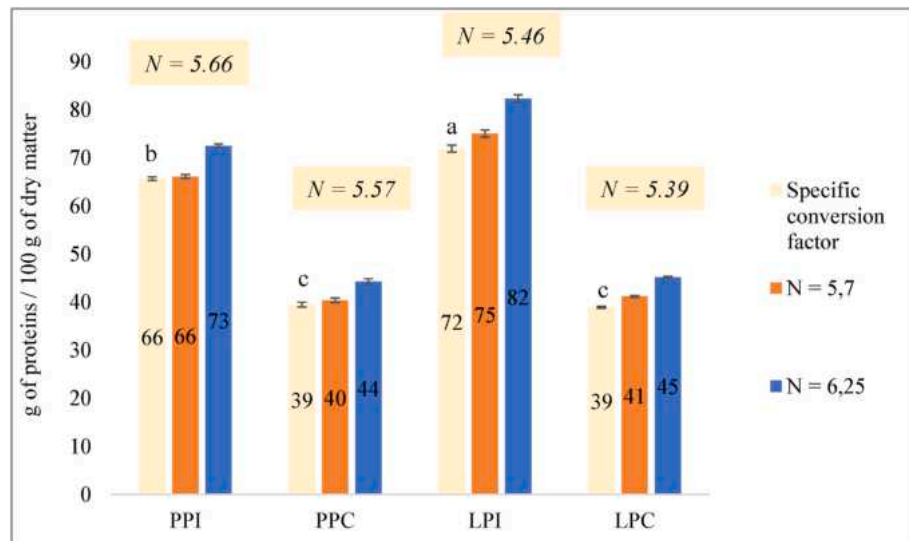


Fig. 2. Protein content calculated using various nitrogen-to-protein conversion factors (N). Results are expressed in g of proteins/100 g of dry matter, as mean \pm standard deviation of three independent measurements. As indicated on the graph, the specific conversion factors are 5.66, 5.57, 5.46 and 5.39 for PPI, PPC, LPI and LPC, respectively ([Supplementary info 2](#)). Different letters indicate significantly different values (p -values < 0.0001).

FAO/WHO, 2019), with previously reported values of N:P factor for pea (5.36) and for lupin (5.44) (Mariotti et al., 2008), and with previous calculations made on the same commercial PPI (Hinderink, 2021). In the case of plant protein samples, a N:P of 5.6 (Mariotti et al., 2008) to 5.7 (notably for soy protein isolates (Mossé, 1990)) was proposed and recognized as relevant by the scientific community. The results of protein content calculated with a N:P of 5.7 (Fig. 2) happened to be very close to those obtained with the specific N:P for each sample. Therefore, one can conclude that 5.7 is a fair reference factor for pea and lupin protein content assessment. The third N:P presented in Fig. 2 corresponds to 6.25. As a usually prevailed N:P, the overestimation of the protein content with this factor is here highlighted, and some authors previously recognized it as irrelevant for plant protein samples (Mariotti et al., 2008). Here, the difference with the protein content values calculated with a N:P of 6.25 is of approximately 9% for all the samples, whereas in the literature, a range of error of 15% was reported for navy bean proteins (Mariotti et al., 2008), and 20% for grains (Mossé, 1990). Non-protein nitrogen can be related to different compounds such as nitrates, nitrites, phospholipids, nitrogenous glycosides (Mariotti et al., 2008) or alkaloids (for instance in lupin (Doxastakis, 2000)). Those results are important when assessing the nutritional outcomes of such ingredients, and their content in non-proteinaceous compounds.

The protein composition of the samples was further investigated by SDS-PAGE (Fig. 3). In the total aqueous suspensions (non-treated by HPH), many proteins of molecular weight (MW) between 50 kDa and 20 kDa were encountered for PPI and PPC, whereas many bands in the range 34–66 kDa were found for LPI and LPC. No distinct difference was highlighted in terms of protein profile between PPI and PPC. Conversely, the protein profile for LPC shows a band at 34 kDa that is not encountered in LPI. The proteins of pea and lupin seeds are mainly storage proteins, named globulins and known as legumin (11S), vicilin (7S) and convicilin (7S) for pea, and as α -conglutin (11S), β -conglutin (7S), γ -conglutin (7S) and δ -conglutin (2S) for lupin. For pea, the band around 75 kDa can be identified as convicilin (Lu, He, Zhang, & Bing, 2020), whereas vicilins have MW around 50, 33, 20 and 17 kDa (Barac et al., 2010). Vicilins are known to be fairly surface-active thanks to their small size and flexibility (Lu et al., 2020). Pea seeds also contain water-soluble albumins (2S), which have low MW around 26, 14 and 6 kDa (Ma, Boye, & Hu, 2017). For lupin, the protein bands of high MW most likely correspond to α -conglutin and β -conglutin, but their subunits have overlapping molecular weights on the SDS-PAGE gels (Burgos-Díaz

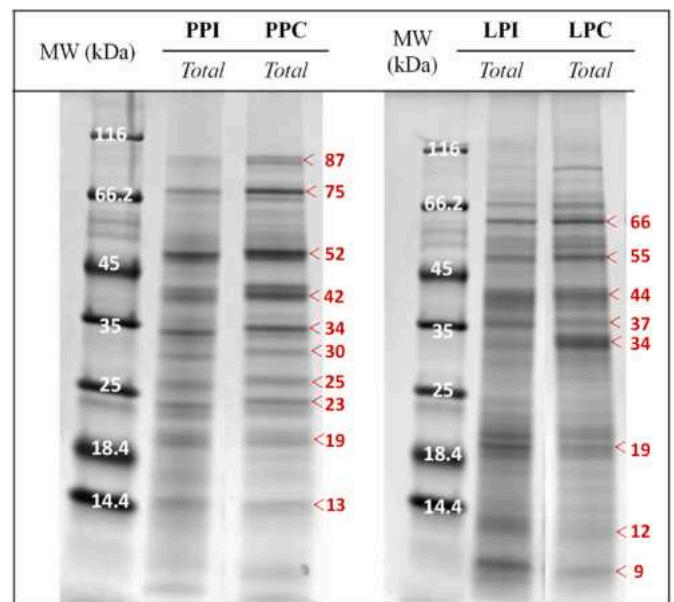


Fig. 3. Protein composition and identification by SDS-PAGE analysis (reducing conditions) in PPI, PPC, LPI and LPC (from left to right) total aqueous suspensions (non-treated by HPH). The first and fourth lanes contain the molecular weight standards (MW are expressed in kDa).

et al., 2016; Schlegel et al., 2019). The profile of the gels are, however, similar to those previously discussed in the literature (Berghout et al., 2014; Fontanari et al., 2012). Protein identification may help in understanding protein functionalities, and the distribution of ‘soluble’ proteins in the supernatants is discussed further in part 3.2.2, along with the effect of high-pressure homogenization on the distribution of the proteins. Nevertheless, it is important to underline that those proteins may be encountered in a multiplicity of colloidal states in the samples, which is also paramount to consider when the functionalities are studied.

