

Characterising the composition and physicochemical properties of legume and oilseed protein concentrates to evaluate their potential for high-moisture extrusion processing

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ABSTRACT

This study represents the initial phase of a larger entity evaluating the applicability of plant protein concentrates from pea, faba bean, flaxseed, and hempseed for high-moisture extrusion processing (HMEP), in which chemical composition and functional properties of the ingredients were analysed. Wheat gluten and pea protein isolate were used as benchmark ingredients. The main emphasis was on properties potentially important for creating fibrous structures in HMEP, although the levels of specific chemical compounds and endogenous enzymes that might influence the nutritional quality and flavour of the extrudates were also analysed. Significant variation was observed among the ingredients, with hemp showing the most similarities to protein isolates in terms of high protein content, low dietary fibre content, and low protein extractability. Flax was comparable to hemp but had a lower protein content. The high protein extractability of legume concentrates may be a barrier for their application in HMEP. The presence of elevated levels of phytic acid, raffinose family oligosaccharides, and lipid-modifying enzymes in certain ingredients need mitigation for HMEP. The findings of this study will serve as a foundation for a subsequent investigation, where the performance of these emerging ingredients will be evaluated in actual HMEP trials.

1. Introduction

In line with the United Nations Sustainable Development Goals, a rapid transition from diets based on animal proteins towards plant protein-based diets is required (Aiking and de Boer, 2020). One approach to boost the intake of plant proteins involves developing affordable, tasty, and nutritious plant-based alternatives that resemble conventional meat products. High-moisture extrusion processing (HMEP) can be used to produce meat analogues that mimic the fibrous structure of whole-cut meat. However, this process generally necessitates the use of costly protein isolates, which impacts the cost competitiveness of plant-based meat analogues, particularly when compared to animal products. For example, non-soy or non-wheat extruded meat analogues cost ca. 20 €/kg, compared to 10 €/kg for minced beef. In fact, the consumers' interest in extruded meat analogues declined after the

initial excitement, partly because of the high price of the products containing refined, highly processed ingredients (e.g. isolates or additives), and poor sensory quality.

At present, protein isolates, sometimes in combination with protein concentrates or flours, derived from soy, wheat, and pea, are the predominant plant proteins utilized in HMEP. However, as global demand for protein continues to rise, there is a need to diversify protein sources. In Northern Europe, pea, faba bean, flaxseed, and hemp stand out as relevant plant-based protein sources as climate resilient crops (Zahari et al., 2020).

Plant protein isolates are commonly produced by wet extraction and drying, a process that requires substantial amounts of water and energy (Schuttyser et al., 2015). In recent years, dry fractionation - which involves milling and air-classification - has gained more interest as a means of protein enrichment due to its lower environmental impact

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compared to wet extraction. Dry fractionation generally yields ingredients with a lower protein content (30-60 %) compared to wet extraction. However, the presence of additional compounds like starch and dietary fibre in the dry fractionated protein concentrates can have benefits in terms of technological functionality and nutritional properties. Along with differences in composition, wet and dry fractionation can also result in ingredients with distinct physicochemical characteristics. Dry fractionation, for example, is known to maintain protein native properties (Schutyser et al., 2015).

HMEP encompasses three fundamental unit operations: 1) mixing and hydration, 2) thermo-mechanical treatment and 3) cooling (Cornet et al., 2022). Although the precise mechanism governing fibrous structure formation remains incompletely understood, various phenomena are speculated to play a part in the process. These phenomena include the unfolding and crosslinking of proteins through disulphide bonding, phase separation of thermodynamically incompatible biopolymers as well as syneresis (Cheftel et al., 1992; Cornet et al., 2022; Liu and Hsieh, 2008; Sandoval Murillo et al., 2019; Tolstoguzov, 1993; van der Sman and van der Goot, 2023; Zhang et al., 2022a). All these phenomena are expected to be influenced by both the ingredient properties and the processing conditions in HMEP. However, it is still uncertain which properties of the ingredients are the most crucial ones for successful structure formation in HMEP. Despite extensive research conducted on HMEP using diverse ingredients, deriving definitive conclusions regarding the influence of ingredient properties on fibrous structure formation remains challenging. This is partially attributed to the complexity of the process but also to the lack of standardised analytical tools for characterising these ingredients (Ma et al., 2022).

This study represents the initial phase of a larger entity evaluating the applicability of plant protein concentrates from pea, faba bean, flaxseed, and hempseed for HMEP. In this phase, we aimed to assess the performance of the ingredients by conducting a thorough characterisation of their chemical composition and physicochemical properties. Subsequently, we compared these properties with those of wheat gluten and pea protein isolate, ingredients more commonly utilised in HMEP. The main emphasis was on properties potentially important for the formation of fibrous structures, although the levels of specific chemical compounds and endogenous enzymes that might influence the nutritional quality and flavour of the extrudates were also analysed. The findings of this study will serve as a foundation for a subsequent investigation, where the performance of these emerging ingredients will be evaluated in actual HMEP trials.

2. Materials

Several interesting plant protein ingredients were first collected based on the following criteria: the ingredient should be 1) commercially available, 2) a protein concentrate, and 3) derived from a plant suitable for cultivation in the Nordic region. From the collected group of ingredients, a smaller subset of the ingredients was selected for this study based on preliminary evaluation of their potential in HMEP (results not reported here). This smaller subset consisted of five protein concentrates from yellow pea, brown pea, faba bean, flaxseed, and hemp. Wheat gluten and pea protein isolate were included in this study as reference ingredients commonly utilised in HMEP. The details of the commercial plant protein ingredients used in this study are presented in Table 1.

3. Methods

3.1. Chemical composition and enzyme activities

The moisture and ash contents of the ingredients were analysed with the gravimetric AACC 44-15.02 method and the AACC 08-01.01 method, respectively. Total nitrogen content was analysed using the Dumas combustion method (Vario MAX CN, Elementar, Germany) and

Table 1
Plant-based protein ingredients used in the study.

| Material | Abbreviation | Brand name, producer | Processing technology |
|--------------------------------|--------------|--|---|
| Wheat gluten | Wheat | Vital Wheat Gluten, Reppal VWG, Lantmännen | Wet extraction, drying |
| Pea protein isolate | Pea-isolate | Nutralys F85M, Roquette | Wet extraction, drying |
| Yellow pea protein concentrate | Pea-yellow | Pea protein F55X, Vestkorn | Dry fractionation |
| Brown pea protein concentrate | Pea-brown | 50.0BP Organic, Aloja-Starkelsen | Dry fractionation |
| Faba bean protein concentrate | Faba | P60, Suomen Viljava Oy | Dry fractionation |
| Flaxseed protein concentrate | Flax | Flaxein, Vegetein | Cold-pressing, alcohol extraction, drying |
| Hemp protein concentrate | Hemp | Hempein, Vegetein | Cold-pressing, alcohol extraction, drying |

the protein content was calculated by multiplying the nitrogen content by the general coefficient 6.25, because specific coefficients were not available for all ingredients.

The dietary fibre content of the samples was analysed in duplicate according to the AOAC 2011.25 method with a semi-automated Dietary Fibre Analyser (ANKOMTDF, Makedon, NY, USA). For the digestible, non-resistant starch determination, a commercial assay kit (Total Starch Assay Kit K-TSTA, Megazyme, Ireland) based on the AACC 76-13.01 method was used.

The analysis of total amino acid content was performed as described by Nordlund et al. (2018), including acid hydrolysis, derivatization and UPLC analysis. For the determination of tryptophan and tyrosine, a separate alkaline hydrolysis was performed. Two replicate derivatisations and UPLC runs were made for each sample hydrolysate.

Free glucose, sucrose, raffinose, stachyose, and verbascose were measured as described by Tuccillo et al. (2022). In brief, samples (0.1 g) were extracted with water (5.0 mL), centrifuged, and supernatants were boiled for 10 min. Thereafter, supernatants were re-centrifuged and deproteinized with ultrafiltration (10 kDa, Merck Millipore, Darmstadt, Germany). After filtering, extracts were diluted in water (1:2 or 1:5 v/v) and the internal standard 2-deoxy-D-galactose (Sigma-Aldrich, St. Louis, USA) was added 500 ng per injection of 10 µL. Compounds were analysed with a high-performance anion exchange chromatograph connected to a pulse amperometric detector as described by Kantanen et al. (2024).

