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Assessing the digestibility and estimated bioavailability/ bioaccessibility of plant-based proteins and minerals from soy, pea, and faba bean ingredients

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ABSTRACT

Concerns have been raised about the nutritional adequacy of plant-based foods due to the presence of anti-nutrients and overall low protein digestibility. Therefore, this study characterizes the estimated bioavailability/ bioaccessibility of iron and zinc and the protein digestibility of 11 commercially available plant-based ingredients to assess their potential in the future development of nutritious plant-based foods. The accessibility of iron and zinc was limited in all ingredients, with only faba bean isolate, pea isolate, faba bean concentrate and texturized pea containing accessible iron. Faba bean isolate was found to have the highest amount of accessible iron (67.4 mg/kg) whereas textured pea showed the lowest amount (0.5 mg/kg). The estimated bioavailability of iron and zinc, based on the calculated molar ratio of phytate, was low for all studied ingredients, with isolates showing the highest overall tendency for available iron and zinc. The amino acid composition data revealed limitations regarding valine and/or isoleucine in all protein concentrates and texturized proteins, soy isolate, and faba bean flour. In contrast, no significant differences were found in overall protein digestibility, suggesting that all tested raw materials, including faba bean, can be considered good protein sources.

1. Introduction

Iron deficiency is the most common nutritional disorder in the world and is a public health problem in both industrialised and non-industrialised countries. In 2016, 41.7% of children younger than five years, 40.1% of pregnant women and 32.5% of non-pregnant women worldwide were anaemic (Pasricha, Tye-Din, Muckenthaler, & Swinkels, 2021; WHO, 2017a, 2017b). Inadequate nutritional iron uptake is a major cause of iron deficiency. While haem iron is efficiently absorbed, non-haem iron has a lower bioavailability and its uptake is influenced by numerous factors such as the presence of antinutrients e.g. phytate, that is abundant in plant foods (Rousseau, Kyomugasho, Celus, Hendrickx, & Grauwet, 2020).

Phytate (*myo*-inositol hexakisphosphate, IP6) inhibits iron and zinc absorption from plant-based foods, e.g. legumes, cereals and seeds. The phosphate groups on the inositol ring can form insoluble complexes with cations, reducing uptake of minerals in the gastrointestinal tract (Lönnerdal, Sandberg, Sandström, & Kunz, 1989; Rousseau et al., 2020; Urbano et al., 2000). In addition, phytate can bind to proteins through electrostatic charges at low pH or through salt bridges at high pH. This, together with other external factors (e.g. pH, temperature, ionic strength conditions) and internal factors (e.g. protein amino acid profile, protein folding and crosslinking), has a negative influence on the digestibility of plant-based proteins (Herreman, Nommensen, Pennings, & Laus, 2020; Joye, 2019; Kumar, Sinha, Makkar, & Becker, 2010). The amount of phytate in different raw materials and foods differs between crops (Zhang, Stockmann, Ng, & Ajlouni, 2022), varieties (Kumar et al., 2005; Mayer Labba, Frøkiær, & Sandberg, 2021; Oomah et al., 2011), growing conditions (Urbano et al., 2000) and processing conditions for the raw materials (Al-Wahsh, Horner, Palmer, Reddy, & Massey, 2005; Taherian et al., 2011).

To investigate the bioavailability of minerals and proteins, *in vitro* methods and animal and human studies can be used (Dias, Costa, Nutti, Tako, & Martino, 2018; Fuller & Tomé, 2005). Although human studies are preferable, static *in vitro* digestion models are generally able to predict outcomes of *in vivo* digestion (Bohn et al., 2018). However, large

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variations between *in vitro* methodologies often limit comparison of results from different studies (Sulaiman, Givens, & Anitha, 2021).

The INFOGEST protocol is a standardized static *in vitro* digestion method (Brodkorb et al., 2019) that is affordable and relatively easy to use, allowing for wide-scale screening of different plant-based ingredients and products (Zhou, Tan, & McClements, 2023). The protocol has been widely used to study macronutrient digestion (Santos-Hernández et al., 2020; Sousa et al., 2023), but more research is needed to identify *in vitro-in vivo* correlations regarding digestibility and bioavailability of micronutrients.

Calculated phytate/mineral molar ratio provides an estimate of mineral bioavailability that can be useful for comparing and classifying foods based on nutrient bioavailability (Hurrell & Egli, 2010; Panel & Nda, 2014). *In vitro* methods are useful for preliminary screening to assess mineral bioaccessibility in a range of foods and staple crops, evaluate the effects of processing conditions and assess other approaches such as fortification to improve iron bioavailability (Sulaiman et al., 2021). Bioavailability refers to the proportion of a compound that is absorbed by intestinal cells and reaches the target tissues in intact or metabolised form, whereas bioaccessibility measures the proportion of a compound that is released from the food matrix during digestion and is accessible for absorption (Rodrigues et al., 2022).

In this study, 11 commercially available plant-based ingredients were screened for their bioaccessibility of iron and zinc, by measuring the soluble mineral fractions obtained in the supernatants after *in vitro* digestion. The *in vitro* results were compared with the estimated mineral bioavailability obtained from calculations of the mineral: phytate molar ratios. Furthermore, the degree of protein hydrolysis (DH) of the commercial ingredients was measured after *in vitro* digestion to estimate the overall protein digestibility. The main purpose of the current work was to characterize and compare the different plant-based ingredients to assess their potential in future development of plant-based foods with improved nutritional properties.

2. Material and methods

2.1. Raw materials

A total of 11 commercially available plant-based raw materials obtained from soy, pea and faba bean from five different suppliers were included in the study (Table 1). Based on specifications from the manufacturers and/or total protein content, the raw materials were categorized into flours (<300 g/kg protein), concentrates (400–700 g/kg protein) isolates (>700 g/kg protein) and textured protein. According to the specifications from the manufacturers, the textured proteins were described as extruded proteins however no detailed information on the process was provided.

Table 1
Overview of the raw materials analysed, product description and supplier. Products were categorized into flours, concentrate, isolates (based on their protein content) and textured protein.