3.1.2. Lipid content and composition

The total lipid content in the different samples is given in Fig. 4A and

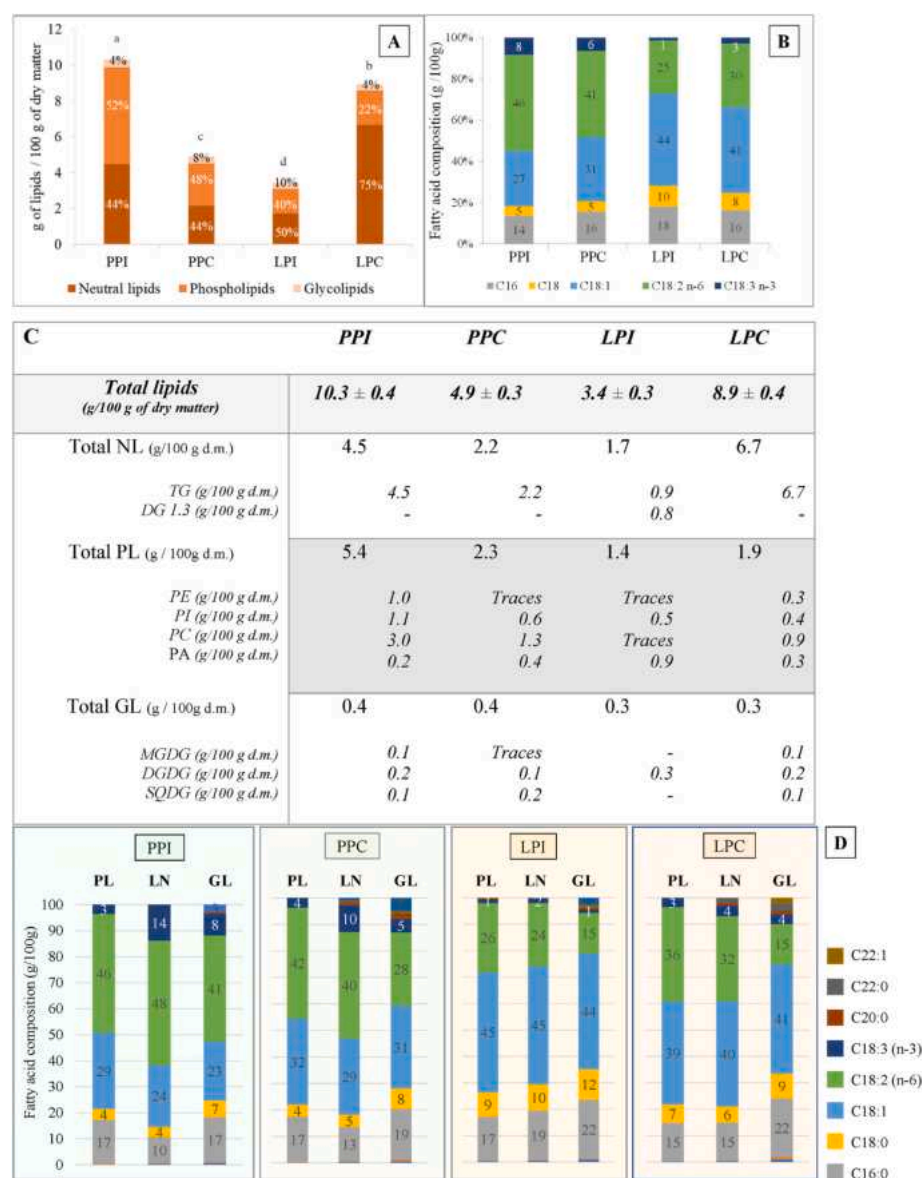


Fig. 4. Lipid content and composition in the protein powders. A: Lipid classes' distribution measured after lipid extraction and fractionation, expressed in g of lipids per 100 g protein powder, (dry basis). The inner percentages represent the fractions of the different lipid classes, relative to the total lipid amount (g/100 g lipid extract). Different letters indicate significant differences between the samples (p -values < 0.0001). B: Fatty acid composition of the total lipid extracts (g/100g). The results were obtained from five independent measurements. C: Lipid classes expressed as absolute percentages of the different classes (g/100 g protein powder, dry basis). TG: triglycerides; DG 1.3: diglycerides (1,3); PE: phosphatidylethanolamine, PI: phosphatidylinositol, PC: phosphatidylcholine, PA: phosphatidic acid, SQDG: sulfoquinovosyldiacylglycerol, DGDG: digalactosyldiacylglycerol, MGDG: monogalactosyldiacylglycerol. D: Fatty acid composition of the different lipid fractions, expressed as percentage (g/100g) of the total fatty acids in the respective lipid class (either neutral lipids (NL), phospholipids (PL), or glycolipids (GL)).

C. Substantial proportions were found in the different samples, ranging from 3.4 to 10.3 g/100 g (d.m.) powder. Regarding pea protein ingredients, the amount of lipids is twice higher when wet fractionated (10.3 ± 0.4 g/100 g (d.m.)) compared to dry fractionated (4.9 ± 0.3 g/100 g (d.m.)). Interestingly, such substantial lipid contents have not been systematically observed: some previous studies report values of 0.9 ± 0.3 g/100 g (d.m.) in the same commercial PPI (Pelgrom, Boom, & Schutyser, 2015), which may be due to incomplete lipid extraction, especially for polar lipids. Regarding lupin-based ingredients, a higher lipid content was determined in LPC, compared to LPI. Yet, as lupin seeds initially contain a high lipid content (around 5–15 g/100 g (d.m.), Table 1), it is presumable that LPI was defatted over processing. Still, a measurable amount of remaining lipids was found in this sample (3.4 ± 0.3 g/100 g (d.m.)). Regarding the fatty acid composition of the full lipid extracts, lupin protein enriched-ingredients contain more saturated and monounsaturated fatty acids than pea ingredients (Fig. 4B). The $\omega 6/\omega 3$ ratio is noticeably lower in pea samples compared to lupin ones (Table 3). The fatty acid composition is discussed into more details in the next paragraph that pertains to the fractionated extracts.

The lipid extracts were fractionated on silica cartridges to analyze in more depth the specific lipid components (phospholipids, neutral

lipids and glycolipids) (Fig. 4A). Phospholipids accounted for about 50 g/100 g of the lipids extracted from PPI and PPC, whereas their relative importance was somewhat lower for lupin (40 g/100 g for LPI and 22 g/100 g for LPC). Four different phospholipids were identified (phosphatidylcholine, phosphatidylinositol, phosphatidic acid and phosphatidylethanolamine). It is worth pointing out that both phospholipids and glycolipids have an amphiphilic structure and potential surface-active properties (Fig. 4B). The lipid extract from LPC had, comparatively, a higher content in neutral lipids, mainly triglycerides (75 g/100 g). When looking at the type of neutral lipids found in the different extracts, only triglycerides were detected for PPI, PPC and LPC, whereas triglycerides and diglycerides were found for LPI. Glycolipids were also present, yet as a minor fraction of the lipid extracts. More specifically, three classes were detected: MGDG, DGDG and SQDG. This provides new data on the nature and typical proportion of glycolipids present in such samples. Finally, the fatty acid composition of the fractionated lipid extracts was analyzed. A homogeneous repartition of fatty acids was found between the phospholipid, glycolipid and neutral lipid fractions (Fig. 4C).