Lipids were extracted using ethanol as the solvent by an accelerated solvent extraction technique. Fatty acids were hydrolysed from different lipid classes and methylated, followed by GC-FID analysis as presented earlier (Tuccillo et al., 2022). Total fat was calculated as the sum of fatty acid methyl esters.

The phytic acid content was analysed in samples (0.5 g) with a commercial kit (Phytic acid assay kit K-PHYT, Megazyme, Ireland) according to the instructions of the manufacturer, and free, soluble phenolic compounds were measured using a spectrophotometric method with Folin-Ciocalteu reagent from extracts obtained after extraction of samples (0.1 g) with 80 % ethanol (Tuccillo et al., 2022). The content of condensed tannins was determined with a modified vanillin assay method as reported by Tuccillo et al. (2022). In brief, samples (0.1 g) were extracted with 1 % sulfuric acid in methanol, centrifuged and supernatants were mixed with the vanillin reagent. After incubation, the absorbances were measured at 500 nm. Results were expressed as catechin equivalents.

Endogenous enzyme activities were measured by spectrophotometric methods. Lipase activity was measured using *para*-nitrophenyl

butyrate as the substrate, whereas lipoxygenase activity was measured using linoleic acid as the substrate as described by Tuccillo et al. (2022). Each sample was extracted in duplicate, and each extract was measured twice.

All analyses were carried out in triplicate unless otherwise stated.

3.2. Physicochemical properties

The particle size distribution of the protein ingredients was measured by laser diffraction with a Mastersizer 3000 (Malvern Instruments Ltd., Worcestershire, UK). All samples were measured both as dispersed in water and in dry form as previously described by Nisov et al. (2022). Two sets of replicate samples were analysed, with each sample undergoing either five (wet module) or three (dry module) sub runs during the measurement.

Microscopic evaluation was conducted by examining all ingredients unstained as dispersed in ethanol using an Axio Imager.M2 microscope (Carl Zeiss GmbH, Göttingen, Germany) with a 20x objective (Zeiss EC Plan-Neofluar, numerical aperture 0.50). Micrographs were obtained in brightfield using an Axiocam 506 CCD colour camera (Zeiss) and the Zen imaging software (Zeiss). Additionally, pea-isolate was examined as dry with cover slip using a confocal laser scanning microscopy (CLSM) equipment consisting of a LSM 710 (Zeiss, Jena, Germany) attached to an Axio Imager.Z microscope (Zeiss). A diode laser line of 405 nm was used for excitation of autofluorescence, and emission was collected at 425-621 nm. Images were assembled from the optical sections taken using a 40x objective (Zeiss EC Epiplan-Neofluar, numerical aperture of 0.75) to the depth of 57-82 µm with 0.88 µm z step. Images were captured with a resolution of 1024 × 1024 using ZEN software (Zeiss). Representative images were selected for publication.

Protein and total extractability were measured at pH values from 3 to 9. Protein ingredients were dispersed in water (5 % solids, w/w), stirred magnetically for 30 min, and pH-adjusted with 1M NaOH or HCl. After another 30 min of stirring and final pH adjustment, the mixture was further stirred for 30 min. Then, 35 g of the mixture was centrifuged (10,000 x g, 15 min, 22 °C) in a 50 mL tube and the supernatant was collected. The protein content of the supernatant (in liquid form, sample size 2.5 g) was determined using the Dumas combustion method (section 3.1). Solids content of the supernatant was analysed by oven-drying at 105 °C overnight. Protein extractability was defined as the ratio of protein content in the supernatant to that in the initial dispersion, and total extractability as the ratio of dry matter content in the supernatant to that in the initial dispersion. The extractions were carried out once and protein and solid contents were analysed in triplicate. It is important to note that some of the compounds present in the supernatant may not be truly soluble but rather exist in a colloidal state. Therefore, it is more appropriate to refer to extractability instead of solubility.

The water hydration capacity (WHC) of the protein ingredients was measured in duplicate using the AACC 56-30.01 method. This determines the maximum amount of water the sample can retain without forming a supernatant during centrifugation (2000 g, 10 min) (Quinn and Paton, 1979). Unlike methods that use excess water, this approach ensures the outcome is not influenced by differences in solubility of the materials studied.

Oil binding capacity (OBC) was determined according to the AACC method 56-11.02 using 2.5 g sample and rapeseed oil as solvent. OBC was calculated as the amount of oil (g) bound in the pellet divided by the initial sample weight (g dry matter).

The viscosity of protein ingredients was measured at a dry matter content of 15 % and sample weight of 28.5 g with a Rapid Visco Analyser (RVA Super 4, Newport Scientific, Warriewood, Australia). The RVA procedure consisted of the following steps: (1) 15 min at 25 °C, (2) heating from 25 to 99 °C at 10 °C/min, (3) 5 min at 99 °C, (4) cooling from 99 to 25 °C at 10 °C/min and (5) 5 min at 25 °C. Throughout all the steps, a steady mixing rate of 160 rpm was applied. From the viscosity profiles, the viscosities after 15 min at 25 °C, 5 min at 99 °C and at the

end of the measurement (final viscosity) were reported.

The thermal behaviour of the protein ingredients was analysed by differential scanning calorimetry (DSC) using a Mettler Toledo DSC2 (Greifensee, Switzerland). A small amount (7-8 mg) of sample was weighed into a medium pressure crucible (stainless steel) and MilliQ water was added to reach a moisture content of about 50 %. The crucible was sealed, and the sample was allowed to equilibrate at 4 °C overnight before heating it from 20-180 °C at a rate of 5 °C/min. Two heating-cooling cycles were performed. Each sample was measured in duplicate.

All analyses were carried out in triplicate unless otherwise stated.

3.3. Principal component analysis

The principal component analysis (PCA) models were created with The Unscrambler version 10.5.1 (CAMO Software AS, Norway) with data that was averaged over replicates, mean-centered and autoscaled. The analysis was done in two parts to create models without missing values. In the first part only chemical composition data for all ingredients was included. In the second part both chemical and physical properties data was included for all ingredients except for Wheat for which data on physical properties was missing.

4. Results and discussion

4.1. Chemical composition and enzyme activities

4.1.1. Protein and amino acids

The protein contents of the studied ingredients varied widely (Table 2). Among the protein concentrates, Hemp had the highest protein content (76 %), while Flax had the lowest (47 %). The reference ingredients Wheat and Pea-isolate contained almost 90 % protein. A raw material with a relatively high protein content is considered a prerequisite for successful structure formation in HMEP. Guyony et al. (2022) recommended that the raw material used in HMEP should contain at least 70 % protein. According to this criterium, the best ingredients for HMEP in the present study would be Wheat, Pea-isolate and Hemp. Although having a high protein content is crucial, it does not necessarily guarantee effective performance in HMEP by itself. The composition, physical characteristics, and functional properties of the proteins and other components within the ingredient may also contribute to the formation of fibrous structures.

The relative amino acid composition (Table S1) of the protein ingredients varied depending on the protein source. Ingredients derived from legumes exhibited a comparable amino acid profile, with glutamic acid, aspartic acid, and arginine as the predominant amino acids. The amino acid profile of oilseed-derived proteins was quite comparable to that of legume-derived proteins. However, the oilseed proteins had higher proportions of cysteine, methionine, and tryptophan and smaller proportions of lysine compared to the legume proteins. The amino acid composition of wheat was notably distinct from the other studied ingredients. Wheat had low proportions of arginine, aspartic acid, and lysine but high proportions of glutamic acid and proline compared with the other ingredients. It is generally known that wheat protein is low in lysine, while legume proteins lack sufficient methionine and cysteine (Day, 2013; Goldstein and Reifen, 2022).

In HMEP, especially the cysteine content is of interest as it affects the protein's ability to form disulfide bonds, which is considered to play a key role in fibrous structure formation (Liu and Hsieh, 2008). In our study, the relative proportion of cysteine was the highest in Wheat, Flax and Hemp. Nasrollahzadeh et al. (2022) showed that high-moisture extruded meat analogues from hemp proteins exhibited a better fibrous structure compared to those derived from pea protein. One possible explanation for the difference was the higher cysteine content in hemp as compared to pea. It is good to note that the temperature at which disulfide-mediated polymerization of proteins occurs is affected by the pH of the local environment (Monahan et al., 1995). Nisov et al.