Category	Description according to specification	Producer/Company
Pea flour	Pea flour F200X	Vestkorn
Faba bean flour	Faba bean flour F200X	Vestkorn
Pea concentrate	Pea protein F55X	Vestkorn
Faba bean concentrate	Faba bean protein 60 - Deflavoured	AGT Foods
Soy concentrate	Soy protein concentrate 066–400 Arcon S	ADM
Pea isolate	Pisane C9	Cosucra Groupe Warcoing
Faba bean isolate	Faba bean protein – 90C -EU	AGT Foods
Soy isolate	SUPRO 595 IP	Solae
Pea texturized	Textured pea protein P6501M	Vestkorn
Faba bean texturized	Textured faba bean protein F6501M	Vestkorn
Soy texturized	Soy protein concentrate T158 Arcon T	ADM

2.2. Chemical analysis

The concentrations of fat, starch, neutral detergent fibre (NDF) and acid detergent fibre (ADF) in the different raw materials were measured at the Analysis Laboratory, Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Ultuna. Total fat content was determined as described in the Official Journal of the European Communities, Commission Directive 152/2009 EC (2009), with a Hydrotec 8000 Soxtec Extraction Unit (Foss Analytical A/S Hillerød, Danmark) used for extraction. Starch content was determined using a method described by Larsson and Bengtsson (1983). Briefly, water-soluble carbohydrates were extracted in acetate buffer (60 °C). Non-water soluble starch was enzymatically hydrolysed in two steps using alpha-amylase (95 °C) and amyloglucosidase (95 °C). Glucose was then phosphorylated to glucose-6-phosphate. Finally, glucose-6-phosphate was oxidized by glucose-6-phosphate dehydrogenase to gluconate-6-phosphate, reducing NADP to NADPH. The absorbance for NADPH was measured at 340 nm and is directly proportional to glucose concentrations. The final starch content was then calculated from the glucose concentrations obtained from the water-soluble carbohydrate fraction and the hydrolysed non-water-soluble starch fraction. Concentration of NDF was determined using a method described by Van Soest, Robertson, and Lewis (1991), while AOAC official method 973.18 was used to determine acid detergent fibre (ADF). All analyses were performed in duplicate.

2.2.1. Protein

Crude protein content in the materials was determined by the Kjeldahl method, using a conversion factor of 6.25 (FAO/WHO, 2011). The measurements were performed in duplicate, using a DT 220 Digestor system followed by a Kjeldahl protein-determining Kjeltec 8200 system (Foss Analytical A/S, Hillerød, Denmark).

2.2.2. Ash and dry matter content

Ash content was measured according to AOAC official method 942.05. In brief, samples were weighed, incinerated in a muffle furnace (Model 62700, Barnstead Thermolyne Corporation, Ramsey, USA) at 550 °C for 12 h, cooled in a desiccator for 1 h and re-weighed. Dry matter content was determined according to AOAC official method 934.01, by drying the samples to constant weight (>16 h) in a convection oven (Model 2000655, J:P: Selecta, Barcelona, Spain) at 105 °C. Both analyses were performed in duplicate.

2.2.3. Amino acid composition

Amino acid composition was determined using the method described by Özcan and Şenyuva (2006) with minor modifications. In brief, proteins were hydrolysed by adding 8 mL 6 mol/L HCl to 0.1 g of sample, followed by incubation for 24 h at 110 °C. The volume was then adjusted to 10 mL using Milli-Q water (18.2 MΩ cm) and the samples were centrifuged for 3 min at 20,000×*g* (Thermo IEC Micromax Centrifuge with Thermo IEC 851 rotor, Waltham, USA) and injected into the LC-MS system [Agilent 1260–1290 Infinity LC System with a Phenomenex (Phenomenex Inc., Torrance, USA) column (C18 (2) 250 mm × 4.6 mm, 3 μm), coupled to an Agilent 6120 single Quadrupole MS in the SIM-positive mode] (Agilent Inc., Santa Clara, CA, USA), using an injector volume of 2 μL. Mobile phase A consisted of 30 ml/L MeOH, 2 ml/L formic acid and 0.1 ml/L acetic acid (HAc), while mobile phase B contained 500 ml/l MeOH, 2 ml/L formic acid and 1 ml/L HAc. The initial gradient was held for 8 min and comprised 94% A and 6% B. The gradient was gradually changed until it reached 80% A and 20% B after 20 min. This gradient was held for 27 min before gradually being altered to reach 94% A and 6% B at a run time of 28 min, which was held for a total run time of 40 min. To derive the standard curve, 18 amino acids

(20088 Amino Acid Standard H, Thermo Scientific™, Waltham, USA), supplied at 2.5 mmol/L (except cysteine, 1.25 mmol/L), each in 0.1 mol/L HCl, were diluted in a concentration range of 1–20 mg/L using 0.2 mol/L HAc. Each measurement was performed in triplicate. During the acid hydrolysis, tryptophan is decomposed and could therefore not be quantified. Although the acid hydrolysis is not optimal for all amino acids, we used this procedure for all protein samples to enable direct comparisons between the various protein sources.

2.2.4. Minerals

The concentrations of iron and zinc in the raw materials were determined in triplicate by atomic absorption spectrometry (240/280 Series AA Systems; Agilent, Santa Clara, USA). For the calibration, a standard curve with concentration range 0.125–0.5 mg/L was used for iron (iron Standard for AAS, 16596 Supelco, Bellefonte, USA) and concentration range 0.2–0.8 mg/L for zinc (Zinc 2% HNO_3, P10010532, CAS 7440-66-6, SPEX CertiPrep™, Metuchen, USA). All measurements were carried out as recommended by the manufacturer. Before measurement, samples were microwave-digested (Milestone Microwave Laboratory System, EthosPlus, Sorisole, Italy) under acidic conditions, as described by Fredrikson, Carlsson, Almgren, and Sandberg (2002). For this, 0.15 g of sample were mixed with 7 mL Milli-Q water, 1.75 mL concentrated HNO_3 (Nitric Acid TraceMetal™ Grade, Fisher Chemical™, Waltham, USA, A509-P500, CAS 7697-37-2) and 0.35 mL HCl 34–37% (Hydrochloric Acid TraceMetal™ Grade, Fisher Chemical™, Waltham, USA, A508-P1, CAS 7647-01-0) in a Teflon vial. The samples were digested at 180 °C for 20 min, followed by a cooling down phase of 20 min, decanted into test tubes and the volume was adjusted to 12 mL using Milli-Q water.

2.2.5. Phytate analysis

Phytate (inositol hexakisphosphate, IP6) concentrations were measured using high-performance ion chromatography (HPIC) coupled with a UV–vis detector (UV-4075; Jasco, Oklahoma City, OK, USA) as described previously (Carlsson, Bergman, Skoglund, Hasselblad, & Sandberg, 2001). In the extraction step, 0.5 g of dry matter was mixed with 10 mL 0.5 mol/L HCl for 3 h. The extract was then centrifuged at 12,000×*g* for 5 min and transferred to an HPLC vial. To elute IP6, an isocratic eluent (800 ml/L 1 mol/L HCl, 200 ml/L Milli-Q water) was used (HPLC pump: 14.5 MPa; model PU-400oi; Jasco Inc., Easton, MD, USA) at a flow rate of 0.8 mL/min. The injection volume was 50 µL. The eluent was mixed with ferrous nitrate at 14.5 MPa, flow rate 0.4 mL/min, using an HPLC pump (model PU-4180; Jasco, Oklahoma City, OK, USA) equipped with a PA-100 guard column and a DIONEX CarboPac PA-100 column (Thermo Scientific™, Waltham, USA). After the post-column reaction, IP6 was detected at 290 nm in a UV–visible HPLC detector. The total run time of each sample was 7 min and the IP6 concentration was calculated using external standards with concentration range 0.1–0.8 mmol/L. The analysis was performed in triplicate.