The proportions of the different lipid classes of PPI and PPC are consistent with those found in pea (*Pisum sativum*) seeds (between 52 and 61 g/100 g phospholipids and 31–40 g/100 g triacylglycerols,

assessed with the Folch extraction methodology) (Yoshida, Tomiyama, Saiki, & Mizushima, 2007). The proportions found in LPC are relevant to those found in the whole lupin seeds (*Lupinus angustifolius*), (about 7 g/100 g of triglycerides, 15 g/100 g of phospholipids and 4 g/100 g of glycolipids, also extracted according to Folch) (Hansen & Czochanska, 1974). Conversely, the composition of the lipids extracted from LPI differs substantially from that in lupin seeds, which can be explained by the expected defatting step included in the protein fractionation process.

These results highlight several points. The first major implication is about the proportions of lipids encountered in these commercial pulse protein ingredients. Such substantial lipid contents have, to the best of our knowledge, not been highlighted hitherto in the literature in pulse protein powders. Hence, it seems that protein extraction (or isolation) processes also have a prominent effect regarding the transfer of lipids (resulting sometimes in a substantial accumulation of the latter in the final protein ingredient, such as in PPI). Indeed, pea seeds contain initially very few lipids (1–4 g/100 g, Table 1), yet the lipid content increased from pea concentrates (about 5 g/100 g (d.m.) of protein powder) to pea isolates for which it reached about 10 g/100 g (d.m.) of protein powder. Regarding lupin samples, lupin seeds are initially quite high in lipids (5–15 g/100 g, Table 1), yet a very low content was found for LPI, contrasting with the almost 9 g/100 g for LPC. Those results highlight the critical role of processing on the final ingredients' composition in non-proteinaceous compounds, in that case, lipids. Yet, some authors did assess the lipid contents in plant protein ingredients (Funke et al., 2022; Karaca et al., 2011; Lqari, Vioque, Pedroche, & Millán, 2002; Ntone et al., 2021; Tsoukala, Papalamprou, Makri, Doxastakis, & Braudo, 2006), sometimes during different steps of protein fractionation (Berghout et al., 2014; Cui et al., 2020; Geerts, Strijbos, van der Padt, & van der Goot, 2017), but systematically with the methodology of Soxhlet. This methodology consists in an extraction by hexane at high temperatures, and may induce some chemical alterations of labile lipid molecules, and most likely does not allow for reaching a full recovery of all lipid classes (López-Bascón-Bascon & Luque de Castro, 2019). Therefore, it seems critical to be aware of the range and nature of the lipid species that are accessible with a given lipid extraction methodology. In our opinion, the transfer of lipids (accumulation or loss) during the fractionation processes aiming at plant protein ingredients deserves more consideration, and should be approached with a proper cold solvent-based extraction such as the Folch extraction methodology. The second major implication relates to the functional properties of the samples: which role may those endogenous lipids play in the emulsifying properties of the plant-based ingredients? Some of these lipids are low-molecular-weight emulsifiers (phospholipids, the main constituent of lecithins, but also diglycerides and glycolipids for instance (Berton-Carabin, Sagis, & Schroën, 2018)). This also raises the question of how proteins may partition when it comes to covering the oil-water interface, as the simultaneous presence of surface-active lipids and proteins may lead to a competitive process of adsorption at the interface (Fang & Dalgleish, 1996). The third major implication concerns the sensory properties of the protein ingredients. Lipids play a pivotal role in the sensory characteristics of food (i.e., texture, aroma tanks), and they are sensitive to oxidative reactions, that may lead to modifications of ingredient's flavours (Gläser et al., 2020; Liu, Cadwallader, & Drake, 2023; Sharan et al., 2022). A better knowledge of their presence and control over processing would be helpful for the formulation of food products with plant protein-enriched ingredients.

The content of tocopherols, which are endogenous lipophilic antioxidants, is also an important parameter to consider for lipid-containing ingredients. The lipid extracts of PPI and PPC were surprisingly very rich in tocopherols (Table 4). Lupin-based extracts contained lower contents, yet the concentrate was richer than the isolate, which could be linked to the defatting of LPI. In pea seeds, about 90 mg total tocopherols/kg of seeds were found (Yoshida et al., 2007), with mainly γ -tocopherol and some δ -tocopherol. No detection of α -tocopherol in pea samples was noticed in our case, whereas some authors did, but also in minor proportions (Yoshida et al., 2007). In lupin seeds, 69–90 mg γ -tocopherol/kg of dry matter were found, along with traces of α -tocopherol (1.4–5.5 mg/kg dry matter) (Czubinski et al., 2021). As for total lipids, one may wonder how the loss or accumulation of such lipophilic molecules in the final ingredients may be affected through protein fractionation processes. In a similar fashion, we also analyzed the presence and relative importance of lipophilic pigments in the lipid extracts by spectrophotometry (Supplementary information 4). Different peaks could be distinguished between 400 and 500 nm, corresponding to carotenoid pigments, for all the samples, and at 676 nm for LPI (may be corresponding to chlorophyll *a*) (Lichtenthaler & Buschmann, 2001). The less purified samples (PPC and LPC) contained more pigments than the others, whereas LPI displayed a different spectrum (probably linked to its potential defatting).

3.1.3. Polysaccharide content and composition

Cell wall polysaccharides (i.e., cellulose, hemicelluloses and pectins) were quantified and identified after acidic hydrolysis by gas chromatography. Traces of cell wall polysaccharides, around 2.6 ± 0.4 and 3.7 ± 0.2 g/100 g (d.m.) were found in PPI and LPI, respectively (Table 2). Conversely, pea and lupin protein concentrates displayed higher proportions around 15.3 ± 0.6 and 14.9 ± 0.3 g/100 g (d.m.), respectively. Starch was measured in pea fractions, and 5.2 ± 0.1 g/100 g (d.m.) was found in PPC (Table 2). This result was confirmed by microscopy, after both coloration with iodine water, and using a polarized light filter. Among the four samples, only PPC appeared to contain starch. These results show that the type of process (wet vs dry fractionation) has a strong effect on the extent to which cell wall polysaccharides are discarded. In fact, non-soluble polysaccharides tend to precipitate through the centrifugation step of wet fractionation, whereas air classification keeps residual amounts in the protein fraction. Carbohydrate contents in pea and lupin protein isolates were very similar before and after ethanol precipitation (Supplementary info 6). This result indicates that very few soluble carbohydrates were recovered in these ingredients. Conversely, for both concentrates, a difference close to 10 g/100 g (d.m.) was detected between samples subjected or not to preliminary ethanol washing, meaning that more soluble carbohydrates are present in the concentrates than in the isolates. Further specific analyses of free monosaccharides would be required to deepen those aspects. To sum up, those results provide with an absolute quantification of polysaccharides, which contrasts with the usual methodology that consists in estimating carbohydrates by difference with the combined contents of proteins, lipids and ashes (Fig. 1). Yet, the latter methodology may lead to the accumulation of uncertainties, as discussed in the previous sections.