Table 2

Chemical composition and enzyme activities of plant protein ingredients (nd=not detected). Values are presented as the average \pm standard deviation of 3 replicates (except for dietary fibre).

| Compound | Wheat | Pea-isolate | Pea-yellow | Pea-brown | Faba | Flax | Hemp |
|---------------------------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Protein (% dm) | 87 \pm 0 | 89 \pm 0 | 54 \pm 0 | 54 \pm 0 | 64 \pm 0 | 47 \pm 0 | 76 \pm 1 |
| Starch (% dm) | 9.8 \pm 0.1 | 0.4 \pm 0.0 | 4.4 \pm 0.0 | 14 \pm 0 | 7.6 \pm 0.2 | 2.4 \pm 0.1 | 0.5 \pm 0.1 |
| Total dietary fibre (% dm) | 10 | 7.0 | 26 | 21 | 23 | 37 | 14 |
| Insoluble DF (% dm)* | 0.7 \pm 0.1 | 3.1 \pm 0.0 | 17 \pm 0 | 12 \pm 0 | 12 \pm 0 | 28 \pm 0 | 4.3 \pm 0.6 |
| Soluble DF (% dm) | 9.5 | 3.9 | 9.5 | 9.6 | 11 | 9.7 | 9.3 |
| Soluble high Mw DF (% dm)* | 1.6 \pm 0.1 | 1.5 \pm 0.1 | 3.0 \pm 0.3 | 2.2 \pm 0.1 | 3.8 \pm 0.0 | 8.3 \pm 0.1 | 6.5 \pm 0.0 |
| Soluble low Mw DF (% dm) | 7.9 | 2.4 | 6.5 | 7.4 | 6.8 | 1.4 | 2.8 |
| Glucose (% dm) | nd | nd | 0.3 \pm 0.1 | 0.2 \pm 0.0 | nd | nd | nd |
| Sucrose (% dm) | 0.1 \pm 0.0 | 0.6 \pm 0.1 | 2.3 \pm 0.1 | 2.1 \pm 0.1 | 1.1 \pm 0.1 | 2.8 \pm 0.2 | 3.1 \pm 0.3 |
| Raffinose (% dm) | nd | 0.2 \pm 0.0 | 1.2 \pm 0.3 | 0.8 \pm 0.1 | 0.2 \pm 0.0 | 0.6 \pm 0.0 | 0.9 \pm 0.1 |
| Stachyose (% dm) | nd | 0.8 \pm 0.1 | 4.6 \pm 0.3 | 2.9 \pm 0.1 | 0.9 \pm 0.1 | 1.0 \pm 0.3 | 1.1 \pm 0.2 |
| Verbascose (% dm) | nd | 0.5 \pm 0.1 | 4.0 \pm 0.3 | 3.9 \pm 0.2 | 3.7 \pm 0.2 | nd | nd |
| Fat (% dm) | 5.0 \pm 0.4 | 6.8 \pm 0.1 | 3.5 \pm 0.3 | 2.7 \pm 0.1 | 3.2 \pm 0.1 | 0.8 \pm 0.1 | 0.6 \pm 0.0 |
| C16:0 (% of fat) | 19 \pm 0 | 13 \pm 0 | 12 \pm 0 | 13 \pm 0 | 14 \pm 0 | 10 \pm 1 | 15 \pm 0 |
| C18:1 (% of fat) | 7.9 \pm 0.5 | 26 \pm 1 | 25 \pm 3 | 26 \pm 1 | 28 \pm 1 | 22 \pm 3 | 6.2 \pm 0.4 |
| C18:2 (% of fat) | 66 \pm 0 | 46 \pm 1 | 49 \pm 0 | 47 \pm 0 | 52 \pm 0 | 36 \pm 0 | 53 \pm 0 |
| C18:3 (% of fat) | 4.1 \pm 0.0 | 7.7 \pm 0.2 | 10 \pm 0 | 8.3 \pm 0.3 | 3.6 \pm 0.1 | 29 \pm 2 | 19 \pm 0 |
| Ash (% dm) | 0.7 \pm 0.0 | 4.6 \pm 0.2 | 5.9 \pm 0.1 | 5.0 \pm 0.1 | 6.4 \pm 0.0 | 9.0 \pm 0.0 | 15 \pm 0 |
| Phytic acid (mg/g dm) | 1.8 \pm 0.0 | 21 \pm 0 | 24 \pm 1 | 21 \pm 2 | 37 \pm 2 | 47 \pm 2 | 98 \pm 2 |
| Free phenolics (mg GAE/g dm) | 0.8 \pm 0.0 | 0.6 \pm 0.0 | 1.1 \pm 0.0 | 0.9 \pm 0.0 | 5.9 \pm 0.2 | 2.6 \pm 0.0 | 0.8 \pm 0.0 |
| Condensed tannins (mg CE/g dm) | nd | 0.2 \pm 0.0 | 0.3 \pm 0.0 | 0.3 \pm 0.0 | 0.6 \pm 0.0 | 0.7 \pm 0.0 | 0.1 \pm 0.0 |
| Lipase (μ mol/g/min) | 2.6 \pm 1.4 | 0.2 \pm 0.0 | 37 \pm 1 | 30 \pm 5 | 7.3 \pm 0.3 | 0.3 \pm 0.0 | 0.2 \pm 0.0 |
| Lipoxygenase (μ mol/g/min) | 15 \pm 6 | 5.8 \pm 4.4 | 807 \pm 246 | 202 \pm 94 | 149 \pm 11 | 2.1 \pm 1.3 | 0.3 \pm 0.2 |

* Values presented as average \pm average deviation of the residues in the first step of the analysis

(2022) for example found that fibrous structure formation of plant proteins during HMEP was more extensive at pH 7 than at pH 5 and attributed the difference to enhanced disulfide bond formation at the higher pH.

4.1.2. Starch, dietary fibre and sugars

Each ingredient in the study contained varying amounts of starch and/or dietary fibre (Table 2). Among these, Pea-brown exhibited the highest starch content (14 %), whereas Flax, Hemp and Pea-isolate were almost starch-free. Wheat contained almost 10 % starch, followed closely by Faba, which contained 7.6 % starch. All protein concentrates were richer in dietary fibre than the reference ingredients. The dietary fibre content was 37 % in Flax and 14 % in Hemp, while it ranged from 21-26 % in the protein concentrates from legumes. The content of insoluble dietary fibre exceeded that of soluble dietary fibre in all concentrates except in Hemp, whereas the reference ingredients had a higher concentration of soluble than insoluble dietary fibre. The high proportion of soluble fibre (66 %) in Hemp was unexpected since it typically accounts for less than 20 % of the total dietary fibre (Callaway, 2004; Mattila et al., 2018). Dietary fibre naturally present in ingredients may influence HMEP, as several previous studies have shown beneficial effects of incorporating hydrocolloids or fibre (up to a certain level) into plant protein-based ingredient mixtures in HMEP. For example, fibrous structure formation has been improved by adding pectin (Dekkers et al., 2018), sodium alginate (Dou et al., 2022) or soy fibre (Grabowska et al., 2016) to soy protein.

All ingredients contained small amounts of glucose and sucrose. The concentrations of the raffinose family oligosaccharides (RFOs) raffinose, stachyose, and verbascose were the highest in legume protein concentrates (5-10 % in total), but they were also detected in Flax (5 % in total) and Hemp (2 % in total). RFOs are types of FODMAP compounds, which can trigger digestive problems in people with functional bowel disorders (Nyyssölä et al., 2021). In the dietary fibre assay used in this study, RFOs are expected to be included in the soluble low molecular weight dietary fibre fraction (Table 2).

The presence of polysaccharides, such as starch or dietary fibre, in ingredients and ingredient mixtures used in HMEP is most likely important since the phase separation of thermodynamically incompatible biopolymers is considered a key mechanism in fibrous structure formation (Cornet et al., 2022; Tolstoguzov, 1993). Biopolymer

incompatibility is one of the most characteristic properties of biopolymer mixtures and it can also be observed in mixtures of proteins belonging to different classes of the Osborne classification. The incompatibility of proteins with polysaccharides typically increases with salt concentration, temperature, and polysaccharide molecular weight, as well as with protein denaturation (Tolstoguzov, 1993). It is likely that the structuring potential of a multicomponent ingredient in HMEP is related to both molecular (e.g. disulfide bonds) and colloidal mechanisms involving interactions between proteins, polysaccharides, and multivalent ions (Nasrollahzadeh et al., 2023). An additional hypothesis is that the fibrous structure formation is a result of the formation of protein-rich and water-rich domains (syneresis) in the cooling die, driven by crosslinking of proteins during solidification of the material (van der Sman and van der Goot, 2023).