2.3. Calculation of iron and zinc bioavailability

To obtain estimates of relative iron and zinc bioavailability in the raw materials, molar ratio of phytate to minerals (Phy:Fe; Phy:Zn) was calculated using molecular mass for phytate of 660.3 g/mol. For iron, Phy:Fe is suggested to be < 1, or preferably <0.4, to significantly improve non-haem iron absorption from plant-based meals (Hurrell & Egli, 2010). According to the European Food Safety Authority (EFSA), Phy:Zn < 5 corresponds to high zinc absorption, Phy:Zn = 5-15 is defined as moderate absorption and ratios >15 represent low bioavailability (Panel & Nda, 2014).

2.4. In vitro digestion

2.4.1. Chemicals and enzymes

Chemicals and enzymes were purchased from Sigma-Aldrich, St. Louis, USA and comprised bile extract porcine (B8631, CAS 8008-63-7), pancreatin from porcine pancreas 8xUPS (P7545, CAS 8049-47-6) and pepsin from porcine gastric (P7012, CAS 9001-75-6). To determine enzyme activity assays were carried out as described in supplementary information provided by Brodkorb et al. (2019). However, to measure trypsin activity, small adjustments were made as described by Sousa et al. (2023). In brief, pancreatin was suspended in simulated intestinal fluid at a concentration of 1.67 µkat trypsin/mL digest and vortexed for approximately 10 s, followed by ultrasound treatment (Ultrasound Bath Elma S15, 50/60 Hz, 35 W, Elma Schmidbauer GmbH, Singen, Germany) at room temperature for 5 min. Thereafter, the suspension was centrifuged (SORVALL LYNX 6000 Centrifuge, Thermo Fisher Scientific, Waltham, USA) for 5 min at 2000×*g* and 4 °C. The supernatant was transferred to a new tube, immediately placed on ice and used for trypsin activity measurements. The same preparation method was used during the digestion experiments. The concentration of bile salts in the bile extract was determined using a Bile Acid Assay Kit (Sigma-Aldrich MAK309).

2.4.2. Sample preparation

An amount of substrate corresponding to 0.2 g of protein was used in each digestion. Before digestion, powders were suspended in Milli-Q water. The texturized samples were ground with mortar and pestle (particle size <2 mm) before water was added. All samples were stirred at 4 °C for at least 12 h before digestion.

2.4.3. In vitro digestion protocol

The *in vitro* digestion was carried out as described previously (Brodkorb et al., 2019), with small adjustments as described by Sousa et al. (2023). All digestion experiments were performed in triplicate, including one blank consisting of simulated fluids (prepared by diluting electrolyte stock solutions as described by Brodkorb et al. (2019)), and enzymes (pepsin activity 32.12 µkat/mg, trypsin activity in pancreatin 0.13 µkat/mg, bile acid concentration 1.84 mmol/g) but with samples replaced by water. The samples were incubated at 37 °C in a shaking water bath (Julabo SW23, Jumbo GmbH, Seelbach, Germany) at 100 rpm.

In the oral phase of *in vitro* digestion (2 min, 37 °C), 5 g of suspension (40 g/kg protein) was mixed with 4 mL simulated salivary fluid (pH 7), 25 µL 0.3 mol/L $CaCl_2$ and 0.975 mL Milli-Q water. Salivary α-amylase was omitted in the oral phase since it is considered to have limited impact on final protein digestion (Pälchen et al., 2021). In the gastric phase (120 min, 37 °C), 8 mL simulated gastric fluid (SGF) and 5 µL 0.3 mol/L $CaCl_2$ were added, the pH was adjusted to 3 using 1 mol/L HCl, and 0.5 mL pepsin with 33.33 µkat/mL digesta was added to the mixture. Finally, Milli-Q water was added to the mixture to reach a total volume of 20 mL. In the intestinal phase (120 min, 37 °C), 8.5 mL simulated intestinal juice (SIF) and 40 µL 0.3 mol/L $CaCl_2$ were added and the pH was adjusted to 7 using 1 mol/L NaOH. Pancreatin was prepared as described earlier and 5 mL pancreatin diluted in SIF mix (1.67 µkat trypsin/mL of total digesta) and 2.5 mL bile/SIF mix (10 mmol/L of total digesta) were added. Finally, Milli-Q water was added to the mixture to reach a total volume of 40 mL. Weight and pH of the digesta were monitored through the different digestion steps and the final pH after digestion was <7.42 for both the blanks and samples. After 120 min in the intestinal phase, the digestion process was stopped by addition of Pefabloc and/or snap-freezing in liquid nitrogen.

For preparation of samples for determination of degree of protein hydrolysis, 0.5 mL of each digesta sample was mixed with 25 µL (23.96 mg/mL) Pefabloc (Sigma-Aldrich, Pefabloc SC, 76307, CAS 30827-99-7), frozen in liquid nitrogen and stored at −20 °C until further analysis. The remaining sample was snap-frozen using liquid nitrogen and stored at −80 °C before freeze-drying (Heto LyoPro 3000, condenser −53.8 °C, Pressure 0.080 hPa, Thermo Fisher Scientific, Waltham, USA).