The cell wall polysaccharides' composition was performed by gas chromatography and colorimetric measurement (Fig. 5). The proportion of each monosaccharide residue is characteristic of pulse seeds. The main monosaccharide residues come from pectins for both plant sources

Table 4

Tocopherol content in the lipid extracts from the different samples. Results are presented as mean \pm SD of three to five independent measurements. Significant differences are given by the letters (*p*-values <0.0001).

	PPI	PPC	LPI	LPC
Total tocopherols ($\mu\text{g/g}$ lipids)	807.8 \pm 151.3 ^a	502.2 \pm 43.1 ^b	27.0 \pm 2.5 ^c	340.9 \pm 153.6 ^b
γ - tocopherol	766.9 \pm 145.8	470.4 \pm 43.7	27.0 \pm 2.5	324.9 \pm 156.7
δ - tocopherol	36.5 \pm 7.3	29.0 \pm 3.7	-	11.7 \pm 1.3

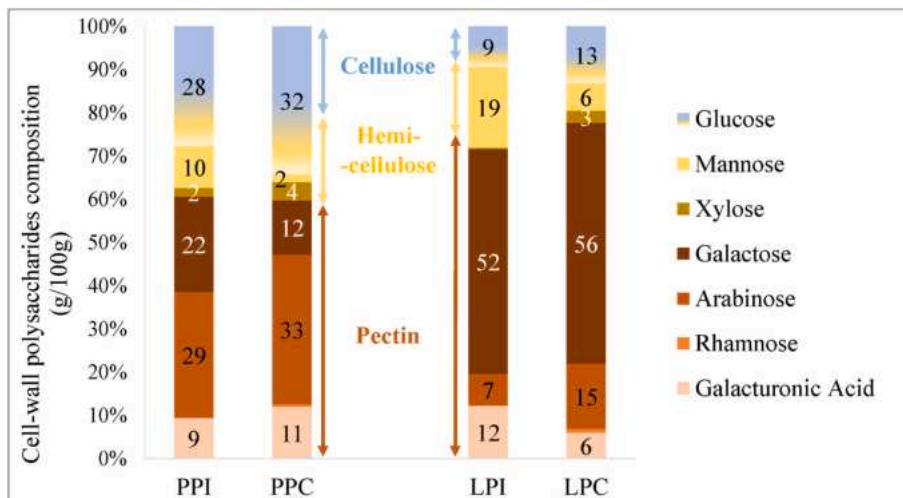


Fig. 5. Cell wall polysaccharides composition measured in the samples (left: pea samples; right: lupin samples). Results are expressed as g/100 g of the total amount of cell wall polysaccharides extracted (results in g/100 g of total cell wall polysaccharides are presented in Table 2). Residues of galacturonic acid, rhamnose, arabinose and galactose represented in shades of orange are totally or mainly associated with pectic polysaccharides. Xylose and mannose residues in yellow are mainly associated with hemicelluloses. Glucose residue amounts are given in shading of yellow to blue, as glucose residues may stem from both cellulose and hemicelluloses.

(about 60 g/100 g for pea samples; against around 75 g/100 g for lupin samples). The more important proportion of galactose in lupin protein powders points to the presence of galactan domains of type-I rhamnogalacturonans (Brillouet & Riochet, 1983) whereas arabinose residues are predominant in pea protein powders, in accordance with the presence of arabinan domains of type-I rhamnogalacturonans (Brillouet & Carré, 1983). These results shed light on the potential interactions that those pectin domains, still present in most protein concentrates, may have on the functionality of the ingredients. Indeed, the surface charge of homogalacturonan pectins (which varies with the degree of methylation on their galacturonic acid backbone) makes them suitable to interact with plant proteins, thus potentially able to affect their properties (notably their solubility) (Einhorn-Stoll, Archut, Eichhorn, & Kastner, 2021).

3.2. Properties of the ingredients in aqueous suspensions – effect of a high-pressure homogenization treatment

3.2.1. Morphology of the samples

The particle size of the powders was first characterized by dry granulometry (Fig. 6). Large structures were encountered for PPI and LPI, with populations centered around 200 and 90 μm respectively, whereas PPC and LPC particles were substantially smaller (around 10 μm), yet with a broad distribution between 50 and 200 μm . Those particle size results are consistent with scanning electron microscopy (SEM) images performed on the dry powders (Fig. 7A). Powders' particles were larger for PPI and LPI compared to PPC and LPC, for which different structures such as starch granules (coloured with lugol) or fibres could

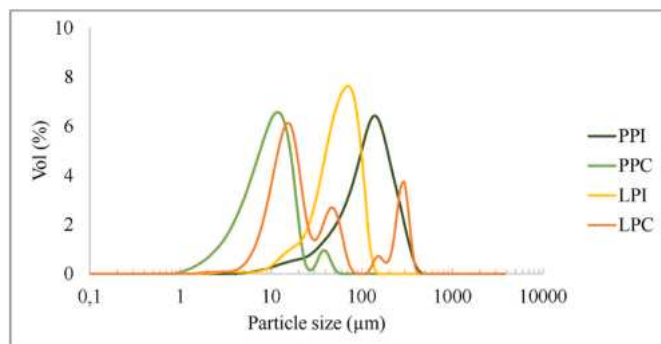


Fig. 6. Particle size analysis of the powders (dry environment). Triplicate analyses were conducted, but for readability one representative curve for each sample is provided here.

be identified. The isolates displayed typical structures encountered in spray-dried protein powders (Cui, Kimmel, Zhou, Chen, & Rao, 2021), with spherical and concave shapes, whereas the concentrate particles looked smaller and more heterogeneous (Möller, van der Padt, & van der Goot, 2021).

The protein powders were then dispersed in aqueous environment (pH 7.0 phosphate buffer). Macroscopic observations showed that sedimentation occurred rapidly when stirring was stopped, even after 24 h stirring (Fig. 8A, top left picture). The observations of the suspensions by optical microscopy revealed contrasted behaviours once in an aqueous environment (Fig. 7B). For PPI and LPI, some granular structures in the aqueous suspensions looked very similar to the particles observed by SEM in the dry powders, suggesting incomplete hydration of the powders. In PPC and LPC, structures corresponding presumably to starch granules and fibres, respectively, could be observed, which is well in line with the composition of these ingredients. Those structures are also consistent with previous observations in pea and lupin isolates and concentrates in the literature (Burger et al., 2021; Möller et al., 2021; Primožic, Duchek, Nickerson, & Ghosh, 2018). The aqueous dispersions were also characterized by confocal fluorescence microscopy (CLSM), to allow for distinguishing between the protein structures and the lipid structures by specific labelling (Fig. 7C). For PPI and LPI, prominent colocalization of both fluorescence signals inside the grain powders suggested that lipids were trapped inside those structures. In contrast, for PPC and LPC, distinct lipid droplets could be noticed, as well as small protein entities (Supplementary info 7 provides the individual images from both laser channels). The preservation of the inherent structural complexity of the mildly processed compounds from dry fractionated ingredients is therefore emphasized. For all samples, a green background corresponding to soluble (or at least finely dispersed) proteins is visible, yet not quantifiable with this technique.