The role of starch in HMEP does not seem to be very clear, although starch is often added to formulations used in the process. Fibrous structures have been successfully produced for example from mixtures of soy protein isolate and wheat starch at a ratio of 9:1 (Lin et al., 2002) and hemp protein concentrate and maize starch at a ratio of 4:1 (Nasrollahzadeh et al., 2022). Chen et al. (2021) studied the effect of amylose and amylopectin addition on HMEP of pea protein isolate (protein-amylose/amylopectin ratio 9:1) and found that fibrillation in HMEP decreased with increasing levels of amylose in the ingredient mixture. On the other hand, Bühlér et al. (2022) found that the type of starch affected the maximum level of inclusion before it interfered with the development of fibrous structures in wheat gluten. Specifically, they discovered that the fibrous structure was compromised by an increased addition of amylopectin-rich starch (at an inclusion level of 5 %), while fibrous structures were highly resilient to amylose-rich starches (up to 20-40 % inclusion). The endogenous starch naturally present in the raw materials could in a similar way as added starch affect structure formation in HMEP.

The formation of covalent bonds through Maillard reactions could also be possible at the high temperatures prevailing in HMEP. The effects of Maillard reactions are, however, not expected to be significant in HMEP due to the low levels of reducing sugars present in the ingredients most commonly used in HMEP (Schmid et al., 2022).

4.1.3. Fat, lipase, and lipoxygenase

The fat content of the legume protein concentrates was 2.7-3.5 %

(Table 2). Flax and Hemp had a very low fat content (<1 %), whereas Wheat and Pea-isolate had higher contents, 5 % and 6.8 %, respectively. The low fat contents of Flax and Hemp suggest that an effective fat removal step was applied to these materials during their production process. The fatty acid compositions of the protein concentrates and isolates showed no significant differences, mirroring the compositions of their respective raw materials (Aluko, 2017; Khrisanapant et al., 2019; Marambe and Wanasundara, 2017). All ingredients were rich in linoleic acid (C18:2), and Flax was also rich in α -linolenic acid (C18:3), both essential fatty acids. The contribution of other fatty acids than those listed in Table 2 was low.

Lipids may affect structure formation in HMEP by acting as plasticisers and lubricants in the process and through interactions with other components present in the formulation (Chen et al., 2023a). The effect of lipids on structure formation can be dependent on how lipids are dispersed in the matrix; lipids naturally present in the raw material may behave differently than plant oils added to the formulation. The fatty acid composition of the lipids may also play a role (Chen et al., 2023b). Meat analogues with fibrous structures have successfully been produced from flour mixtures containing up to 10 % endogenous lipids (see e.g. Jeon et al. (2023)), however, it has been shown that the addition of lipids in the form of plant oil may hamper fibrous structure formation in HMEP (already at 4 % oil content), possibly due to decreased mechanical stresses (and heat) in the system, which arise from decreased viscosity and increased lubrication in the ingredient mixture (Gwiazda et al., 1987; Kendler et al., 2021). The negative effects of lipids can, however, be counteracted to some extent by increasing the screw speed or by adding the oil at the end of the extruder barrel instead of at the front (Kendler et al., 2021).

The activity of lipase and lipoxygenase was clearly higher in legume protein concentrates than in the other ingredients. The lipoxygenase activity was especially high in Pea-yellow (807 $\mu\text{mol/g/min}$). Although the legume concentrates had relatively low-fat contents (<3.5 %), they were rich in polyunsaturated fatty acids (>50 % of total fatty acids), which may cause stability problems during storage in the presence of lipid-oxidizing enzymes. Gao et al. (2020) showed that the lipoxygenase activity in yellow pea protein isolate was affected by the extraction pH and suggested that a higher activity resulted in the formation of beany off-flavours during wet extraction.

4.1.4. Ash

The ash content was the highest in Flax and Hemp, 9 % and 15 %, respectively, whereas legume concentrates had an ash content of only 5–6 % (Table 2). The presence of minerals may affect structuring in HMEP by altering the ionic strength of the matrix and thus affecting the conformational stability of proteins as well as the thermodynamic compatibility of proteins with polysaccharides (Corredig et al., 2011; Nasrollahzadeh et al., 2023). In addition, multivalent ions, such as Ca^{2+} , can affect heat-induced gel formation of proteins and polysaccharides (e.g. pectin). Nasrollahzadeh et al. (2023) suggested that the outstanding fibrous structure formation of pumpkin seed protein in HMEP could be related to its high mineral content that promotes phase separation.

4.1.5. Phytic acid, phenolics and condensed tannins

Phytic acid levels in the protein concentrates ranged from 21 mg/g in Pea-isolate and Pea-brown to 98 mg/g in Hemp (Table 2). The presence of phytic acid may affect structure formation in HMEP through its interaction with minerals. Phytic acid is known to chelate divalent cations and form insoluble complexes which are not dissociated for absorption (Zhang et al., 2022b). In addition, phytic acid has strong interactions with positively charged proteins and polysaccharides via electrostatic connections. Phytate-protein complexes stay intact by digestive enzyme which hinders utilization of amino acids. It has also been demonstrated that interactions between phytate and protein can alter the solubility of proteins (Wang and Guo, 2021), potentially impacting the functionality of proteins in HMEP.

Free phenolic compounds were most abundant in Faba and in Flax, whereas tannin contents of all ingredients were quite low (Table 2). Phenolic compounds (including condensed tannins) can bind to proteins and thus modify the physicochemical properties of proteins (Masoumi et al., 2024), which may affect structure formation in HMEP. The presence of phenolic compounds has also been linked to bitter and astringent tastes of plant-based protein concentrates and isolates (Wang et al., 2022). In addition, phenolic compounds, especially polyphenols, may affect the colour of the extrudates through enzymatic reactions and/or auto-oxidation (Geng et al., 2023). Tannins in legumes are mainly located in the seed coat, which is normally removed in the fractionation process (Singh et al., 2017).

4.2. Physicochemical properties

4.2.1. Particle size and microstructure

The particle size distribution of the ingredients was analysed both in dry form and as dispersed in water (Fig. 1). In dry form, Pea-yellow, Pea-brown and Faba had the smallest and Pea-isolate clearly the largest particle size, which was in accordance with their manufacturing methods; wet-extracted proteins showing higher particle size and dry fractionated clearly lower. When dispersed in water, the particle size of most of the ingredients increased, most likely due to the swelling of hydrated insoluble particles. The relative increase was the highest for Flax, for which the particle size was 2.6-fold higher in water than in dry form. This could be related to the high content of insoluble dietary fibre in Flax. Wheat showed the smallest increase (1.6-fold) in particle size as compared to its dry form. Pea-yellow, Pea-brown and Faba had the smallest particle sizes of all ingredients also when dispersed in water. Pea isolate exhibited significantly different properties compared to other ingredients, as its particle size was considerably smaller in water than in its dry form. The large particle size in dry form may be attributed to thermal processing (drying) of the ingredient during its production, which causes protein denaturation and aggregation. During mixing with water, these aggregates or microgel particles may start degrading in a similar way as gelatinised starch granules.

The observed differences in the measured particle size distributions were confirmed by light microscopic evaluation (Fig. 2). It should be noted that for light microscopy, the ingredients were dispersed in ethanol instead of water to prevent swelling and aggregation of the materials. The microscopy images revealed that Pea-yellow, Pea-brown and Faba contained only very small particles, while in other ingredients larger particles or aggregates were present. Flax and Hemp contained large, dark, or red-coloured sheet-like structures probably originating from the seed outer layers. Native starch granules (based on the presence of Maltese crosses visible in polarized light) were observed only in Wheat, Pea-yellow, Pea-brown and Faba (images not shown). More detailed examination of the Pea-isolate powder in dry form by the reflectance mode in confocal laser scanning microscope (Fig. 2h) revealed the presence of collapsed granule resembling particles.