2.4.4. In vitro protein digestibility and degree of hydrolysis

The digestibilities of the *in vitro* digested raw materials were assessed by measuring free amino groups in the intestinal digests (degree of protein hydrolysis, DH). DH was determined in triplicate, using the *o*-phthaldialdehyde (OPA) method (Nielsen, Petersen, & Dambmann, 2001). For the OPA reagent, 7.62 g sodium tetraborate decahydrate (Sigma-Aldrich, S9640, CAS 1303-96-4) and 0.2 g sodium dodecyl sulphate (SDS, Sigma-Aldrich, L5750, CAS 151-21-3) were dissolved in 150 mL Milli-Q water. Once the reagent components were completely dissolved, 160 mg phthaldialdehyde 97% (OPA, Sigma-Aldrich, P1378, CAS 643-79-8), were dissolved in 4 mL ethanol, and 176 mg DL-dithiothtreitol (DTT, Sigma-Aldrich, D0632, CAS 3483-12-3) were added to the reagent. Finally, the solution was made up to a total volume of 200 mL and stored for <2 h in darkness until use. For the serine standard, a concentration range of 0.185–0.95 mmol/L (DL-Serine, LOT SLBK6776V, CAS 302-84-1) was prepared. For the calibration curve, 400 μL of standard solution were added to a flow-cuvette with 3 mL OPA reagent and the solution was incubated for 120 s at room temperature, after which absorbance was measured at 340 nm. To measure degree of protein hydrolysis in the digesta, the samples were centrifuged at 4 °C for approximately 20 min at 10,000×*g* (Heraeus Pico and Fresco 17, Thermo Fisher Scientific, Waltham, USA) and then absorbance was measured as described for the standard. Degree of protein hydrolysis (DH) was calculated as:

$$DH\ (\%) = \frac{NH_2\ (Sample)}{Total\ NH_2\ (Acid\ hydrolysate)} \times 100$$

where NH_2 (Sample) is concentration of free amino groups in each digested sample after blank correction, expressed as serine equivalents/g protein. Total NH_2 (acid hydrolysate) is total amount of free amino groups after acid hydrolysis, based on amino acid composition analysis of the different raw materials. Acid hydrolysis was conducted at 100 °C for 18 h using 6 mol/L HCl. For faba bean, total free amino acid concentration was 6.56 ± 0.12 mmol/g protein, while for pea and soy it was 7.76 ± 0.78 and 7.03 ± 0.66 mmol/g protein, respectively. This values are in agreement with previously presented values by Marinea, Ellis, Golding, and Loveday (2021) for soy based gels (7.05–7.71 mmol serine equivalents/g of protein) and the theoretical value (7.67 mmol of total amino acids/g of protein) calculated from the amino acid composition of soybeans reported by (Day, 2013).

2.4.5. Estimation of iron and zinc bioaccessibility

Freeze-dried digesta samples were re-suspended in 20 mL Milli-Q water and centrifuged at 13,000×*g* for 20 min at 4 °C (SORVALL LYNX 6000 Centrifuge, Thermo Fisher Scientific, Waltham, USA). The supernatant was removed and the content of iron and zinc was determined in both the supernatant and the pellet, using atomic absorption spectrometry as described in 2.2.4 (the pellet was microwave-digested as described in 2.2.4 before atomic absorption spectrometry). As the enzymes and reagents used during the digestion contained trace elements, all samples were blank-corrected using an average of nine digestion blanks. The content of iron (zinc) found in the supernatant i.e. the amount of minerals that were released from the sample during digestion was considered accessible iron (zinc) (Lemmens et al., 2018) while the combined concentration of each mineral in the pellet and supernatant was used to calculate the recovery of the individual mineral.

2.5. Statistical analysis

The results of the chemical analyses (n = 2), amino acid composition (n = 3) and molar ratio of phytate and mineral (n = 3) are presented as mean and pooled standard deviation. The results were further analysed by one-way analysis of variance (ANOVA, Typ I), followed by Tukey's post-hoc test. The results from the degree of hydrolysis measurements (n = 3, n = 2) were analysed using ANOVA (Typ III) for unbalanced population size. To determine the correlation coefficient between the result from the protein digestion and the amount of phytate found in the different raw materials Pearson's product-moment correlation and a 95% confidence interval was used. All statistical analyses were performed using R studio (Version 4.3.0, RStudio Inc., Boston, USA).

3. Results and discussion

3.1. Chemical analysis

All 11 raw ingredients were analysed for their composition in protein, starch, fat, fibre and moisture (Table 2). To allow for the comparability of protein hydrolysis between the samples, they were normalized according to a protein content of 0.2 g. For simplicity and comparability with other studies, a general protein conversion factor of 6.25 was used for all materials (Sousa et al., 2020; 2023). The amount of protein in pea flour (208 g/kg) and faba bean flour (309 g/kg) was representative of milled crops and similar to values reported by Mayer Labba et al. (2021) for faba bean (228–283 mg/kg) and Martineau-Côté, Achouri, Karboune, and L'Hocine (2022) for pea (181–275 mg/kg). The pea concentrate contained 494 g/kg protein. In comparison, Rekola et al. (2023) found 530 g/kg protein in the same pea concentrate and 824 g/kg in pea isolate from a different supplier. Overall, the total protein content found in concentrates and isolates from pea, faba bean and soy was in agreement with that reported for similar products (de Paiva Gouvêa et al., 2023). For the texturized raw materials, the total protein content largely depended on whether isolate or concentrate was used for the texturising process, making comparison of results impossible. However, the results obtained (pea 597 g/kg, faba bean 622 g/kg, soy 674 g/kg) were in agreement with the composition data (i.e., protein, starch, fat, ash, moisture content) provided by the supplier. As the total fibre content was not determined in this study, the presented composition, limited to the measured amounts of hemicellulose, cellulose, and lignin (NDF and ADF), does not provide an indication of the remaining polysaccharide fractions. Based on the specification of the products the total fibre content can vary between 20 and 190 g/kg depending on the product.

3.2. Amino acid composition

The amino acid composition of the different raw materials is presented in Table 3. As acid was used to hydrolyse the proteins, tryptophan could not be detected (Ozols, 1990). Overall, high amounts of leucine, lysine, aspartic acid, arginine and glutamic acid were found in all raw materials. In contrast, low amounts of cysteine and no methionine were found in all products. The content of the sulphur-containing amino acid methionine is generally low in plant-based proteins, when compared with animal-based products (Herreman et al., 2020), and for all products in the present study, only low amounts of cysteine and no methionine were found. In addition, acid hydrolysis can lead to breakdown of cysteine, methionine and tyrosine, and can influence quantification of these amino acids (Ozols, 1990).

According to recommended protein intake guidelines for adults (FAO/WHO/UNU, 2007), none of the texturized protein materials analysed met the requirements for valine and isoleucine. The texturized faba bean protein contained 23.4 mg isoleucine/g protein (recommended 30 mg/g protein) and 30.3 mg valine/g protein (recommended 39 mg/g protein). Among the faba bean products, only faba bean isolate met the requirements for isoleucine and valine. Soy isolate and soy concentrate met the requirement for isoleucine, but showed limitations for valine. However, since these raw materials are not intended for individual consumption, but used as an ingredient, the limitations can be overcome by product formulation and a balanced diet. The texturized products are used as-is, but combining different plant-based proteins can be a possible means to meet the requirements for isoleucine and valine, as shown in previous studies (Herreman et al., 2020).