We then explored how HPH could impact the ingredients' colloidal state in dispersions by optical microscopy (Fig. 7D) and CLSM (Fig. 7E). HPH treatment significantly modified the morphologies of the aqueous dispersions, through the disruption of the large structures. For all samples, and notably PPI and LPI, the powder grains were broken and disrupted throughout the treatment, as also noted by particle size analysis (Fig. 11 A1 and B1). Only remaining fragments were observed for PPI and LPI. Starch grains from PPC were still present, with diameters analyzed by image analysis around 12–13 μm . Those starch grains seemed to pack with fibrous structures. This tendency of polysaccharide structures to pack was also observed for LPC. This reduction in particle size by HPH was also reported by Primožic et al. (2018) on lentil protein isolate and helped to improve the emulsifying properties of the ingredient. The observation of the treated dispersions by CLSM showed that

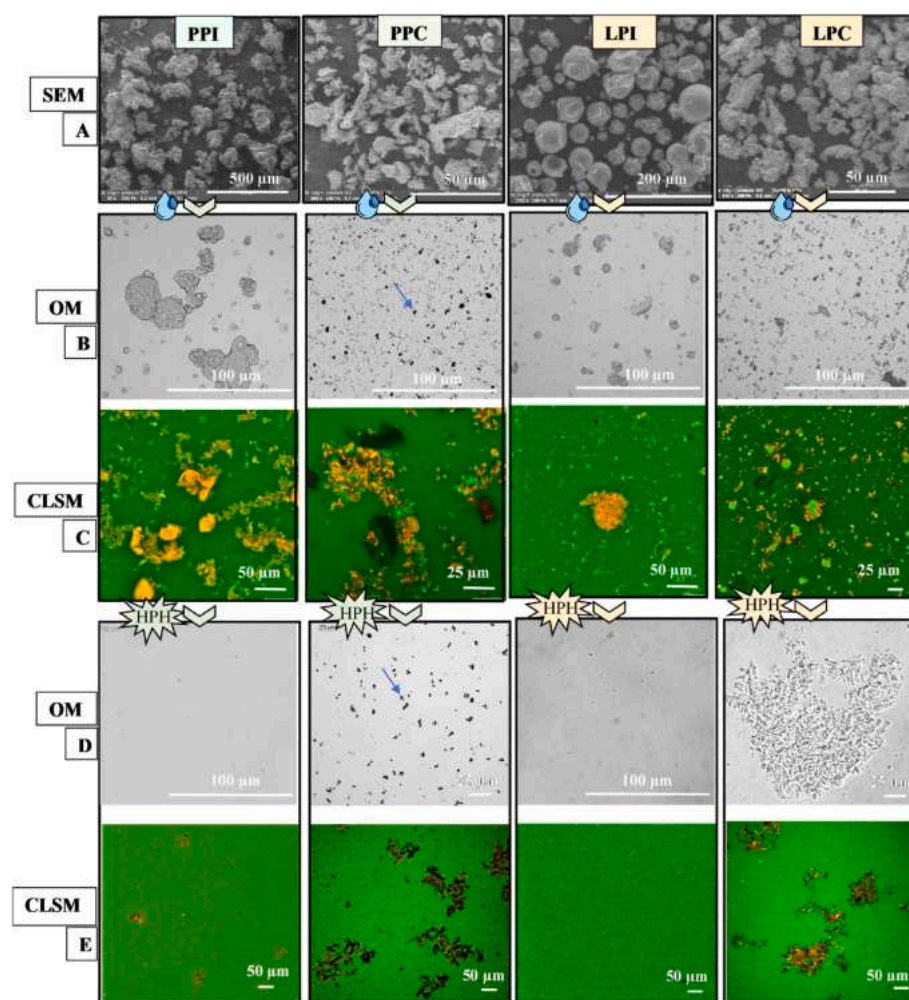


Fig. 7. Row A: Scanning electron microscopy (SEM) images of the powders (dry state); Row B: Optical microscopy (OM) images of the aqueous suspensions (1 g/100 g proteins in pH 7.0 buffer, 90 mM NaCl), non-treated by homogenization, stained with lugol for PPC (the arrows point to starch granules); Row C: Confocal laser scanning microscopy (CLSM) (lipids were stained with Nile Red and appear in red; proteins were stained with Alexa 488 and appear in green) of the same non-homogenized suspensions; Rows D, E: OM and CLSM images of the suspensions after the homogenization treatment (HPH). The blue droplet icon indicates the change of environment from dry powder to aqueous suspensions.

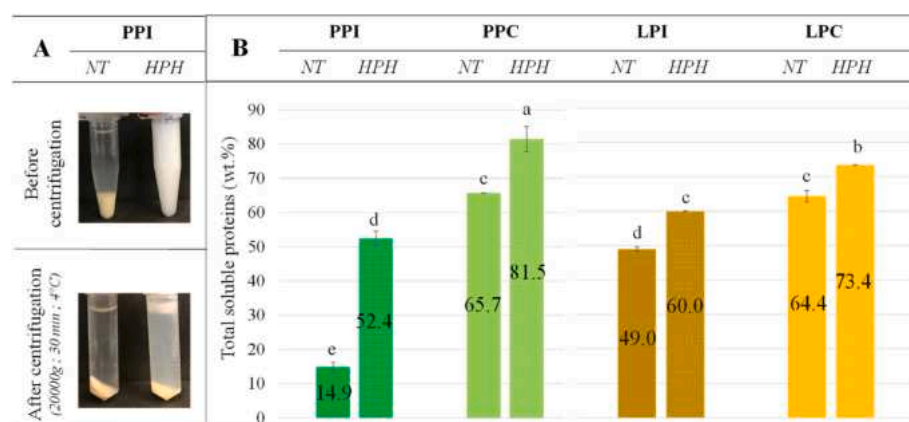


Fig. 8. Partitioning between the insoluble and soluble fraction of the suspensions. A: Pictures of PPI suspension (1 g of proteins/100g) before centrifugation (upper picture), when non-treated by homogenization (NT) (left tube) vs. treated by homogenization (HPH) (right tube), and after centrifugation (lower picture) at 20 000 g; 30 min; 4 °C, NT (left tube) and treated by HPH (right tube). B: Protein solubility (i.e., the fraction that remains in the supernatant after centrifugation at 20 000 g; 30 min; 4 °C) in the aqueous suspensions before HPH, and after (dotted bars). Results are shown as mean \pm SD of three independent measurements. Significant differences are provided by different letters on top of the histograms (p -values < 0.0001).

the lipid structures, first appearing as trapped in the grain powders, were liberated as small droplets. Images from individual channels are provided in [Supplementary info 8](#), to allow for a better distinction of the fluorescence pertaining to lipids. For PPC and LPC, the structures made of starch and/or fibres were also visible, and showed as well some lipid droplets trapped inside.