The particle size distribution of the ingredients may in many ways affect structure formation in HMEP. The particle size is a critical factor in determining the flow of a powder in the feeder of the extruder, in addition to powder morphology, particle-surface interactions and interparticle forces (McGuire et al., 2022). For example, Osen et al. (2014) reported easier processing for larger particles, while Nasrollahzadeh et al. (2023) utilized the knowledge of powder flowability when adjusting the solids feed rate of the extruder. A lower feed rate was chosen for a powder with lower flowability. In our study, the legume protein concentrates possessed particularly small particle sizes in dry form which may hamper their flow in the extruder feeder. The particle size also affects the rate by which particles are hydrated and dissolved in the extrusion process, i.e. the transition from individual particles or granules to a compacted solid to a viscous melt, and eventually the viscosity and flow behaviour of the melt (McGuire et al., 2022). On the other hand, Osen et al. (2014) found no variations in the texture of

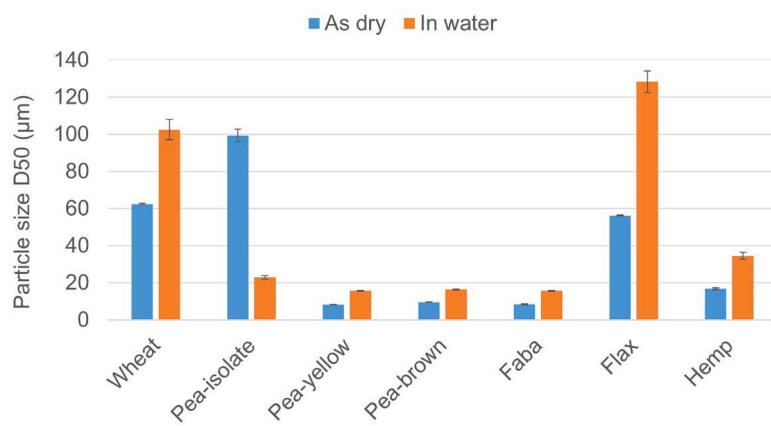


Fig. 1. Particle size (D50) of protein ingredients in dry form and as dispersed in water.

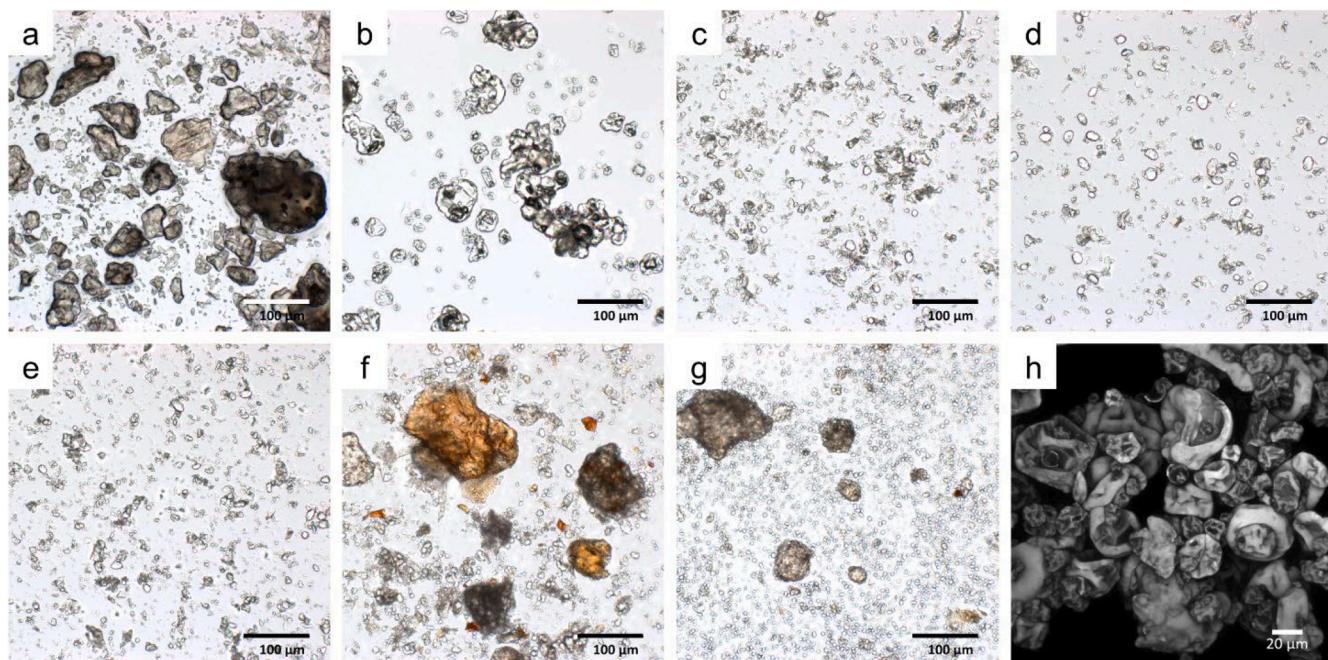


Fig. 2. Brightfield images of a) Wheat, b) Pea-isolate, c) Pea-yellow, d) Pea-brown, e) Faba, f) Flax, g) Hemp and an autofluorescence image by CLSM of h) Pea-isolate. Scale bar represents 100 μm in the brightfield images and 20 μm in the CLSM image.

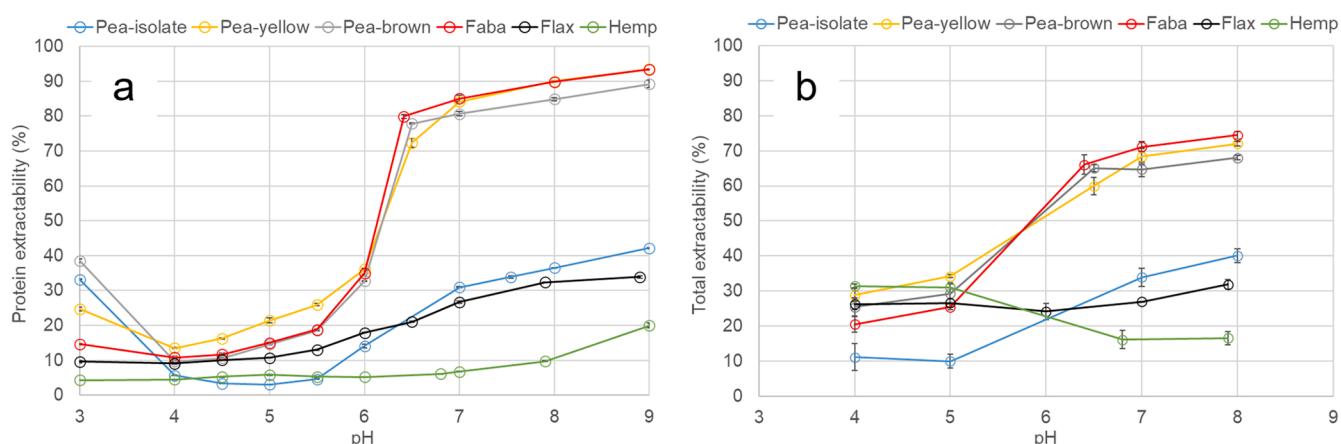


Fig. 3. Protein extractability (a) and total extractability (b) of protein ingredients.

extrudates made from different pea protein isolates with a particle size ranging from 40 to 158 µm (D50). The particle size of a powder is affected by the drying method used. Dry fractionation generally results in a fine powder, which tends to exhibit poor flow characteristics in the extrusion feeder. However, Elshamy et al. (2025) showed that the flow properties of dry fractioned faba bean and yellow pea protein concentrates could be considerably improved by pre-conditioning and pre-processing the powders before HMEP.

4.2.2. Protein and total extractability

There were great differences in the protein extractability (often called solubility in the literature) of the ingredients (Fig. 3a). The protein extractability of all ingredients was below 40 % at pH values ≤ 6. At pH values above 6, the protein extractability of Pea-Yellow, Pea-brown and Faba was relatively high (>70 %) as compared with the other ingredients. A high protein extractability is common for dry fractionated raw materials as the proteins remain in their native state during the process, while other fractionation techniques that require water or solvents with a subsequent drying step, tend to denature the proteins leading to low protein extractability. Hemp had the lowest protein extractability of all ingredients in the studied pH range, with a maximum of 20 % at pH 9. Pea-isolate and Flax showed intermediate protein extractability (18-42 %) at pH values above 6. There were great differences in the protein extractability of the ingredients at their native pH (as 5 % dispersions in water) as shown in Table 3. Hemp had the lowest (6 %) and Faba the highest protein extractability (80 %) at native pH.