Table 2
Chemical composition of the different faba bean, pea and soy raw materials, grouped into flours, concentrates, isolates (based on total protein content) and texturized proteins.

Category	Composition						
	Protein	Starch	Fat	Fibre NDF	Fibre ADF	Ash	Moisture*
Pea flour	208	537	11	26	19	29.5	98.0
Faba bean flour	309	465	11	28	24	32.0	94.7
Pea concentrate	494	47	35	26	6	59.3	80.7
Faba bean concentrate	575	65	17	18	8	65.6	73.5
Soy concentrate	681	14	2	90	56	45.5	84.7
Pea isolate	854	2	57	8	2	62.2	80.5
Faba bean isolate	883	7	69	5	4	37.5	74.1
Soy isolate	859	9	15	10	2	42.3	71.9
Pea texturized	597	39	30	90	4	50.8	74.2
Faba bean texturized	622	53	11	17	12	57.1	78.1
Soy texturized	674	9	1	57	39	57.5	86.3
Pooled standard deviation	7	3	1	2	1	0.5	0.4

Chemical composition expressed as g/kg dry. *Expressed as g/kg sample.

3.3. Minerals and phytate

The amount of iron in the raw materials is presented in Table 4. No significant difference ($p > 0.05$) was found between the flours and the texturized faba bean, which contained low amounts of iron. The iron content in the concentrates varied between 85 and 117 mg/kg, with faba bean concentrate containing significantly ($p < 0.05$) less iron than the pea and soy concentrate. Differences between the soy and faba bean isolates and texturized products were found, where faba bean isolate contained the overall highest amount of iron. However, no significant difference was found between the pea isolate and textured pea product containing 199 respectively 194 mg/kg dry product. This aligns with previously reported values by Mayer Labba et al. (2021) for faba bean flour (18–213 mg/kg), Zhang et al. (2022) for soy (79–116 mg/kg) and) for pea (39 ± 12 mg/kg). However, with great variability between different cultivars (Mayer Labba et al., 2021). The amount of zinc found in the different raw materials varied from 2.6 to 114 mg/kg (Table 4). The highest amount was found in faba bean isolate and faba bean concentrate, while the lowest was found in texturized soy protein, soy protein concentrate and pea flour. Similar values have been reported by Mayer Labba et al. (2021), Millar, Gallagher, Burke, McCarthy, and Barry-Ryan (2019) and Zhang et al. (2022) for faba bean and pea. For soy, a range of 57–92 mg/kg was observed by Zhang et al. (2022). However, as most available data on minerals found in different crops refer to entire products and flours, rather than isolates or concentrates, direct comparisons are not always possible.

Upon comparison with the suggested daily intake of iron, it is evident that the consumption of 100 g of texturized pea or faba bean isolate would likely meet the recommended intake for all individuals, given the assumption that the majority of the mineral is bioavailable. Concerning zinc, while 100 g of faba bean isolate or faba bean concentrate would meet the recommendations for all females, it falls short of meeting the requirements for males in any age group. However, since the bioavailability of minerals (i.e. the amounts that are available for uptake and utilization on the body), depends on the amount of phytate present, as it has a strong inhibitory effect on the mineral uptake, the phytate content also has to be considered when comparing the different raw materials. The amount of phytate present in plant-based raw materials depends on numerous factors, including crop, variety and growing conditions (Urbano et al., 2000). The amount of phytate found in the different raw materials analysed in the present study varied from 9.4 g/kg in the pea flour to 28.9 g/kg in the faba bean concentrate (Table 4). Similar amounts have been reported previously, but with large variations, in e.g. faba beans (1.1–21.0 g/kg) (Carnovale, Lugaro, & Lombardi-Boccia, 1988; Mayer Labba et al., 2021; Millar et al., 2019; Zhang et al., 2022) and soy (11.0–18.8 g/kg) (Al-Wahsh et al., 2005). A value of 5.7 g/kg has been reported previously for peas (Millar et al., 2019) and a range of 14.4–25.5 g/kg for pea products (Carnovale et al., 1988; Chigwedere et al., 2023).

3.4. Estimated mineral bioavailability based on molar ratio of phytate to mineral

To obtain an estimation of the bioavailability of iron and zinc in the raw materials, molar ratios of Phy:Fe and Phy:Zn were calculated (Figs. 1 and 2). All obtained values for both ratios exceeded the limits suggested by Panel and Nda (2014) and Hurrell and Egli (2010), indicating very low bioavailability of iron and zinc in all raw materials if consumed without enhancers such as ascorbic acid or meat.

Faba bean isolate and pea isolate showed the lowest Phy:Fe ratio of the materials tested (Fig. 1A) and can be adequate iron sources if consumed with enhancing compounds or products. For the texturized faba bean and faba bean concentrate, an average Phy:Fe ratio of 28.4 and 28.6, respectively, was obtained and these protein materials were thus estimated to have the lowest bioavailability of iron if consumed individually.

Faba bean isolate and pea isolate also had the lowest Phy:Zn ratio (Fig. 1B), and are likely to provide sufficient bioavailable zinc if consumed within a balanced diet. Texturized soy protein and soy concentrate had the highest Phy:Zn ratio and are therefore unlikely to contain bioavailable zinc if consumed individually. However, the recommendations for zinc refer to the overall diet and not individual products or ingredients, so these results can only provide a rough guide.

3.5. Mineral bioaccessibility after in vitro digestion

To estimate the bioaccessibility of iron and zinc, the amounts of minerals in the supernatant obtained after centrifugation of *in vitro* digested samples were measured (Table 5) and calculated as the ratio of minerals in the soluble fraction (supernatant) to the amount of minerals in the undigested sample.

Accessible iron (between 0.26% and 31.7%) was detected in four of the samples (faba bean concentrate, faba bean isolate, pea isolate, pea texturized). Previous studies on wheat, finger millet, pearl millet and beans have shown lower bioaccessibility values, ranging from 1.10 to 4.94% (Muleya, Young, & Bailey, 2021). In contrast, Lemmens et al. (2018) reported higher bioaccessibility values for wheat, ranging from 4.6% to 36.6%, depending on processing conditions. An increase in bioaccessiblity of iron and zinc has been correlated with the concentration of phytate, which can be reduced during processing (Gupta et al., 2015; Hurrell 2004; Larsson et al., 1997). Further, processing may also influence the food structure and consequently the release of minerals from the food matrix. Thus, the comparison of bioaccessibility values from differently processed ingredients or products is challenging.

Table 3
Amino acid composition of the different raw materials and recommended protein intake for adults (FAO/WHO/UNU, 2007).