Those observations are useful to decipher the morphology of the aqueous dispersions of the studied plant-based ingredients, before and after a homogenization treatment. They provide a general overview of

the structural changes occurring on a plant protein powder dispersion, both regarding proteins and non-proteinaceous compounds. In line with these morphological features, one may expect substantial differences in protein solubility, which is addressed in the next section (3.2.2.). Besides, as the HPH treatment leads to a release of endogenous lipid droplets that were initially trapped inside complex composite structures endogenous, we may expect that the treatment could enhance potential twisting effects of these lipids on the functional properties of the dispersions.

3.2.2. Protein partitioning between the insoluble fraction and the supernatant

To investigate protein solubility, the aqueous suspensions, treated or non-treated by HPH, were centrifuged at 20 000 g, for 30 min, at 4 °C. An upper phase similar to a creamed phase was retrieved after centrifugation, which was more visible for the samples that had been subjected to the HPH treatment (Fig. 8A). As there is a substantial proportion of lipids in the powders, and in line with the disruption of the lipid-containing structures during HPH, this upper phase logically corresponds to lipid droplets and/or lipid-rich colloids.

Protein solubility was determined by cautiously sampling the supernatants below this creamed phase, and measuring their nitrogen content (Fig. 8B). For both PPC and LPC, protein solubility was high (about 65 wt% of the total protein content). Conversely, PPI displayed a much lower protein solubility (14.9 wt%), whereas that of LPI was intermediate (49.0 wt%), which emphasizes how protein solubility is tailored by the processing history of the ingredients. We then explored how HPH modulated the measured protein solubility. For all samples, protein solubility was enhanced after HPH. The effect was substantial for

PPI, for which the solubility was increased by a 3.5-fold factor. Such a rise in protein solubility induced by a HPH treatment of aqueous dispersions was also very recently shown with a similar commercial PPI (Lijuan Luo, Cheng, et al., 2022). An enhancement of protein solubility using HPH was also shown on different commercial pea ingredients (isolates and concentrates) (Burger & Zhang, 2019; Moll et al., 2021), as well as on various plant protein ingredients (quinoa (Lan Luo, Cheng, et al., 2022), lentil (Primozic et al., 2018) and faba bean (Jingqi Yang et al., 2018)). However, it seems that this improvement in plant-protein solubility by HPH is valid within a given range of pressures, beyond which HPH could be detrimental for the functionality (re-aggregation) and digestibility of the proteins (Lijuan Luo, Cheng, et al., 2022; Melchior, Moreton, Calligaris, Manzocco, & Nicoli, 2021; Nikbakht Nasrabadi, Sedaghat Doost, & Mezzenga, 2021; Saricaoglu, 2020). Nevertheless, and considering the residual turbidity of the supernatants after centrifugation, the increase in protein solubility after HPH should be interpreted by considering the colloidal state of the proteins found in this supernatant. To investigate those aspects, dynamic light scattering analyses were carried out on the supernatants, and the results are given

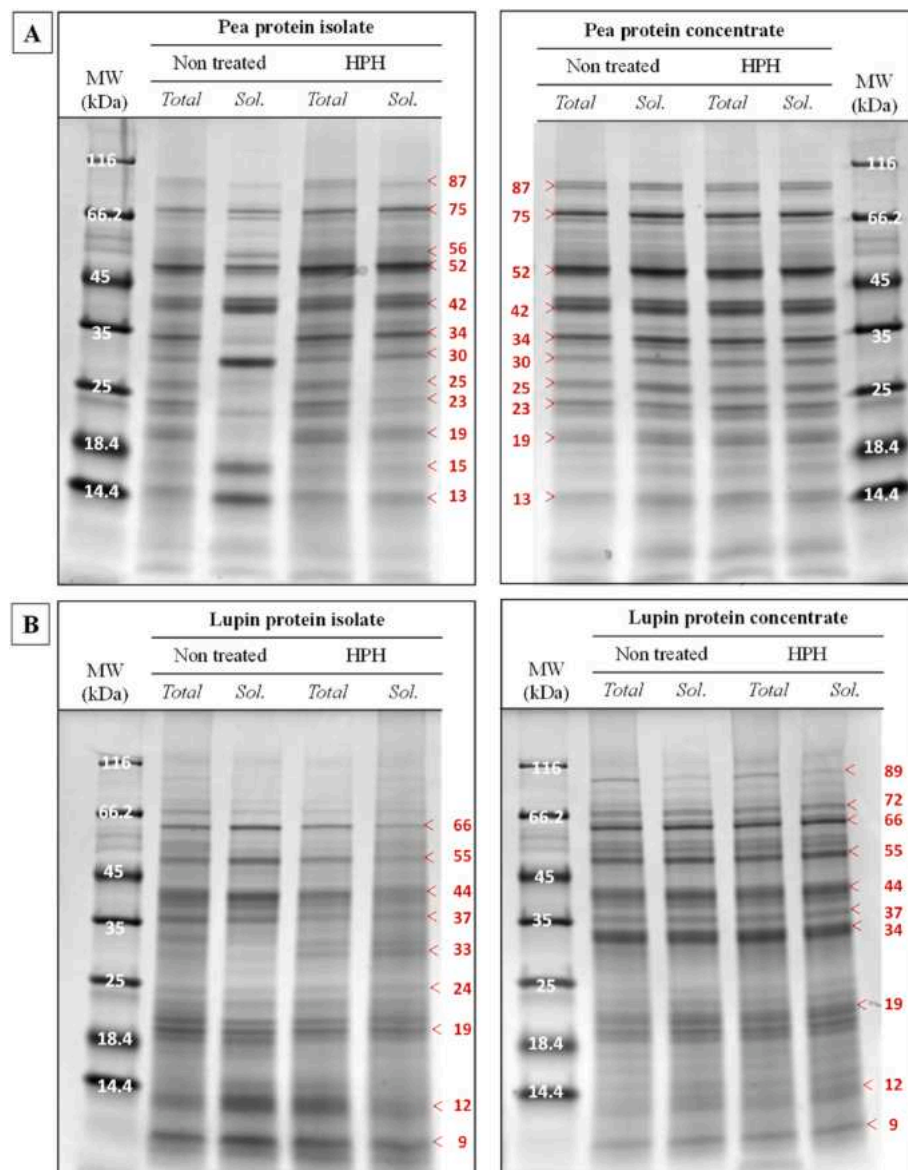


Fig. 9. Protein composition and identification by SDS-PAGE in reducing conditions (A, PPI (left) and PPC (right); B, LPI (left) and LPC (right)). The first and last migration lanes contain the molecular weight standards (MW in kDa, white font). The molecular weight (kDa) of each major protein band identified is given on the right- or left-side of the figures (red font).

in section 3.2.3. UV-visible spectra of the supernatants collected after centrifugation of the treated, and non-treated suspensions, are given in Supplementary info 5.

Protein identification by SDS-PAGE of the supernatants was then achieved for the suspensions either non-treated or treated by HPH, and compared with the protein profiles of the total suspensions (Fig. 9).