The total extractability (Fig. 3b) of the legume ingredients (Pea-isolate, Pea-Yellow, Pea-brown and Faba) followed a similar trend as the protein extractability, i.e. it increased markedly at pH values above 6. For Flax, the total extractability varied only little (24-32 %) in the studied pH range. Hemp behaved differently, as it showed the highest total extractability (31 %) at acidic pH-values and the lowest total extractability (16 %) at pH values above 6. The protein and total extractability of Wheat was not analysed, due to difficulties caused by strong aggregation upon dispersing it in water, but it is generally known that wheat gluten is poorly soluble in water (Arakawa et al., 2008; Hamer et al., 2009).

The solubility of various biopolymers present in the ingredients is considered important for structure formation in HMEP. Protein solubility is dictated by its surface properties, which are influenced by the quantity and arrangement of hydrophilic and hydrophobic amino acid components on the protein surface. Additional factors affecting protein solubility are solvent pH, ionic strength, and temperature. The solubility of proteins in an ingredient can reflect its processing history, as the solubility generally decreases with heat-induced protein denaturation (Lam et al., 2018). The functional properties of plant protein ingredients are, however, not solely determined by the solubility of the proteins. Equally important is the extractability of these proteins from the plant tissue. In plants, proteins are commonly enclosed within cellular

structures that require disintegration to release the proteins. Processing of plant materials can have an impact on both the extractability and the solubility of plant proteins.

In HMEP, opposite to many other plant protein applications, proteins with rather low solubility normally perform the best. For instance, Geerts et al. (2018) reported enhanced fibrillation in HMEP by subjecting a blend of soy protein and soy flour to roasting at 150 °C, which caused a decrease in the nitrogen solubility index from about 90 % to below 40 %. On the other hand, Nisov et al. (2022) did not find a clear link between protein solubility and improved fibrous structure formation by pH-shifting. High levels of other insoluble compounds, such as insoluble dietary fibre, may disturb protein crosslinking and thus be detrimental for fibrous structure formation (Dekkers et al., 2018).

4.2.3. Water hydration and oil binding capacity

The ingredients differed also in their water hydration capacity (WHC) (Table 3). Pea-isolate had the highest (3.3 g/g) WHC, followed by Flax (2.2 g/g). Pea-yellow, Faba and Hemp possessed similar WHCs of 1.1-1.3 g/g. An interesting observation was the low WHC of Pea-brown (0.6 g/g) as compared to Pea-Yellow (1.3 g/g). The WHC of Wheat was not analysed, due to difficulties caused by strong aggregation of the material upon dispersing it in water, but in previous studies rather low WHC values (1.5 -1.9 g/g) have been reported for wheat gluten (Grabowska et al., 2014; Nasrollahzadeh et al., 2023). It should be noted that the method we used for determining the WHC differs somewhat from the conventional method, which uses excess water. In the method we employed, significantly less water is added, avoiding the formation of a liquid phase. This method is considered more suitable for comparing the WHC of plant-based ingredients showing differences in water solubility (Quinn and Paton, 1979).

The WHC of proteins is partly determined by their amino acid composition, with highly charged proteins exhibiting greater electrostatic attraction toward water (Lam et al., 2018). In many studies, an increase in the WHC of plant-based ingredients has been observed after thermal processing (Aguilera et al., 2009; Geerts et al., 2018; Lam et al., 2018; Ma et al., 2011). This could be partly related to increased exposure of hydrophilic groups in the unfolded and denatured proteins, but also to changes in water absorption of the starch and dietary fibre fractions present in the ingredient (Aguilera et al., 2009). WHC is also affected by the porosity of the ingredient matrix (Lam et al., 2018). The high WHC of Pea-isolate in our study could be linked to protein denaturation (wet extracted) and/or to its granular powder structure, while the high WHC of Flax could be a result of its high dietary fibre content. The WHC is associated with the material's capability to create viscous dispersions when introduced to water. Nasrollahzadeh et al. (2023) utilized the WHC data of the ingredients in selecting suitable water feed rates in HMEP. Ingredients with a higher WHC were extruded at higher moisture contents and vice versa.

There were less pronounced differences in the oil binding capacity (OBC) than in the WHC of the ingredients (Table 3). The ingredients with the highest WHC, Pea-isolate and Flax, also showed the highest OBC of 1.5-1.6 g/g. Pea-yellow, Pea-brown, Faba and Hemp had an OBC of 1.2-1.3 g/g, whereas the OBC of Wheat was the lowest (0.9 g/g). Proteins with higher hydrophobicity tend to have higher OBC, but OBC is also affected by the porosity of the ingredient matrix. Thermal processing does not seem to affect the OBC of plant-based ingredients to the same extent as the WHC (Aguilera et al., 2009; Lam et al., 2018; Ma et al., 2011).

4.2.4. Viscosity

The viscosity of the ingredients was studied as 15 % dispersions in water during heating and cooling in the RVA. Examples of viscosity profiles and the appearance of the samples after the heating-cooling cycle are shown in Fig. 4a. Viscosity values extracted from various points of the viscosity profile are presented in Fig. 4b (except for Wheat and Hemp). Before heating, most ingredients exhibited relatively low

Table 3

Protein extractability at native pH (5 % dispersion), water hydration capacity (WHC) and oil binding capacity (OBC) of protein ingredients (na=not analysed).

| Ingredient | Native pH | Protein extractability at native pH (%) | WHC (g water/g sample dm) | OBC (g oil/g sample dm) |
|-------------|-----------|---|---------------------------|-------------------------|
| Wheat | na | na | na | 0.9 ± 0.0 |
| Pea-isolate | 7.5 | 34 ± 0 | 3.3 ± 0.0 | 1.6 ± 0.0 |
| Pea-yellow | 6.5 | 72 ± 1 | 1.3 ± 0.0 | 1.2 ± 0.0 |
| Pea-brown | 6.5 | 78 ± 0 | 0.6 ± 0.0 | 1.3 ± 0.0 |
| Faba | 6.4 | 80 ± 1 | 1.1 ± 0.1 | 1.2 ± 0.0 |
| Flax | 6.0 | 18 ± 0 | 2.2 ± 0.0 | 1.5 ± 0.0 |
| Hemp | 6.8 | 6 ± 0 | 1.1 ± 0.0 | 1.2 ± 0.0 |