	Amino acid composition (mg/g protein)											Pooled standard deviation	p-value[1]	mg/kg BW *per* day[2]	mg/g protein[3]
	Pea flour	Faba bean flour	Pea concentrate	Faba bean concentrate	Soy concentrate	Pea isolate	Faba bean isolate	Soy isolate	Pea texturized	Faba bean texturized	Soy texturized				
Essential amino acids															
Histidine	25	24	25	22	24	23	22	21	23	22	22	3	0.752	10	15
Isoleucine	33^{abc}	27^{bcd}	29^{abcd}	25^{cd}	35^{ab}	36^{a}	31^{abcd}	31^{abcd}	30^{abcd}	23^{d}	28^{abcd}	3	<0.001	20	30
Leucine	72^{abc}	69^{bcd}	76^{abcd}	69^{cd}	80^{ab}	83^{a}	73^{abcd}	69^{abcd}	76^{abcd}	68^{d}	69^{abcd}	5	0.026	39	59
Lysine	78^{a}	63^{bc}	83^{a}	63^{bc}	73^{ab}	80^{a}	61^{bc}	60^{c}	77^{a}	60^{c}	63^{bc}	4	<0.001	30	45
Phenylalanine	46^{abcd}	34^{e}	49^{ab}	37^{cde}	50^{ab}	54^{a}	40^{bcde}	47^{abc}	49^{ab}	36de	45^{abcde}	4	<0.001	25*	30*
Threonine	39^{abcd}	33^{e}	40^{abc}	34^{cde}	44^{a}	40^{abc}	33de	39^{abcde}	38^{bcde}	33de	41^{ab}	4	<0.001	15	23
Valine	42^{a}	32^{ab}	38^{ab}	32^{ab}	37^{ab}	40^{a}	34^{ab}	32^{ab}	37^{ab}	30^{b}	33^{ab}	4	0.00599	26	39
Non-essential amino acids															
Alanine	47^{ab}	39^{ab}	45^{ab}	38^{ab}	48^{a}	43^{ab}	40^{ab}	37^{ab}	43^{ab}	38^{ab}	39^{ab}	4	0.00479	–	–
Arginine	70^{bcd}	91^{a}	82^{abc}	80^{abc}	65^{cd}	71^{bcd}	73^{abcd}	55^{d}	77^{abc}	89^{ab}	56^{d}	7	<0.001	–	–
Aspartic acid	121	122	130	112	135	135	113	109	125	114	107	11	0.0323	–	–
Cysteine	5^{a}	3^{b}	3^{b}	2^{b}	3^{b}	3^{b}	2^{b}	3^{b}	3^{b}	2^{b}	3^{b}	1	<0.001	–	–
Glutamic acid	181^{ab}	175^{ab}	185^{ab}	170^{b}	211^{a}	195^{ab}	172^{ab}	182^{ab}	182^{ab}	170^{b}	178^{ab}	14	0.0447	–	–
Glycine	48^{a}	42^{ab}	40^{ab}	35^{b}	43^{ab}	40^{ab}	34^{b}	37^{ab}	40^{ab}	38^{ab}	38^{ab}	4	0.0306	–	–
Proline	47^{ac}	43^{bc}	46^{bc}	44^{bc}	59^{a}	50^{abc}	43^{bc}	52^{ab}	45^{bc}	43^{c}	52^{ab}	3	<0.001	–	–
Serine	55	51	57	51	60	60	53	52	57	50	54	4	0.0333	–	–
Tyrosine	38	30	35	30	38	38	31	28	36	29	28	4	0.00414	–	–

*Results are expressed as mg/g protein based on dry weight. *Value corresponds to phenylalanine + tyrosine. [1]One-way ANOVA was used to determine differences between the raw materials. Different superscript letters indicate significant differences according to Tukey's test ($p < 0.05$). [2]mg protein required by an adult per kg body weight.[3]Mean nitrogen requirement of* 105 mg/kg *per day (0.66 g protein/kg per day)* (FAO/WHO/UNU, 2007).

Table 4
Amounts of minerals and phytate found in the raw materials and recommended daily intake of iron and zinc.

Category	Composition		
	Iron	Zinc	Phytate*
Pea flour	54^{f}	34^{f}	9.4^{g}
Faba bean flour	63^{f}	45^{e}	13.8^{de}
Pea concentrate	117^{d}	74^{d}	23.2^{b}
Faba bean concentrate	85^{e}	111^{a}	28.9^{a}
Soy concentrate	104^{d}	31^{f}	14.6^{cd}
Pea isolate	200^{b}	88^{b}	13.1^{ef}
Faba bean isolate	389^{a}	114^{a}	18.4^{c}
Soy isolate	139^{c}	51^{e}	10.9^{fg}
Pea texturized	194^{b}	86^{bc}	17.2^{c}
Faba bean texturized	70^{ef}	76^{cd}	23.4^{b}
Soy texturized	114^{d}	26^{f}	16.3^{cd}
Pooled standard deviation	6	4	0.8
Recommended intake[1]	9	12.7	–
Male 18–50 years	9	12.4	–
Male 51–70 years	15[2]	9.7	–
Females 18–50 years	8[3]	9.5	–
Females 51–70 years			

Results are expressed as mg/kg dry weight.*expressed as g/kg dry weight. [1]Recommended intake (RI) in mg/day, according to the Nordic Nutrition Recommendation 2023, assuming a mixed animal/vegetable diet with a phytic acid intake of about 600 mg/day. [2]If large menstruation bleedings, screening of iron status and supplementation as indicated. [3]If still menstruating, the RI for 25–50 y (15 mg/day) should be used (Nordic Council of Ministers, 2023). Lowercase letters indicate significant differences between samples ($p < 0.001$).

Further, the *in vitro* results are in line with the estimates of bioavailability based on molar Phy:Fe ratios, which indicated that pea isolate, faba bean isolate (Phy:Fe < 6) and texturized pea protein (Phy:Fe ~7.5) contain available iron. However, faba bean concentrate had a high Phy:Fe ratio (~29), corresponding to low expected bioavailability. Soy isolate had a relatively low Phy:Fe value (~6), indicating available iron if consumed within composite meals high in ascorbic acid and meat, but no accessible iron was found in the supernatant after *in vitro* digestion. Thus, the results obtained after *in vitro* digestion of faba bean concentrate and soy isolate were contradictory to the estimated bioavailability results. For the remaining samples, no accessible iron was found and overall no accessible zinc was detected in any of the digested products. Estimated bioavailability based on Phy:Zn ratio indicated that moderate absorption of zinc could be expected from pea isolate and faba bean isolate and thus was not in agreement with the *in vitro* results.