Prior to HPH treatment, for PPC and LPC, no major difference in the protein subunits could be observed between the total suspensions and the supernatants. However, for both isolates (in particular, for PPI), the SDS-PAGE profile displayed differences between the total suspension and the supernatant after centrifugation. Indeed, for PPI, the bands corresponding to albumin (13 and 15 kDa (Ma et al., 2017)), vicilin (30 and 52 kDa (Hinderink, Münch, Sagis, Schroën, & Berton-Carabin, 2019)), α -legumin (42 kDa) and convicilin (75 kDa) (Barac et al., 2010)) looked very intense at the expense of those of β -legumin (19 and 23 kDa (Barac et al., 2010)) and other chains of vicilins (25 and 34 kDa (Hinderink et al., 2019)) that were not recovered in the supernatants. Densitometry analysis of the protein composition for pea samples confirmed this underrepresentation of β -legumin at the expense of α -legumin and albumins in PPI's supernatant (Fig. 10). Conversely, for PPC, the protein composition was similar in the whole suspension and in the supernatant, suggesting an even contribution of all protein classes to the insoluble and soluble protein fractions. For LPI, only minor differences were found between the whole suspension and the supernatant, which is in line with the higher protein solubility of this sample (prior to HPH treatment) compared to PPI (49 wt% vs 15 wt%, respectively; Fig. 9). It should be pointed out that for lupin-based samples, the attribution of the protein bands to distinct proteins is more complex than for pea samples, and would require deeper investigations (via advanced protein purification, notably). Indeed, under reducing conditions, α -conglutin consists of many different subunits of low MW (10–23 kDa) (Schlegel et al., 2019), just like δ -conglutin (13 or 22 kDa) (Fontanari et al., 2012). Yet, α -conglutin also includes medium MW (27–36 kDa) and high MW (41–84 kDa) polypeptides, just as the main protein found in *Lupinus angustifolius* which is β -conglutin (Foley et al., 2015), displaying a broad MW range of subunits (27–72 kDa) (Burgos-Díaz et al., 2016; Fontanari et al., 2012; Schlegel et al., 2019). To wrap-up, those results show that the initial soluble protein fraction is not necessarily representative of the full protein composition of the isolate samples, especially for PPI, whereas the opposite was found for the concentrates.

After HPH treatment, for LPC and PPC, an equal distribution and relative importance of the different proteins in the soluble and insoluble fractions was observed. For PPI and LPI, treating the suspensions by HPH resulted in similar protein composition profiles in the total suspension and the corresponding supernatant, which is also well illustrated in the densitometry profiles for PPI (Fig. 10). To the best of our knowledge, although several recent studies did describe the use of HPH as a means to improve plant protein solubility (as detailed earlier), it was not linked to potential changes in the protein composition of the soluble fraction. Yet, it is an important point when such suspensions are meant to subsequent use as functional ingredients. Moreover, as discussed,

HPH was shown to increase protein solubility for every sample (Fig. 8B), and this increase was substantial for PPI. On the other hand, the protein partition of this sample was different between the soluble and the total fraction prior HPH. Therefore, only a minor part of the total proteins of this sample is initially represented in the soluble fraction. Yet, the protein composition in both fractions was recovered after HPH. Therefore, one can conclude on the efficient role of HPH to disrupt large protein aggregates, that were initially less present in the soluble fraction, leading to a more representative overview of the sample's protein composition in the supernatant.

3.2.3. Characterization of the 'soluble' fraction

Particle size measurements were performed by static light scattering (SLS) analysis on total aqueous dispersions either prior to or after HPH treatment (Fig. 11A1 and 11B1). The PPI dispersion displayed a bimodal population of particles, which was the largest from the four tested samples (main peak around 70 μ m), compared to a population around 15 μ m for the other protein dispersions. HPH allowed to reduce the particle size, which is consistent with the microscopy images, as discussed earlier. Yet, the obtained results should be analyzed simultaneously with the microscopy images of the dispersions for a good understanding of the samples' morphologies. For instance, particle size distributions for PPC suspensions looked relatively similar prior to and after HPH, whereas the microscopy images indicated otherwise. In that case, the broadness of the encountered structures' sizes is clearly not captured by light scattering analysis, which emphasizes the need to couple those two methodologies to have a comprehensive overview of such samples with high structural and compositional complexity.

HPH largely promotes the distribution of proteins towards the supernatant of such suspensions (i.e., in the so-called soluble fraction): this effect is reached by decreasing the particle size in the whole suspension, breaking up large aggregates (>10 μ m) into smaller ones. This point is consistent with microscopy observation where HPH helped to break down the non-dispersible structures in aqueous suspension, notably more for the isolates compared to the concentrates. This is relevant with the more important share of protein aggregation for protein isolates, as wet fractionation implies to proceed to a spray-drying step, critical for protein aggregation (Yang et al., 2022). On the contrary, the dry environment of mild fractionation allows to preserve the native colloidal state of the proteins, consistent with the smaller structure observed by microscopy, and with the absence of particle size shift before or after HPH.

The proteins and/or small colloids contained in the supernatants were further studied by dynamic light scattering (DLS), which revealed particle sizes ranging between 8 and roughly 200 nm (Fig. 11A2 and 11B2). Globulins and albumins are reported to have respective diameters of 7–9 nm and 3–5 nm, which confirms that proteins are largely present as small aggregates in the supernatants (Erickson, 2009; Yang et al., 2022). Other authors found main peaks around 30–40 nm in non-treated total suspensions of PPI (passed through 1- μ m filters), that were either freeze-dried or spray-dried (Yang et al., 2022). No

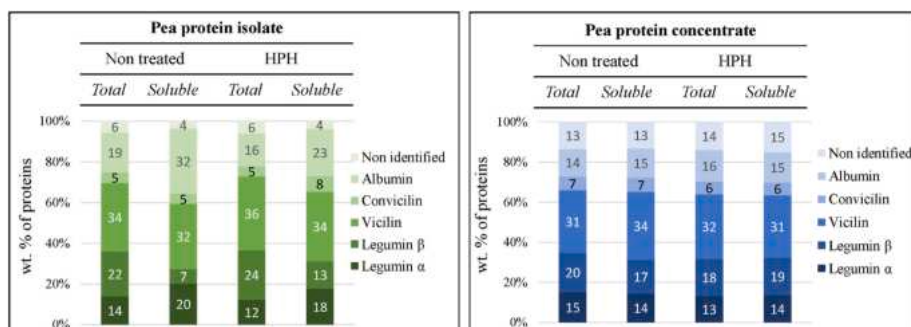


Fig. 10. Densitometry analysis of the SDS-PAGE profiles for PPI (left) and PPC (right), providing the percentage of the different protein classes in the samples.