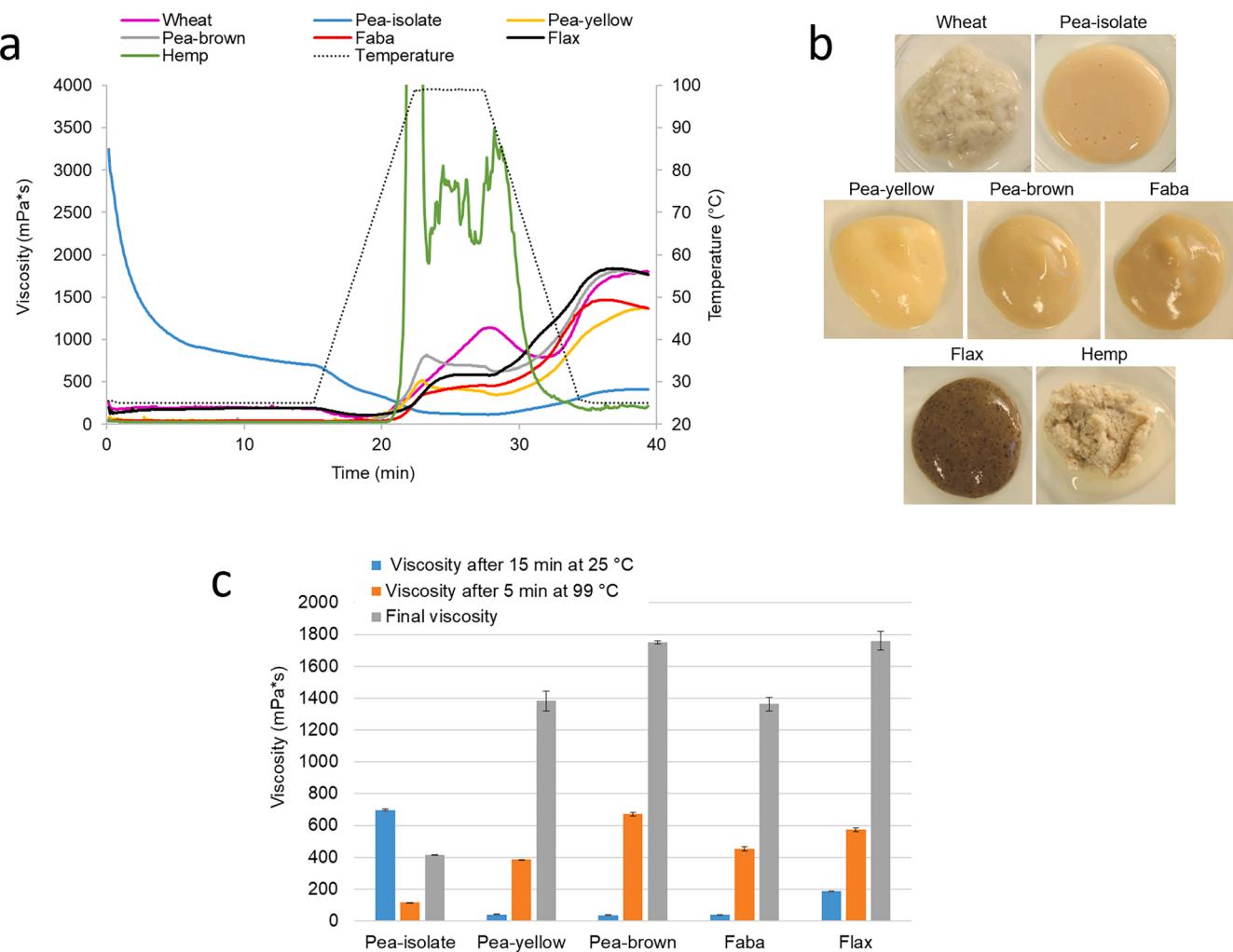


Fig. 4. Results of viscosity measurements with the Rapid Visco Analyser. a) Examples of viscosity profiles of 15 % protein ingredient dispersions during heating and cooling, b) photos of the dispersions after heating and cooling and c) viscosity values extracted from the profiles after 15 min at 25 °C (initial viscosity), after 5 min at 99 °C and after cooling to 25 °C (final viscosity).

viscosity, but a notable rise in viscosity occurred upon heating. During cooling a further increase in viscosity was typically noted. The viscosity of Pea-isolate stood out from the rest of the ingredients due to its notably high initial viscosity, which subsequently decreased during the initial phase at 25 °C and during heating. Similar behaviour was reported in the study by Osen et al. (2014). This phenomenon may be attributed to the gradual hydration and disintegration of the granular powder. During cooling a slight increase in viscosity was observed for Pea-isolate. Most ingredients formed homogeneous, smooth dispersions after heating and cooling (Fig. 4b), except Wheat and Hemp which showed an aggregated appearance. Wheat aggregated directly upon mixing with water at room temperature while Hemp aggregated during heating. The aggregation caused variability in the RVA results, and no viscosity values were therefore extracted from the viscosity profiles of these samples. It was, however, an interesting observation that Wheat and Hemp showed such a strong tendency to phase separation already at concentrations and temperatures much lower than those applied in HMEP. Similar aggregation of hemp protein and variability in RVA profiles have also been reported in previous studies (Nasrollahzadeh et al., 2022; Zahari et al., 2023, 2020). This could be related to the high content of free sulphydryl groups in hemp protein, which increases its tendency to aggregate in water through the formation of disulfide bonds (Tang et al., 2006) in a similar way as for wheat gluten (Hamer et al., 2009). The aggregation tendency seems to be stronger for gluten, as it aggregates spontaneously

already after water addition (Unbehend et al., 2004), whereas heating is needed to induce the aggregation of hemp protein. Gluten is also rich in glutamine, which has a strong tendency to participate in hydrogen bonding (Kasarda, 1999).

The extrusion process is highly affected by the rheological properties of the ingredient mixture. The shear stresses (and heat) generated by screw rotation inside the extruder are dependent on the viscosity of the ingredient mixture and the structure of the extruded product is governed by the flow behaviour of the melt in the cooling die (Wittek et al., 2021). Rheological measurements are, however, challenging to perform under conditions relevant for HMEP. Although the RVA measurements in our study were performed at much lower concentration, temperature, and shear rates than those encountered in HMEP, they could still be useful for comparing viscosities among various ingredients and in choosing appropriate water feeds for HMEP. A better understanding of the rheological properties of materials at temperatures relevant to HMEP could be obtained with a newer version of the RVA capable of heating up to 140 °C (Yuan et al., 2021) or with a closed cavity rheometer (Wittek et al., 2021), although HMEP conditions cannot fully be mimicked in those instruments either.

4.2.5. Differential scanning calorimetry (DSC)

DSC measurements were conducted to get information on the extent of protein denaturation and starch gelatinisation in the ingredients.

Clear endothermic peaks in the DSC thermograms were observed in the first heating run for Faba, Pea-brown, Pea-yellow and Hemp (Fig. S1-S5). The peaks were not observed in the second heating, indicating that the thermal transitions resulted from irreversible changes in the materials. For plant-based ingredients, such thermal transitions are typically attributed to starch gelatinisation, protein denaturation, or the dissociation of amylose-lipid complexes. In this study, the legume protein concentrates exhibited two overlapping peaks at temperatures ranging from 75 to 120 °C (Fig. S2-S4). These peaks likely indicate the denaturation of various protein fractions in the ingredients (Arntfield et al., 1986; Osen et al., 2014; Sim et al., 2019), suggesting mild temperatures were applied during their processing. This is an expected result for legume protein concentrates produced by dry fractionation. The legume protein concentrates also contained small amounts of starch, which is assumed to be at least partly in native form in the dry fractionated ingredients. Starch gelatinisation typically occurs at lower temperatures compared to protein gelatinisation; however, no distinct endothermic peaks at temperatures typical for starch gelatinisation were observed in the DSC thermograms for the legume concentrates. The DSC instrument might have lacked the sensitivity to detect thermal transitions in the small starch amounts present in the samples.

Hemp exhibited a distinct endothermic peak between 80-115 °C (Fig. S5), which was attributed to protein denaturation. Previous studies have shown that even harsh wet extraction processes do not always fully denature hemp proteins ((Hadnadev et al., 2018; Nasrollahzadeh et al., 2022). The considerable amount of undenatured protein in Hemp was surprising considering the low protein extractability observed for the ingredient (see section 4.2.2).

The lack of endothermic peaks in Pea-isolate and Flax indicates that the proteins in these ingredients were fully denatured during their production process (Osen et al. 2014). No peaks were detected in the thermograms for Wheat either. It is known that gluten does not exhibit a clear denaturation peak in DSC analysis (Hamer et al., 2009), which means that DSC measurements do not provide information on any heat treatments that may have been applied during the production of gluten ingredients. For flax, a broad exothermic peak was observed above 130 °C. The cause of this peak remains uncertain. However, if it is not an artefact, it may be associated for example with crosslinking reactions taking place at elevated temperatures (Lukin et al., 2024). For other ingredients, a slight exothermic heat flow increase was also detected above 130 °C. It is important to recognise that measuring thermal transitions in multi-component plant materials is complex, requiring careful interpretation of the results.

4.3. Comparison of ingredients

Principal component analysis (PCA) was used as a tool to compare the plant protein ingredients with each other. In the first analysis (Fig. S6), only the chemical composition of all ingredients was included. According to the analysis, wheat-, legume- and oilseed-based ingredients were clearly separated from each other, which suggests major differences in their chemical composition. Wheat was linked to a high protein, proline and glutamic acid content, while Pea-yellow, Pea-brown and Faba to high contents of lysine, stachyose, verbascose, as well as lipase and lipoxygenase activities. Hemp was associated with a high level of methionine and cysteine, while Flax had the highest levels of several amino acids such as tryptophan and glycine.

In the second analysis (Fig. S7), all analysed properties were included for all ingredients except for Wheat (due to missing physico-chemical data). The results showed much clearer separation of Pea-isolate from the other legume-based ingredients than when only chemical data was considered. The oilseed ingredients were also clearly separated into their own group. Characteristics typical for Pea-isolate were a high protein content, high water- and oil-binding capacity, high initial viscosity, and large particle size in dry form. Some of the typical characteristics for legume protein concentrates were high

contents of tyrosine, starch, stachyose, high lipase and lipoxygenase activity and high protein extractability. Characteristics specific for the oilseed ingredients were high contents of methionine, cysteine, arginine, glycine, soluble high Mw dietary fibre, phytic acid and ash, as well as a large particle size in water.