Overall recovery was 76% for iron and 94 % for zinc. The lower recovery of iron can be partly attributed to formation of insoluble iron oxides (Ems, St Lucia, & Huecker, 2024), which are incompletely atomised in the flame during atomic absorption spectroscopy (Harris, 2010, p. 716). Differences in recovery can also be a consequence of variations within the blanks, which can cause uncertainty in the results (Muleya et al., 2021). On average, 1.61 ± 0.25 mg Fe/L and 4.06 ± 0.79 mg Zn/L were found in the blanks. This is in agreement with results presented by Muleya et al. (2021) indicating that an approximate concentration of 1.73 mg Fe/L and 3.36 mg Zn/L can be expected.

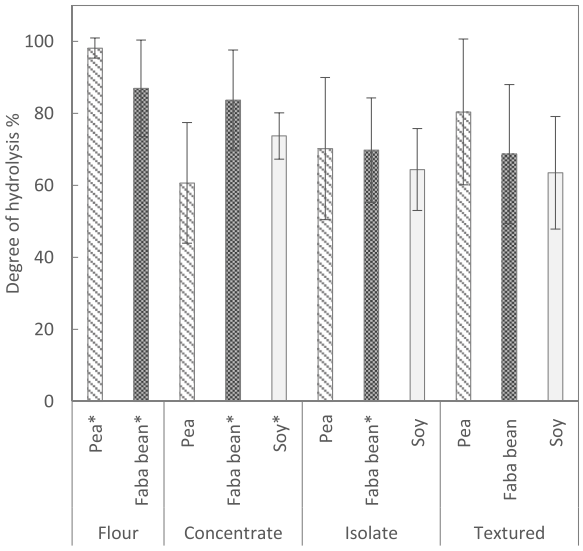

Fig. 2. Degree of hydrolysis (DH) in % for the different raw materials (n = 3 or n* = 2).

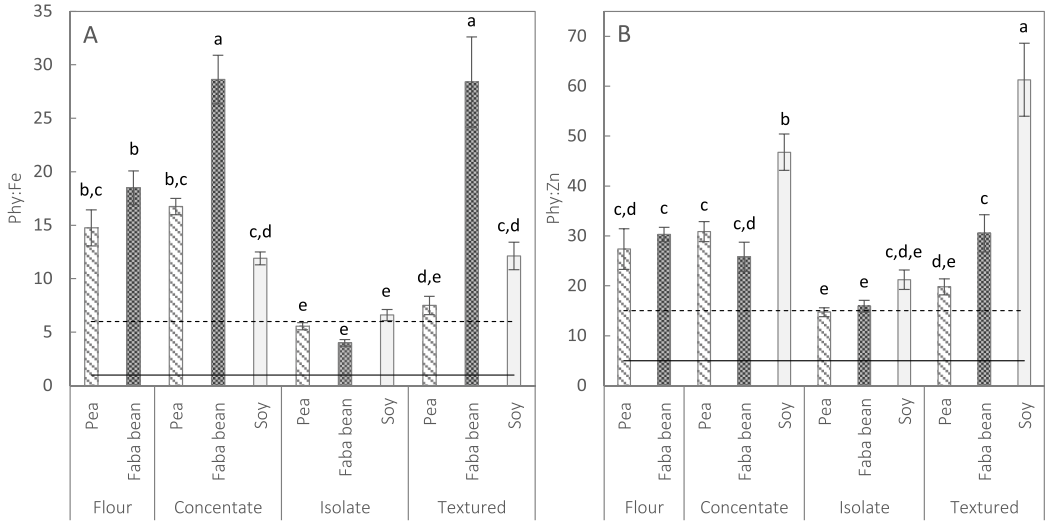

Fig. 1. Estimated mineral bioavailability based on the molar ratio of phytate to iron/zinc. (A) Molar ratio of phytate to iron (Phy:Fe), where Phy:Fe < 1 (solid line), or preferably <0.4, is needed for adequate iron absorption from plain cereal or legume-based meals without absorption enhancers. Phy:Fe = 6 (dashed line) can be considered adequate in composite meals high in ascorbic acid and meat (Hurrell & Egli, 2010). (B) Molar ratio of phytate to zinc (Phy:Zn), where Phy:Zn < 5 (solid line) corresponds to high zinc absorption and Phy:Zn = 5-15 (dashed line) corresponds to moderate absorption (Panel & Nda, 2014). Lowercase letters indicate significant differences between samples ($p < 0.001$).

Table 5
Amount of iron and zinc found in the different fractions, i.e. supernatant and pellet, of digesta samples and calculated recovery from both fractions.

	Iron			Zinc		
	Supernatant[a]	Pellet[a]	Recovery %	Supernatant[a]	Pellet[a]	Recovery %
Pea flour	ND	37 ± 3	69 ± 5	ND	27 ± 2	79 ± 6
Faba bean flour	ND	39 ± 2	62 ± 4	ND	36 ± 4	81 ±
Pea concentrate	ND	95 ± 8	81 ± 6	ND	65 ± 4	87 ± 6
Faba bean concentrate	14 ± 1.2	52 ± 4	77 ± 5	ND	96 ± 5	87 ± 5
Soy concentrate	ND	105 ± 3	100 ± 3	ND	29 ± 1	95 ± 4
Pea isolate	31.7 ± 1.4	107 ± 14	69 ± 7	ND	90 ± 5	102 ± 6
Faba bean isolate	67.4 ± 6.8	188 ± 12	66 ± 3	ND	115 ± 6	100 ± 6
Soy isolate	ND	96 ± 3	69 ± 2	ND	51 ± 5	101 ± 9
Pea texturized	0.5 ± 1.3	126 ± 5	65 ± 3	ND	86 ± 11	101 ± 12
Faba bean texturized	ND	53 ± 4	76 ± 6	ND	76 ± 6	100 ± 8
Soy texturized	ND	114 ± 4	100 ± 3	ND	26 ± 4	97 ± 16

[a] mg/kg protein powder based on dry weight ± standard deviation. ND- Not Detected.

Introducing additional minerals (added in the digestive fluids) into the system can also make the characterisation susceptible to inaccuracies, as it is not possible to distinguish between in-sample and added minerals. To evaluate the contribution of iron and zinc in reagents used in the INFOGEST method, Muleya et al. (2021) used isotopic labelling to discriminate between reagent-derived and sample-derived iron and zinc. This approach can improve the accuracy of the results, but requires changes from the original protocol, while the need for working with radioactive substances limits its applicability. For this reason, blank correction can be a more applicable approach.