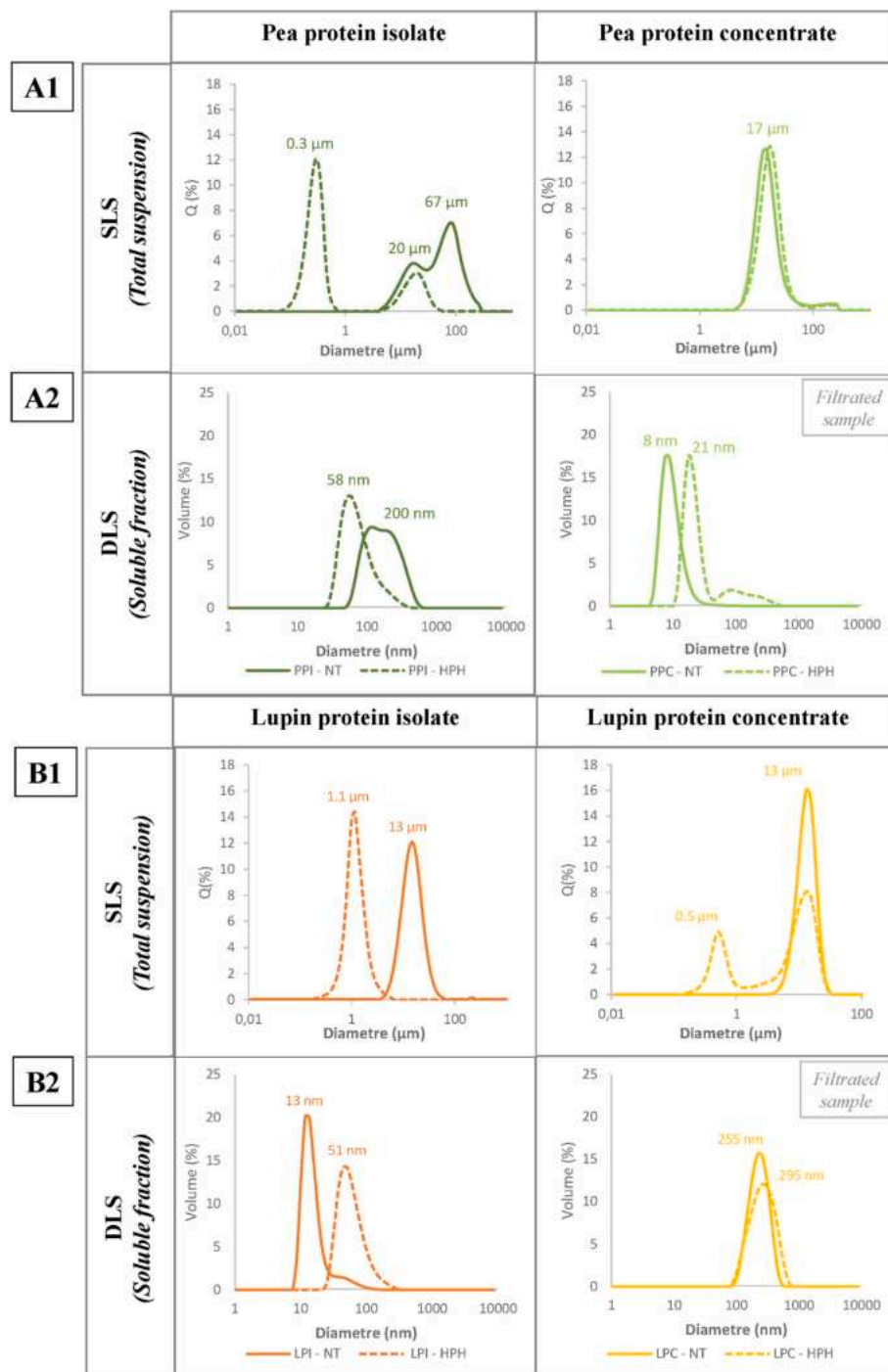


Fig. 11. Particle size distribution in the total aqueous suspensions, obtained by static light scattering (SLS) (A1 and B1), and in the supernatants obtained by dynamic light scattering (DLS) (A2 and B2). The samples were non-treated (full lines) or treated by HPH (dotted lines), for pea (A) and lupin (B) samples. Data correspond to the d_{median} (in μm or nm). Prior to DLS measurements, some samples (PPC and LPC) were filtrated on 0.45- μm cellulose filters (noted 'Filtrated sample'). For clarity, representative profiles from two independent repetitions are presented.

systematic effect of the homogenization step was observed on the particle size studied by DLS. Those results should be regarded cautiously: for practical reasons, the samples were 11-fold diluted, and some of them (PPC and LPC) had to be filtrated to yield a scattering signal relevant to proper analysis. Hence, it is important to note that the intensity of the signal response is tightly linked to the proportion of the analyzed particles, i.e., when homogenized, the amount of soluble aggregates was more important (as the protein concentration in the supernatants increased, Fig. 8B) and may therefore overcome the signal obtained for fewer small structures detectable before HPH.

To wrap up, the treatment by HPH only moderately impacts the size of the small, so-called 'soluble aggregates' present in the supernatants, as shown with the DLS results, but their proportion is enhanced, as the

solubility measures shown (Fig. 8). Their small size allows them to remain in suspension in the supernatants of the solutions after centrifugation, which leads to consider them as 'soluble', though being small aggregates or protein fragments. The aggregated state of the proteins prior HPH, mostly visible for PPI, can be efficiently altered by HPH process, which allowed for a good recovery of the protein composition in the supernatant, and an increase in proportion. Those observations support the previous quantitative results on the reversibility of the aggregated state of those proteins via solubility measurements. This effect of HPH can most likely be attributed to the hydrodynamic conditions and high shear rates occurring during the treatment, thus favouring interactions between proteins and solvent (Lijuan Luo, Cheng, et al., 2022). Other mechanisms were reported according which HPH

would allow the exposure of the disulfide bonds of the aggregates, with further disruption of these bonds through the process (Melchior et al., 2022). To wrap up, the improvement of commercial protein ingredients' solubility by HPH processing is a straightforward and efficient means to optimize the use of ingredients in food products formulation without additive- or high temperature-based interventions.

4. Conclusion

This comprehensive investigation of the composition of commercial pea and lupin protein ingredients reaches beyond the current characterization strategies normally encountered in this field of research, thus providing new insights to understand their behaviour as aqueous suspensions. The composition of these ingredients is highly complex, in particular, substantial amounts of endogenous lipids were found, sometimes much higher than in the initial seeds, and of which an important proportion is phospholipids that have surface-active properties. The role of such components in the functional properties of these ingredients, and in particular, the emulsifying properties, must therefore be deepened, as well as the mechanisms underlying their transfers/accumulation during protein fractionation processes. When HPH is applied to aqueous suspensions of these ingredients, the colloidal morphology is altered and the protein solubility (defined as the protein content remaining in the supernatant) is enhanced. The amount of small protein aggregates in this 'soluble' fraction is increased after HPH, but their size hardly changes. Polysaccharide-based structures are reduced in size by HPH but tend to pack, whereas small lipid-containing structures (appearing as small droplets) are released from large composite particles. The remaining presence of polysaccharides, and their identification, is also an original outcome and points out to the interest of investigating their role on the overall functionalities of the ingredients into more depth. Hence, according to the sum of the macro-nutrients measured in the samples through this study, there is still a proportion of non-characterized compounds that must be remembered. We hope that this research will encourage the food science community to consider the structural and compositional complexity of commercial or lab-made plant protein-rich fractions as an integral part of related research. For instance, deconvoluting the respective roles of proteins and of non-protein compounds, and their interactions, is probably critical to interpret correctly, and in turn control, the functionalities of these ingredients.

Author statement

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Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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