The PCA results indicated that the protein concentrates were not particularly similar to the reference ingredients in the analysed properties. However, notable differences between the reference ingredients Wheat and Pea-isolate were also observed. A summary of the properties potentially relevant for fibrous structure formation is shown in Table 4. Some common properties of the reference ingredients were identified, i. e. high protein content (87-89 %), high fat content (5.0-6.8 %), low dietary fibre content (7-10 %), low protein extractability (up to 34 %), large particle size in dry form (D50 60-100 µm) and high degree of protein denaturation (no peaks in DSC) as compared to the other ingredients. Hemp exhibited similarities to the reference ingredients in terms of its high protein content, low dietary fibre content and low protein extractability. Hemp was also rich in cysteine residues and ash and showed strong phase separation (aggregation) after heating, which according to literature (as discussed above), could be additional properties advantageous for the formation of fibrous structures in HMEP. In fact, extrudates with promising textural properties have in some previous studies been produced from hemp protein alone (Amagliani et al., 2023) or in combination with other plant proteins (Nasrollahzadeh et al., 2022; Zahari et al., 2023, 2020). Flax, like Hemp, had low protein extractability, high cysteine levels, and significant protein denaturation. However, its low protein and high insoluble fibre content may limit its functionality in HMEP. The high protein extractability of the legume concentrates may be a barrier for their applicability in HMEP.

Nutritional quality and flavour-related factors cannot be ignored when assessing the suitability of a plant-based ingredient for HMEP. The composition of plant protein concentrates gives, in general, nutritional benefits over more protein-rich isolates, being richer in dietary fibre and micronutrients. In this study, the concentrates were clearly richer sources of dietary fibre than the reference isolates and their somewhat higher ash content indicated higher mineral contents. In an earlier study, contents of several B vitamins were also markedly higher in legume concentrates than isolates (Siitonен et al., 2024). Legume proteins generally have less methionine than good quality reference proteins but are rich in lysine as seen also in this study. On the other hand, the oilseed proteins, Hemp and Flax, had a favourable essential amino acid composition in comparison with the other ingredients.

A drawback of dry-fractionated plant protein concentrates is the retention of antinutrients and FODMAP compounds in the ingredients, unlike in protein isolate production where they are usually substantially reduced or eliminated (Vogelsang-O'Dwyer et al., 2020). Among the studied ingredients, the high phytic acid contents in Hemp and Flax and the high content of RFOs in pea protein concentrates can be challenging. Extrusion processing may lower the levels of some antinutrients (Nikmaram et al., 2017), but for example RFOs present in dry fractionated pea protein concentrate have been found to be unaffected by HMEP (Nyssölä et al., 2021). Considering nutrition, phytic acid may also have positive effects, e.g. due to its antioxidant action (Kumar et al., 2021).

Flavour-related factors evaluated in this study included lipid modifying enzymes, lipase and lipoxygenase, soluble phenolic compounds, and condensed tannins. All the ingredients had a relatively low fat content, primarily consisting of polyunsaturated fatty acids. This composition together with a high lipoxygenase activity, as observed in the legume concentrates, could induce off-flavours during storage if the enzymes are not inactivated during processing. Phenolic compounds, associated with bitter taste and astringency, were found at the highest level in Faba. In addition to their effects on flavour, some of the phenolic compounds have been documented as bioactive compounds with several potential health benefits (Singh et al., 2017). Interestingly, both lipoxygenase activity and free phenolic content were especially low in

Table 4

Summary of ingredient characteristics potentially relevant for structure formation in HMEP. The magnitude of each property is marked as follows: * low, ** medium and *** high value (within the range of each property). Note that the star ratings are not indicative of the importance of the listed properties.

| | Wheat | Pea-isolate | Pea-yellow | Pea-brown | Faba | Flax | Hemp |
|-------------------------------------|-------------------|-------------|------------|-----------|------|-------|-------------------|
| Protein content | *** | *** | * | * | ** | * | ** |
| Proportion of cysteine | *** | * | ** | * | ** | *** | *** |
| Starch content | *** | * | ** | *** | ** | * | * |
| Dietary fibre content | * | * | ** | ** | ** | *** | * |
| Fat content | *** | *** | ** | ** | ** | * | * |
| Ash content | * | ** | ** | ** | ** | *** | *** |
| Protein extractability ¹ | * | ** | *** | *** | *** | ** | * |
| Total extractability ¹ | * | ** | *** | *** | *** | ** | * |
| WHC | ** | *** | ** | * | ** | *** | ** |
| OBC | * | *** | ** | ** | ** | *** | ** |
| Viscosity at 25 °C | aggr ² | *** | * | * | * | ** | * |
| Viscosity at 99 °C | aggr ² | * | ** | *** | ** | *** | aggr ² |
| Viscosity after heating and cooling | aggr ² | * | ** | *** | ** | *** | aggr ² |
| Particle size dry | ** | *** | * | * | * | ** | * |
| Particle size wet | *** | * | * | * | * | *** | ** |
| Peaks in DSC | no | no | yes | yes | yes | (yes) | yes |

¹) at native pH;

²) sample aggregated

Hemp. The low content of condensed tannins present in the ingredients is not expected to affect sensory quality.

5. Conclusions

This study represented the first phase of an investigation aimed at evaluating the applicability of various plant protein concentrates for HMEP. In this phase the assessment was carried out by comparing the chemical composition and physicochemical properties of the protein concentrates with those of two reference ingredients (protein isolates) more commonly used in HMEP. The results showed that none of the protein concentrates fully matched the properties of the reference ingredients in the analysed properties, however, notable differences between the two reference ingredients were also observed. This suggests that fibrous structures in HMEP can be formed from ingredients with diverse properties, potentially through varied mechanisms or under different processing conditions. Among properties potentially important for fibrous structure formation, Hemp was found to be similar to the reference ingredients in terms of its high protein content, low dietary fibre content and low protein extractability. In addition, Hemp was rich in cysteine residues and ash, exhibiting strong aggregation during heating. Flax also showed potential due to its low protein extractability, high proportion of cysteine and a high extent of protein denaturation, although its low protein content may restrict its functionality in HMEP. The high protein extractability of the legume concentrates may be a barrier for their application in HMEP. From a nutritional point of view, protein concentrates may bring benefits through their higher content of dietary fibre, although the high levels of RFOs in pea and phytic acid in hemp, can be a disadvantage. In addition, the high level of phenolic compounds may cause bitterness in faba bean concentrates and the high activities of lipase and lipoxygenase off-flavours in legume concentrates.

A more comprehensive understanding of how these ingredients perform in HMEP will emerge through the second phase of this investigation in which the ingredients are used in real HMEP trials (reported in an upcoming paper). An increased understanding of the interplay between ingredient properties and the formation of fibrous structures in HMEP would facilitate the selection of the most suitable ingredients for the process without the need for extensive screening trials under real processing conditions. Additionally, it would enable the market introduction of new plant protein ingredients that are specifically tailored for HMEP.

Ethical statement

The authors declare that no human or animal subjects were involved in the experiments conducted for this paper.

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Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT (OpenAI) and Microsoft Copilot in order to improve the readability and language of the manuscript. After using these tools, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

CRediT authorship contribution statement

Martina Lille: Methodology, Visualization, Investigation, Writing – original draft. **Minnamari Edelmann:** Methodology, Supervision, Investigation, Writing – original draft. **Heikki Aisala:** Writing – review & editing, Formal analysis, Visualization. **Ulla Holopainen-Mantila:** Visualization, Writing – review & editing, Investigation. **Anna-Maija Lampi:** Methodology, Writing – original draft. **Anni Nisov:** Writing – review & editing, Conceptualization. **Vieno Piironen:** Writing – original draft, Methodology. **Pinja Pöri:** Writing – review & editing, Investigation. **Fabio Tuccillo:** Methodology, Investigation. **Yaqin Wang:** Methodology, Investigation. **Kaisu Honkapää:** Supervision, Funding acquisition, Writing – review & editing, Project administration, Conceptualization. **Nesli Sozer:** Writing – review & editing, Project administration, Conceptualization, Supervision, Funding acquisition.

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.

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Supplementary materials

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Data availability

Data will be made available on request.

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