Furthermore, the addition of minerals, especially calcium can influence the formation and stability of phytate mineral complexes (Wang & Guo, 2021) which can affect mineral distribution between supernatant and pellet. Despite the fact that phytate and calcium show a much lower complex stability than iron or zinc the fact that calcium is present in much higher concentrations can overpower the lower affinity as a consequence of mass action (Angel, Tamim, Applegate, Dhandu, & Ellestad, 2002). However, as the interactions between phytate and other food components not only depend on the mineral concentrations but also pH, ionic strength, supporting electrolyte and temperature (Wang & Guo, 2021) it is difficult to evaluate how and to what extent these different factors influence the final result.

The *in vitro* method used in this study for estimation of iron and zinc bioaccessibility is based on the simulation of the gastro-intestinal digestion for estimation of the amount of iron and zinc that can be absorbed in the digestive tract, by measuring the fraction of iron and zinc that is obtained in the supernatant of the centrifuged digested samples. Although the obtained values from the *in vitro* experiments are relative rather than absolute estimates of mineral absorption, due to the absence of several of the physiological factors that can affect bioavailability, such relative estimates can still be useful and suffice to form a strategy to obtain an enhanced mineral availability from plant-based foods. However, since the total iron (zinc) fraction may not be readily available for absorption, a combination of *in vitro* digestion with uptake studies using e.g., Caco-2 cells would provide a tool to study both passive diffusion and active absorption of iron (zinc). This will be an interesting approach in further studies using food products.

3.6. *In vitro protein digestibility and degree of hydrolysis*

The protein digestibilities of the *in vitro* digested materials were determined by quantification of free amino groups in the supernatant of the digested samples (degree of hydrolysis, DH) using the OPA method (Fig. 2). The values shown are based on the number of bonds hydrolysed in the digesta and the total number of peptide bonds *per* protein equivalent. As for some samples, the DH exceeded 100% the outlier values have been excluded. Therefore, results are presented in duplicates for those samples resulting in DH >100%. This overestimation can be attributed to the autolysis of digestive enzymes once the food substrate is fully digested and is often accruing in single protein systems (Marinea et al., 2021; Sousa et al., 2023).

Although the faba bean and pea flour showed the highest overall DH, no significant differences were found between the raw materials ($p = 0.342$). Depending on the type of pea product, DH varied between 60.7 ± 16.8% for pea concentrate and 98.1 ± 2.8% for pea flour, with pea isolate and texturized pea protein showing intermediate values (70.2 ± 19.8% and 80.4 ± 20.3%, respectively). For faba bean, an overall mean DH of 80.2 ± 9.4% was found, with faba bean flour and faba bean concentrate showing the highest values and faba bean isolate and texturized faba bean protein the lowest. The degree of protein hydrolysis for soy ranged on average between 73.7 ± 6.4% for soy concentrate and 63.5 ± 15.6% for texturized soy protein (Fig. 2).

In comparison, Reynaud, Lopez, Riaublanc, Souchon, and Dupont (2020) found DH values ranging between 25% and 85% for the same type of pea isolate, depending on the processing of the isolate. They also found that pea emulsions are better hydrolysed than protein isolates, which they attributed to the high-pressure processing during emulsification (Reynaud et al., 2020), which underlines the effect of processing on the digestibility of proteins. In general, the isolation process can influence the structure of proteins, including partial denaturation, which can increase digestibility. Nevertheless, results presented by Sousa et al. (2023) on pigeon peas (DH 100%) and black beans (DH 86%), among others highlight the trend of overestimating the total digestibility in pure protein systems. This observation has led to suggestions to include different nutrients during digestion to mimic real food and avoid potential overestimation (Sousa et al., 2023). Besides this, the INFOGEST protocol stands out as the best tool for a standardized comparison, even of a single nutrient system once the content of the studied nutrient is normalized.

Comparing the obtained result with intervention studies in pigs (Herreman et al., 2020), soy and pea proteins are expected to have higher digestibility than faba bean protein. This could not be confirmed by our results and could be a consequence of processing (Mathai, Liu, & Stein, 2017; Sá, Moreno, & Carciofi, 2019) improving the digestibility of faba bean (Martineau-Côté et al., 2022). Further, fewer data is available on faba beans than on peas and especially soy, which limits the generalisability of the findings (Herreman et al., 2020).

Besides the potential impact of the processing on protein digestibility, phytate can reduce the bioavailability of proteins (Angel et al., 2002; Wang & Guo, 2021). However, no correlation ($r = 0.45$) was found between the DH and the amount of phytate in the *in vitro* digested sample (Figure A14). This can result from the fact that no significant differences were found between the DH of the different raw materials but also due to the presence of, divalent cations e.g. iron, zinc or calcium that can compete with protein for complex formation with phytate and thereby increase the bioavailability of the protein (Prattley, Stanlez, & Voort, 1982; Wang & Guo, 2021). The impact of the interactions between proteins and phytate on protein digestibility is still not fully

understood (Wang & Guo, 2021) and needs further investigation.

4. Conclusions

All 11 raw materials studied had a high content of phytate and low estimated bioavailability of iron or zinc, if consumed individually. Isolates showed the lowest molar ratio of phytate: mineral and therefore the highest tendency for available iron and zinc making this product most suitable as an ingredient for the development of plant-based foods with improved nutritional properties. A similar trend was reflected in the results obtained after *in vitro* digestion, although four of the raw materials were found to have accessible iron. The results underline the need for, development of processing methods to reduce the amount of phytate to improve the bioavailability of minerals in plant-based raw materials and foods.

The recommendations for isoleucine and valine were not met by all materials, with faba bean products containing the lowest amounts. Therefore, adjustments within the product formulation are needed to overcome this limitation and to improve the overall protein quality.

The *in vitro* protein digestibility was estimated via degree of protein hydrolysis (DH), average DH after *in vitro* digestion was similar for all ingredients, indicating no significant differences among the analysed materials. Despite the fact that faba beans are often considered low-quality protein, DH results indicate otherwise, implying that faba bean protein can have a digestibility similar to that of pea or even soy depending on the processing. However, degree of protein hydrolysis does not provide information on the digestibility of individual amino acids, but rather reflects breakdown of peptide bonds. Therefore, further refinement of the methodology will be useful for assessment of the digestibility of protein and individual amino acids in plant-based raw materials and products.

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CRediT authorship contribution statement

Jaqueline Auer: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marie Alminger:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Conceptualization. **Marina Marinea:** Writing – review & editing, Validation, Methodology, Investigation. **Mathias Johansson:** Writing – review & editing, Investigation. **Galia Zamaratskaia:** Writing – review & editing, Supervision. **Anders Högberg:** Writing – review & editing, Supervision. **Maud Langton:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

We declare that the research was conducted in the absence of any financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.115893.

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